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Citrus Research International, Nelspruit

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## 1 MARKET ACCESS TECHNICAL COORDINATION

By Vaughan Hattingh and Elma Carstens (CRI)

### 1.1 SUMMARY

Twelve years of exchange of technical information has resulted in the opening of a new export market for South African fresh citrus fruit with the signing of a bilateral workplan between South Africa and the Philippines. The USA published a rule which removed the restriction on ports for entry of South African fresh citrus fruit. South Africa received 20 notifications of non-compliance from the EU, 14 for FCM and 6 for CBS. There was no progress on the long outstanding market access issues with Vietnam, Japan and the USA and the amended protocol for fresh citrus fruit exports to China had not been signed. Concerns related to mealybug interceptions on fresh citrus fruit for export to South Korea were addressed. CRI supported the eSwatini National Plant Protection Organisation with compliance measures for the EU regulations pertaining to FCM.

### OPSOMMING

Twaalf jaar se uitruiling van tegniese inligting het tot die opening van 'n nuwe uitvoermark vir Suid-Afrikaanse vars sitrusvrugte gelei met die ondertekening van 'n bilaterale werkplan tussen Suid-Afrika en die Filippyne. Die VSA het 'n reël gepubliseer wat die beperking op hawens vir toegang van Suid-Afrikaanse vars sitrusvrugte verwyder het. Suid-Afrika het 20 kennisgewings van “non-compliance” van die EU ontvang, 14 vir VKM en 6 vir SSV. Daar was geen vordering met die lang uitstaande marktoegangskwessies met Viëtnam, Japan en die VSA nie. Die gewysigde protokol vir uitvoere van vars sitrusvrugte na China is nie onderteken nie. Kommer wat verband hou met wtluis-onderskeppings op vars sitrusvrugte vir uitvoer na Suid-Korea is aangespreek. CRI het die eSwatini Nasionale Plantbeskermingsorganisasie ondersteun met maatreëls om die EU-regulasies met betrekking tot VKM na te kom.

### 1.2 EUROPEAN UNION (EU)

#### FCM

In 2020 South Africa received the first notifications of non-compliance for FCM in June and in reaction to the FCM interceptions, the European Commission sent a letter to DALRRD on 10 July 2020 requesting investigation reports. By the end of August South Africa had received 11 notifications of FCM interceptions. Based on the number of notifications, the CGA FCM and CBS Disaster Management Committee agreed that the industry request DALRRD approval to implement the following temporary measure from 1 September 2020 for the remainder of the 2020 season: For the export of oranges (*Citrus sinensis*) to the EU, PPECB will only allow exporters to make a booking for such exports in containers under shipping regime codes as specified under Option C in the Citrus FMS and that FMS Category A oranges will also be allowed to be shipped under shipping regime code ECW0 out of the Cape Town port.

The EU reported 14 FCM interceptions on South African citrus by the end of the 2020 season. CRI coordinated the actions of the FMS Rapid Response group to conduct desktop investigations on all the interceptions and provided the reports to DALRRD. CRI also provided inputs on the DALRRD investigation reports that were submitted to the EU.

In accordance with the procedure as agreed with DALRRD, CRI coordinated a process of reviewing the FMS in light of the 2020 season results. The FMS Core Technical Team and the FMS Working Group met in October and CRI discussed the recommended FMS amendments with DALRRD on 02 November 2020. The revised FMS was communicated to the industry at the annual DALRRD export coordinating meeting of 12 November 2020.

CRI and CGA participated in a collaborative project with the Netherlands importers association to undertake additional FCM sampling of selected consignments of South African citrus arriving in Netherlands, with the objective of potentially strengthening the FMS.

The approved FMS for the 2021 export season was circulated by DALRRD on 20 November 2020 and CRI sent out a Cutting Edge to highlight the amendments to the FMS for the 2021 export season. Several meetings were held with PhytClean to implement the changes.

The European Commission (EC) requested EFSA to provide an opinion on the FCM systems approach used by South Africa. EFSA engaged with DALRRD who in turn referred the matter to CRI to prepare a response to the questionnaire received from EFSA, for return to EFSA by end January 2021. CRI prepared the response and provided the first draft to DALRRD on 13 January 2021. A virtual meeting took place on 19 January 2021 to discuss the draft. The revised response to EFSA was supplied to DALRRD on 29 January 2021. DALRRD submitted the completed questionnaire to EFSA. Feedback was pending from EFSA by the end of the reporting period.

## **CBS**

Based on the three CBS interceptions and as a pro-active matter, the CGA FCM and CBS Disaster Management Committee agreed that the industry request DALRRD approval to implement the following temporary measure for the remainder of the 2020 season: PPECB not to inspect any citrus fruit of the affected types and production regions after midnight on 12 September 2020. DALRRD indicated their support and it was agreed that the intervention can be effected through the automatic withdrawal of EU registration of affected orchards on the PhytClean system. The following production areas and types of citrus fruit were excluded from the early closure:

1) The CBS free areas of the Western and Northern Cape. 2) The low-risk Gamtoos and Katriver production regions. 3) Mandarins (soft citrus) - a low-risk citrus type.

DALRRD notified the EU in September 2020 about the early termination of citrus exports from SA of certain citrus types from specific production areas. In the communication DALRRD erroneously referred to the low-risk Gamtoos and Katriver production regions as CBS free areas. The EU notified DALRRD that according to their systems, these two areas are not recognised as CBS free areas and requested the necessary clarifications. CRI was requested for inputs and submitted a response to DALRRD in March 2021.

By the end of the 2020 export season, the EU had reported 6 CBS interceptions on South African citrus in 2020.

At the CBS-RMS Working Group meeting on 19 October 2020 and the CRI-DALRRD meeting on 2 November 2020 no changes were made to the CBS-RMS for the 2021 citrus export season. The CBS RMS for the 2021 export season was circulated by DALRRD on 20 November 2020.

Ensuing from discussions between CRI and DALRRD, DALRRD confirmed that CRI-Phytrisk is an approved CBS risk management system. Cutting Edge (No 304) communicated the updated spray programmes for CBS for 2020/2021 and the systems available to determine the associated risk. CRI provided inputs on the 5 DALRRD CBS investigation reports that were submitted to the EU.

## **FRUIT FLIES**

The EU did not report any fruit fly interceptions on South African citrus in the 2020 export season. At the FMS Working Group meeting on 19 October 2020 and the CRI-DALRRD meeting on 2 November 2020 no changes were made to the FFMS for the 2021 citrus export season.

### **1.3 JAPAN**

In 2020 there was again no response from Japan-MAFF on the three long outstanding market access requests: access for all mandarins (except Satsumas), under the current protocol for Clementines (pending from November 2009); revision of the current cold treatment conditions for the export of all eligible citrus types to Japan by the inclusion a cold treatment of 1.4°C or lower for 16 consecutive days (pending from November 2009) and inclusion of all navel orange cultivars in the current protocol (pending from September 2016). Their

explanation for the lack of response remains the same - Japan-MAFF only work on one market access request from a country at a time and the protocol for South African Avocado exports to Japan has not been finalised yet.

#### 1.4 USA

In this reporting period none of the long outstanding matters – the equivalence between USA domestic CBS regulations and USA import regulations (access for fruit from CBS areas in South Africa); recognition and access for CBS pest-free places of production in an area of low pest prevalence; and inclusion of other Western Cape magisterial districts in the export programme; the updated pest list and an updated work plan, was concluded despite the ongoing engagement by the South African Embassy staff, DALRRD and various industry partners.

The new point for discussion with USDA-APHIS was the request from SA to include additional ports of entry for export of citrus fruit from SA and on 4 November 2020 USDA-APHIS announced that the restriction on ports for entry of SA citrus has been removed and the new rule will be effective after publication in the Federal Register on 5 November 2020. The revised conditions were included in the Fruits and Vegetables Import Requirements.

#### PRE-CLEARANCE PHYTOSANITARY INSPECTIONS

Due to the COVID-19 pandemic affecting global travel, DALRRD sent letters in March 2020 to the USA to propose alternatives for conducting phytosanitary preclearance inspections. CRI provided inputs into the letter. The USDA-APHIS indicated that inspections can be conducted by DALRRD and the permanent USDA staff in South Africa.

#### 1.5 CHINA

DALRRD received the final amended protocol to import fresh citrus fruit from the GACC and provided the document to CRI on 06 October 2020. The amended protocol reflected all the changes as requested by DALRRD pertaining to the requirement for information about region and province on the carton labels and the quarantine pest control measures for *E. ceratoniae*. This amended protocol allows for lemons to be shipped at a different in transit cold temperature -  $\leq 3^{\circ}\text{C}$  for 18 days. A virtual technical meeting between GACC and DALRRD was scheduled for 09 November 2020 to sign the amended protocol, but the meeting was cancelled and DALRRD has not been able to secure a re-scheduled meeting and despite several follow ups by DALRRD and various industry partners, the amended protocol had not been signed by the end of this reporting period.

#### 1.6 SOUTH KOREA

#### PRE-CLEARANCE PHYTOSANITARY INSPECTIONS

Due to the COVID-19 pandemic affecting global travel, DALRRD sent letters to South Korea in March 2020 proposing alternatives for conducting phytosanitary preclearance inspections and calibration of equipment for in transit cold treatments. CRI provided inputs into the letter. Despite letters and follow up by SA Embassy in Japan, South Korea maintained their position that no exports would take place in the absence of a South Korean Inspector in South Africa. Meetings and further communication took place and on 24 April 2020 South Korea indicated that on submission of the following documents they will be in a position to make a decision: results of the field inspection of export production units during the growing season; confirmation of checks of regular disinfection and sanitary conditions of packing houses and storage facilities to ensure conformance with set requirements; confirmation of checks of cold treatment facilities and the estimated export volume and cold treatment facility location from 1 May to 12 July 2020. The documents were submitted to South Korea and on 1 May 2020, South Korea indicated that inspections can be conducted by DALRRD and that a bigger sample will be inspected on arrival in South Korea. They also indicated that continuation of this arrangement would be reviewed on a monthly basis.

## MEALYBUGS

During the previous two export seasons, concerns were raised by South Korea about rejections for mealybugs and incorrect carton markings. Citrus mealybug is the most abundant species, but is not a quarantine pest for South Korea. However, identification of mealybugs to species level is not always possible at the phytosanitary inspection point, requiring laboratory analysis and causing logistical bottlenecks. Accordingly, the inspection standard for mealybugs was amended to a zero tolerance for all mealybug species in inspections on fruit for export to South Korea in 2020.

On 1 July 2020 concerns were raised by the South Korean authorities about interceptions of mealybug eggs. An industry-DALRRD meeting was held and a notification was sent to parties producing, packing and exporting citrus to South Korea, highlighting the need to remain vigilant and to take all measures to prevent fruit infested with mealybugs being presented for export to this market.

No further concerns were raised by the South Korean authorities for the remainder of the export season and the interim measure was maintained for the duration of the 2020 season. CRI re-evaluated a molecular mealybug diagnostic procedure that was developed in the early stages of opening the Korean export market. It was found that with some minor adjustments the technique can still be used for identifying the non-quarantine citrus mealybug. The opportunity to conduct such identifications will be available for the next export season with the objective of reducing unnecessary rejections.

At the 2020 Annual Citrus Coordinating Meeting, DALRRD again highlighted the concern about interceptions of mealybugs on citrus fruit destined for the South Korean market. A decision was taken that a workshop will be held with producers to find solutions to safeguard the future of the South Korean export market. At the Coordinating meeting it was decided that a workgroup, including DALRRD, PPECB, CGA and CRI will draft documents for discussion with producers. A virtual meeting of the workgroup took place on 14 January 2021 and two documents 1) Proposed requirements for the management of mealybugs for exports to South Korea (DALRRD) and 2) Proposed corrective actions, were drafted for discussion at the producer workshop. The virtual producer workshop took place on 21 January 2021 and the documents were discussed and accepted. The two documents were included as part of the South Korea one pager that was sent out on 11 March 2021.

Further meetings took place between CRI and DALRRD to draft a Standard Operating Procedure (SOP) for the identification of mealybugs on citrus fruit intended for export to this market and a CRI Cutting Edge (with DALRRD co-authors) explaining the SOP was sent out in March 2021. CRI also circulated a Cutting Edge with a colour chart and diagnostic keys to assist with the identification of mealybugs.

### 1.7 INDIA

During this reporting period there was no response from the Indian Authorities on three outstanding issues: trial consignments sent in 2017 and 2018 for India to accept in-transit cold treatment for fruit flies in all crops exported from SA to India; the erroneous report of *Elsinoë australis* interception on citrus fruit from South Africa; and the reported presence of *Phyllosticta citricarpa* in India. DALRRD sent a letter to the Special Secretary at the Indian Plant Protection Division in June 2020. The Special Secretary at the Indian Plant Protection Division did acknowledge receipt of the letter.

### 1.8 VIETNAM

The two outstanding issues to complete the workplan for fresh citrus fruit exports to Vietnam, remained the incomplete Pest Risk Analysis and a verification visit to South Africa. The outstanding issues pertaining to the PRA entail finalizing the list of quarantine pests and packhouse procedures. Despite several follow ups by DALRRD and Industry, no communication was received from the Plant Protection Department of the Ministry of Agriculture and Rural Development of Vietnam (PPD) by the end of the reporting period. DALRRD sent a letter to PPD on 08 July 2020 requesting an update. Despite ongoing engagement by the South African embassy staff, industry partners and DALRRD an official response from PPD to DALRRD remains pending.

## 1.9 THE PHILIPPINES

On 20 May 2020 DALRRD received the draft workplan from the BPI and after consultation with CRI, DALRRD responded to the BPI on 19 June 2020, seeking agreement on three issues (no delisting of packhouses and orchards based on export inspection in SA; details of the responsibilities of PPECB and the correct stipulation of the 22-day cold treatment). In this communication DALRRD also requested the BPI to allow for trial shipments, as the workplan stated that the BPI must be present in SA when the first consignment is exported. On 15 July 2020 CRI received the final workplan for citrus exports to the Philippines from DALRRD and all the concerns had been addressed.

DALRRD submitted a signed work plan to the BPI on 24 July 2020 and on 31 August 2020, after 12 years of technical information exchanges, SA received the signed workplan from the BPI. The negotiations for market access for all fresh citrus fruit types from South Africa to the Philippines started in 2009 when SA first submitted a Pest Information Package to the Philippine authorities (BPI).

DALRRD made several follow up enquiries with BPI as to when exports could commence (permits be issued) but was informed that specific administrative procedures in the Philippines first needed to be concluded before permits could be issued.

The administrative procedures in the Philippines were concluded in March 2021. The cut-off date for registration for export to the special citrus markets was set at 31 March 2021 and on 7 April 2021 DALRRD supplied BPI with a list of PUCs and orchards approved by DALRRD, as well as a list of approved packhouses. On 15 April 2021, DALRRD submitted a list of exporters approved by DALRRD. Despite several further follow ups by DALRRD and various industry partners, no import permits were issued by the BPI by the end of this reporting period, but issuance of permits commenced shortly thereafter.

## 1.10 AUSTRALIA

South Africa submitted an information data package, prepared by CRI, in 2005 in support of an application for access to this market. After numerous enquiries by SA, a response was received from Australia in February 2007. Additional scientific data to support the cold disinfestation protocol for the two fruit fly species namely Natal fruit fly (*Ceratitis rosa*) and the five spotted fruit fly (*Ceratitis quinaria*) were requested by Australia. The scientific data generated by CRI to support the cold disinfestation protocol for the Natal fruit fly (*Ceratitis rosa*) was submitted to DoA in September 2007. As part of this response, information was again submitted to confirm that the five spotted fruit fly is not a known pest of citrus in SA and therefore no mitigation measures can be legitimately required. The priorities of the market access submissions from SA were discussed during a visit of a South African delegation to Australia in 2007 and were finalised as follows: Citrus, Table grapes, Pome fruit and Stone fruit. However, despite this meeting, none of the South African plant products that had applied for access were listed by AQIS in their Biosecurity Australia Policy Memorandum that was published in September 2007. SA queried this anomaly, but with no response from Australia. In 2014, after no feedback/response from Australia for 4 years and no apparent further interest in exporting to this market, it was removed from the agenda of the Market Access Working Group for Fresh Fruit and Vegetables.

In September 2020 DALRRD received a request from Australia to confirm South Africa's first and second priority for market access requests. In October 2020 another survey was conducted by FPEF and the outcome confirmed the decision taken in 2014 - no interest in exporting to Australia.

## 1.11 eSwatini

CRI provided assistance to eSwatini in drafting a response to the EU to explain the management measures for false codling moth (FCM) in citrus fruit exports to the EU. CRI also provided assistance on how to respond to the EU regarding eSwatini compliance with EU phytosanitary regulations (Commission Implementing Directive (EU) 2019/523 of 21 March 2019 that amends Annexes I to V to Council Directive 2000/29/EC EU Regulation).

## 1.12 CITRUS ONE PAGERS

CRI provided inputs to the PHYTOSANITARY REQUIREMENTS AND WORKING PROCEDURES FOR EXPORT OF CITRUS FROM RSA to Taiwan, Thailand, South Korea, USA, Philippines and Japan (Citrus One Pagers). The documents were circulated to the industry.

## 2 BIOSECURITY

By Solomon Gebeyehu, Wayne Kirkman, Elma Carstens and Vaughan Hattingh (CRI)

### 2.1 SUMMARY

A draft Biosecurity Master Plan for the citrus industry was prepared, has undergone a few revisions in the course of 2020, with a final working version planned to be completed in the course of 2021. Following the publication of presence of HLB/Las in coastal Kenya in early 2020, engagement took place with DALRRD including providing a briefing document by CRI to outline necessary actions to engage Kenyan partners. DALRRD subsequently communicated with KEPHIS (NPPO of Kenya) with proposals on next steps in eradicating or containing HLB.

In light of information communicated to CRI by CGA members in Zimbabwe about the possible development of commercial citrus plantations by Chinese investors in partnership with the government of Zimbabwe, an information pack on the risks of HLB and ACP introduction with uncertified planting materials from China was provided to empower the growers as they engage with the regulators. CRI engagements also continued with contacts in other African countries including Angola, Mozambique, Tanzania, Botswana, eSwatini, Kenya, and Ethiopia in a bid to pursue collaboration and regional awareness relating to HLB and ACP.

The HLB and ACP Action Plan and HLB Safe System has undergone significant revisions to align with the new regulation on HLB and ACP that was enacted by DALRRD in February 2021 (Regulation No. 44188 of 12 Feb 2021), and CRI provided critical inputs to successive drafts of the regulation. In August/September 2020 field training was conducted for 12 DALRRD inspectors on surveys for citrus greening (HLB and African greening) and ACP in Swellendam in the Southern Cape, as a continuation of the theoretical and laboratory training provided to the same group in December 2019.

A framework was developed to draft mini PRAs of the Top 10 Biosecurity Threats. Three of these PRAs were completed and sent to the Biosecurity Advisory Committee (BAC) for inputs. The plan is to continue the process and conduct mini PRAs for the remaining seven of the Top Biosecurity Threats. In this reporting period three pest reports relating to *B. dorsalis* were sent to the IPPC, two of which were on the detection and the third eradication of the pest in the pest free areas of South Africa.

Citrus Leprosis (CL), affected farms in the Sundays River Valley, Kleinplaas, Halaron, Bellevue, and Elim East were audited for compliance to the CLRP in July 2020, and will be audited again in July 2021. The audits focussed on pruning, spray programmes, weed control, mite presence, controlled movement of people and record keeping. CRI continued its contribution to the Phyto Risk Forum, and in this reporting period three virtual meetings took place. Various issues were discussed including results of the Greening survey conducted in October 2020 in citrus Greening free buffer zone in the Western Cape in the Knysna magisterial district were reported, the reporting of *Phytophthora palmivora* on papayas in Tzaneen and Malelane in SA, as well as generation of the necessary data in responding to EU's concern relating to *Xylella fastidiosa* which culminated in a notification from the EU that South Africa is recognised as a country free from *Xylella fastidiosa*.

A follow up survey was conducted in September and October 2020 in the town of Knysna, which is part of the Greening free buffer zone in the Western Cape and 37 samples were collected. Due to the continued findings of positive trees in most neighbourhoods of Knysna, a proposal has been submitted to DALRRD to restrict the Greening free buffer zone only to the area between Keurbooms River and Nature Valley in the Knysna magisterial district. A survey will be conducted in June-August 2021 to determine if this area would be suitable to be used as a buffer zone.

## OPSOMMING

'n Konsep Biosekuriteit Meestersplan vir die sitrusbedryf is opgestel en het gedurende 2020 verskeie hersienings ondergaan, met 'n finale werksweergawe wat beplan word om in 2021 afgehandel te word. Ná die publiserings van die teenwoordigheid van HLB / Las in die kusstreke van Kenia, vroeg in 2020, het daar samesprekings met DALRRD plaasgevind. CRI het 'n inligtingsdokument opgestel met die nodige aksies om die Keniaanse vennote te betrek. DALRRD het met KEPHIS (NPPO van Kenia) gekommunikeer met voorstelle oor die volgende stappe hoe om HLB uit te roei of te bekamp.

Na aanleiding van inligting wat CGA-lede in Zimbabwe aan CRI verskaf het oor die moontlike ontwikkeling van kommersiële sitrus-aanplantings deur Chinese beleggers in vennootskap met die Zimbabwiese regering, is 'n inligtingspakket oor die risiko's van HLB en ACP introduksie deur ongesertifiseerde plantmateriaal vanuit China opgestel om produsente te bemagtig wanneer hulle met die regering in gesprek tree. Die CRI samesprekings het ook met kontakte in ander Afrika-lande, insluitend Angola, Mosambiek, Tanzanië, Botswana, eSwatini, Kenia en Ethiopië voortgegaan, in 'n poging om samewerking en streeksbewustheid rakende HLB en ACP te bewerkstellig.

Die HLB- en ACP-aksieplan en die HLB Safe System het verskeie hersienings ondergaan om in te pas by die nuwe regulasie oor HLB en ACP wat in Februarie 2021 deur DALRRD uitgevaardig is (Regulasie nr. 44188 van 12 Februarie 2021). CRI het kritieke insette op konsepte van die regulasie gelewer. In Augustus / September 2020 is veld-opleiding vir 12 DALRRD-inspekteurs oor opnames vir sitrusvergroening (HLB en Afrika-vergroening) en ACP in Swellendam in die Suid-Kaap aangebied, as 'n voortsetting van die teoretiese en laboratorium-opleiding wat in Desember 2019 aan dieselfde groep gebied is.

'n Raamwerk is ontwikkel om mini-PRA's vir die top 10 Biosekuriteitsbedreigings op te stel. Drie van hierdie PRA's is voltooi en aan die "Biosecurity Advisory Committee (BAC)" vir insette gestuur. Die plan is om voort te gaan en mini-PRA's vir die oorblywende sewe Top Biosekuriteitsbedreigings op te stel. In hierdie verslagperiode is drie verslae met betrekking tot *B. dorsalis* aan die IPBK gestuur, waarvan twee oor opsporing in 'n pesvrye gebied was en die derde oor die uitwissing van die plaag in die pesvrye gebied van Suid-Afrika.

Sitrus Leprose (CL), wat plase in die Sondagsriviervallei, Kleinplaas, Halaron, Bellevue en Elim-Oos affekteer, is in Julie 2020 geoudit vir die nakoming van die CLRP en sal in Julie 2021 weer geoudit word. Die oudits het op snoei, spuitprogramme, onkruidbestryding, teenwoordigheid van myte, beheerde beweging van mense en rekordhouding gefokus. CRI het sy bydrae tot die Phyto Risk Forum voortgesit, en in hierdie verslagperiode het drie virtuele vergaderings plaasgevind. Verskeie kwessies is bespreek, insluitend die resultate van die Vergroeningsopname wat in Oktober 2020 in die sitrusvergroeningsvrye buffersone in die Wes-Kaap in die Knysna landdrostdistrik uitgevoer is, verslagdoening oor *Phytophthora palmivora* op papajas in Tzaneen en Malelane in SA, asook die generering van die nodige data om te antwoord op die EU se kommer met betrekking tot *Xylella fastidiosa*, wat gelei het tot 'n kennisgewing van die EU dat Suid-Afrika erken word as 'n land wat vry van *Xylella fastidiosa* is.

'n Opvolgopname is in September en Oktober 2020 in Knysna, wat deel van die sitrusvergroeningsvrye buffersone in die Wes-Kaap vorm, uitgevoer en 37 monsters is versamel. Omrede positiewe bome noual in meeste woonbuurte van Knysna gevind is, is 'n voorstel by DALRRD ingedien om die vergroeningsvrye buffersone slegs tot die gebied tussen Keurboomsrivier en Nature Valley in die Knysna magistraatsdistrik te beperk. 'n Opname sal in Junie-Augustus 2021 gedoen word om vas te stel of hierdie gebied geskik is om as buffersone te dien.

## 2.2 **Develop and maintain a comprehensive citrus industry biosecurity plan - to ensure overall mitigation of the Southern African citrus industry's biosecurity risks**

### 2.2.1 Project 1. Develop a Southern Africa citrus industry biosecurity master plan

In a bid to develop a comprehensive citrus industry biosecurity master plan that would serve as a road map to guide industry in addressing biosecurity threats in the context of increasing threats from invasive pests and diseases, biosecurity strategic plans and published articles written by similar industries around the world were reviewed. A draft Master Plan appropriate for the context of the Southern African citrus industry had been written and submitted to Vaughan Hattingh for review and input. It has since undergone a few revisions, with a further revision being done currently to produce a substantially improved draft that will be circulated to the Biosecurity Advisory Committee (BAC) for input in coming months. The plan is to produce a final working version of the Master Plan after comments are received from BAC.

### **2.3 Biosecurity portfolio: Design, develop and oversee the implementation and operation of appropriate biosecurity structures, engagements, procedures, co-operations, resources, projects and other appropriate actions**

#### **2.3.1 Project 2. Identify, assess and initiate engagement with international funding providers, for future support of Southern African biosecurity projects**

Efforts were made to engage USDA-APHIS/Pretoria to fund a regional training workshop on citrus greening and its vectors at ICIPE/Kenya. Funding was secured and contract documents signed, paperwork to effect the necessary advanced payment to ICIPE was finalized in early 2020, but implementation of training could not take place due to travel restrictions related to COVID-19. The plan is to select 14-16 trainees from strategic countries in Africa with mutual interest to collaborate on surveillance of HLB and ACP. Contact has been made recently with the new APHIS attaché at the US Embassy in Pretoria, Mr Mario Ambrosino, who has stated that the Covid-19 situation has resulted in funding re-prioritization by APHIS, and thus although the desire to implement the training stands, the training may only be implemented in 2021 provided that the Covid situation and funding availability allows.

### **2.4 Networking and awareness: Obtain supportive participation of relevant stakeholders and interested parties**

Following the publication of presence of HLB/Las in coastal Kenya in early 2020, engagement took place with DALRRD including providing a briefing document by CRI to outline necessary actions to engage Kenyan partners. DALRRD subsequently communicated with KEPHIS (NPPO of Kenya) with proposals on next steps in eradicating or containing HLB. In parallel, a Cutting Edge publication (number 296) was prepared and disseminated to citrus growers within the CGA umbrella and nursery operators to highlight the discovery of Las in Kenya, and encourage their active involvement in detection surveys of HLB/Las and ACP. A virtual bilateral meeting was held between the NPPO of SA (DALRRD) and the NPPO of Kenya (KEPHIS) in September 2020, with invited participation by CRI and ICIPE to provide technical inputs in the discussion. One of the key points of the meeting was the undertaking by KEPHIS to better understand the status of HLB/Las in coastal Kenya where it was recently reported, with the aim to scope the possibility of eradication or containment. South Africa undertook to provide technical assistance that may be needed to accomplish this objective. The Kenyan counterparts have committed to provide feedback to South Africa once they complete the scoping exercise.

In light of information obtained from CGA members in Zimbabwe about the possible development of commercial citrus plantations by Chinese investors in partnership with the government of Zimbabwe, an information pack was provided by CRI Biosecurity and IPM teams to CGA partners on the risk of HLB and ACP associated with the possible importation of citrus propagation materials to Zimbabwe directly from China. The intention was to empower the Zimbabwe commercial citrus growers as they engage with the Ministry of Agriculture/ the NPPO to encourage use of propagation materials from CIS-certified nurseries in South Africa and Zimbabwe. A few meetings have since taken place between grower representatives and government officials who have expressed willingness to engage to address the concerns and have shown interest to be guided by scientific principles.

In Namibia, CRI Biosecurity was made aware of new citrus developments with ties to South Korea. Through contact with consultants and the developers it has been ascertained that all citrus planted to date originates

from South Africa, albeit not from a CIS-registered nursery. Assurance has been received that plant material for the next phase of development, Mexican Lime plantings, will be sourced from South Africa. Contact has been made with influential Namibian growers, and they have been encouraged to put pressure on the Namibian NPPO to prevent importation of citrus material from high pathogen-risk sources.

**2.5 Ensure successful implementation of processes, procedures and interactions to ensure the timely identification and assessment of biosecurity threats facing the Southern African citrus industry**

**2.5.1 Project 3. Develop and oversee implementation of Southern African citrus industry pest-specific action plans for priority biosecurity pests**

Draft mini PRAs of three of the Top 10 Biosecurity Threats and a draft PRA framework has been sent to the BAC for inputs and approval. The three pests are *Candidatus Liberibacter asiaticus* (Las), *Citrus yellow vein clearing virus* (CYVVCV) and *Xylella fastidiosa* subsp. *pauca*. Inputs and approval were received from BAC. The plan is to continue the process and conduct mini PRAs for the remaining seven Top Biosecurity Threats.

*Phytophthora palmivora*, currently listed as a quarantine pest for South Africa on several hosts including citrus, was detected in papaya samples in the Tzaneen and Malalane areas. A DALRRD/Industries meeting to discuss the way forward took place on 2 February 2021 and several workgroups were formed to draft documents needed to conduct follow up surveys. The next meeting will take place in May 2021.

In order to ensure that the import conditions for Citrus are updated, a list of all the mites occurring on citrus had been compiled. Mites had been identified that comply with the definition of a quarantine pest – *A quarantine pest is a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled (ISPM 5, FAO)*. According to the list (list of mites that comply with definition of a q-pest) the 'dangerous mites' - mites known to cause economic losses and which can result in market access problems are already listed as part of our import conditions. Four new mites, not currently part of the import conditions, were identified that might be of quarantine importance to South Africa. A request will be sent to the BAC in the next reporting period to advise on the inclusion of these four mites.

**2.6 Ensure effective implementation of processes, procedures, interactions to advance actions required to successfully mitigate the risks and consequences of biosecurity incursions**

**2.6.1 Project 4: Develop and oversee implementation of a Southern African citrus industry HLB action plan and safe tree production system**

The HLB Steering Committee continued to meet virtually in the course of the reporting period almost every three months, with the 8th meeting held virtually on March 24, 2021. The HLB and ACP Action Plan has undergone some revisions to align the plan with the new regulation on HLB and ACP that was enacted by DALRRD in February 2021 (Regulation No. 44188 of 12 Feb 2021), and CRI provided critical input to successive drafts of the regulation. Also, revisions to the action plan have been done to sections relating to detection delimitation surveys to make the plan practically feasible to implement. The HLB Safe System document has also been revised, specifically with regard to the practical operations, audit and risk assessment of the HLB Safe Nurseries. The updated Action Plan and HLB Safe System documents are being finalised by CRI, and will be presented to the HLBSC for discussion/approval at the next meeting scheduled to take place virtually in July 2021.

A follow up engagement was made with Jan Hendrik Venter of DALRRD to progress the emergency registration of insecticides in preparation for the control of Asian citrus psyllid, ACP in the event of an incursion and early detection in South Africa. Upon realising that there was no progress on this issue, SG approached CGA's Paul Hardman to write a letter to the chemical registrar of DALRRD in March 2021 highlighting the need for registration of selected chemistries suggested by CRI, so that the companies that hold the registration licences of these chemicals can come forward and apply for registration. Accordingly, the letter was sent by

CGA to the registrar as well as the input suppliers of South Africa (Crop Life) in April 2021. Progress in this regard will be reported on in the next annual report. In August/September 2020 field training was conducted for 12 DALRRD inspectors on surveys for citrus greening (HLB and African greening) and ACP in Swellendam in the Southern Cape, as a continuation of the theoretical and laboratory training provided to the same group in 2019. The Swellendam area was chosen as a suitable field training site due to the wide spread occurrence of African greening in these orchards. As part of the field training, 2 scenarios of the HLB and ACP Action Plan were also tested. Twelve DALRRD officials were trained on identification of disease symptoms and on tap sampling to find ACP. CRI appointed a biosecurity field officer as part of the 4Y plan to increase capacity to expand surveys and preparedness to respond to incursions by HLB and ACP.

#### 2.6.2 Project 5. Ensure that HLB and ACP surveillance is undertaken in Eastern and Southern Africa

During the reporting period, the following activities have taken place in various countries:

##### Angola

CRI became aware of the locations of large orchards planted with trees imported from Brazil, particularly Minas Gerais, where the nurseries are not housed in insect-secure structures. A survey visit to Angola was planned by Paul Fourie and Wayne Kirkman to assess the status of the trees and the HLB risk that they pose to southern African citrus production. Angola Alliance and Edifox were again extremely helpful in the logistical planning of the surveys, and also in providing locations and contact details of other farms with imported trees. Meetings with the Angolan Ministry of Agriculture were arranged to follow up on previous engagements and present the survey schedule, and also to debrief the Ministry of the findings.

Angola Alliance also arranged for Drs Daniel Bassimba (Instituto de Investigación Agronómica, Chianga, Huambo) and Camillo Jose (PlantCare, Luanda), both trained plant pathologists, to participate in the surveys and meetings.

Given the time constraints, all citrus trees/orchards could not be inspected. Owners/managers of farms were requested to take the survey team to diseased or problem areas on the farms. On smaller farms, orchards were surveyed in a transect pattern, while on larger farms, orchards were inspected from the back of a truck to identify possible survey points. Trees were visually inspected for signs and symptoms of citrus pests and diseases, particularly those that resemble HLB and its vectors, and other exotic diseases, such as Citrus Variegated Chlorosis, Citrus canker, Sudden Death, Leprosis Virus, and post-bloom fruit drop. Leaf and twig samples with suspect symptoms were taken for laboratory analysis at CRI in South Africa. Yellow sticky traps for insects were also placed at the various localities. These were collected approximately 3 weeks later by Nelus de Waal and sent to CRI for inspection. All plant and insect samples were transported to South Africa with the relevant permits from both countries.

Various tests were conducted on the leaf and twig samples and three laboratories participated in different aspects to confirm results. DNA/RNA was extracted from petiole and midrib tissue of leaves and various PCR and sequencing analyses conducted using approved methods (see attached reports). Following the surveys, but prior to the sample analyses, a debriefing meeting was held with the Secretary of State for Agriculture, Jose Carlos Lopes da Silva Bettencourt, and National Director of Agriculture, Antonio Sozinho. This meeting was attended by the survey team, as well as Rui Lopes of Edifox. Dr Bassimba explained the preliminary findings of the survey, highlighting the concern that we found typical HLB / Greening symptoms in some of the orchards planted with trees from Minas Gerais. To our knowledge and that of Drs Bassimba and Jose, African Greening was not yet described in Angola, and given the remote locations of some of these farms, we dreaded that finds were HLB.

The following recommendations were made to the Ministry by CRI:

1. Refrain from granting import permits for citrus trees from countries with exotic diseases.
2. Urgently compile a list of all such previous imports and conduct thorough surveys to ascertain whether any exotic pests or diseases might have entered with the trees (these surveys should be repeated annually for at least 3 years, since the symptoms might be latent).

3. Complete analyses on the 2019 survey samples, and implement control measures accordingly. It was noted that we could not be certain whether the typical symptoms were caused by HLB or African Greening.
4. Nurseries in Angola should participate in the SA-CIS to ensure that certified and disease-free citrus propagation material is used. It was suggested that a delegation of key stakeholders should visit South Africa and the SA-CIS to promote this initiative.

Mr Bettencourt agreed to write an order immediately to ban imports of citrus material from Brazil. He also requested that a project proposal be compiled for surveys of all suspect orchards in the North and South of Angola. A workshop should also be held with all relevant role players. Dr Fourie recommended that the Ministry invite the relevant experts from Fundecitrus (Sao Paulo, Brazil) to assist them with this workshop; Fundecitrus is world-renowned in their HLB management systems and importantly also speaks Portuguese. Mr Bettencourt also agreed that Citrus should be added onto the list of crops in a development programme with special government funding. This should assist in supporting biosecurity initiatives, particularly control measures where needed.

Dr Fourie pledged CRI's ongoing support for citrus biosecurity initiatives in Angola, but stressed that CRI does not have the capacity to conduct the required surveillance throughout Angola. The Ministry should invest in building the required capacity for surveillance and diagnostics. CRI could support these initiatives through technical support and training in South Africa or Angola. Dr Fourie noted that Drs Bassimba and Jose's participation in the surveys was a highlight and that they are ideally equipped to lead the survey project.

The meeting also discussed problems regarding the availability of agrochemicals. It was proposed that Angola recognises South African registrations, to which Mr Sozhino agreed. A preliminary laboratory report was provided to the survey team on 12 August 2019, which indicated that HLB was not detected, but that African Greening was detected in some samples. It was noted that the laboratory was doing some more confirmatory tests as well as tests for other pathogens. Comprehensive analyses of the samples were concluded (see attached reports), which indicated that African Greening was detected, but no HLB or CVC was detected. All ACP traps were collected and evaluated. No *D. citri* nor *T. erytrae* were detected.

Dr Fourie recently contacted the Angolan team to follow up on the development of quarantine and diagnostic services in Angola, and import requirements from HLB countries. He also welcomed the Angolan nurserymen to visit the CIS and encouraged them to procure disease-free material from the CIS. Mr José Carlos Bettencourt, the Secretary of State for Agriculture, received the message well and pledged his support to the fight against HLB and other diseases.

#### Mozambique

The first trip to Mozambique's southernmost province (Maputo province) was in May 2019. No *D. citri* or *T. erytrae* were found at any of the sites by visual inspection or tap sampling. Traps were collected one month after hanging, and were sent to CRI for evaluation. No *D. citri* and *T. erytrae* were detected on any of the traps.

The second trip to Mozambique's Inhambane province was done in March 2020. Results from leaf samples and traps collected showed no Las or ACP or *Trioza* spp. However, other *Diaphorina* species like *D. punctulata* and *D. zebrana* were identified, as well as a few other Psylloidea specimens.

Subsequently in a bid to continue monitoring, two hundred ACP traps were given to a missionary who operates in the Cabo Delgado province, right up to the Tanzanian border, where it is not possible for CRI employees to visit due to political unrest. Half the traps were to be placed after the beginning of the rainy season, at the end of January 2021, and the others at the end of February 2021. Placements of these traps has not been possible due to violent unrest in Cabo Delgado.

#### Tanzania

Initial contact has been made with researchers at Morogoro in Tanzania, to arrange collection of ACP samples twice a year, which will be tested for Las, as an indicator of the southward spread of HLB.

### South Africa

CRI has placed about 200 ACP traps since July 2020 in strategically selected frontier citrus farms in Limpopo and Mpumalanga. These traps are regularly serviced once a month and traps read in the trap reading lab at CRI. Additional ACP traps were placed in the Hoedspruit area in March 2021, and currently approximately 50 ACP traps are being received from growers in Letsitele after they were encouraged to trap during visits in March. In addition, DALRRD has deployed about 160 traps in various parts of SA, and these traps are also read at CRI, and reports submitted to DALRRD and growers on an ongoing basis. CRI is investigating the outsourcing of monitoring for growers and screening of ACP traps, as trap reading is the obvious bottleneck in the system at the moment.

### eSwatini

The major citrus producing Ngonini and Tambuti Estates send ACP traps from time to time, which are read and reports generated at CRC. The latest batch of 60 ACP traps that were collected from the Ngonini Estate from April-October 2020 showed 25 adult *Trioza* sp. No Las or ACP were found on these traps.

### Botswana

Engagement with the NPPO of Botswana and FAO office in the course of 2020 to raise awareness about HLB and ACP, as well as current activities elsewhere in Africa seems to have yielded some progress in that FAO has provided the NPPO with 100 ACP traps which the NPPO is preparing to deploy in selected commercial farms in the Tuli Block where most citrus is produced in Botswana. In parallel CRI is engaging the developers of a new citrus production in Selebi Phikwe area in Eastern Botswana. This involves 1500 ha being planted with citrus and we are engaging with the growers/investors to conduct surveillance and monitoring for HLB and ACP.

#### 2.6.3 Project 6: Facilitate initiation of an HLB eradication plan in Ethiopia

A meeting between CRI (Wayne Kirkman, Paul Fourie, S. Gebeyehu) and the contact from a commercial citrus estate in Ethiopia Mr Barry Smales that was planned for April 2020 in Port Elizabeth had to be cancelled due to COVID travel restrictions. The meeting is being planned again in Port Elizabeth sometime 2021. One of the agenda items for discussion is to explore the feasibility of eradicating HLB infected citrus orchards and replace them with clean, disease-free planting material from a CIS-certified nursery in SA. A parallel discussion is underway with managers of a nearby commercial citrus farm owned by Midroc Group. They have also expressed interest to replace old trees with planting materials possibly sourced from SA. This is positive if implemented as it is a step towards eradication of HLB in that area. Discussion is also underway with a potential consultant from SA to be contracted to provide technical assistance to Midroc management as they work towards replacing old citrus trees with new plant materials and expand area of production-including selection of rootstock and budwood possibly sourced from a nursery in SA in the CIS network.

#### 2.6.4 Project 7: Ensure in close collaboration with relevant government officials that regulations of relevance to biosecurity risk mitigation are appropriately updated and compliance effectively implemented

In this reporting period three pest reports were sent to the IPPC. Two of the reports were on detections of *B. dorsalis* (previously *B. invadens*) in pest free areas in South Africa, while the third report was to notify that *B. dorsalis* was eradicated from the pest free areas. The status of the fruit fly however remained the same – the pest is considered to be present in specified regions, actionable and under official control in South Africa.

Country specific import conditions for citrus propagation material and seeds were drafted and submitted to DALRRD. DALRRD submitted the import conditions to the specific countries. By the end of this reporting period, comments were only received from Spain. CRI provided inputs as requested by DALRRD.

Inputs were provided to the **DETAILED PROTOCOL FOR THE IMPORTATION AND POST-ENTRY QUARANTINE MANAGEMENT OF PROPAGATION MATERIAL OF CITRUS SPP (BUDWOOD, TISSUE CULTURES AND SEED)**. The final protocol was circulated to the workgroup for implementation in April 2021.

In 2020, 36 *B. dorsalis* were trapped in the Sundays River Valley. Eradication actions were successfully conducted on over 18 000 ha, and the pest was declared eradicated.

#### 2.6.5 Project 8: Monitoring and Control of Leprosis

Citrus Leprosis (CL), one of the oldest citrus diseases, is known to occur in South and Central American countries, but has not previously been reported on citrus in South Africa. CL is caused by several RNA viruses, commonly referred to as either the cytoplasmic type (CL-C), or the nuclear type (CL-N). Symptoms similar to that of CL were observed on three farms in the Addo area of the Eastern Cape Province. Molecular diagnostics were conducted by CRI Nelspruit, with initial results reported on 07, 14 and 24 May 2018. Duplicate testing was conducted by ARC Tropical and Subtropical Crops, reported on 12 May 2018. The presence of a single virus belonging to the *Dichorhavirus* genus was detected in samples from all three orchards. Limited sequence data indicated that the associated virus is a strain of *Orchid fleck virus* (OFV) with closest sequence identity to a strain previously characterised on cymbidium orchids (*Cymbidium* spp.), but not previously reported on citrus. *Brevipalpus californicus* mites, which are known vectors of some leprosis-causing viruses, were observed in all three orchards, and their identity was confirmed. This resulted in activation of precautionary actions by CRI, while further clarification of virus identity and confirmation of causative association was being sought. Clarification of identity was required to determine the official regulatory status of the virus on citrus in South Africa (e.g. quarantine pest or non-quarantine regulated pest). The discovery was reported to the Department of Agriculture, Forestry and Fisheries (DAFF) as is required, and the regulatory status of the pest is pending.

The virus persists around mite feeding sites, and mites become infected when feeding on symptomatic tissue, and transmit the virus by subsequent feeding. The virus cannot spread systemically, which increases the chances of containment and eradication of this disease.

CRI responded by forming a Citrus Leprosis Advisory Panel. This panel studied all applicable literature and through much workshopping developed the Citrus Leprosis Response Plan (CLRP) to control and contain the disease. The affected growers were made aware of actions required, focussing on removal of CL-N inoculum (symptomatic material), and the containment and control of mites, and these actions were implemented according to the CLRP. Growers were monitored and assisted with implementation of the CLRP by CRI and SRCC.

A delimitation survey procedure and survey methods were developed. These surveys were initiated to determine the spread of the disease and infected mites. New findings came to light as a result of the surveys and were confirmed by molecular diagnostics. This information was communicated to the affected growers, and the response plan was implemented. Owners of neighbouring orchards were also informed of their responsibilities according to the response plan. Surveys were continually coordinated, conducted and communicated by CRI and SRCC. All suspicious samples were collected by CRI, inspected for the presence of flat mites, dipped in an acaricide and sent to Nelspruit for molecular diagnosis. No flat mites were found in any of the affected orchards during the reporting period.

The affected farms in the Sundays River Valley, Kleinplaas, Halaron, Bellevue, and Elim East were audited for compliance to the CLRP in July 2020, and will be audited again in July 2021. The audits focussed on pruning, spray programmes, weed control, mite presence, controlled movement of people and record keeping.

Halaron complied fully with all requirements of the CLRP, and it has been recommended that the Red1 status be downgraded. The affected orchards have subsequently been destroyed. There were once again no major non-compliances on Bellevue and Elim East. Bellevue was purchased by SRCC, and the Delta and Midnight Valencia orchards where leprosis was first discovered were removed and destroyed. The remaining affected orchards, Cara Cara navels, are under good management and no new symptoms were detected. Once again the major finding at Kleinplaas was that the weeds and ground cover (Wandering Jew) had not been removed, and so the status of the farm remains Red-1. However, much pruning and destruction of affected trees has been accomplished, and significant progress has been made, with very few new symptoms.

#### 2.6.6 Project 9: Phytosanitary Risk Forum

In this reporting period three virtual meetings took place. Results of the Greening survey conducted in October 2020 in citrus free Greening buffer zone in the Western Cape in the Knysna magisterial district were reported. Inputs were provided to the document “Interceptions of pests on commodities imported into South Africa” and the document will be updated to indicate actions taken when a quarantine pest is intercepted. Procedures will also be updated to ensure that samples will be identified before the consignment is released.

The detection of *Phytophthora palmivora* was discussed as the disease was detected in papaya samples in the Tzaneen and Malalane areas. Currently *P. palmivora* is listed as a quarantine pest for South Africa on many hosts including citrus.

The EU requested South Africa to provide proof that *Xylella fastidiosa* is not present in South Africa. DALRRD requested the implicated industries to submit 5 samples at the DALRRD Diagnostic Laboratory in Stellenbosch to be tested. CRI submitted five samples which tested negative for *Xylella fastidiosa*. DALRRD received a notification from the EU that South Africa is been recognised as a country free from *Xylella fastidiosa*.

#### 2.6.7 Project 10: Greening surveys (African greening - *Candidatus Liberibacter africanus* & Asiatic greening - *Candidatus Liberibacter asiaticus*)

A follow up survey was conducted in September and October 2020 in the town of Knysna, which is part of the Greening free buffer zone in the Western Cape and 37 samples were collected. The Greening free buffer zone in the Western Cape includes two magisterial districts namely, Knysna and Uniondale. Ten of the 37 samples tested positive for African Greening. A follow up visit was planned for November but due to the COVID 19 situation, the visit was postponed. Due to the continued findings of positive trees in most neighbourhoods of Knysna, a proposal has been submitted to DALRRD to restrict the greening free buffer zone only to the following part of the Knysna magisterial district: area between Keurbooms River and Nature Valley. A survey will be conducted to determine how many citrus trees are in that specific area, and if it is suitable to be used as a buffer zone.

#### 2.6.8 Project 11: Citrus Free Zone (5 km) outside the Citrus Foundation Block (CFB) in the Eastern Cape Province in the magisterial district of Uitenhage

In January 2011 legislation was published which prohibited the keeping, cultivation and planting of specific plants, including *Citrus* species, in the area of 5 km radius outside the citrus foundation block (CFB) in the Eastern Cape province in the magisterial district of Uitenhage. Surveys were conducted in 2011 to identify farms with citrus trees and orders were given to all the implicated owners. Most of the trees were removed but one of the owners refused to remove the trees despite an order issued and follow up visits by SA-DAFF. In November 2018 SA-DAFF reported the case to SAPS in Uitenhage. In January 2019 the SAPS indicated that the case was referred to the Senior Prosecutor for a decision. In November 2019 further information was provided to the Senior Prosecutor on request. In November 2020, the Public Prosecutor informed DALRRD that Mr Harbron has provided written consent for the trees on Vrede Farm to be removed. The trees were removed by DALRRD and a team from the CFB in December 2020.

### 3 PORTFOLIO: INTEGRATED PEST MANAGEMENT

#### 3.1 PORTFOLIO SUMMARY

By Sean D Moore (Portfolio Manager: IPM, CRI)

The Integrated Pest Management (IPM) Portfolio, and indeed CRI as a whole, did not escape the effect of the prevailing theme underscoring the last year, being the Covid-19 pandemic. As the past research cycle (year) began, Level 5 lockdown was imposed, causing us all to reschedule our programmes and manage our time a lot more carefully, particularly to avoid unnecessary exposure to one another. Fortunately, this did not dramatically affect activity within our research portfolio, testimony to the commitment and resourcefulness of

the IPM research team. Furthermore, within a few weeks, the government officially declared us an essential service.

The IPM Portfolio consists of three programmes: False Codling Moth (FCM), Fruit Flies and Other Pests. These are coordinated respectively by Sean Moore, Aruna Manrakhan and Tim Grout. At least every second year, CRI's extension team solicit's the citrus growers' opinions on their research needs and priorities, a process that is conducted region by region. This is combined with CRI's knowledge of market access forces and impending biosecurity threats. Research proposals are finally vetted by specialist research committees with strong CGA and citrus technical representation. Through this process, the top ranked research priority has remained FCM, closely followed by fruit flies, thus warranting full research programmes dedicated to each of these pests or pest groupings. Also ranked highly were thrips and mealybugs. The ranking of biosecurity increased, due to the now well heralded impending threat of the arrival of the Asian citrus psyllid and the devastating bacterial disease (Asian greening or HLB) that it vectors. Residue issues were also widely recognised as a significant market access hurdle, giving impetus to the call for and pursuit of biological control alternatives.

The FCM research portfolio included 18 research projects. Fourteen of them addressed issues pertaining to preharvest management and the remaining four addressed postharvest management issues. Although FCM levels in the field are significantly reduced relative to previous years, due to improved management application, the phytosanitary status of FCM for most export markets, drives most of our research focus. There are several highlights in this research programme from the last year. A two year project, monitoring FCM activity in Limpopo Province concluded that temperatures begin increasing any time from early July, with an associated escalation in FCM activity. As a result, another study is being initiated to investigate the efficacy of a mating disruption programme initiated much earlier than traditionally recommended. Monitoring of FCM levels on organic and conventional farms and the reasons for differences recorded, concluded that there were higher levels of soil-dwelling predators in organic orchards, and significantly higher levels of magnesium and calcium in conventional fruit, which could be contributing to the difference in pest levels. Some interesting breakthroughs in achieving improved efficacy with the FCM granulovirus were the discovery of synergism between the virus and yeasts extracted from the insect gut, and synergism between the granulovirus and the nucleopolyhedrovirus from litchi moth. A four-year postharvest detection project came to an end, identifying volatile emissions reliably indicative of FCM infestation. The most promising technology identified was Selected Ion Flow Technology (SIFT) MS, and consequently, a new study has been initiated to explore this further.

The aim of the fruit fly research programme is to collate information on the biology, ecology and control of fruit fly pests in order to effectively mitigate the risk of these pests in citrus from southern Africa. There is a zero tolerance of fruit fly in export citrus. A total of eight research projects were conducted within the programme and as with the FCM programme, so too are there several highlights within the fruit fly programme. The efficacy of several attract and kill products was tested, demonstrating that they all effectively suppressed fruit fly populations. In a postharvest cold treatment trial, Medfly was shown to be the most cold-tolerant of all citrus associated species. Consequently, all cold treatments for Medfly should be equally effective against the other species. Furthermore, the efficacy of a 3.5°C cold treatment was determined for all species.

The Other pests programme covers a broad range of pests, including those considered as key IPM pests, biosecurity pests, potential phytosanitary pests and minor and sporadic pests. Within this programme, a total of 11 research projects were conducted during the last year. A two-year survey for oleander scale in commercial citrus orchards in different production regions was completed, showing that this species is not in citrus orchards in South Africa. Several research projects are being conducted on pest dynamics and IPM under nets, revealing results that are sometimes variable between sites but mealybug and red scale populations appear to increase under the nets while damage from thrips under nets often declines. Work is also being conducted on *Aphytis melinus* augmentation for the control of red scale and *Anagyrus vladimiri* augmentation for control of mealybug, but these projects are still ongoing and results are not yet conclusive. A study is also being conducted in Kenya on the control of the Asian citrus psyllid, using systemic products, but progress has been delayed, in part due to the pandemic.

During the past year, a number of these studies within the portfolio were conducted in collaboration with other research entities, including long-standing relationships such as those with Rhodes University, Pretoria University and Stellenbosch University, and some newer collaborations, such as the Agricultural Research Council, ICIPE (Kenya), InsecTech and the University of KwaZulu-Natal. The IPM team is gearing up for the increased levy funding during the 2021-22 financial year, including forming new research partnerships.

Unfortunately the pandemic prevented the occurrence of much in-person technology transfer. However, the IPM team did participate in several technology transfer sessions on virtual platforms, both with growers and with various roleplayers in the Agchem industry. The researchers within the team also continued to be productive in publishing several articles in peer-reviewed scientific publications, the SA Fruit Journal and the Cutting Edge.

We look forward to an extremely productive and exciting research year ahead. The significantly increased grower levy will allow us to expand our research focus beyond what was previously possible and hopefully expedite solutions to a range of problems, particularly those of a market access nature.

## **PORTEFEULJE OPSOMMING**

Die Geïntegreerde Plaag Beheer (IPM) –portefeulje, en inderdaad CRI in sy geheel, het nie die effek van die heersende tema wat die afgelope jaar ontkom nie, naamlik die Covid-19 pandemie. Soos die vorige navorsingsiklus (jaar) begin het, is Vlak 5 inperkings ingestel, wat veroorsaak het dat ons almal ons programme moes herskeduleer en ons tyd versigtig bestuur, veral om onnodige blootstelling aan mekaar te voorkom. Gelukkig het dit nie die aktiwiteit in ons navorsingsportefeulje dramaties beïnvloed nie, wat getuig van die toewyding en vindingrykheid van die IPM-navorsingspan. Verder het die regering ons binne 'n paar weke tot 'n noodsaaklike diens verklaar.

Die IPM portefeulje bestaan uit drie programme: Valskodlingmot (VKM), Vrugtevlieë en Ander Plae. Hierdie word onderskeidelik deur Sean Moore, Aruna Manrakhan en Tim Grout gekoördineer. Ten minste elke tweede jaar vra CRI se uitbreidingspan die sitrus kwekers se opinies oor hul navorsingsbehoefte en prioriteite, 'n proses wat streek vir streek uitgevoer word. Dit word met CRI se kennis van marktoegankragte en dreigende biosekuriteit bedreigings gekombineer. Navorsingsvoorstelle word uiteindelik deur spesialis navorsingskomitees met sterk CGA- en sitrus tegniese verteenwoordiging gekeur. Deur hierdie proses het VKM die hoogste navorsing prioriteit gebly, nou gevolg deur vrugtevlieë, wat volledige navorsingsprogramme toegewy aan elk van hierdie plae of plaaggroeperings regverdig. Blaaspootjie en witluis is ook hoog ingedeel. Die rangorde van biosekuriteit het toegeneem as gevolg van die aangekondigde dreigende gevaar van die koms van die Asiatiese sitrus bladvlooi en die verwoestende bakteriese siekte (Asiatiese vergroening of HLB) wat dit oordra. Residu kwessies word ook algemeen erken as 'n betekenisvolle hindernis vir marktoegang, wat stukrag gee aan die oproep na en strewe na biologiese beheer alternatiewe.

Die VKM navorsingsportefeulje het 18 navorsingsprojekte ingesluit. Veertien van hulle het kwessies rakende voor-oesbestuur aangespreek en die oorblywende vier het na-oes bestuur kwessies geadresseer. Alhoewel VKM vlakke in die veld aansienlik laer is in vergelyking met vorige jare, as gevolg van verbeterde bestuurtoepassing, dryf die fitosanitêre status van VKM vir die meeste uitvoermarkte steeds die grootste deel van ons navorsingsfokus. Daar is verskeie hoogtepunte in hierdie navorsingsprogram van die afgelope jaar. 'n Twee jaar projek wat VKM aktiwiteit in die Limpopo Provinsie moniteer, het tot die gevolgtrekking gekom dat temperature begin toeneem enige tyd vanaf vroeg in Julie, met 'n gepaardgaande toename in VKM aktiwiteit. As gevolg hiervan word 'n ander studie begin om die doeltreffendheid van 'n paringsontwrigting program te ondersoek wat baie vroeër begin as wat tradisioneel aanbeveel word. Monitering van VKM vlakke op organiese en konvensionele plase en die redes vir verskille wat aangeteken is, het tot die gevolgtrekking gekom dat daar hoër vlakke van roofdiere in die grond in organiese boorde was, en aansienlik hoër vlakke van magnesium en kalsium in konvensionele vrugte, wat tot die verskil in plaagvlekke kon bydra. 'n Paar interessante deurbreke in die bereiking van verbeterde doeltreffendheid met die VKM granulovirus was die ontdekking van sinergisme tussen die virus en giste wat uit die insek maag onttrek is, en sinergisme tussen die granulovirus en die nukleopolihidrovirus van lietsjie mot. 'n Vier jaar na-oes opsporingsprojek het tot 'n einde gekom, wat vlugtige emissies identifiseer wat betroubaar dui op VKM besmetting. Die belowende tegnologie wat geïdentifiseer is

was die Geslekteerde loon Vloei Tegnologie (SIFT) MS, en gevolglik is 'n nuwe studie begin om dit verder te ondersoek.

Die doel van die vrugtevlieg navorsingsprogram is om inligting oor die biologie, ekologie en beheer van vrugtevlieë plaë te versamel om die risiko van sitrus uit suidelike Afrika effektief te verminder. Daar is geen toleransie vir vrugtevlieg in uitvoer sitrus nie. Altesaam agt navorsingsprojekte is binne die program uitgevoer en soos met die VKM-program, is daar ook verskeie hoogtepunte in die vrugtevliegprogram. Die doeltreffendheid van verskeie lok- en doodmaakprodukte is getoets, wat getoon het dat hulle almal vrugtevliegpopulasies effektief onderdruk. In 'n na-oes kouebehandelingsproef is Medvlieg aangewys as die mees koue verdraagsame vrugtevlieg van alle sitrus geassosieerde spesies. Gevolglik moet alle koue behandelings vir Medvlieg ewe effektief wees teen ander spesies. Verder is die doeltreffendheid van 'n 3.5°C koue behandeling vir alle spesies bepaal.

Die Ander Plaë programme dek 'n wye verskeidenheid plaë, insluitend die wat beskou word as die belangrikste plaë vir IPM, biosekuriteit, moontlike fitosanitêre plaë en minder belangrike en sporadiese plaë. Binne hierdie program is daar die afgelope jaar altesaam 11 navorsingsprojekte gedoen. 'n Twee jaar opname vir oleander dopluis in kommersieële sitrusboorde in verskillende produksiestreke is voltooi, wat toon dat hierdie spesie nie in sitrus boorde in Suid-Afrika voorkom nie. Verskeie navorsingsprojekte word uitgevoer oor plaëdinamika en IPM onder nette, wat resultate onthul wat soms wisselend is tussen persele, maar die populasies van witluis en rooidopluis neem toe onder nette, terwyl skade deur blaaspootjies onder nette dikwels afneem. Daar word ook gewerk aan *Aphytis melinus* loslatings vir die beheer van rooidopluis en *Anagyrus vladimiri* loslatings vir beheer van witluis, maar hierdie projekte duur nog voort en die resultate is nog nie deurslaggewend nie. 'n Studie word ook in Kenia gedoen oor die beheer van die Asiatiese sitrus bladvlooi met behulp van sistemiese produkte, maar die vordering is vertraag, deels as gevolg van die pandemie.

Gedurende die afgelope jaar is 'n aantal van hierdie studies in die portefeulje uitgevoer in samewerking met ander navorsing entiteite, insluitende jarelange verhoudings soos die met Rhodes Universiteit, die Universiteit van Pretoria en Stellenbosch Universiteit, en sommige nuwer samewerkings, soos die Landbounavorsingsraad, ICIPE (Kenia), InsecTech en die Universiteit van KwaZulu-Natal. Die IPM-span maak gereed vir die verhoogde heffing in bevonding gedurende die boekjaar 2021-22, insluitend die vorming van nuwe navorsingsvennootskappe.

Ongelukkig het die pandemie die voorkoms van baie in-persoon oordrag van tegnologie voorkom. Die IPM-span het wel aan verskeie tegnologie oordrag sessies op virtuele platforms deelgeneem, beide met produsente en met verskillende rolspelers in die Agchem industrie. Die navorsers in die span het ook voortgegaan om produktief te wees in die publikasie van verskeie artikels in wetenskaplike eweknieë-hersiende jurnale, die SA Vrugte Joernaal en die Snykant.

Ons sien uit na 'n uiters produktiewe en opwindende navorsingsjaar wat voorlê, Die aansienlike verhoogde produsenteheffing sal ons in staat stel om ons navorsingsfokus verder uit te brei as wat voorheen moontlik was, en hopelik oplossings vir 'n reeks probleme te bespoedig, veral die wat marktoegang van natuur is.

## 3.2 **PROGRAMME: FALSE CODLING MOTH**

Programme coordinator: Sean D Moore (CRI)

### 3.2.1 **Programme summary**

False codling moth (FCM) remains not only the most important phytosanitary pest for the southern African citrus industry, but consequently, as the top priority pest for research, due to the ongoing importance of retaining market access. Although the management of FCM both in the field and postharvest (both in the packhouse and during shipping) has improved dramatically over the last few years, as seen by the generally low levels of pest presence and infestation, its phytosanitary status for most markets means that it is likely to remain a research priority for some time to come. Consequently, in the last research cycle, 18 research projects were registered on FCM. Fourteen of them addressed issues pertaining to preharvest management and the remaining four addressed postharvest management issues.

Of the projects that investigated preharvest management issues, four focussed on various aspects of pest monitoring. The first aimed at the identification of a female attractant, as an alternative to the currently available pheromone tool for monitoring male moths (3.2.3). The project has moved from a field cage setup to a laboratory-based wind tunnel. No significant attractant has been identified yet. Another project had a similar objective but is taking a different approach, which is to isolate and characterise the pheromones emitted from the three androconiae known to occur in male moths and known to be involved in the mating process (3.2.12). Due to lockdown, no progress has been possible yet on this project. The objective of the third monitoring project was to track the phenology of FCM activity and temperature patterns in citrus orchards in Limpopo over a two year period (3.2.5). It was determined that temperatures begin increasing any time from early July, with an associated escalation in FCM activity. As a result, another study is being initiated to investigate the efficacy of a mating disruption programme initiated at this time, rather than only at the traditional timing of October. The last of the monitoring-type projects is exploring the reasons for lower FCM activity on organic farms than on conventional farms (3.2.17). Certain consistent and significant differences have been recorded, such as higher levels of staphylinid predators in the soil in organic orchards, differences in entomopathogenic fungal species dominance and higher levels of magnesium in conventional fruit.

Two of the preharvest projects had the aim of better understanding mating disruption. The first was to investigate the the rate of pheromone release from the four different mating disruption products and the influence of temperature (3.2.14). Unfortunately, no progress was possible, due to lockdown. However, this is proceeding in 2021. Once this has been done, the optimal density of dispensers in an orchard will be determined. The second mating disruption project is comparing the efficacy of mating disruption under nets with that in open orchards (3.2.16). More wild moths were caught in Checkmate-treated blocks under the nets than in open orchards, which was not the case with Isomate. However, moth activity was generally higher under nets, even in control orchards. Unfortunately, fruit infestation levels were negligible.

Another project is investigating a combination of mating disruption with the sterile insect technique (SIT) (3.2.15). When mating disruption was added to an SIT programme, moth catches were reduced by 75%. Mating disruption on its own reduced moth catches by 90-95% relative to untreated orchards. Unfortunately, FCM levels were very low and no fruit infestation has been detected yet. A second SIT-focussed project investigated potential sterility challenges with irradiated moths (3.2.6). It was found that if moths were allowed to mate before irradiation, then the sterilising effect of the irradiation was compromised.

Three projects focussed on improving the efficacy of baculovirus biopesticides. The first investigated baculovirus-yeast synergism, finding a significant synergistic effect between CrleGV and certain yeast species, thus improving virulence against FCM (3.2.2). Additionally, these yeasts proved to be female attractants. The yeast-virus combinations were tested in semi-field trials. However, the trial protocol needs to be improved, after which the trials will be repeated. The second baculovirus project investigated two unrelated factors, but both aiming at improving virulence of the novel litchi moth virus, CrpeNPV (3.2.4). The first component was repeated passage of CrpeNPV through FCM, considered a heterologous host, to try and naturally select for the genotypes in the isolate that are most virulent to FCM. There was no improvement in virulence, nor any genetic changes in the isolate, indicating that the virulence of the isolate is already optimal for FCM. The second component investigated synergism between CrpeNPV and CrleGV against FCM. The combination of the viruses resulted in a significant improvement in virulence but did not improve speed of kill. The final baculovirus project is a continuation of a previous project that successfully selected for a UV-resistant isolate of CrleGV (3.2.19). The objective of this project is to determine if the genetic integrity of the UV-resistant strain can be maintained through *in vivo* bulking up of the virus and to test the efficacy of the virus in the field.

The final two preharvest projects addressed other aspects of FCM control. The first had the general objective of conducting field trials (3.2.18). In this project, semi-field bioassays were conducted with a range of biological options. Results were unconvincing, due to a need to improve the protocol used. In another trial, the addition of molasses to late season sprays for FCM, was investigated as a means to overcome the challenge in achieving adequate spray coverage of dense trees. Unfortunately, results were again inconclusive. The final preharvest project investigated synergism between various active ingredients for improved control of FCM (3.2.11). Promising results were recorded between Neem and CrleGV.

Of the postharvest projects, two looked at postharvest treatments and two investigated postharvest detection technologies. The first postharvest treatment trial was the now long ongoing project on development of cold treatments, in which a range of trials were conducted (3.2.8). Results included extending the duration of exposure to 4°C to a point where 100% mortality was recorded, being 28 days; and identifying larval characteristics after exposure to cold treatment, which could reliably indicate whether larvae were likely to die or survive thereafter. The other postharvest treatment project evaluated the efficacy of vapour heat treatments and found that 6 h exposure to 45°C or 47°C, caused a mean corrected mortality of 83.9% and 88.0% in fifth instars (3.2.9).

The first postharvest detection project is a now completed one on identification of volatile emissions associated with FCM infestation in citrus fruit (3.2.7). The project could have terminated the previous year, but was retained in order to collaborate with an Italian company on X-ray tomography. Unfortunately, because of lockdown, this could happen. Nonetheless, promising volatile results were obtained over a four-year period and investigation of these will be continued in a new project used Selected Ion Flow Technology (SIFT) MS in collaboration with the University of Leuven in Belgium. The final postharvest detection project aims to use the antennal response of the FCM larval parasitoid, *Agathis bishopi*, to identify key volatiles indicative of FCM fruit infestation (3.2.13). Progress on this project was not possible, due to lockdown, but is now progressing.

## Programopsomming

Valskodlingmot (VKM) bly nie net een van die mees belangrike fitosanitêre plaë vir die Suid-Afrikaanse sitrus bedryf nie, maar gevolglik, die hoogste prioriteitsplaag vir navorsing, as gevolg van die aangaande belangrikheid van die behoud van marktoegang. Alhoewel die beheer van VKM beide in die veld en na-oes (beide in die pakhuis en gedurende verskeping) dramaties verbeter het oor die laaste paar jaar, soos gesien deur die algemene lae vlakke van plaag teenwoordigheid en besmetting, beteken die fitosanitêre status vir meeste marke dat dit waarskynlik 'n navorsingsprioriteit gaan bly vir nog 'n geruime tyd. Gevolglik in die laaste navorsingsiklus is 18 navorsingsprojekte op VKM geregistreer. Veertien van die projekte adresseer kwessies rakende vooroes beheer en die oorblywende vier adresseer na-oes beheer kwessies.

Van die projekte wat vooroes beheer kwessies ondersoek, het vier op verskeie aspekte van plaag monitering gefokus. Die eerste poog om 'n wyfie lokmiddel te identifiseer, as 'n alternatief tot die huidige beskikbare feromoon stelsel vir die monitering van mannetjie motte (3.2.3). Die projek het beweeg vanaf 'n veld hok opstelling na 'n laboratorium gebaseerde wind-tonnel. Geen beduidende lokmiddel is nog geïdentifiseer nie. Nog 'n projek het 'n soortgelyke doel maar neem 'n ander benadering, dit is om die feromone wat uit die drie androkonië wat in mannetjie motte voorkom, en wat bekend is om in paring betrokke te wees, te isoleer en karakteriseer (3.2.12). As gevolg van die inperkings is nog geen vordering met hierdie projek moontlik nie. Die doel van die derde moniteringsprojek was om die fenologie van VKM aktiwiteit en temperatuur patrone in sitrus boorde in Limpopo oor 'n twee jaar periode na te volg (3.2.5). Daar is vasgestel dat temperature enige tyd vanaf vroeg Julie begin toeneem, met 'n geassosieerde verhoging in VKM aktiwiteit. As gevolg daarvan word nog 'n studie geloods om die doeltreffendheid van 'n paringsontwrigting program te ondersoek wat gedurende hierdie tyd geloods is, eerder as die tradisionele tyd van Oktober. Die laaste van die moniterings tipe projekte ondersoek die redes vir laer VKM aktiwiteite op organiese plase teenoor konvensionele plase (3.2.17). Verskeie bestendige en betekenisvolle verskille is aangeteken, soos hoër vlakke Staphylinidae roofdiere in die grond in organiese boorde, verskille in entomopatogeniese swam spesie oorheersing en hoër vlakke magnesium in konvensionele vrugte.

Twee van die vooroes projekte het gepoog om paringsontwrigting beter te verstaan. Die eerste was om die tempo van feromone losgelaat vanaf die vier verskillende paringsontwrigting produkte te ondersoek asook die effek van temperatuur (3.2.14). Ongelukkig was geen vordering moontlik nie as gevolg van die inperking. Dit werk gaan egter voort in 2021. Sodra dit voltooi is, gaan die optimale digtheid van die vrystellers in 'n boord bepaal word. Die tweede paringsontwrigting projek vergelyk die doeltreffendheid van paringsontwrigting onder nette met die in oop boorde (3.2.16). Meer wilde motte is in Checkmate behandelde blokke onder die nette gevang as in oop boorde, wat nie die geval was met Isomate nie. Alhoewel mot aktiwiteit oor die algemeen hoër onder nette was, selfs in kontrole boorde. Ongelukkig was vrug besmetting weglaatbaar min.

Nog 'n projek ondersoek 'n kombinasie van paringsontwrigting met die steriele insek tegniek (SIT) (3.2.15). Wanneer paringsontwrigting tot 'n SIT program gevoeg is, is mot vangste met 75% verminder. Paringsontwrigting op sy eie het mot vangste verlaag met 90-95% relatief tot die onbehandelde boorde. Ongelukkig was VKM vlakke baie laag en geen vrug besmetting is nog bespeur nie. 'n Tweede SIT-gefokusde projek het die potensiele steriliteit uitdagings met bestraalde motte ondersoek (3.2.6). Daar is gevind dat wanneer motte toegelaat word om voor bestraling te paar, die sterilisering effek van die bestraling in gedrang gebring word.

Drie projekte fokus om die doeltreffendheid van die bakulovirus bioplaagdoders te verbeter. Die eerste het bakulovirus-gis sinergisme ondersoek, en het betekenisvolle sinergistiese effekte tussen CrleGV en sekere gis spesies gevind, en so die virulensie teen VKM verbeter (3.2.2). Hierdie giste blyk ook wyfie lokmiddels te wees. Die gis-virus kombinasie is getoets in semi-veld biotoetse. Die proef protokol moet egter verbeter word, waarna die proewe herhaal sal word. Die tweede bakulovirus projek het twee onverwante faktore ondersoek, maar beide poog om die virulensie van die nuwe lietsjiemot virus, CrpeNPV, te verbeter (3.2.4). Die eerste komponent was die herhaalde passering van CrpeNPV deur VKM, beskou as 'n heteroloë gasheer, om te probeer om die genotipes in die isolaat wat die mees virulent is teen VKM natuurlik te probeer selekteer. Daar was geen verbetering in die virulensie nie, asook geen genetiese veranderinge in die isolaat nie, wat aandui dat die virulensie van die isolaat reeds optimaal is vir VKM. Die tweede komponent het die sinergisme tussen CrpeNPV en CrleGV teen VKM ondersoek. Die kombinasie van die virusse het in 'n betekenisvolle verbetering in die virulensie gelei, maar het nie die doodmaak tempo verbeter nie. Die finale bakulovirus projek is 'n voortdoring van 'n vorige projek wat suksesvol 'n UV-bestande isolaat van CrleGV geselekteer het (3.2.19). Die doel van die projek is om te bepaal of die genetiese integriteit van die UV-bestande stam volhou kan word deur *in vivo* opbouing van die virus en om die doeltreffendheid van die virus in die veld te toets.

Die finale twee vooroes projekte adresseer ander aspekte van VKM beheer. Die eerste het die algemene doel om veldproewe uit te voer (3.2.18). In hierdie projek, is semi-veld biotoetse uitgevoer met 'n reeks biologiese opsies. Resultate was onoortuigend, weens 'n behoefte om die huidige protokol te verbeter. In 'n ander proef is die byvoeging van melasse tot die laatseisoen bespuitings vir VKM ondersoek as 'n manier om die uitdaging om voldoende spuitbedekking van digte bome te bewerkstellig, te oorkom. Ongelukkig was die resultate weer onoortuigend. Die finale voor oes projek het die sinergisme tussen verskeie aktiewe bestanddele vir verbeterde beheer van VKM ondersoek (3.2.11). Belowende resultate is tussen Neem en CrleGV aangeteken.

Van die na-oes projekte het twee gekyk na na-oes behandelinge en twee het na-oes opsporing tegnologieë ondersoek. Die eerste na-oes behandeling proef was die nou lang volgehoue projek vir die ontwikkeling van koue behandelinge, waarin 'n reeks proewe uitgevoer is (3.2.8). Resultate sluit die verlenging van die blootstellingstydperk van 4°C tot 'n punt waar 100% mortaliteit aangeteken is, dit is 28 dae; en om die larvale eienskappe te identifiseer na blootstelling aan koue behandeling, wat betroubaar kan aandui of larwes waarskynlik daarna sal sterf of oorleef. Die ander na-oes behandeling projek evalueer die doeltreffendheid van damphittebehandelings en het gevind dat 6 uur blootstellings aan 45°C of 47°C, gelei het tot 'n gemiddelde gekorrigeerde sterftesyfer van 83.9% en 88.0% in vyfde instars (3.2.9).

Die eerste na-oes opsporings projek is 'n voltooide projek oor die identifikasie van vlugtige vrystellings geassosieer met VKM besmetting in sitrus vrugte (3.2.7). Hierdie projek kon die vorige jaar beëindig word, maar is aangehou om saam met 'n Italiaanse maatskappy te werk aan X-straal tomografie. Ongelukkig kon dit nie gebeur nie as gevolg van die inperkings. Nietemin is belowende vlugtige resultate verkry oor 'n vier jaar periode en gaan verder ondersoek word in 'n nuwe projek deur gebruik van Geselekteerde loon Vloei Tegnologie (SIFT) MS in samewerking met die Universiteit van Leuven in België. Die finale na-oes opsporing projek poog daarin om die antennale reaksie van die VKM parasitoïed, *Agathis bishopi*, te gebruik om die sleutel vlugtige stowwe te identifiseer wat dui op VKM besmetting (3.2.13). Vordering op hierdie projek was nie moontlik nie as gevolg van die inperking, maar vorder nou.

### **3.2.2 FINAL REPORT: Yeast-baculovirus synergism: Investigating mixed infections for improved management of the false codling moth, *Thaumatotibia leucotreta***

## Summary

*Thaumatotibia leucotreta* is a phytophagous insect endemic to southern Africa. The baculovirus *Cryptophlebia leucotreta granulovirus* (CrLeGV-SA) forms an integral component of an IPM programme for control of *T. leucotreta* and is highly effective. Due to the mutualistic association identified between *Cydia pomonella* and epiphytic yeasts, resulting in a significant increase in larval mortality, we proposed to determine which yeast species occur naturally in the gut of *T. leucotreta* larvae and to examine whether the isolated yeasts in combinations with CrLeGV-SA, enhance its effectiveness. Infested Navel oranges were collected from orchards across South Africa. This led to the isolation and identification of six yeast species via PCR amplification and sequencing of ITS region and D1/D2 domain of the LSU. Larval development and attraction assays were conducted with the isolated yeast species, which were shown to accelerate larval development, reduce mortality and attract neonate *T. leucotreta* for feeding. Oviposition preference assays were conducted with adult *T. leucotreta* females on fruit inoculated with and without yeast. Significantly, more eggs were deposited on yeast-inoculated fruit in two-choice tests. Detached fruit bioassays were then performed to determine the optimal yeast:virus ratio and to further enhance yeast/virus formulation through the addition of an adjuvant and surfactant. The optimal yeast concentration to use alongside CrLeGV-SA was determined and the inclusion of an adjuvant and surfactant to the formulation greatly enhanced its efficacy. Semi-field trials were initiated with promising preliminary results being obtained. The results obtained provide a platform for further research into the application of a yeast/virus treatment as a novel control and monitoring option for *T. leucotreta*.

## Opsomming

*Thaumatotibia leucotreta* is 'n fitofagiese insek wat endemies is aan Suid-Afrika. Die bakulovirus *Cryptophlebia leucotreta granulovirus* (CrLeGV-SA) vorm 'n integrale onderdeel van 'n IPM-program vir die beheer van *T. leucotreta* en is baie effektief. As gevolg van die mutualistiese verband wat tussen *Cydia pomonella* en epifitiese giste geïdentifiseer is, wat 'n beduidende toename in larwemortaliteit tot gevolg gehad het, het ons voorgestel om vas te stel watter gisspesies natuurlik in die derms van *T. leucotreta*-larwes voorkom en om te ondersoek of die geïsoleerde giste in kombinasies met CrLeGV-SA, die doeltreffendheid daarvan verbeter. Besmette Navel-lemoene is versamel uit boorde regoor Suid-Afrika. Dit het gelei tot die isolasie en identifikasie van ses gisspesies via PCR-versterking en volgorde van die ITS-streek en D1/D2-domein van die LSU. Larwe-ontwikkeling en aantrekkingstoetse is uitgevoer met die geïsoleerde gisspesies, wat getoon het dat dit larwe-ontwikkeling versnel word, mortaliteit verminder word en die pasuitgeboreide *T. leucotreta* lok vir voeding. Eierleggings-voorkeuroetse is uitgevoer met volwasse *T. leucotreta*-wyfies op vrugte wat met en sonder gis geënt is. Betekenisvol meer eiers is op gis-ingeënte vrugte gelê in tweekeuse-toetse. Afgeskeide vrugte-bioetse is daarna uitgevoer om die optimale gis:virus-verhouding te bepaal en om gis/virusformulering verder te verbeter deur die toevoeging van 'n byvoegmiddel en benatter. Die optimale giskonsentrasie om saam met CrLeGV-SA te gebruik, is bepaal en die insluiting van 'n byvoegmiddel en benatter in die formulering het die doeltreffendheid daarvan verbeter. Semi-veldproewe is begin met belowende voorlopige resultate. Die behaalde resultate bied 'n platform vir verdere ondersoek na die toepassing van 'n gis/virusbehandeling as 'n nuwe beheer- en moniteringsopsie vir *T. leucotreta*.

## Introduction

The agricultural sector in South Africa is of great importance as it contributes significantly to the country's gross domestic product (GDP). The horticulture sector's total gross value in 2017/18 was around 78 billion ZAR (5.3 billion US dollars). The citrus industry was the largest contributor, with 19 billion ZAR (1.3 billion US dollars) and employed more than 100 000 people (DAFF, 2019). The majority of the citrus industry's total income comes from exports, nearly 92 %, with a relatively small amount coming from local markets (CGA, 2019). The economic value of the citrus industry in South Africa is clear, and thus the industry must be protected.

Some of the main risks that the industry has identified include plant diseases and the loss of citrus fruit due to damage caused by insect pests. There are a large number of insect pests including locusts, aphids, scale

insects, thrips, flies, ants, beetles and moths that affect the citrus industry in South Africa (Moore et al., 2008). One of the most detrimental pests is *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), or otherwise commonly known as the false codling moth (FCM). It is a major pest of citrus, which can cause substantial fruit damage throughout larval feeding (Mkiga et al., 2019). When neonate larva bore into the fruit, it results in the rind's discolouration, and these penetrations sites are also subject to secondary infection by other microbial agents (Newton, 1998). The fruit's physiological state is also altered by larval penetration resulting in premature ripening, which leads to abscission (Newton, 1998). When larvae exit the fruit, they leave behind a distinctive trail of excrement or "frass" as it is commonly referred to. This affects the fruit's marketability, resulting in significant financial loss each year (Moore, 2002). Additionally, and most importantly, *T. leucotreta* is classified as a phytosanitary pest in regions to which South Africa exports, such as the European Union (EU) and the United States of America (USA). *Thaumatotibia leucotreta* is endemic to Sub-Saharan Africa and poses a serious risk to South African citrus exports (Gilligan et al., 2011). The detection of a single larva within fruit destined to be exported can result in an entire shipment being rejected (Moore, 2002).

An integrated pest management programme has been implemented to control *T. leucotreta*. The baculovirus *Cryptophlebia leucotreta* granulovirus (CrleGV-SA) forms one component of this programme and is highly effective (Moore, 2019). It has been formulated into three commercially available products, viz. Cryptogran™, Cryptex® and Gratham® (Moore, 2021). A mutualistic association between *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) and yeasts belonging to the genus *Metschnikowia* has previously been demonstrated (Witzgall et al., 2012). Larval feeding galleries inoculated with *M. andauensis*, reduced larval mortality and enhanced larval development. Additionally, adult *C. pomonella* female oviposition preference was also shown to be influenced by the volatiles produced by *M. andauensis*. This mutualistic relationship was manipulated for biological control purposes, by combining *M. pulcherrima* with the baculovirus *Cydia pomonella* granulovirus (CpGV) Knight and Witzgall (2013). The combination of *M. pulcherrima* with brown cane sugar and CpGV in laboratory assays and field trials resulted in a significant increase in larval mortality. A similar observation was made when *M. pulcherrima* was substituted for *Saccharomyces cerevisiae* (Knight et al., 2015). This indicates that yeasts harbour the potential for use in biological control, especially when combined with other well-established biocontrol methods. Thus, they could potentially represent a rich resource of new biological agents for the control of *T. leucotreta*.

In this study, we proposed to determine which yeast species occur naturally in the gut of *T. leucotreta* larvae and to examine whether any of the isolated yeast species, when combined with the CrleGV-SA, enhance its effectiveness. For a more comprehensive and detailed report of the study, please consult the PhD thesis of Marcel van der Merwe, available through Rhodes University.

## Stated objectives

- A – To isolate yeast from the digestive tract of *T. leucotreta* larvae, collected from geographically distinct citrus producing regions.
- B – To identify the species of yeasts isolated, via PCR amplification and sequencing of the ITS region and D1/D2 domain of LSU.
- C – To determine if the isolated yeast have any effect on *T. leucotreta* larval development.
- D – To determine whether any of the isolated yeast species act as an oviposition stimulate for female *T. leucotreta* moths.
- E – To conduct detached fruit bioassay using CrleGV and a variety of yeast mixtures, to determine whether yeast improves the virulence of CrleGV.
- F – To identify the yeast species present on and within the oranges after the completion of laboratory bioassays.
- G – To obtain a fully annotated genome sequence for the best performing yeast isolate.
- H – Yeast formulation and field trials.

## Materials and methods

### Isolating yeast from *Thaumatotibia leucotreta* larvae

*Thaumatotibia leucotreta* larva were harvested from field collected infested oranges. Larva were surface sterilised, dissected and had their gut contents gathered using a sterilised inoculating loop and streaked onto YPD agar plates inoculated with penicillin and streptomycin. Plates were incubated at 28 °C for 3 days and emerging colonies of potential yeast isolates were selected and re-isolated on fresh penicillin/streptomycin YPD plates to obtain pure yeast colonies.

#### Identification of yeast associated with *Thaumatotibia leucotreta* larvae

Yeast isolates were grown in liquid YPD medium inoculated with penicillin/streptomycin and incubated at 28 °C on a shaker for 3 days. DNA was isolated from yeast cultures using the ZR Fungal/Bacterial DNA MiniPrep™ kit. The internally transcribed spacer (ITS) region of rDNA and the D1/D2 domains of the large ribosomal subunit (LSU) were amplified using universal ITS1/4 and NL1/4 primers. Inqaba Biotechnical Industries sequenced the PCR products. The sequences were identified by comparison to GenBank database of non-redundant sequences using BLAST.

#### *Thaumatotibia leucotreta* larval development assay

Five newly hatched *T. leucotreta* neonates were placed onto treated Navel orange. The treated Navel oranges were then maintained in a 25°C CE room for 35 days. Assays were conducted on four dates, with nine Navel oranges per treatment. After 10 days, cotton wool was placed between the Navel oranges to provide emerging 5<sup>th</sup> instar larvae with a pupation site. The cotton wool was checked daily thereafter, and the number of pupated *T. leucotreta* larvae recorded. After 35 days, each Navel orange was dissected to check for any remaining *T. leucotreta* larvae.

#### *Thaumatotibia leucotreta* larval feeding assay

A two-choice bioassay was conducted whereby two 50 µl drops of yeast culture and blank YPD medium were pipetted across from one another, ± 1 cm from the edge of a plastic petri dish. Red and blue colourants were used at 1:25 dilution to distinguish between neonate *T. leucotreta* that fed on yeast medium, blank medium or both for two hours. Ten starved *T. leucotreta* neonates were collected 24 to 48 hours after hatching and placed in the centre of a petri dish using a fine paintbrush. The petri dish was then covered with a glass lid, to prevent the neonates from escaping, and was left for two hours for the larvae to feed. The colouration of the neonate's gut was then checked under a dissecting microscope.

#### Evaluating the oviposition preference of adult *Thaumatotibia leucotreta* females

Trials were divided into two-choice and multiple-choice tests. Ten two-choice tests were conducted per yeast species. Two Navel oranges were placed opposite one another in a container, one yeast inoculated and one control. To determine which yeast species influenced adult *T. leucotreta* female's oviposition preference. Thereafter, fifteen multiple-choice tests were performed using the best performing yeast species from two-choice tests. Treated Navel oranges were placed in each corner of the container.

Sterilised Navel oranges were dipped three times into a specific treatment and allowed to air dry in a 25°C CE room. Treated Navel oranges were then transferred onto 4 cm tall plastic stands randomly placed in a plastic container (60 × 40 × 40 cm) covered with muslin cloth. A single pair of mated *T. leucotreta* adults were then released into the container, which was then sealed to prevent them from escaping. The mated pair of *T. leucotreta* adults were exposed to the treated Navel oranges for 48 hours. Once the choice test was completed, the Navel oranges were removed, and the number of eggs oviposited carefully counted. Navel oranges were checked under well-lit conditions for eggs and marked with a Sharpie.

#### Detached fruit bioassays

Three sets of detached fruit bioassays were conducted to determine i) The optimal yeast concentration to use alongside CrleGV-SA; ii) Assess the effectiveness of combining each of the isolated yeasts with CrleGV-SA

and iii) To further enhance the efficacy of the yeast/virus mixture through the addition of adjuvants such as molasses and BREAK-THRU® S 240.

Navel oranges that had reached colour break were collected from orchards in Sundays River Valley, Eastern Cape, SA. The collected fruit was washed in 0.5% bleach solution, rinsed twice with ddH<sub>2</sub>O and allowed to air dry in CE room at 28°C overnight. The following morning, Navel oranges were placed onto a sterile metal rack and sprayed with a specific treatment until thoroughly covered. The treated oranges were then placed onto cardboard egg cartons, transported to a 25°C CE room and allowed to dry for 30 – 45 minutes. Once dried, five *T. leucotreta* neonates were then added to each fruit. A total of 30 Navel oranges were used per treatment, with each treatment replicated three times. Detached fruit bioassays would run for 14 days after which Navel oranges were dissected with a serrated knife and inspected for the presence of live *T. leucotreta* larvae.

### Semi-field trials

Semi-field trials were conducted in citrus orchards with a relatively low *T. leucotreta* presence. All treatments were applied after sundown to avoid any immediate inactivation of biological agents due to UV radiation exposure. Molasses and BREAK-THRU® S 240 were used with most treatments at concentrations of 0.25 % and 0.005 %, respectively, per 100 L of water. Treatments were prepared in a Janisch spray machine and applied as medium cover film sprays (until the point of run-off) using handheld spray guns with 2 mm nozzles at a pressure of 20 Bar. Eight to 10 adjacent trees were sprayed per treatment, and three unsprayed buffer trees were incorporated between each treatment. Thirty oranges were collected from both the northern and southern sides of trees to establish whether different sunlight exposure affects efficacy. Oranges were collected 1 day and 1, 2 and 4 weeks after application. Each orange was inoculated with four *T. leucotreta* neonates in the laboratory and maintained in a 25 °C CE room for 14 days. After 14 days oranges were dissected and inspected for live *T. leucotreta* larvae.

### **Results and discussion**

<b>Objective / Milestone</b>	<b>Achievement</b>
A. Yeast isolation.	Achieved: Yeast isolates were successfully isolated from the digestive tract of <i>T. leucotreta</i> larva obtained from Addo, Nelspruit and Stellenbosch.
B. Yeast identification.	Achieved: A total of six yeast species were identified down to species level.
C. Artificial diet assay.	This objective was removed because it does not mimic field conditions and would not provide any additional information about yeast/virus synergism.
Additional. Yeast effect on larval development.	Achieved: It was found that Navel oranges treated with yeast significantly decreased larval development time and larval mortality.
Additional. Neonate movement assay.	Achieved: Neonate <i>T. leucotreta</i> behaviour was influenced by the isolated yeasts species.
D. Oviposition assay.	Achieved: Significantly more eggs were oviposited on Navel oranges inoculated with the isolated yeasts species than those without during two-choice tests.
Additional. Female <i>T. leucotreta</i> Y-tube choice test.	This objective was removed due to difficulties experienced with the Y-tube apparatus, <i>T. leucotreta</i> would not migrate down the tube.
Additional. GCMS of yeast isolates.	Current: GCMS work has been initiated; the exposure time of the fibre to yeast isolates and the run time of the sample has been optimised. The isolated yeast species now need to be examined.
E. Detached fruit bioassays using varying yeast concentrations.	Achieved: Lowering the yeast concentration resulted in higher mortality rates. The optimal yeast concentration was determined to be $2 \times 10^6$ cells/ml.
Additional. Detached fruit bioassay using all isolated yeast species.	Achieved: All isolated yeast species performed similarly under laboratory conditions, including <i>S. cerevisiae</i> .

Additional. Detached fruit bioassay including Break-Thru S 240® and molasses to the formulations.	Achieved: The addition of molasses and BREAK-THRU® S 240 to <i>Pichia kudriavzevii</i> and <i>S. cerevisiae</i> 's significantly increased larval mortality increased compared to treatments that excluded molasses and BREAK-THRU® S 240.
F. Yeast persistence on fruit after application.	This objective was removed as it would not provide any additional information about yeast/virus synergism.
G. Whole genome sequencing of the best performing yeast isolate.	This objective was removed as it would not provide any additional information on the best performing yeast isolate.
H. Semi field trials.	Current: Semi field trials have been initiated, with two replicates being completed.

#### Objectives: A and B

The overall aim was to isolate and identify yeast from the gut of *T. leucotreta* larvae via the PCR amplification and sequencing of the ITS region and LSU (D1/D2 domain) of nuclear rDNA. Navel oranges infested with *T. leucotreta* larvae were collected from three citrus-producing regions across South Africa. The guts of over 30 *T. leucotreta* larvae were screened for yeast, which resulted in the isolation and identification of six yeast species, viz. *M. guilliermondii*, *H. uvarum*, *C. lusitaniae*, *K. marxianus*, *P. kudriavzevii* and *P. kluyveri*.

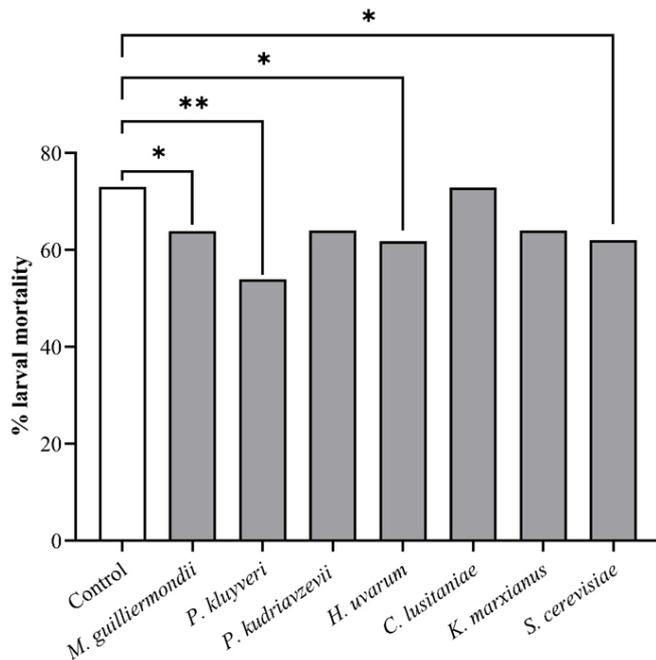
#### Additional: Yeast effect on larval development

It was found that Navel oranges treated with yeast significantly decreased larval development time (Table 3.2.2.1). *Pichia kluyveri* ( $P = 0.0001$ ), *H. uvarum* ( $P = 0.0092$ ) and *S. cerevisiae* ( $P = 0.0024$ ) performed the best out of all the applied yeast treatments. Although *C. lusitaniae* ( $P = 0.002$ ) also significantly decreased larval development time, the mortality of *T. leucotreta* was high at 76.67 %.

**Table 3.2.2.1.** Pupation rate of *T. leucotreta* larvae for 25 day detached fruit bioassays, where sterilised Navel oranges ( $n = 36$ ) were inoculated with yeast or left untreated. \* and \*\* indicate significant differences from the control according to a Fisher's exact test ( $P < 0.05$  and  $P < 0.01$ , respectively).

Treatments	Survived	Pupated		Percentage before	Significance	P-value
		Before	After			
Control (ddH <sub>2</sub> O)	41	15	26	37 %		
<i>M. guilliermondii</i>	59	35	24	59 %	*	0.0414
<i>P. kluyveri</i>	78	58	20	74 %	**	0.0001
<i>P. kudriavzevii</i>	58	35	23	60 %	*	0.0253
<i>H. uvarum</i>	63	40	23	63 %	**	0.0092
<i>C. lusitaniae</i>	42	30	12	71 %	**	0.0020
<i>K. marxianus</i>	58	38	20	66 %	**	0.0075
<i>S. cerevisiae</i>	62	42	20	68 %	**	0.0024

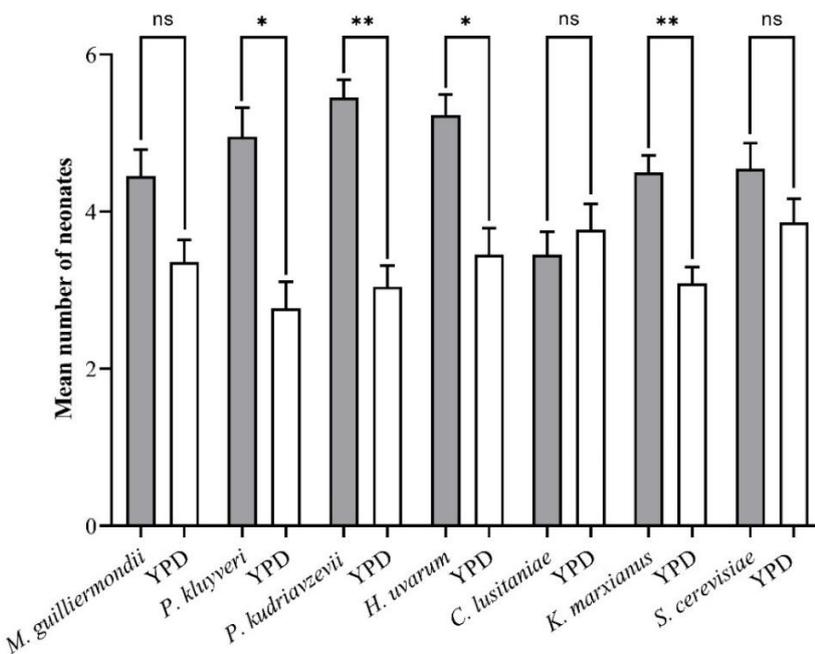
*Thaumatotibia leucotreta* larval mortality was significantly lower on Navel oranges treated with *M. guilliermondii* ( $P = 0.0452$ ), *P. kluyveri* ( $P < 0.0001$ ), *H. uvarum* ( $P = 0.0144$ ) and *S. cerevisiae* ( $P = 0.0194$ ) (Figure 3.2.2.1.1). Three yeast treatments, viz. *C. lusitaniae* ( $P > 0.9999$ ), *P. kudriavzevii* ( $P = 0.0586$ ), and *K. marxianus* ( $P = 0.0586$ ) did not decrease larval mortality. Overall, *P. kluyveri* performed best, as it decreased larval mortality by 20.55 % and 74.00 % of larvae pupated before 25 days.



**Figure 3.2.2.1.** *Thaumatotibia leucotreta* larval mortality after 35 days on sterilised Navel oranges ( $n = 36$ ), inoculated with yeast or left untreated. \* and \*\* indicate significant differences from the control according to a Fisher's exact test ( $P < 0.05$  and  $P < 0.01$ , respectively).

Additional: Neonate movement assay

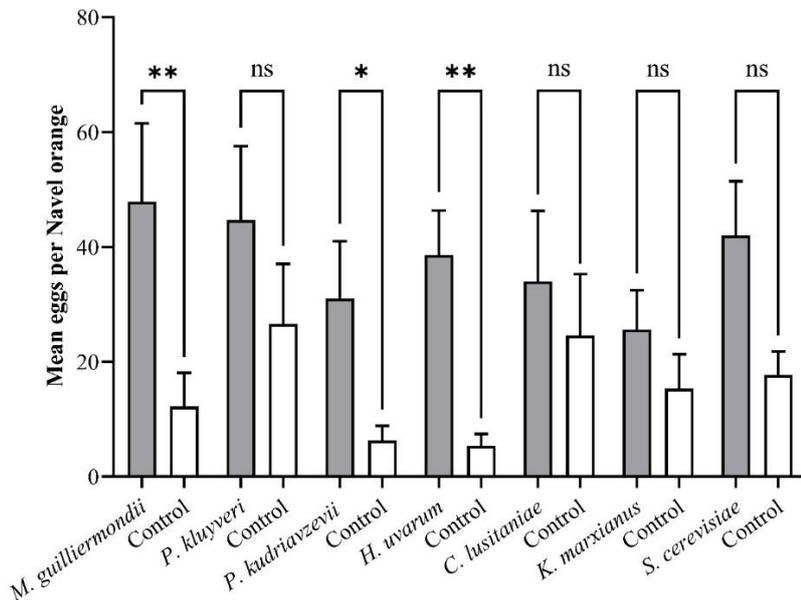
The only time larvae preferred the YPD medium was in the *C. lusitaniae* ( $P = 0.5907$ ) treatment. Two yeast species, *M. guilliermondii* ( $P = 0.0606$ ) and *S. cerevisiae* ( $P = 0.2579$ ), had no significant effect on larval attraction and feeding. Neonate *T. leucotreta* behaviour was influenced by four yeasts, viz. *P. kluyveri* ( $P = 0.0043$ ), *H. uvarum* ( $P = 0.0037$ ), *P. kudriavzevii* ( $P = <0.0001$ ) and *K. marxianus* ( $P = 0.0005$ ) (Figure 3.2.2.2).



**Figure 3.2.2.2.** Neonate *T. leucotreta* larval attraction and feeding in response to seven yeasts species ( $n = 22$ ). \* and \*\* indicate significant differences from the control (YPD) according to a paired Student t-test ( $P < 0.05$  and  $P < 0.01$ , respectively), ns indicates that differences were not significant.

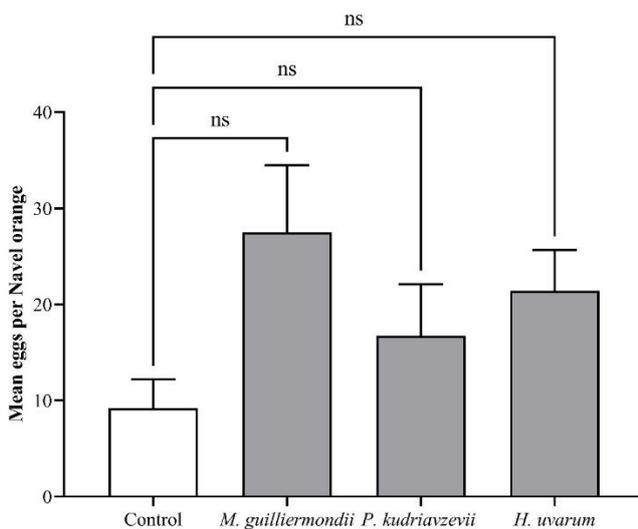
Objective D: Oviposition assay

Significantly more eggs were oviposited on Navel oranges inoculated with *M. guilliermondii* ( $P = 0.0090$ ), *P. kudriavzevii* ( $P = 0.0471$ ) and *H. uvarum* ( $P = 0.0013$ ) compared to the control during the two-choice tests (Figure 3.2.2.3). *Pichia kluyveri* ( $P = 0.3768$ ), *C. lusitaniae* ( $P = 0.5729$ ), *K. marxianus* ( $P = 0.2838$ ) and *S. cerevisiae* ( $P = 0.0517$ ) did not influence the oviposition preference of adult *T. leucotreta* females.



**Figure 3.2.2.3.** Oviposition preference of adult *T. leucotreta* females for different gut-associated yeasts species during two-choice tests ( $n = 10$ ). \* and \*\* indicate significant differences from the control according to a paired Student t-test ( $P < 0.05$  and  $P < 0.01$ , respectively), ns indicates that differences were not significant.

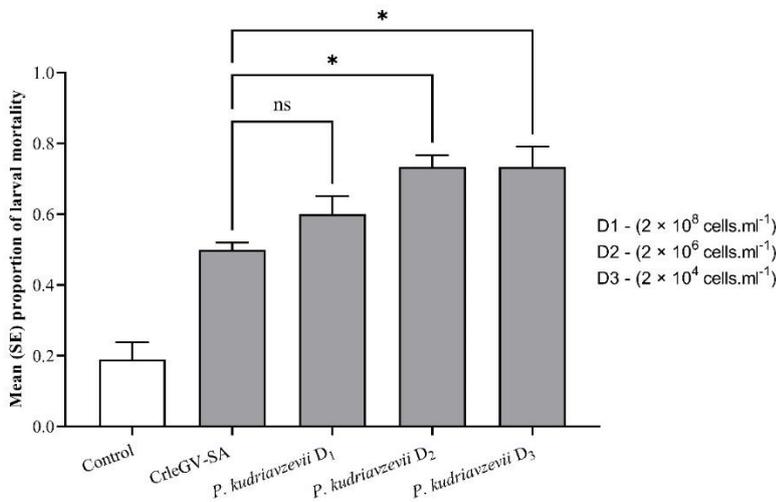
Navel oranges inoculated with *M. guilliermondii*, *P. kudriavzevii*, and *H. uvarum* did not significantly increase the number of eggs oviposited on them. No significant differences were found between yeast and control treatments during multiple-choice tests (Figure 3.2.2.4).



**Figure 3.2.2.4.** The oviposition preference of adult *T. leucotreta* females for Navel oranges inoculated with different treatments during multiple-choice tests ( $n = 15$ ). A Kruskal–Wallis test was used to determine significance and multiple comparison of mean ranks post hoc test was used to determine where the significant differences occurred, ns indicating not significant.

Objective E: Detached fruit bioassays using varying yeast concentrations

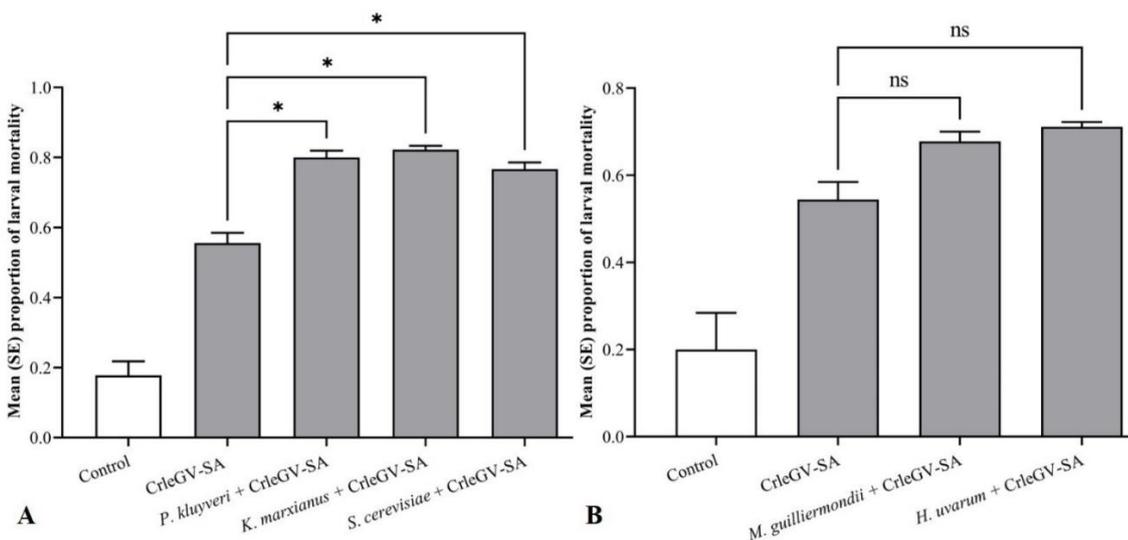
No significant difference was recorded between CrleGV-SA and *P. kudriavzevii* applied at  $2 \times 10^8$  cells.ml<sup>-1</sup> ( $P = 0.6372$ ). A significant difference was, however, recorded between *P. kudriavzevii* applied at  $2 \times 10^6$  cells.ml<sup>-1</sup> ( $P = 0.0442$ ) and  $2 \times 10^4$  cells.ml<sup>-1</sup> ( $P = 0.0415$ ), when compared to CrleGV-SA alone (Figure 3.2.2.5).



**Figure 3.2.2.5.** *Thaumatotibia leucotreta* larval mortality in 14-day detached fruit bioassays ( $n = 3$ ) on Navel oranges treated with ddH<sub>2</sub>O, CrleGV-SA at  $9.31 \times 10^7$  OBs.ml<sup>-1</sup> and *P. kudriavzevii* at varying concentrations ranging from  $2 \times 10^8$  to  $2 \times 10^4$  cells.ml<sup>-1</sup> plus CrleGV-SA. \* indicates that differences were significant, and ns indicates that differences were not significant, according to an analysis of variance (ANOVA) using Tukey's test ( $P < 0.05$ ).

Additional: Detached fruit bioassay using all isolated yeast species

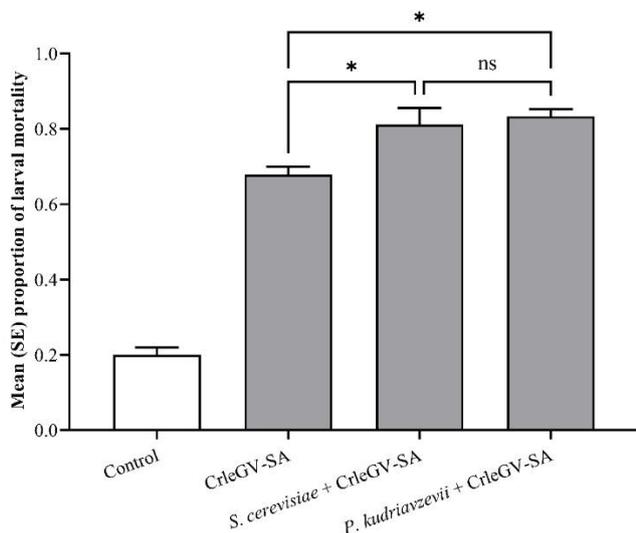
A significant difference was recorded between Navel oranges treated with *P. kluyveri* ( $P = 0.0012$ ), *K. marxianus* ( $P = 0.0005$ ) and *S. cerevisiae* ( $P = 0.0041$ ) in combination with CrleGV-SA compared to CrleGV-SA alone (Figure 3.2.2.6). *Meyerozyma* ( $P = 0.2784$ ) and *H. uvarum* ( $P = 0.1443$ ) did not enhance the efficacy of CrleGV when applied in combination.



**Figure 3.2.2.6.** Larval mortality of *T. leucotreta* in 14-day detached fruit bioassays ( $n = 3$ ). A) Navel oranges treated with *P. kluyveri*, *K. marxianus* and *S. cerevisiae* combined with CrleGV-SA. B) Navel oranges treated with *M. guilliermondii* and *H. uvarum* combined with CrleGV-SA. \* indicates that differences were significant, and ns indicates that differences were not significant, according to an analysis of variance (ANOVA) using Tukey's test ( $P < 0.01$ , respectively).

Additional: Detached fruit bioassay including Break-Thru S 240® and molasses to the formulations

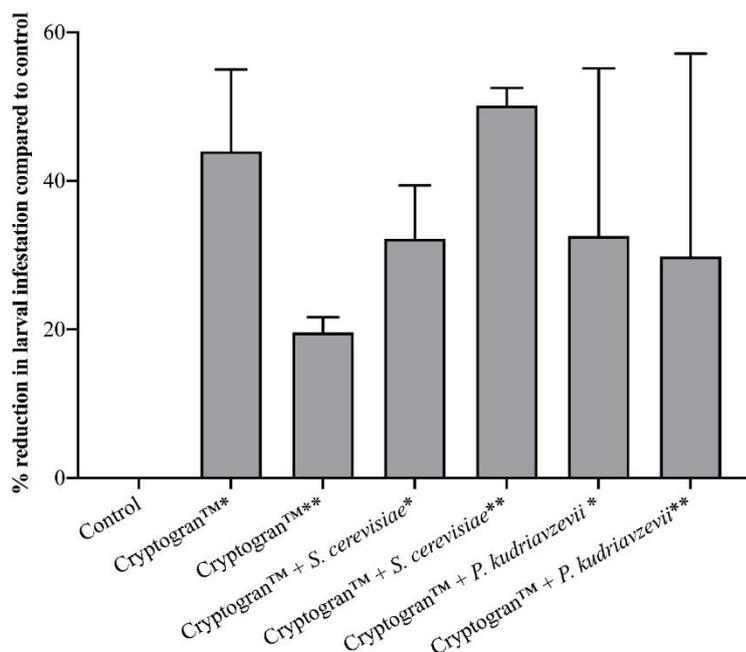
The addition of molasses and BREAK-THRU® S 240 significantly enhanced the efficacy of *P. kudriavzevii* ( $P = 0.0195$ ) and *S. cerevisiae* ( $P = 0.0418$ ) formulations compared to CrleGV-SA (Figure 3.2.2.7). *Pichia kudriavzevii* and *S. cerevisiae*'s larval mortality increased by 12.71 % and 14.71 %, respectively, compared to treatments that excluded molasses and BREAK-THRU® S 240.



**Figure 3.2.2.7.** *Thaumatotibia leucotreta* larval mortality in 14-day detached fruit bioassays ( $n = 3$ ) on Navel oranges treated with ddH<sub>2</sub>O, CrleGV-SA at  $9.31 \times 10^7$  OBs.ml<sup>-1</sup>, *P. kudriavzevii* and *S. cerevisiae* at  $2 \times 10^6$  cells.ml<sup>-1</sup> plus CrleGV-SA. Except for the control, all treatments had the addition of molasses and BREAK-THRU® S 240 at 0.25 % and 0.005 %, respectively. \* indicates that differences were significant, and ns indicates that differences were not significant, according to an analysis of variance (ANOVA) using Tukey's test ( $P < 0.05$ ).

Objective H: Semi field trials

Due to the limited number of trials that could be performed due to the restricted time available before fruit harvesting, no statistical analyses were conducted on the collected data. Even though mean reduction in infestation, relative to the control, was determined with the data from the two sites. The residual efficacy of the applied treatments seemed to drop off after two weeks in the semi-field trials; thus, data from week four was excluded when determining the reduction in larval infestation. The data obtained does provide valuable insight into how *P. kudriavzevii* and *S. cerevisiae* perform in the field. Overall, the performance of *S. cerevisiae* was impressive and consistent, resulting in the least variability (Figure 3.2.2.8). The efficacy of *P. kudriavzevii* during the second semi-field trial was comparable to that of *S. cerevisiae*. Still, problems with its formulation during the first semi-field trial resulted in poor performance.



**Figure 3.2.2.8.** Mean reduction in larval infestation relative to each treatment's control during the first two weeks of the four-week period over which the two semi-field trials were conducted at Pennyholme and Tregoss Skinner farms. \* and \*\* indicate treatments that included BREAK-THRU® S 240 and molasses plus BREAK-THRU® S 240, respectively.

## Conclusion

In conclusion, this study's primary aim was to identify a yeast species that would enhance the efficacy of CrleGV-SA against *T. leucotreta*. Six yeast species were successfully isolated and identified from *T. leucotreta* larvae collected from geographically distinct citrus-producing regions. Larval feeding and oviposition assays were conducted with the isolated yeast species to evaluate *T. leucotreta* neonate and adult female behavioural response. Detached fruit bioassays were then conducted to assess the efficacy of combining CrleGV-SA with the isolated yeast species. The mixture of *P. kudriavzevii* with CrleGV-SA significantly increased the efficacy of the virus in a laboratory setting and therefore, this yeast isolate was used in semi-field trials. However, additional semi-field trials need to be conducted to confirm the formulation's efficacy in the field. Future research into the efficacy of *P. kudriavzevii* in combination with CrleGV-SA in the field is required to develop a novel biopesticide formulation.

## Future research

Future work arising from this study would involve increasing the number of larvae sampled and expanding the bioprospecting process to other citrus-producing regions across South Africa, such as Limpopo Province. The process whereby yeast species were isolated can be streamlined through the use of more selective plated media to limit the growth of non-yeasts. Analysing the haemolymph and specialised cells such as fat bodies within *T. leucotreta* larvae may further indicate which of the isolated yeast species are mutualistic (Gibson and Hunter, 2010; Vega and Dowd, 2005). Interactions between insects and microbes with a mutualistic relationship are greater than those that interact by chance, as they have evolved together (Becher *et al.*, 2018; Madden *et al.*, 2018). The results obtained during the ovipositional preference trials create various opportunities for future research. Performing ovipositional preference trials in much larger cages and conducting field trials may result in a more definite answer. Conducting field trials with the isolated yeast species may also inadvertently be attractive towards other citrus pests and lead to the development of a broader pest monitoring system. The volatile profiles of the isolated yeasts species should be purified via column chromatography and analysed via GCMS to identify the metabolites they produce. Analysing the volatile profiles produced by the isolated yeast species was initiated but could not be completed due to Covid-19 lockdown induced time constraints. Analysing adult *T. leucotreta* female flight response in wind tunnels without a visual cue could provide a better indication of their attractiveness to volatiles produced by the isolated

yeast species (Witzgall *et al.*, 2012). Additionally, analysing the antennal response of adult *T. leucotreta* females via GC-EAD may indicate the specific compound or bouquet of compounds that is/are responsible for eliciting an olfactory response (Gökçe *et al.*, 2018). Identifying the unique odour profiles that yeasts produce and the isolation of these compounds' physiologically active constituents could potentially define the host's attraction (Scheidler *et al.*, 2015). A repeat of the semi-field trials would also be of considerable interest to determine whether the results obtained in this study can be replicated. Additional replicates need to be performed to obtain data that can be statistically analysed. The trial duration should also be extended slightly to determine if the residual efficacy of *P. kudriavzevii* and *S. cerevisiae* extend beyond the four weeks tested. Once semi-field bioassays are completed, a high infestation field site should be sought where a more conventional replicated field trial can be conducted.

## Technology transfer

- Society for Invertebrate Pathology Annual Conference, 12 – 16 August 2018, Oral presentation.
- 10th Citrus Research Symposium, 19 – 22 August 2018, Oral presentation.
- Analytical Methods in Chemical Ecology, August 5-16, 2019, GCMS workshop.

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### 3.2.3 FINAL REPORT: Improvement of the quality and quality control testing of sterile moths for FCM SIT

Project 1164 (April 2017 – March 2021) by Sean Moore, Wayne Kirkman (CRI), Mellissa Peyper, Clarke van Steenderen, Tammy Marsberg, Martin Hill (RU), Nevill Boersma, Ciska Kruger, Craig Chambers and Sampie Groenewald (Xsit)

#### Summary

The sterile insect technique (SIT) for FCM has been commercially implemented in citrus in South Africa since 2007 with generally good success. However, a few possible problems have been identified and there is a continual pursuit to improve the quality and performance of the sterile moths. Thus, this study focused on various aspects. Firstly, the lack of activity of sterile moths at cooler temperatures compared to wild moths. Previous work identified trehalose as an effective cryoprotectant for sterile moths, if added to the larval diet. Consequently, a field trial was conducted during autumn and winter of 2018 and 2019, comparing recaptures of moths reared on a trehalose augmented diet with those reared on the normal diet. Recaptures were higher for trehalose moths for both years. Additionally, trap catches indicated that trehalose-fed moths survived longer in the field than control moths. Furthermore, a reliable quality control test to measure the mating competitiveness of sterile male moths was required. This was investigated in laboratory trials by determining the spermatophore transfer between: sterile males to wild females and wild males to wild females. Simultaneously, choice-trials were conducted in a field net to compare the mating incidents between sterile and wild moths. Wild males were found to have a significantly higher spermatophore transfer than sterile males, with sterile males showing no preference for sterile females. A statistically significant correlation was also recorded between mating incidents in cages involving sterile males and spermatophore transfer in laboratory trials. Therefore, laboratory based spermatophore transfer trials can be used as a reliable quality control measure for sterile males. Lastly, further investigation is required to determine a suitable technique to create a genetic “fingerprint” of the different wild and sterile crosses, in order for the F1 generation to be identified in the field. However, a sterile F1 generation will only ever be possible in the presence of wild females and will always be at a dramatically reduced level relative to the F1 generation of a wild-wild crossing.

#### Opsomming

Die steriele insektegniek (SIT) vir VKM word sedert 2007 kommersieel in sitrus in Suid-Afrika geïmplementeer met oor die algemeen goeie sukses. 'n Paar moontlike probleme is egter geïdentifiseer en daar word voortdurend daarna gestreef om die kwaliteit en prestasie van die steriele motte te verbeter. Hierdie studie het dus op verskillende aspekte gefokus. Eerstens die gebrek aan aktiwiteit van steriele motte by koeler temperature in vergelyking met wilde motte. Vorige werk het trehalose geïdentifiseer as 'n effektiewe kouebeskermsmiddel vir steriele motte, indien dit by die larvale dieet gevoeg word. Gevolglik is 'n veldproef gedurende die herfs en winter seisoene van 2018 en 2019 gedoen, wat die hervangste van motte wat op 'n trehalose aanvullende dieet geteel is, vergelyk met die wat op die normale dieet geteel is. Hervangste was vir albei jare hoër vir trehalose-motte. Daarbenewens het hervangste aangedui dat motte wat met trehalose gevoed is, langer in die veld oorleef as kontrole motte. Verder was 'n betroubare kwaliteitskontrole-toets nodig

om die paringsmededingendheid van steriele mannetjie motte te meet. Dit is ondersoek in laboratoriumproewe deur die spermatofoor oordrag te vergelyk tussen: steriele mannetjies tot wilde wyfies en wilde mannetjies tot wilde wyfies. Gesamentlik is keuringsproewe in 'n veld-net uitgevoer om die paringsvoorvalle tussen steriele en wilde motte te vergelyk. Daar is gevind dat wilde mannetjies 'n aansienlike hoër spermatofoor oordrag het as steriele mannetjies, terwyl steriele mannetjies geen voorkeur vir steriele wyfies toon nie. 'n Statisties beduidende korrelasie is ook aangeteken tussen parings voorvalle in hokke wat steriele mannetjies insluit en oordrag van spermatofoor in laboratoriumproewe. Daarom kan laboratoriumgebaseerde spermatofoor-oordragproewe gebruik word as 'n geskikte beheer tegniek vir steriele mannetjies. Verdere ondersoek is nodig om 'n genetiese 'vingerafdruk' van die verskillende wilde en steriele kruise te skep, sodat die F1 generasie in die veld geïdentifiseer kan word. 'n Steriele F1 generasie sal egter eers moontlik wees in die teenwoordigheid van wilde wyfies en sal altyd teen 'n dramaties verlaagde vlak wees in vergelyking met die F1 generasie van 'n wilde-wilde kruising.

## Introduction

The sterile insect technique (SIT) for FCM has been commercially implemented in citrus in South Africa (Western and Eastern Cape) since 2007 with generally good success. The moths are reared in the Xsit production facility in Citrusdal and then transported in a cold immobilised state to release sites. In the case of the Eastern Cape, moths can remain in this cold immobilised state for 24 h or more. It is understood that this treatment can compromise the fitness and hence performance in the field of the sterile moths. This is a case of adding insult to injury, as the ionizing radiation used to sterilise the moths already causes oxidative stress (Calabrese *et al.*, 2007).

Therefore, if an alternative means of immobilising the moths can be developed, this may make a significant difference to the efficacy of SIT in the field, as the moths would become more competitive with wild male moths. A potential means of doing this is through anoxia or hypoxia (López-Martínez and Hahn, 2012; López-Martínez *et al.* 2014). Not only would this avoid harmful cold immobilisation, but anoxia (mild oxidative stress) has been demonstrated to actually increase activity of anti-oxidant enzymes, thus reducing the damage (extreme oxidative stress) caused by the radiation (López-Martínez and Hahn, 2012).

An additional shortcoming of the SIT programme for FCM is that the laboratory-reared irradiated moths are not able to remain active at temperatures as low as wild moths can. Wild moths are known to have an activity threshold of between 10 and 15°C (Daiber 1980), whereas irradiated moths experience markedly reduced activity at temperatures below 20°C (Stotter 2011). Daniel (2016) recently demonstrated in laboratory and small-scale field trials that the addition of trehalose, as a cryoprotectant, to the FCM larval diet, can significantly increase moth activity at low temperatures. In the laboratory, activity at 15°C was improved from zero to 40% and in the field, four times as many trehalose fed than normal diet fed moths were recaptured. Consequently, this was tested on a semi-commercial scale.

It is not only important to improve the quality of the moths being released, but also to improve our ability to accurately measure this quality. Currently, the final or ultimate measure of the quality of released sterile male moths is a point release-recapture test, which is conducted with sterile moths produced by Xsit on a daily basis. A certain percentage of the released moths must be recaptured in order to indicate that the moths are of acceptable fitness. However, this test is conducted using synthetic pheromone dispensers used for monitoring and is therefore more strongly attractive to the moths than virgin females (unpublished data). Additionally, even if they locate the source of the pheromone, it does not necessarily indicate that the moths are mating competitive or will mate at all. Therefore an improved quality control test must be developed, which more accurately measures these factors.

## Stated objectives

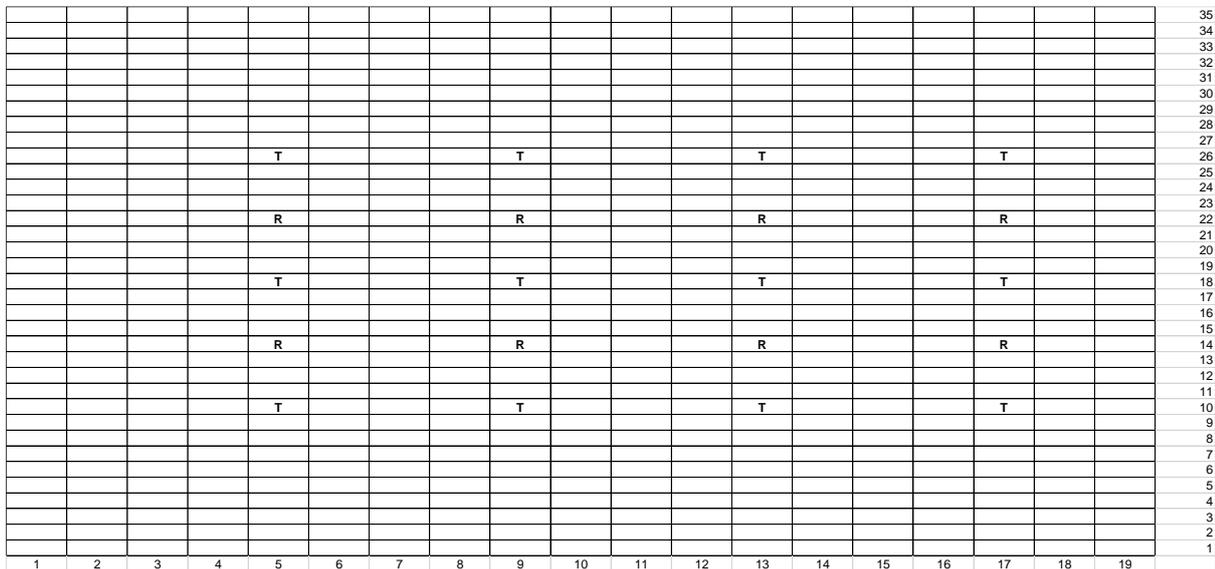
- Large scale determination of the effect of trehalose on sterile moth performance
- Laboratory trials on the effects of trehalose
  - Critical thermal limits
  - Biological parameters

- Development of a QC test to measure mating competitiveness of sterile male moths
- Anoxia for immobilisation of sterile insects
- Potential infestation by the F1 generation of released sterile moths

**Materials and methods**

Large scale determination of the effect of trehalose on sterile moth performance

Twice a week, 40 jars of larvae were produced at Xsit on the normal larval diet and 40 jars with trehalose augmented diet. Trehalose was added at 7 g per 100 g of other dietary ingredients, including water. Additionally, Calco Red was added to the normal moth diet, as is normal practice in the Xsit facility, and it was excluded from the trehalose moth diet. This was in order to be able to differentiate between moths in the field. Whenever sufficient moths had eclosed for release of a few thousand per hectare, which was approximately twice a week, moths were dispatched from Citrusdal to Addo with Xsit’s normal cold transportation. Moths from each of the two treatments were then weighed out into eight identical portions, each portion in a petri dish and taken to the field for release. Numbers of moths released was estimated by dividing the total mass by the mean mass of a moth, determined to be 0.228 g. The release site consisted of two identical lemon orchards on the farm Rietfontein in the Sundays River Valley. Each orchard was approximately 1 ha in size and the two orchards were separated by another lemon orchard of similar size. The layout in each orchard was identical, with eight release points and 12 trapping points (Figure 3.2.3.1). Each petri dish of normal moths was released by hand at one of the eight release points in one of the orchards and the eight petri dishes of trehalose moths were released in the same way in the other orchard. Yellow delta traps were used, loaded with Chempac FCM lures and sticky floors. Each week, traps were read and cleaned and treatments per orchard were swapped. Moths were identified as normal or trehalose by squashing them and observing the colour (red or orange) and were then counted. Releases were initiated on 8 June and will be continued for at least eight weeks. A small quantity of moths from each treatment is held back on each occasion and the quality assessed and compared between treatments.



**Figure 3.2.3.1.** Layout of release orchards. Each square represents a tree. Spacing between rows was 6 m and between trees was 3 m, thus 555 trees per ha. (T = traps; R = moth release points).

Laboratory trials on the effects of trehalose on FCM

- *Critical thermal limits*

The sterile male pupae were kept in labelled petri dishes in a Freezepoint Eco Panel controlled environment room at 24°C with 12/12h light/dark cycle until eclosion. When pupae began turning black, indicating that they would eclose soon. Moistened cotton wool was added to the petri dishes to keep emerged adults alive and hydrated until experimentation. To control for age, the petri dishes were checked twice a day for eclosion, and

adults removed for experiments as soon as a minimum of 10 individuals from each diet had emerged. All experiments were completed within 24 h of one another.

For each experiment, 20 freshly-eclosed adults (10 reared on the standard diet, and 10 on the trehalose-dosed diet) were isolated in individual Eppendorf tubes, which were inserted into a high density foam float such that only the lids of the tubes emerged above the top of the float. The float was placed in a Grant R4 water bath with GP200 digital immersion thermostat filled with a 50/50 distilled water/glycol mixture to allow for sub-zero temperatures to be reached without freezing of the liquid. Previous experiments performed by Porter *et al.* (2019) using the same equipment and methodology confirmed that the temperatures inside the Eppendorf tubes mirrored the water bath temperatures with no lag.

The temperature ramping protocol used here followed that used in Boardman *et al.* (2012) for FCM larvae by starting with a 30 minute acclimation period at 15°C, followed by a constant downwards temperature ramp of 0.25°C per minute, to allow for comparison of the results between the two studies. At each 1°C change in temperature, the insects were removed from the water bath, tipped onto their backs, and their ability to self-right checked alongside evidence of muscle spasm. This was completed as quickly as possible so that the insects were not exposed to ambient temperatures for too long. The individuals that were able to self-right or that exhibited no spasms were returned to the water bath and the experiment continued until no insects remained. Those that were unable to self-right or showed a lack of muscle control upon checking were removed and kept at ambient temperature in the laboratory. The temperature at which the insects were removed from the experiment was recorded as the CT<sub>min</sub> for each individual. The FCM were checked for recovery following one hour after the experiment had ceased, and only those that recovered to normal behaviour were used in the final dataset. The whole experiment was repeated three times using new, freshly-eclosed individuals each time, resulting in a total sample size of 30 individuals for each diet.

#### - *Biological parameters*

FCM artificial diet for the control was prepared by adding 50 g of dry ingredients of the diet to 50 g of distilled water in a 352 ml Consol glass jar. For the 1X rate, 7 g of trehalose was added per 100 g of FCM diet (50 g diet and 50 g water), 14 g trehalose for 2x and 21 g trehalose added for the 3X rate. Jars were plugged with a cotton wool stopper. Diet was then autoclaved for 18 min at 121°C. Once the jars had cooled down, approximately 200 FCM eggs were placed into each jar. Jars were kept at 27°C until pupation.

#### *Developmental rate*

The developmental rate of the various trehalose treated larvae was determined by placing 30 larvae individually into glass vials. The dates of inoculation, pupation and eclosion were recorded. The average developmental times were then calculated for each treatment. This was repeated thrice.

#### *Pupal weight and fecundity*

Pupae were removed from the cotton wool wads and sexed. Twenty male and female pupae from each treatment were then weighed and coupled. Couples were then placed into 30 ml pill vials lined with wax paper. A moistened cotton wool ball was placed in the top of the pill vials. Couples were left in the vials until the female died, wax sheets were replaced daily and the number of eggs oviposited were counted and totalled up. Eggs sheets were then left for six days, the number of unhatched eggs were then counted using a dissecting microscope. This was repeated for each treatment and for three replicates.

#### *Flight ability*

To determine flight ability of each treatment 20 pupae from each treatment was placed at three different temperature, 16°C, 20°C and 27°C. Pupae were placed inside a flight chamber. Chambers were then sealed and left for approximately two weeks to allow for the moths to eclose and exit the flight chamber. After the two week period, the flight chamber was opened and the number of moths/pupae remaining were counted.

#### Development of a QC test to measure mating competitiveness of sterile male moths

In order to test the reliability of the laboratory mating competitiveness trials, field cage studies were conducted. A field cage (3 m x 3 m x 2.3 m (height)) was erected over four small potted citrus trees. Twenty of each of

sterile males, sterile females, fertile males and fertile females were released into the cage, starting at 17h00 or 17h30 on each of six occasions in February and March 2019. Thereafter, evening temperatures became too cold. Releases were conducted by placing each moth individually in a 30 ml sample bottle in the cage and allowing the moths to leave the bottles voluntarily. Males were placed on the downwind side of the cage and females on the upwind side of the cage. Every 30 min an inspection was conducted to determine departures from bottles and mating and to record these. Mating pairs were collected and identified. This was continued until two simultaneous inspections revealed no further mating. Temperature and humidity were logged every 30 min in the cages using a Maxim iButton (Fairbridge Technologies, Johannesburg). Simultaneously, spermatophore transfer trials were conducted with sterile moths from the same batches used in the cage, exactly as described in the previous section. The trial was thus replicated successfully six times. A regression analysis was conducted between spermatophore transfer and mating incidents in cages.

#### Anoxia for immobilisation of sterile insects

The protocol for this study will be based on López-Martínez & Hahn (2012) and López-Martínez *et al.* (2014). Firstly, the appropriate length of anoxia for FCM will be determined i.e. the maximum duration of exposure after which 100% survival is still achieved. Anoxia conditioning will be performed by placing groups of 50 moths in small petri dishes, perforated to allow air flow, and placing them in polypropylene bags. The bags will then be flushed with nitrogen for 2 min, long enough to displace all the oxygen, and sealed. A second nitrogen-flushed bag will be placed over the first bag and sealed to ensure anoxia. After anoxia, moths will be irradiated and then reperfused with oxygen. Moths in the normoxia treatments will be placed in perforated petri dishes that are sealed in polypropylene bags that are thoroughly perforated to facilitate airflow. The treatment groups will thus be normoxia and no radiation (NxNr), normoxia and 150 and/or 200 Gy (Nx150/200, the current standard treatment for FCM SIT), and anoxia and 150 and/or 200 Gy (Ax150/200).

The comparative effect of these treatments on moth fitness will then be tested. This will include flight-ability, mating success and longevity. Flight-ability will be measured according to standard protocols currently used by Xsit. Mating success is not currently a QC measurement used by Xsit. This will therefore have to be developed, as described below. Longevity will be determined both by keeping moths communally and solitarily after treatment.

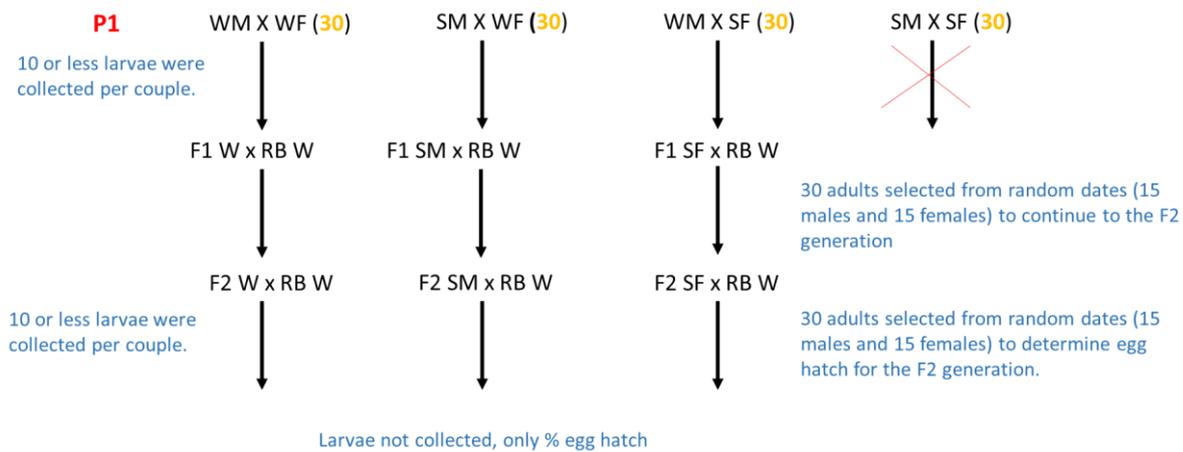
Finally, the effect of the treatments on moth sterility will be measured by recording fecundity (eggs laid) and fertility (egg hatch) of untreated female moths paired with treated male moths.

Similar trials can be conducted with hypoxia.

#### Potential infestation by the F1 generation of released sterile moths

##### - *Laboratory trials with various couplings of wild and sterile moths*

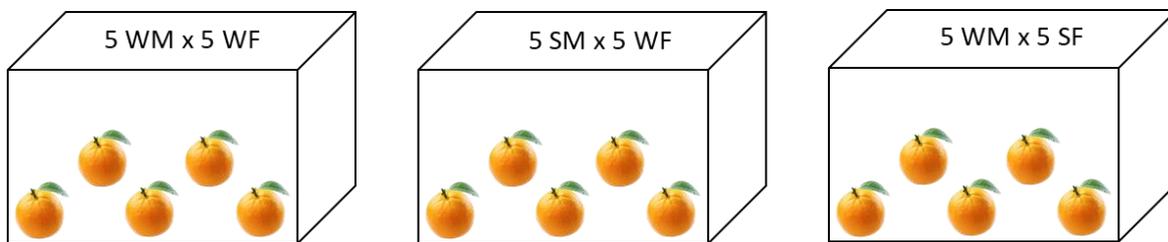
Laboratory trials with various crossings of sterile and wild (larvae/pupae collected from River Bioscience) moths were conducted in 20 ml pill vials to determine fertility of sterile moth (Figure 3.2.3.2). Crosses used were; wild male (WM) x wild female (WF), wild male (WM) x sterile female (SF), sterile male (SM) x wild female (WF) and sterile male (SM) x sterile female (SF). Thirty couples of each crossing were used for the P1 generation. Strips of wax paper were placed into each pill vial for oviposition and the pill vial was closed with cotton wool moistened with water. Wax paper was removed daily, eggs were counted and placed in separate vials. This was done daily until the female moth died. Egg sheets were placed at  $\pm 26^{\circ}$  C to allow for hatching. Total hatched and unhatched eggs were counted and recorded. Ten larvae were collected per vial and placed singularly in a glass vial containing FCM artificial diet. Larvae were left to develop and pupate at  $\pm 26^{\circ}$  C. Fifteen female and fifteen male moths were randomly collected from the F1 emerging moths. The F1 moths were then coupled with the relevant wild moth and the above process repeated. This continued until the F2 generation and was replicated three times.



**Figure 3.2.3.2.** Diagram representing how the laboratory trials with couplings of wild and sterile moths was set up.

- *Laboratory cage trials with various couplings of wild and sterile moths with Navel oranges*

Laboratory cage trials were conducted with ripe Navel oranges and WM x WF, SM x WF and WM x SF crossing. Ten oranges were placed in 40 x 40 x 60 cm mesh cages and five couples of one of the crosses were placed in the cage, this was repeated five times for each crossing (Figure 3.2.3.3). Moths were removed from the cages and dissected to determine the number of spermatophores transferred. The number of eggs oviposited on each orange was counted and recorded. Oranges were placed back in the cages and left for a further four days. Penetration holes were then counted and recorded. Oranges were then left for a further 7 days, after which they were dissected and the number of larvae found in the orange was recorded. This was replicated three times.



**Figure 3.2.3.3.** Diagram representing the setup of laboratory cage trials with Navel oranges.

- *Genetic finger print*

Sterile moths will be released by ground (quad bike) in netted citrus orchards and in orchards outside of nets at commercially used rates. Pheromone baited traps will be used to gauge wild and sterile moth activity in orchards. Infested fruit will be collected on a regular basis. A genetic 'fingerprint' will then be created from the larvae collected from infested fruit and compared to the genetic 'fingerprint' of wild FCM and the SIT laboratory culture. Methods for creating a genetic 'fingerprint' are still under investigation.

### Results and Discussion

Objective / Milestone	Achievement
A. Large scale determination of the effect of trehalose on sterile moth performance	Another large scale field trial was completed and a higher number of trehalose moths was once again recaptured, this time, significantly so.
Laboratory trials on the effects of trehalose on FCM	Critical thermal limits were determine for each trehalose treatment, as well as the biological parameters.

C. Development of QC test to measure mating competitiveness of sterile male moths	Semi-field and laboratory trials have been completed. These trials determined a suitable method to test the QC of mating competitiveness of sterile moths.
D. Anoxia for immobilisation of sterile insects	Will be reported in 1221
E. Potential damage caused by the F1 generation of released sterile moths	Laboratory trials have been completed to determine the fecundity and fertility of sterile and wild couples. Cage trials with Navel oranges have been completed. Monitoring and fruit and larval collects have been completed for a second consecutive season. An alternative method for genetic 'fingerprinting' is under investigation. As this project is now terminated, this objective has been transferred to a new project (1299).

#### A. Large scale determination of the effect of trehalose on sterile moth performance

Between 725 and 11765 moths were released on each occasion per ha. Overall recaptures of trehalose moths to date was higher compared to normal moths (Table 3.2.3.1). Recaptures of trehalose moths in the orchard in which they were not released during the preceding week were almost double that of normal moths. This indicates that either trehalose moths survived longer than normal moths, or they dispersed further (i.e. from the adjacent orchard) than did the normal moths. Although the latter is distinctly possible, as trehalose is recognised as a fuel for flight in insects, the former appears more likely in this case, as during the first week of monitoring, no moths other than those released in each orchard were caught in that orchard.

**Table 3.2.3.1.** Number control and trehalose treated moths released on each occasion in 2018.

Week of trial	Release dates	Moths released per ha	Date trap inspected	Moths recaptured			
				Trehalose moths		Normal moths	
				In trehalose release block	In normal release block	In trehalose release block	In normal release block
1	8 June	725	16 June	638	0	428	0
	11 June	4202					
	12 June	3922					
2	16 June	11765	25 June	360	23	396	28
	18 June	3922					
	20 June	3922					
	22 June	2717					
3	25 June	4202	30 June	37	14	32	2
	26 June	1401					
4	30 June	3641	18 July	107	22	70	2
		5 July					
Total				1142	59	926	32
				1201		958	

A minimum of 1450 moths and a maximum of 9843.81 moths were released per ha. Overall recaptures for trehalose moths were higher than those of control moths. A higher number of trehalose moths were also found in the opposite release site, indicating that trehalose moths lived longer or dispersed further than the control moths (Table 3.2.3.2).

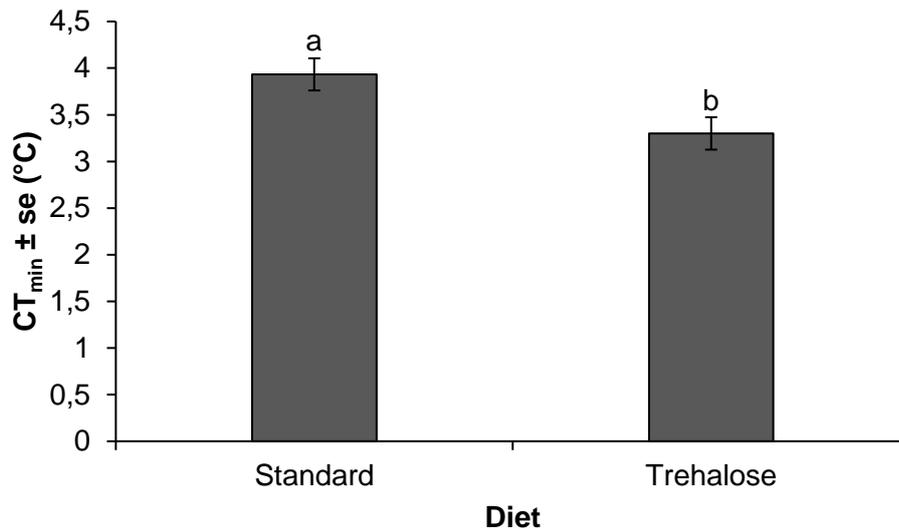
**Table 3.2.3.2.** Number of control and trehalose treated moths released on each occasion in 2019.

Week of trial	Release dates	Moths released per ha	Date trap inspected	Moths recaptured			
				Trehalose moths		Normal moths	
				In trehalose release block	In normal release block	In trehalose release block	In normal release block
1	10/05/2019	4546.29	16/05/2019	150	0	0	322
2	16/05/2019	4396.04					
	18/05/2019	3761.37					
3	21/05/2019	2515.35	21/05/2019	193	32	5	41
	23/05/2019	3323.58	25/05/2019	12	0	2	15
	25/05/2019	2929.83					
4	29/05/2019	3030.86	29/05/2019	84	4	2	8
	30/05/2019	1891.05	03/06/2019	41	27	5	47
5	03/06/2019	9843.81					
	05/06/2019	3885.71	07/06/2019	98	13	1	35
	07/06/2019	3730.29					
6	10/06/2019	2901.33	12/06/2019	3	0	1	6
	12/06/2019	2512.76					
	13/06/2019	1450.67	18/06/2019	0	0	0	0
7	18/06/2019	5180.95	24/06/2019	17	2	0	15
	24/06/2019	5180.95					
				01/07/2019	8	2	0
Total				606	80	16	491
				686		507	

## B. Laboratory trials on the effects of trehalose on FCM

### *Critical thermal limits*

Adult FCM reared on a standard diet as larvae had a statistically significantly higher  $CT_{min}$  of  $3.93 \pm 0.17^{\circ}C$  (SE) than those reared on a trehalose augmented diet, which exhibited a  $CT_{min}$  of  $3.30 \pm 0.17^{\circ}C$  (SE) ( $t_{(58)} = 2.59$ ,  $P = 0.01$ ) (Figure 3.2.3.4). Both sets of adults ranged in  $CT_{min}$  from a minimum of  $2.00^{\circ}C$  to a maximum of  $5.00^{\circ}C$ , although both the mode and median were  $4.00^{\circ}C$  for the adults of larvae fed on a standard diet, but both  $3.00^{\circ}C$  for those reared on a trehalose augmented diet.



**Figure 3.2.3.4.** The critical thermal minima (CT<sub>min</sub>s) of *Thaumatotibia leucotreta* adults reared on either a standard or trehalose augmented diet as larvae. Letters indicate statistically significant differences.

*Biological parameters*

Developmental rate

The average developmental rate overall increased slightly as the rate of trehalose increased, however this did not negatively affect the FCM for further testing (Table 3.2.3.3).

**Table 3.2.3.3.** The average developmental rates for FCM reared on three different concentrations of trehalose augmented and standard artificial diet.

Treatment	Average days		
	Rep 1	Rep 2	Rep 3
Standard	26.78	29.37	33.15
1 X	25.07	29.6	32.96
2 X	26.90	32.67	36.35
3 X	29.00	34.30	38.78

Pupal weight and fecundity

The average pupal weight for those reared on trehalose was less than that of the average weight of pupae reared on the standard artificial FCM diet (Table 3.2.3.4). Additionally, there were no significant differences recorded in the fecundity and percentage hatch of eggs with trehalose treated FCM compared to the standard FCM (Table 3.2.3.5).

**Table 3.2.3.4.** Average weight (mg) for male and female FCM moths on the three different concentrations of trehalose augmented and standard diet

Treatment	Average weight (mg)							
	Rep 1		Rep 2		Rep 3		Overall Average	
	Male	Female	Male	Female	Male	Female	Male	Female
Standard	18.77	25.43	21.54	24.43	27.24	34.44	22.51	28.10
1 X	18.71	22.91	22.21	26.65	24.68	29.40	21.87	26.32
2 X	18.49	22.14	18.74	22.19	25.43	28.19	20.88	24.17
3 X	19.04	22.41	20.80	23.58	25.08	30.14	21.64	25.37

**Table 3.2.3.5.** The average percentage fecundity of FCM on the three different concentrations of trehalose augmented and standard diet

<b>Average Percentage Egg Hatch</b>				
<b>Treatment</b>	<b>Rep 1</b>	<b>Rep 2</b>	<b>Rep 3</b>	<b>Average</b>
<b>Standard</b>	86.74	90.06	91.10	89.30
<b>1 X</b>	86.71	91.48	91.32	89.84
<b>2 X</b>	89.24	95.19	89.99	91.47
<b>3 X</b>	81.66	92.08	92.77	88.83

Flight ability

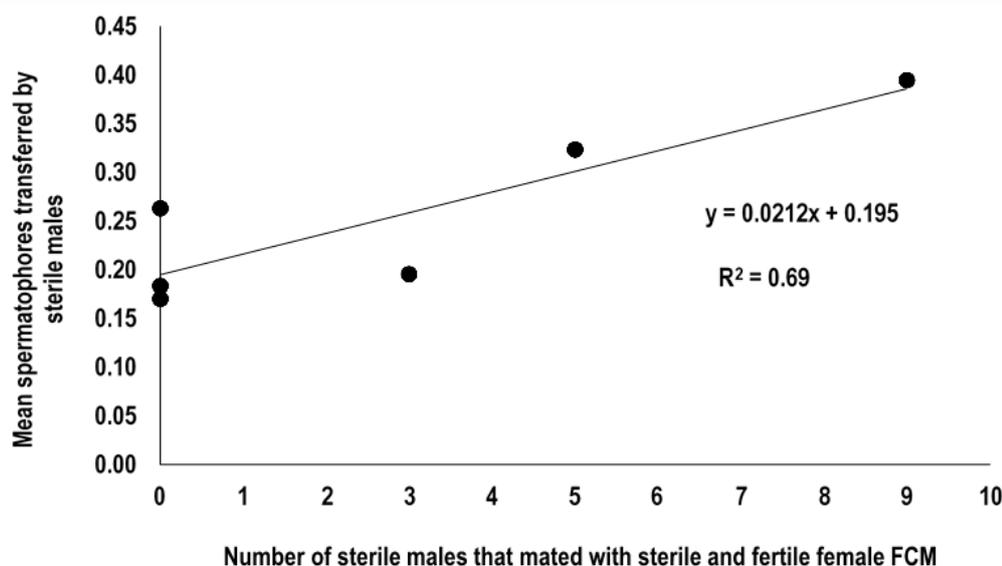
The flight ability of the FCM adults was improved when larvae were reared on artificial diet augmented with trehalose, especially at the lower temperature and at the highest concentration of trehalose (Table 3.2.3.6). Thus, trehalose would be a beneficial additive during the cooler months of the year.

**Table 3.2.3.6.** The average percentage flight ability of FCM on the three different concentrations of trehalose diet and standard diet

<b>Average Percentage flight</b>			
<b>Treatment</b>	<b>16°C</b>	<b>20°C</b>	<b>27°C</b>
<b>Standard</b>	28.33	85.00	86.67
<b>1 X</b>	26.67	76.67	76.67
<b>2 X</b>	25.00	81.67	96.67
<b>3 X</b>	41.67	68.33	95.00

**C. Development of QC test to measure mating competitiveness of sterile male moths**

Six replicates were completed for laboratory and field cage mating trials. Five of the six replicates showed ideal temperatures (above 20°C) for moth activity (Boersma *et al.* 2018). Mating couples of various combinations were observed after 19h30 and continued until 01h30. A statistically significant correlation was recorded between mean spermatophore transfer from sterile males to females (both fertile and sterile) and mating incidents involving sterile males in field cages ( $F = 12.13$ ;  $P = 0.025$ ) (Figure 3.2.3.5). The results obtained from this study confirms that spermatophore transfer can be used to measure the mating competitiveness of sterile males.



**Figure 3.2.3.5.** Mean number of spermatophores transferred by sterile *Thaumatotibia leucotreta* males compared to the number of sterile males that mated with wild and sterile females.

#### D. Anoxia for immobilisation of sterile insects

All moths were immobilised within a few seconds of anoxia. A further 15 seconds of nitrogen fumigation was applied. All moths appeared to fully recover thereafter. No further work was conducted with anoxia in their project. However, work with anoxia did continue within a separately registered project (1221).

#### E. Potential infestation by the F1 generation of released sterile moths

##### *Laboratory trials with various couplings of wild and sterile moths*

Coupling laboratory trials showed that wild x wild crosses oviposited a higher number of eggs than the other crosses; the majority of these eggs hatched (Table 3.2.3.7 and 3.2.3.8). From initial observation the SM x WF pupae that were collected were mostly males and eggs oviposited from these male couples were sterile with the exception of one or two couples. All crosses except for SM x SF made it through to the F2 generation. It must be noted that the quality of the sterile moths improved during the course of the experiment, as the percentage egg hatch from sterile crosses reduced from the start of the experiment.

**Table 3.2.3.7.** Oviposition results for all combinations of wild and sterile moths for two generations and comparison to original studies completed by Bloem *et al.* 2001

Average Oviposition					
Results from 200 Gy	Present study				Bloem et al
	Rep 1	Rep 2	Rep 3	Average	
<b>P1</b>					
WM x WF	237.43	215.1	357.13	269.89	0
WM x SF	146.07	23.03	118.67	95.92	400
SM x WF	232.67	208.2	318.87	253.25	180
SM x SF	140.4	29.93	120.8	97.04	220
<b>F1</b>					
WM x WF	260.71	215.03	380.8	285.51	0
WM x SF	224.55	183.4	280	229.32	180
SM x WF	163.89	231.85	246.57	214.10	400

<b>F1 SM x F1 SF</b>	0	0	0	0.00	200
<b>F2</b>					
<b>WM x WF</b>	260.71	362.9	288.7	304.10	0
<b>WM x SF</b>	334.5	380.27	359.07	357.95	0
<b>SM x WF</b>	306.09	436.27	221.37	321.24	0
<b>SM x SF</b>	0	0	0	0.00	0

**Table 3.2.3.8.** Percentage egg hatch results for all combinations of wild and sterile moths for two generations and comparison to original studies completed by Bloem *et al.* 2001

<b>% EGG HATCH</b>					
Results from 200 Gy	Present study				Bloem et al
	Rep 1	Rep 2	Rep 3	Average	
<b>P1</b>					
<b>WM x WF</b>	96.04	96.08	97.37	96.50	NA
<b>WM x SF</b>	2.38	1.59	2.25	2.07	0
<b>SM x WF</b>	23.99	28.66	39.19	30.61	24
<b>SM x SF</b>	0	0	0	0.00	0
<b>F1</b>					
<b>WM x WF</b>	86.04	95.08	93.3	91.47	NA
<b>WM x SF</b>	66.63	81.03	95.83	81.16	0
<b>SM x WF</b>	2.73	63.27	6.44	24.15	0
<b>SM x SF</b>	0	0	0	0.00	0
<b>F2</b>					
<b>WM x WF</b>	86.04	93.25	89.6	89.63	NA
<b>WM x SF</b>	84.31	90.55	92.12	88.99	NA
<b>SM x WF</b>	89.13	95.57	85.62	90.11	NA
<b>SM x SF</b>	0	0	0	0.00	NA

*Laboratory cage trials with various couplings of wild and sterile moths with Navel oranges*

Results from the laboratory cage trails indicate that the number of eggs oviposited on oranges by WM x WF is higher compared to SF x WM and SM x WF (Table 3.2.3.9). Number of eggs oviposited by WM x WF was significantly more than SF x WM. There is no significant difference between SM x WF and the other two couplings (One way ANOVA  $F(2, 42) = 4.7466, p = 0.1385$ ). Penetrations holes were considerably higher for WM x WF compared to the other two couplings. The results show that penetration holes were significantly different between all treatments (Kruskal-Wallis test  $H(2, N = 45) = 37.73140, p = 0$ ). The number of larvae found in the oranges was once again higher in the WM x WF crossing compared to the other two. The number of larvae found in oranges was significantly different between WM x WF and SF X WM; WM x WF and SM x WF, but not significantly different between SF x WM and SM x WF (Kruskal-Wallis test  $H(2, N = 45) = 30.11739, p = 0$ ). There were no significant differences observed in spermatophore transfer for all couplings.

**Table 3.2.3.9.** Results obtained from laboratory cage trials with Navel oranges.

		Number of Eggs	Penetration	Number of larvae	Spermatophore counts
SF x WM	Rep 1	58.22	0.00	0.12	2.20
SF x WM	Rep 2	12.22	0.02	0.04	0.72
SF x WM	Rep 3	20.24	0.00	0.04	0.80
SM x WF	Rep 1	87.72	4.10	1.42	1.24
SM x WF	Rep 2	32.04	0.58	0.08	0.80
SM x WF	Rep 3	17.76	0.12	0.00	0.64
WM x WF	Rep 1	103.90	26.56	13.38	1.32
WM x WF	Rep 2	58.14	11.22	3.66	1.36
WM x WF	Rep 3	34.76	8.90	3.74	1.08

#### Genetic finger print

Restriction endonuclease analysis was attempted on test samples, however the enzymes did not cut the DNA. Thus, RENs will not be used for genetic fingerprinting but AFLPs will be used to determine a difference between populations. Test samples for wild, Xsit and Xsit x wild larvae have been completed and the results are currently being analysed. If samples give a positive indication between populations. AFLPs were also used to create a genetic 'fingerprint', however this method proved to be unreliable in clearly separating the different categories. Nonetheless an alternative method is under investigation in order to create the genetic 'fingerprint'.

#### Conclusion

Trehalose was tested in a large scale field trial in 2018 and 2019 as a cryoprotectant or flight fuel in order to determine if FCM flight, measured by recaptures, was improved. A higher number of trehalose moths were recaptured compared to control moths. The trehalose moths also appeared to remain in the area longer or fly further than control moths. Further laboratory studies were completed to determine the effect of trehalose on the moths' fitness. The results from the critical thermal limits show that moths reared on trehalose had a lower  $CT_{min}$ , than moths reared on the standard diet. Changes in the biological parameters were also observed.

A laboratory trial, investigating the transfer of spermatophores from sterile and non-sterile male moths to the bursa copulatrix of female moths, indicated that sterile males are mating competitively with non-sterile males. Semi-field cage and laboratory cage trials confirmed the mating competitiveness of sterile males and the spermatophore transfer method used in this study is an ideal quality control method.

With regards to the genetic 'fingerprint' to determine the fertility of the F1 generation, several genetic methods were tested however no reliable results were obtained. Thus, we have formed a collaboration with researchers at Stellenbosch University in order to identify a more suitable method. However, it is important to note that a sterile F1 generation can only occur in the field if wild female are indeed present and if this does occur, it will always be at a dramatically reduced level relative to the F1 generation from a wild-wild pairing.

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### 3.2.4 FINAL REPORT: Improving baculovirus virulence against the false codling moth by repeated passage and virus combinations.

Project 1199 (2019 – 2020/21) by D Taylor, PP Iita, M Jukes, C Knox, M Hill (Rhodes University), and S Moore (CRI)

## Baculovirus synergism (D Taylor)

### Summary

CrleGV-SA-based biopesticides have long been used as part of an IPM strategy for the control of *T. leucotreta* in citrus orchards in South Africa. This study investigated whether a synergistic interaction could improve baculovirus-based management of *T. leucotreta*. The interaction between CrleGV-SA and CrpeNPV was first investigated in terms of lethal concentration. This was calculated using 7-day surface-dose bioassays for each virus and a 1:1 mixture of OBs against *T. leucotreta* neonates. The mixed infection performed significantly better than either virus by itself, while each virus by itself did not differ significantly from the other. The LC<sub>50</sub> for CrleGV-SA, CrpeNPV and the mixed infection were  $1.53 \times 10^4$ ,  $1.15 \times 10^4$ , and  $4.38 \times 10^3$  OBs/mL, respectively. The LC<sub>90</sub> of CrleGV-SA, CrpeNPV and the mixed infection were calculated to be  $4.10 \times 10^5$ ,  $1.05 \times 10^5$ , and  $4.09 \times 10^4$  OBs/mL, respectively. The second aspect between CrleGV-SA and CrpeNPV that was investigated was the speed of kill, using time-response bioassays. Each virus by itself did not differ significantly from the other, while the mixed infection took significantly longer to kill 50 % and 90 % of the larvae, suggesting competition for resources between viruses during the systemic phase of infection. The LT<sub>50</sub> for CrleGV-SA, CrpeNPV and the mixed infection were 117.5, 113.5, and 139.0 hours respectively. The LT<sub>90</sub> for CrleGV-SA, CrpeNPV and the mixed infection were 153.2, 159.3, and 193.4 hours respectively. A multiplex PCR analysis was done on viruses recovered from the time-response bioassays. The presence of both CrleGV-SA and CrpeNPV in larvae inoculated with only one of the two viruses suggests either stock contamination or covert infections of CrleGV-SA and CrpeNPV in the *T. leucotreta* population used in this study.

### Opsomming

Biologiese-plaagdoders wat op CrleGV-SA gebaseer is, word lank reeds gebruik as deel van 'n IPM-strategie vir die bestryding van *T. leucotreta* in sitrusboorde in Suid-Afrika. In hierdie studie is ondersoek ingestel of 'n sinergistiese interaksie die bakulovirus-gebaseerde bestuur van *T. leucotreta* kan verbeter. Die interaksie tussen CrleGV-SA en CrpeNPV is eers ondersoek in terme van dodelike konsentrasie. Dit is bereken deur gebruik te maak van 7-dae oppervlaktedosis biotoetse vir elke virus en 'n 1:1 mengsel van OBs teen *T. leucotreta* pasuitgeborede larwes. Die gemengde infeksie het aansienlik beter gevaar as enige virus alleen, terwyl elke virus op sy eie nie noemenswaardig van die ander verskil het nie. Die LC<sub>50</sub> vir CrleGV-SA, CrpeNPV en die gemengde infeksie was onderskeidelik  $1,53 \times 10^4$ ,  $1,15 \times 10^4$  en  $4,38 \times 10^3$  OBs/ml. Die LC<sub>90</sub> van CrleGV-SA, CrpeNPV en die gemengde infeksie is onderskeidelik bereken op  $4,10 \times 10^5$ ,  $1,05 \times 10^5$  en  $4,09 \times 10^4$  OBs/ml. Die tweede aspek tussen CrleGV-SA en CrpeNPV wat ondersoek is, was die spoed van mortaliteit, deur gebruik te maak van tydresponso-biotoetse. Elke virus op sy eie het nie betekenisvol van die ander verskil nie, terwyl die gemengde infeksie aansienlik langer geneem het om 50% en 90% van die larwes dood te maak. Die LT<sub>50</sub> vir CrleGV-SA, CrpeNPV en die gemengde infeksie was onderskeidelik 117,5, 113,5 en 139,0 ure. Die LT<sub>90</sub> vir CrleGV-SA, CrpeNPV en die gemengde infeksie was onderskeidelik 153,2, 159,3 en 193,4 ure. 'n Multiplex PCR-analise is gedoen op virusse wat herwin is uit die tydresponso-biotoetse. Die aanwesigheid van beide CrleGV-SA en CrpeNPV in larwes wat slegs met een van die twee virusse geënt is, is of 'n aanduiding van kontaminasie in die inentings monster of 'n bedekte infeksies van CrleGV-SA en CrpeNPV in die *T. leucotreta* populasie wat in hierdie studie gebruik is.

## Improved virulence (P Iita)

### Summary

CrleGV-SA has been successful at reducing *T. leucotreta* populations in the field for almost two decades. There is however, a growing need for additional baculovirus variants. CrpeNPV offers a unique opportunity for an additional biopesticide in IPM systems for control of *T. leucotreta*. This study investigated the repeated passage of CrpeNPV through a heterologous host, *T. leucotreta*, in order to determine the potential for improved virulence or speed of kill. A high dose of  $1.6 \times 10^4$  OBs/ml was used to perform 12 repeated passages through *T. leucotreta* larvae in surface-dose bioassays. The biological activity of the passaged virus was evaluated against neonate *T. leucotreta* in surface-dose bioassays. Results showed that the virulence of CrpeNPV did not improve after 12 passages. The LC<sub>50</sub> values were estimated at  $1.96 \times 10^4$  and  $1.58 \times 10^4$

OBs/ml and the LC<sub>90</sub> values were estimated at  $3.46 \times 10^4$  and  $3.68 \times 10^4$  OBs/ml for the passaged and wildtype viruses respectively. Results from time-response bioassays showed the speed of kill of CrpeNPV did not improve after 12 passages. The LT<sub>50</sub> values were 88.44 and 83.74 hours, whereas the LT<sub>90</sub> values were 115 hours and 102 hours for the passaged and wildtype viruses respectively. Whole genome sequencing and *in silico* REN profiling was performed to identify genetic changes. These analyses indicated that the genotype of CrpeNPV was maintained following 12 passages. The results from this study suggest that CrpeNPV may already be optimally suited to the heterologous host *T. leucotreta* as it persists under these conditions without significant changes to the genome. These results have positive implications for the genetic integrity of CrpeNPV as a potential biocontrol agent in the field.

## Opsomming

CrleGV-SA is al amper twee dekades suksesvol gebruik in die vermindering van *T. leucotreta*-bevolkings in die veld. Daar is egter 'n toenemende behoefte aan addisionele bakulovirus-variante. CrpeNPV bied 'n unieke geleentheid vir 'n addisionele biologiese-plaagdoder in IPM-stelsels vir die beheer van *T. leucotreta*. Hierdie studie het die herhaalde deurgang van CrpeNPV deur 'n heterologiese gasheer, *T. leucotreta*, ondersoek om die potensiaal vir verbeterde virulensie of spoed van mortaliteit te bepaal. 'n Hoë dosis van  $1,6 \times 10^4$  OBs/ml is gebruik om 12 keer deur *T. leucotreta*-larwes in biotoetse in oppervlak-dosis deur te voer. Die biologiese aktiwiteit van die deurgevoerde virus is geëvalueer teen die pastuitgebroeide *T. leucotreta* in oppervlak-dosis biotoetse. Resultate het getoon dat die virulensie van CrpeNPV na 12 deurvoerings nie verbeter het nie. Die LC<sub>50</sub> waardes is geskat op  $1,96 \times 10^4$  en  $1,58 \times 10^4$  OBs/ml en die LC<sub>90</sub> waardes is onderskeidelik geskat op  $3,46 \times 10^4$  en  $3,68 \times 10^4$  OB / ml. Resultate van tydrespons biotoetse het getoon dat die spoed van doodmaak van CrpeNPV nie verbeter het na 12 deurvoerings nie. Die LT<sub>50</sub>-waardes was 88,44 en 83,74 ure, terwyl die LT<sub>90</sub>-waardes onderskeidelik 115 en 102 ure was vir die deurgevoerde- en wildtipe-virusse. Volledige genoom sekvensering en *in silico* REN-profilering is uitgevoer om genetiese veranderinge te identifiseer. Hierdie ontledings het aangedui dat die genotype van CrpeNPV na 12 deurvoerings behou is. Die resultate van hierdie studie dui daarop dat CrpeNPV alreeds optimaal geskik is vir die heterologiese gasheer *T. leucotreta*, aangesien dit onder hierdie toestande voortduur sonder dat die genoom betekenisvol verander nie. Hierdie resultate het positiewe implikasies vir die genetiese integriteit van CrpeNPV as 'n potensieële biologiese beheermiddel in die veld.

## Introduction

Baculoviruses have been used in the field for control of insect pests since the 1960s (e.g., Ignoffo and Couch 1981; Tanada and Kaya 1993; Cunningham 1995; Moscardi 1999; Szewczyk *et al.* 2006, 2009). *Cryptophlebia leucotreta* granulovirus (CrleGV-SA) has been used to control *T. leucotreta* in citrus orchards for 15 years as part of an integrated pest management (IPM) system (Moore *et al.* 2015). Although this is the only baculovirus registered for use against *T. leucotreta* in citrus orchards, *Cryptophlebia peltastica* nucleopolyhedrovirus also infects and kills *T. leucotreta* (Marsberg *et al.* 2017). Not only is this an alternative to CrleGV, but it provides the opportunity to test a mixture of baculoviruses to determine whether they can work synergistically to be more effective against *T. leucotreta*. Several studies have shown that baculovirus mixtures lead to improved virulence (e.g., increased speed of kill) or have synergistic effects compared to a single virus in isolation (Arrizubieta *et al.* 2015, Biedma *et al.* 2015). This also seems to be the case with CrleGV and CrpeNPV. For example, initial tests with mixtures of CrleGV and CrpeNPV in laboratory bioassays significantly reduced the occlusion body (OB) concentration required to kill 90 % of *T. leucotreta* neonates (Jukes *et al.*, 2017). However, speed of kill was not investigated in this study. Should a combination of viruses lead to a change in lethal concentration or speed of kill, a more effective baculovirus-based biopesticide for the control of *T. leucotreta* could be developed. Furthermore, mixtures of viruses with improved efficacy could prove to be an effective management tool should host resistance to CrleGV arise in the field. The first part of this study extended previous studies by Jukes *et al.*, (2017) to investigate the virulence and speed of kill of different mixtures of CrleGV-SA and CrpeNPV against *T. leucotreta* in laboratory bioassays. The ultimate goal is to determine whether baculovirus mixtures, as opposed to individual virus, should be considered as an active ingredient in future biopesticides to provide improved control of *T. leucotreta* in citrus orchards.

A second approach to potentially improve the efficacy of a biopesticide is to passage the virus repeatedly through a heterologous host. For example, the Mexican isolate of the *Cydia pomonella* granulovirus (CpGV-M) has demonstrated some moderate efficacy against the oriental fruit moth, *Grapholita molesta* (Busck) (Graillot *et al.*, 2016). However, to obtain a more effective virus for *G. molesta* control, a combination of genotypically distinct CpGV isolates was passaged through larvae of a *G. molesta* laboratory culture. After 12 successive passages, the insecticidal efficacy of the virus population improved significantly. The concentration of virus OBs required to kill 90% of neonate larvae was 450-fold lower than that of the original isolate mixture, and 120-fold lower than that of the CpGV-M isolate alone. Following adaptation to this heterologous host (*G. molesta*), the efficacy against its homologous host, codling moth, *C. pomonella*, was conserved. This mixed isolate population can be produced in codling moth without loss of efficacy, which is useful from a commercial production perspective (Graillot *et al.*, 2016).

Although the above study used prepared genotypic mixtures for passage experiments, it is known that wild type baculoviruses, and in particular NPVs, isolated from field insect populations can exist in multiple genotypes (Erlandson, 2009). Likewise, since CrpeNPV is also thought to consist of mixed genotypes, it is possible that repeated passages through the heterologous host, *T. leucotreta*, could also lead to changes in virulence or infectivity. Consequently, the second part of this study is to determine whether the virulence of CrpeNPV can potentially be improved against *T. leucotreta* by repeated passaging through this heterologous host, the homologous host being the litchi moth, *C. peltastica*.

For more comprehensive and detailed reports on the two studies, see the MSc theses of David Taylor and Petrus Iita, available through Rhodes University.

## **Stated objectives**

### **Objectives (Baculovirus synergism; D Taylor)**

- A. Literature review and experimental design
- B. Determine the LC<sub>50</sub> and LC<sub>90</sub> of each virus in isolation against *T. leucotreta* by 7-day surface-dose bioassays.
- C. Determine the LC<sub>50</sub> and LC<sub>90</sub> of each virus mixture against *T. leucotreta* by 7-day surface-dose bioassays.
- D. Determine the LT<sub>50</sub> and LT<sub>90</sub> of each virus in isolation and 50:50 mixtures against *T. leucotreta* by surface-dose time-response bioassays.
- E. Quantify any differences in the LT<sub>50</sub> and LT<sub>90</sub> of virus mixture when compared to viruses in isolation.
- F. Determine which viruses are present in cadavers collected after each treatment by multiplex PCR.

### **Objectives (improved virulence by repeated passage; P Iita)**

- A. Literature review and experimental design
- B. Determine whether virulence of CrpeNPV can be improved by repeated passage in the heterologous host (in this case *T. leucotreta*)
- C. Analyse the genomes of CrpeNPV and determine genetic differences between the stock virus and virus recovered after repeated passage.

## **Materials and methods**

### **Baculovirus synergism (D Taylor)**

1. Determining and comparing lethal concentrations of baculovirus mixtures (covering objectives B & C).

The protocol used by Jukes (2017) will be followed. Lethal concentrations of virus mixtures will be determined by surface-dose bioassays performed in 24-well trays with artificial diet. Viral mixtures that will be tested against *T. leucotreta* are shown in Table 3.2.4.1. The bioassays will use neonates and run for 7 days after which mortality will be determined. Bioassays will be done in triplicate and analysed using a probit model. The dose-response curves of the viral mixtures will then be compared to the dose-response curves of the single

viruses to determine whether the viruses can work synergistically to reduce the concentrations required to kill the pests.

**Table 3.2.4.1.** Virus mixtures to be tested against *T. leucotreta*.

Mixture	Virus composition (%)	
	CrleGV-SA	CrpeNPV
1	100	0
2	0	100
3	50	50

2. Determining and comparing the speed of kill of baculovirus mixtures (covering objectives D & E).

The protocol described by Marsberg (2016) will be used to determine the time taken for the mixtures to kill 50% and 90% of the larvae. Surface-dose time-response bioassays will be done on artificial diet in glass vials using the LC<sub>90</sub> of each mixture described in Table 3.2.4.1. A single neonate will be placed into each vial and vials will be checked for mortality every eight hours until no more mortality is observed. The diet will then be dissected to search for any remaining live larvae. The time-response bioassays will be done in triplicate and the time-response will be analysed using a logit regression to determine the LT<sub>50</sub> and LT<sub>90</sub> of each mixture. The time-response curves will be compared to determine whether the viruses can work synergistically to increase the speed of kill.

3. Molecular comparison of virus stock and recovered virus (covering objective F).

Cadavers from each time-response bioassay will be collected and stored. OBs will be collected from these cadavers and DNA will be extracted from these OBs. Multiplex PCR will be used to determine which viruses are present in the cadavers of each treatment.

### Improved virulence (*P lita*)

1. Analysis of improved virulence.

To determine whether virulence of CrpeNPV can be improved by repeated passage in *T. leucotreta*, a virulence selection protocol was used based on Graillet *et al.* (2016). The virus was passaged 12 times through 3<sup>rd</sup>/4<sup>th</sup> instar *T. leucotreta* obtained fresh at each passage. After each passage, the first symptomatic larvae (alive or dead) were collected and stored. Viral OBs were then extracted from these larvae at each passage and used for the following passage. At the end of the experiment, the virus obtained from the final passage was evaluated by bioassay in comparison to the original stock virus to determine LC<sub>50</sub> and LT<sub>50</sub>.

2. Genetic characterisation of passaged virus.

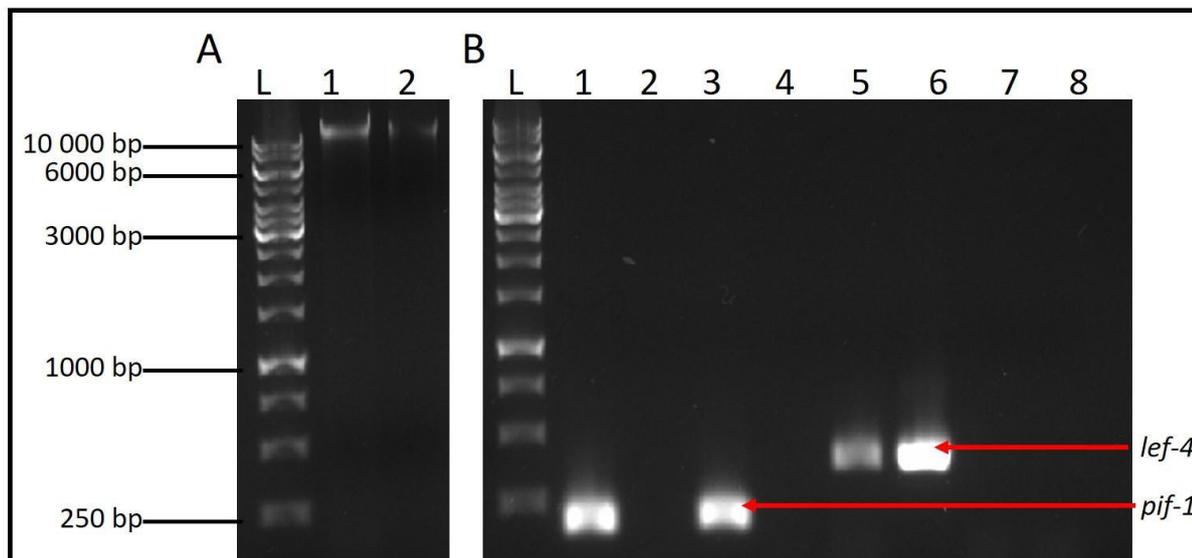
Since CrpeNPV is a wild-type virus, it consists of several genotypes within the isolate. The selective process during continued passage of virus may alter the combination of genotypes, selecting for certain and against others. This could result in genetic changes in the virus population. Consequently, if any change in virulence were to be recorded, isolates could be characterised by restriction endonuclease (REN) analysis and whole genome sequencing (by next generation sequencing (NGS) and compared the original virus.

## Results and discussion

### Baculovirus synergism (D Taylor)

1. Virus Stock Screening:

Following agarose gel electrophoresis (AGE), the DNA extractions from both CrpeNPV and CrleGV-SA stocks produced bands of greater than 10 000 bp (Figure 3.2.4.1A). The *pif-1* PCR product of CrpeNPV created a band of 170-200 bp while the *pif-1* PCR product of CrleGV-SA showed no sequence had been amplified. The *-pif-1* positive control showed a PCR product of 170-200 bp and the *pif-1* negative control showed that no sequence had been amplified. The *lef-4* amplicon of CrleGV-SA was 350-450 bp while the *lef-4* reaction of CrpeNPV produced no amplicon. The positive control of *lef-4* was shown to have a sequence length of 350-450 bp. The negative control of *lef-4* produced no bands after AGE (Figure 3.2.4.1B). This confirmed that the virus stocks were pure as far as is possible to determine by PCR, and that each stock contained the desired virus.



**Figure 3.2.4.1.** AGE of the initial screening of the CrleGV-SA and CrpeNPV virus stocks. The abbreviation bp denotes base pairs. Gel A, Lane L: Generuler 1kb ladder. Lane 1: Genomic DNA from the CrpeNPV stock. Lane 2: Genomic DNA from the CrleGV-SA stock. Gel B, Lane L: GeneRuler 1kb ladder. Lane 1: CrpeNPV *pif-1*. Lane 2: CrleGV-SA *pif-1*. Lane 3: *pif-1* positive control. Lane 4: *pif-1* negative control. Lane 5: CrleGV-SA *lef-4*. Lane 6: *lef-4* positive control. Lane 7: CrpeNPV *lef-4*. Lane 8: *lef-4* negative control.

## 2. Dose-response biological assays:

Following a probit analysis of the data generated by the dose-response biological assays, the LC<sub>50</sub> and LC<sub>90</sub> doses for CrleGV-SA against *T. leucotreta* were determined to be  $1.53 \times 10^4$  and  $4.10 \times 10^5$  OBs/mL respectively, being the least virulent of the three treatments (Table 3.2.4.2). CrpeNPV was the second most virulent treatment with the LC<sub>50</sub> and LC<sub>90</sub> doses being  $1.15 \times 10^4$  and  $1.05 \times 10^5$  OBs/mL respectively (Table 3.2.4.2). The most virulent treatment was the 1:1 mixture of CrleGV-SA and CrpeNPV with the LC<sub>50</sub> and LC<sub>90</sub> values being  $4.38 \times 10^3$  and  $4.19 \times 10^4$  OBs/mL respectively (Table 3.2.4.2).

**Table 3.2.4.2.** Lethal concentrations of CrleGV, CrpeNPV and the mixed inoculation against *T. leucotreta* for seven-day surface dose biological assays. SE = Standard error.

	CrleGV		CrpeNPV		50/50 GVNPV	
	Concentration (OBs/mL)	SE	Concentration (OBs/mL)	SE	Concentration (OBs/mL)	SE
<b>LC<sub>50</sub></b>	$1.53 \times 10^4$	$3.021 \times 10^3$	$1.15 \times 10^4$	$1.46 \times 10^3$	$4.38 \times 10^3$	$6.36 \times 10^2$
<b>LC<sub>90</sub></b>	$4.10 \times 10^5$	$1.57 \times 10^5$	$1.05 \times 10^5$	$2.22 \times 10^4$	$4.19 \times 10^4$	$8.53 \times 10^3$

The ANOVA revealed that there was at least one significant difference among the treatments (Df = 4, LR = 52.87,  $p < 0.0001$ ). The EDcomp function revealed that the mixed infection had a significantly lower LC<sub>50</sub> than both CrleGV-SA ( $t = 2.91$ ,  $p = 0.0036$ ) and CrpeNPV ( $t = -8.40$ ,  $p < 0.0001$ ). However, the LC<sub>50</sub> values for

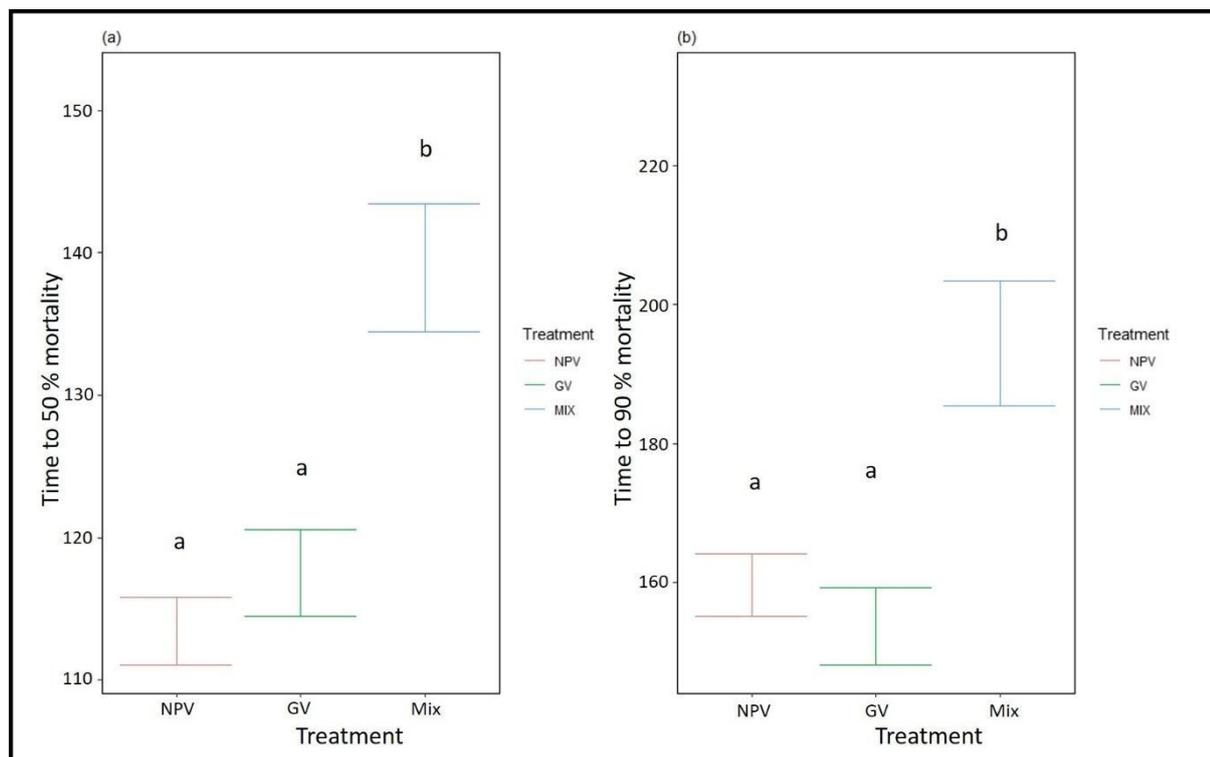
CrleGV-SA and CrpeNPV did not differ significantly from each other ( $t = 1.06$ ,  $p = 0.288$ ). The  $LC_{90}$  value of the mixed infection was also significantly lower than the  $LC_{90}$  values of both CrleGV-SA ( $t = 2.07$ ,  $p = 0.038$ ) and CrpeNPV ( $t = -5.08$ ,  $p < 0.0001$ ), but the  $LC_{90}$  values of CrleGV-SA and CrpeNPV did not differ significantly ( $t = 1.60$ ,  $p = 0.110$ ).

### 3. Time-response biological assays:

The purpose of this experiment was to determine and compare the  $LT_{50}$  and  $LT_{90}$  of CrleGV-SA, CrpeNPV and a 1:1 mixture of CrleGV-SA and CrpeNPV OBs when used on *T. leucotreta* neonates. Surface-dose time-response biological assays were performed on *T. leucotreta* using the  $LC_{90}$  values reported in chapter 3 (Table 3.2.4.4.1). A logit GLM was then used to calculate and compare the  $LT_{50}$  and  $LT_{90}$  values among the three treatments.

A Wald's chi squared test with type ii sum of squares revealed that both time ( $\chi^2 = 578.98$ ,  $df = 1$ ,  $P < 0.001$ ) and treatment ( $\chi^2 = 16.28$ ,  $df = 2$ ,  $P < 0.001$ ) were significant predictors of mortality.

The logit regression model showed that virus with the fastest speed of kill at the 50 % mortality point was CrpeNPV with a  $LT_{50}$  of 113.5 hours, followed closely by CrleGV-SA with a  $LT_{50}$  of 117.5 hours, although this difference was not statistically significant (Figure 3.2.4.2). At the point of 90 % mortality, the fastest-killing treatment was CrleGV-SA with a  $LT_{90}$  of 153.2 hours, followed closely by CrpeNPV with a  $LT_{90}$  of 159.3 hours, although once again, this difference was not statistically significant (Figure 3.2.4.2). The slowest-killing treatment at both the points of 50 % mortality and 90 % mortality was the mixture, with a  $LT_{50}$  of 139.0 hours and a  $LT_{90}$  of 193.4 hours. Both times are statistically significantly longer than both the  $LT_{50}$  and  $LT_{90}$  of CrleGV-SA, and the  $LT_{50}$  and  $LT_{90}$  of CrpeNPV (Figure 3.2.4.2).



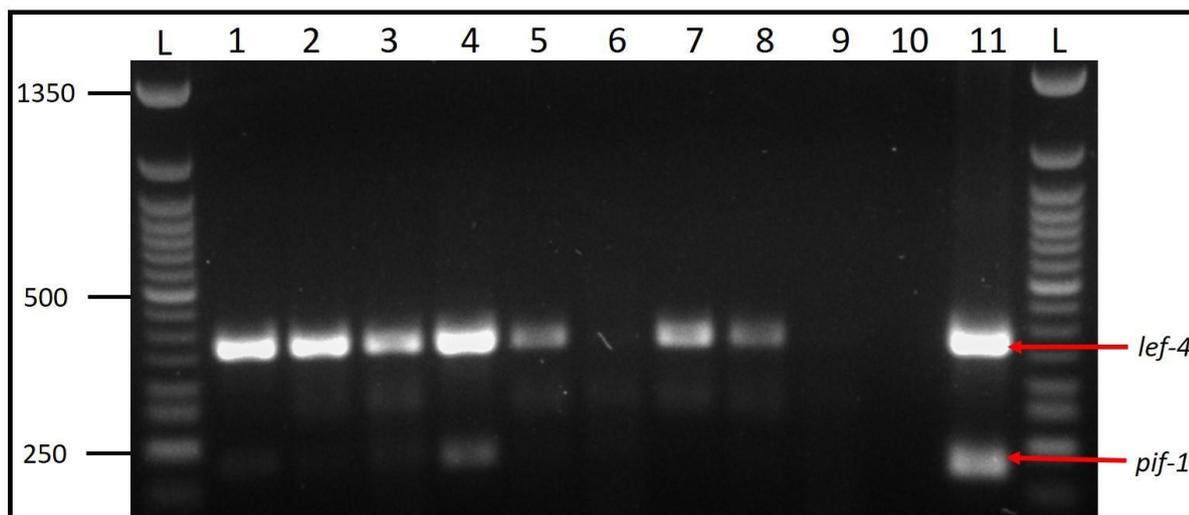
**Figure 3.2.4.2.** Confidence intervals ( $\alpha = 0.05$ ) for the  $LT_{50}$  (left) and  $LT_{90}$  (right) of CrleGV-SA (green), CrpeNPV (red) and a mixed infection of CrleGV-SA and CrpeNPV (blue) on *T. leucotreta* neonates. Different letters denote statistically significant differences.

### 4. Molecular screening:

A multiplex PCR reaction described by Jukes (2018) was performed to determine which viruses were present in the cadavers of each reaction following a DNA extraction using the modified CTAB extraction technique

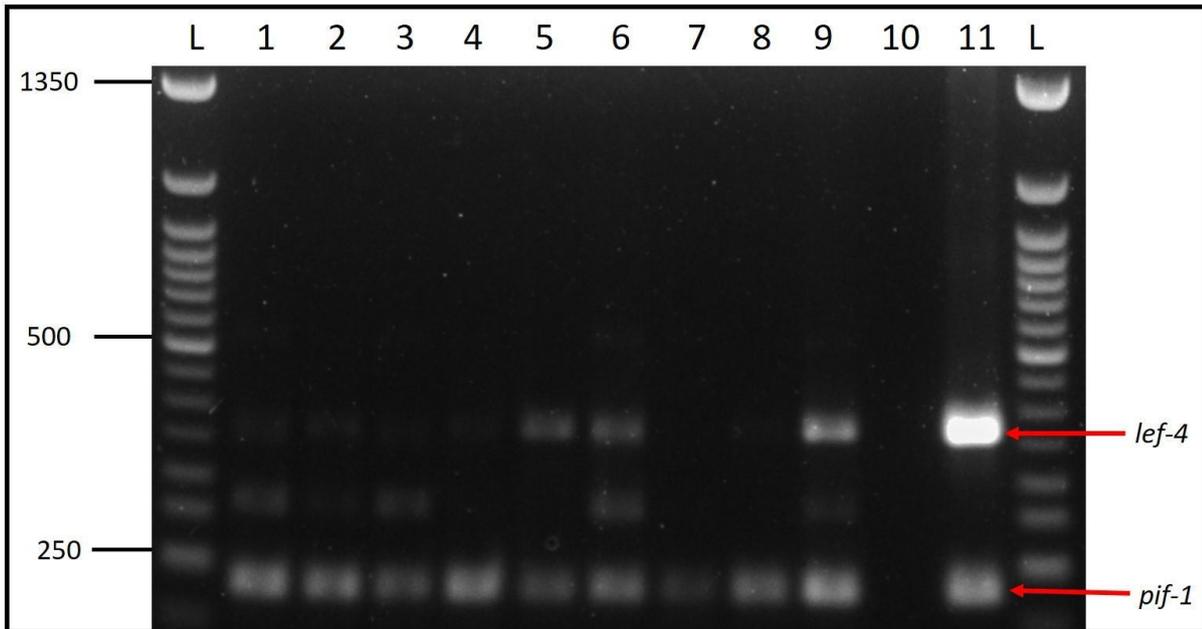
(Method 3) developed in section 5.2.1. A 187 bp region of the *pif-1* gene of CrpeNPV and a 378 bp region of the *lef-4* gene of CrleGV-SA were screened to determine which viruses were present in each case.

Following PCR of the DNA extractions of the larvae treated with CrleGV-SA, 7 out of 9 showed *lef-4* bands (Figure 3.2.4.3). In 4 out of 9 cases, faint bands consistent with the expected size of the *pif-1* amplicon were present, suggesting the presence of CrpeNPV in the cadavers (Figure 3.2.4.3). Lanes 1-5, 7 and 8 showed amplicons consistent with *lef-4*, while lanes 9 and 10 showed no bands. Lane 6 showed a faint unidentified band of between 250-300 bp. In addition to the *lef-4* bands, lanes 1-4 also showed faint bands consistent with *pif-1* and lanes 2, 3, 5, 7 and 8 showed faint unidentified bands of between 250-300 bp (Figure 3.2.4.3). Lane 11 (positive control) showed bands of both *lef-4* and *pif-1*.



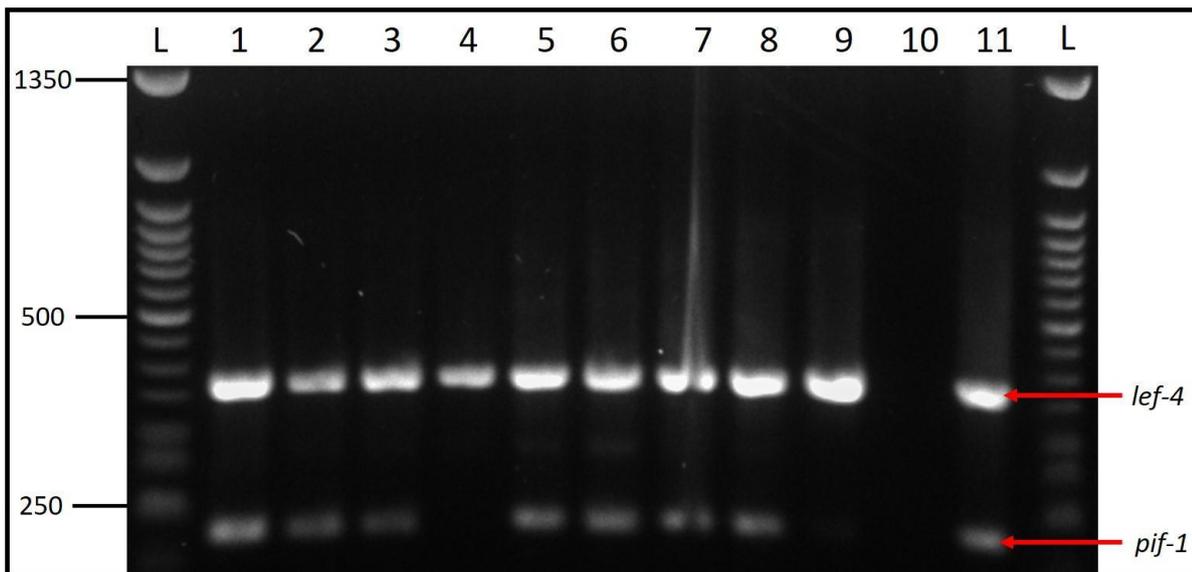
**Figure 3.2.4.3.** AGE with ethidium bromide staining of the mPCR amplicons resulting from gDNA extracted from larval cadavers inoculated with CrleGV-SA. Lanes 1 to 9: amplicons from DNA extracted from cadavers from the CrleGV-SA time-response biological assays, lane 10: no-template control, lane 11: positive control, lanes L: NEB 50bp ladder.

The AGE of the PCR products of larvae treated with CrpeNPV showed that *pif-1* amplicons were present in all 9 cases (Figure 3.2.4.4). Faint *lef-4* bands appeared in 5 out of 9 cases, and bright bands of the same size appeared in 3 out of 9 cases, suggesting the presence of CrleGV-SA in 7 out of 9 cases. Lanes 1-9 showed *pif-1* band. Additionally, lanes 1, 2, 5, 6 and 9 showed *lef-4* bands, suggesting the expression of covert infections of CrleGV-SA. Lanes 1, 2, 3, 6 and 9 also showed faint unidentified bands of between 250-300 bp. Lane 10 (no-template control) showed no bands, while lane 11 (positive control) showed both *pif-1* and *lef-4* (Figure 3.2.4.4).



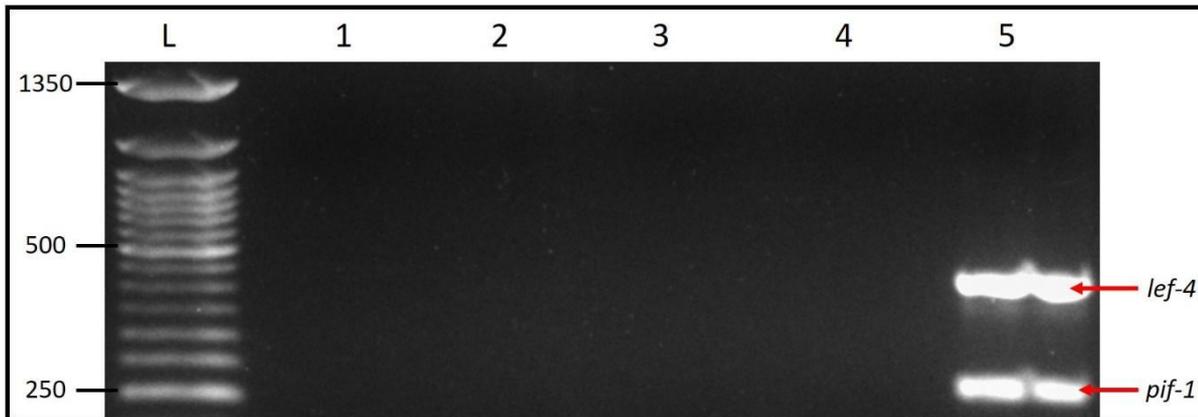
**Figure 3.2.4.4.** AGE with ethidium bromide staining of the mPCR amplicons resulting from gDNA extracted from larval cadavers inoculated with CrpeNPV. Lanes 1 to 9: amplicons from DNA extracted from cadavers from the CrpeNPV time-response biological assays, lane 10: no-template control, lane 11: positive control, lanes L: NEB 50bp ladder.

The AGE of the PCR products from the larvae treated with a 1:1 mixture of CrleGV-SA and CrpeNPV OBs produced *lef-4* bands in all 9 cases, and fainter *pif-1* bands in 8 out of 9 cases, suggesting the presence of both CrleGV-SA and CrpeNPV in 8 out of 9 cases and only CrleGV-SA in 1 out of 9 cases (Figure 3.2.4.5). In all cases the *lef-4* band was brighter than the *pif-1* band, suggesting that either CrleGV-SA becomes dominant over CrpeNPV in mixed infections or the DNA extraction methods or mPCR protocol favours one of the two viruses. Lanes 1-3 and 5-8 produced bands of both *lef-4* and *pif-1*, suggesting both CrleGV-SA and CrpeNPV were present in these cases. Lanes 4 and 9 showed only *lef-4*, suggesting the presence of only CrleGV-SA. Lanes 5 and 6 showed additional faint unidentified bands of between 250-300 bp. Lane 10 (NTC) showed no bands, and lane 11 (positive control) showed both *lef-4* and *pif-1* (Figure 3.2.4.5).



**Figure 3.2.4.5.** AGE with ethidium bromide staining of the mPCR amplicons resulting from gDNA extracted from larval cadavers inoculated with a 1:1 mixture of CrleGV-SA and CrpeNPV. Lanes 1 to 9: amplicons from DNA extracted from cadavers from the mixed infection time-response biological assays, lane 10: no-template control, lane 11: positive control, lanes L: NEB 50bp ladder.

A faint unidentified band of between 250 and 300 bp appeared on all three gels (Figures 3.2.4.3, 3.2.4.4, 3.2.4.5). This band was present in 6 out of 9 cases on the CrleGV-SA gel, including in one lane where no other bands appeared (Figure 3.2.4.3). On the CrpeNPV gel, this band appeared in 5 out of 9 cases (Figure 3.2.4.4), and on the mixed infection gel this band appeared in 3 out of 9 instances (Figure 3.2.4.5). It was never present in the positive control and it was never present in the no-template control. Multiplex PCR using *T. leucotreta* gDNA as the template produced no bands, discounting the possibility that the primers were binding to genomic DNA from *T. leucotreta* or gut bacteria from *T. leucotreta* larvae (Figure 3.2.4.6). Lanes 1-3 (*T. leucotreta* gDNA) and lane 4 (NTC) produced no bands and lane 5 (positive control) showed both *lef-4* and *pif-1* (Figure 3.2.4.6).



**Figure 3.2.4.6.** Products from the mPCR using *T. leucotreta* gDNA as a template. Lanes 1 to 3: products of the PCR reaction containing *T. leucotreta* gDNA, lane 4: no-template control, lane 5: positive control, Lane L: NEB 50 bp ladder.

### Improved virulence (P lita)

#### 1. Dose-response bioassays:

Probit analysis of data obtained from surface dose-response bioassays of the original virus (termed CrpeNPVpx0) against neonate *T. leucotreta* indicated an LC<sub>50</sub> value of  $1.58 \times 10^4$  OBs/ml and LC<sub>90</sub> of  $3.68 \times 10^4$  OBs/ml with 95% confidence interval (Table 3.2.4.3).

**Table 3.2.4.3.** The LC<sub>50</sub> and LC<sub>90</sub> values of CrpeNPVpx0 against neonate *T. leucotreta*.

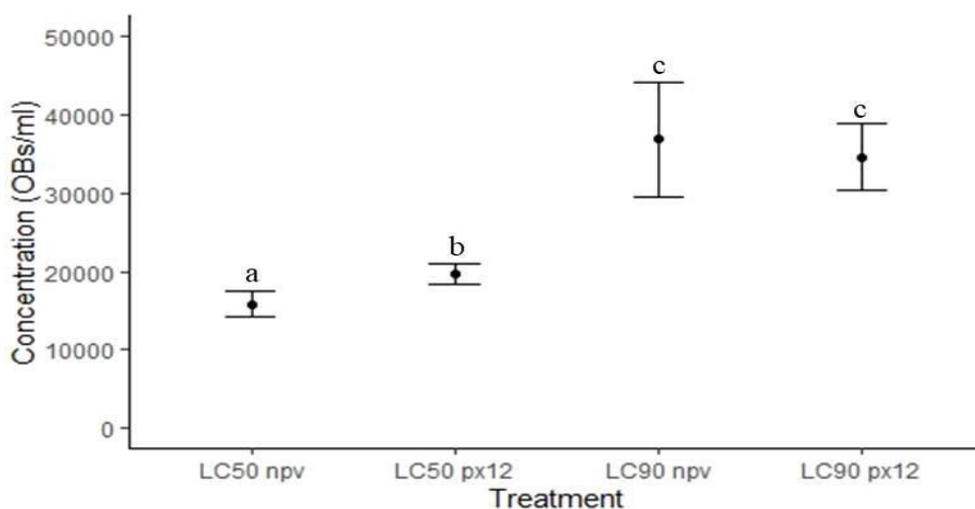
Lethal concentration	Concentration OBs/ml	Standard Error (SE)	95% Confidence limits	
			Lower SE	Upper SE
LC <sub>50</sub>	$1.58 \times 10^4$	824.04	$1.41 \times 10^4$	$1.74 \times 10^4$
LC <sub>90</sub>	$3.68 \times 10^4$	3735.6	$2.94 \times 10^4$	$4.4 \times 10^4$

Probit analysis of data obtained from surface dose-response bioassays of the passaged virus (termed CrpeNPVpx12) against neonate *T. leucotreta* indicated a LC<sub>50</sub> value of  $1.96 \times 10^4$  OBs/ml and LC<sub>90</sub> value of  $3.46 \times 10^4$  OBs/ml with 95% confidence interval (Table 3.2.4.4).

**Table 3.2.4.4.** The LC<sub>50</sub> and LC<sub>90</sub> values of CrpeNPVpx12 against neonate *T. leucotreta*.

Lethal concentration	Concentration OBs/ml	Standard Error (SE)	95% Confidence limits	
			Lower SE	Upper SE
LC <sub>50</sub>	$1.96 \times 10^4$	649.82	$1.83 \times 10^4$	$2.09 \times 10^4$
LC <sub>90</sub>	$3.46 \times 10^4$	2162.8	$3.03 \times 10^4$	$3.88 \times 10^4$

The ratios from probit analysis at LC<sub>50</sub> and LC<sub>90</sub> for CrpeNPVpx0 and CrpeNPVpx12 were compared. Results showed that there was no significant difference between the LC<sub>90</sub> for both viruses, whereas the LC<sub>50</sub> differed significantly (Figure 3.2.4.7). The virulence of CrpeNPVpx12 was significantly lower than that of CrpeNPVpx0.



**Figure 3.2.4.7.** Relationship between probit ratios of neonate *T. leucotreta* at LC<sub>50</sub> and LC<sub>90</sub> with CrpeNPVpx0 and CrpeNPVpx12. The LC<sub>50</sub>npv and LC<sub>90</sub>npv represent the LC<sub>50</sub> and LC<sub>90</sub> values for CrpeNPVpx0, whereas LC<sub>50</sub>px12 and LC<sub>90</sub>px12 represent the LC<sub>50</sub> and LC<sub>90</sub> values for CrpeNPVpx12, respectively. Error bars with the same letter indicate no significance, whereas error bars with different letters represent significant difference.

2. Time-response bioassays:

Three replicates of time-response bioassays of CrpeNPVpx0 and CrpeNPVpx12 against neonate *T. leucotreta* were each carried out using their respective LC<sub>90</sub> determined as inoculum to determine LT<sub>50</sub> and LT<sub>90</sub> of the virus. The recorded mortality for neonate *T. leucotreta* from time-response bioassays with CrpeNPVpx0 is shown in Table 3.2.4.5 for each replicate.

**Table 3.2.4.5.** Mortality for neonate *T. leucotreta* in time-response bioassays using the LC<sub>90</sub> of CrpeNPVpx0.

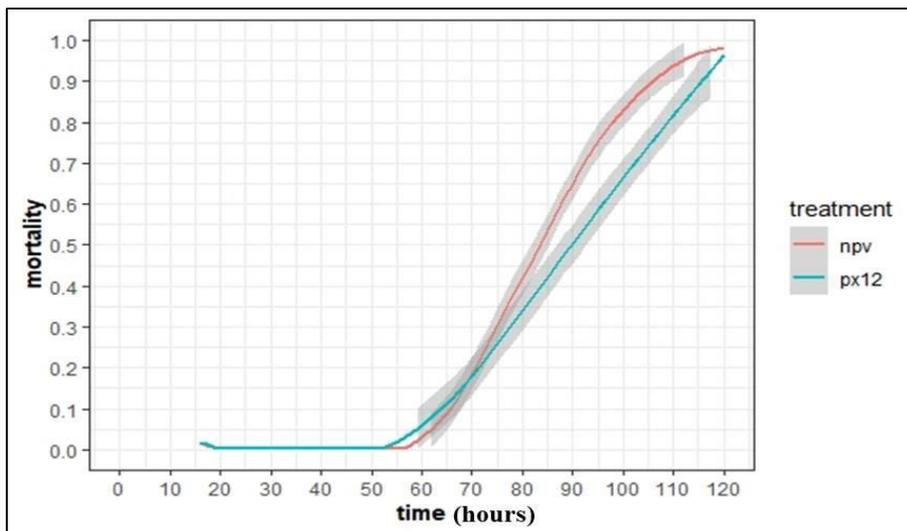
Time (Hours)	Mortality (%)		
	Replicate 1 (n = 50)	Replicate 2 (n = 50)	Replicate 3 (n = 50)
16	0	0	0
24	0	0	0
32	0	0	0
40	0	0	0
48	0	0	0
56	0	0	0
64	2	0	0
72	30	22	12
80	48	54	30
88	52	74	58
96	80	90	78
104	86	94	86
112	92	96	98
120	96	96	100
128	96	100	
136	100		

The first larval mortality was recorded 64 to 72 hours post treatment, and observations continued until 100% mortality was recorded for each replicate (Table 3.2.4.5). No mortality was recorded in the controls. Logit regression analysis of time-mortality relationship for CrpeNPVpx0 against neonate *T. leucotreta* showed the means for the three replicates as  $LT_{50} = 83.74$  hours (3 days 12 hours) and  $LT_{90} = 102$  hours (4 days 6 hours). The recorded mortality for neonate *T. leucotreta* from time-response bioassays with CrpeNPVpx12 is shown in Table 3.2.4.6 for each replicate.

**Table 3.2.4.6.** Mortality of neonate *T. leucotreta* in time-response bioassays using the  $LC_{90}$  of CrpeNPVpx12.

Time (Hours)	Mortality (%)		
	Replicate 1 (n = 49)	Replicate 2 (n = 50)	Replicate 3 (n = 50)
16	0	0	0
24	0	0	0
32	0	0	0
40	0	0	0
48	0	0	0
56	0	0	0
64	0	6	6
72	22	30	34
80	28	30	38
88	38	44	54
96	52	60	68
104	74	74	72
112	80	92	92
120	86	96	98
128	90	100	100
136	96		
144	96		
152	98		

The first larval mortality was recorded 72 hours post treatment in replicate 1 and continued until 98% mortality was recorded, as one larva failed to respond to the treatment and pupated, hence n = 49 (Table 3.2.4.6). Furthermore, the first larval mortality was recorded 64 hours post treatment in replicate 2 and 3, and observations continued until 100% mortality was recorded for each replicate (Table 3.2.4.6). No mortality was recorded in the controls. The time-mortality relationship for CrpeNPVpx12 against neonate *T. leucotreta* was analysed by logit regression. From the analysis, the means for the three replicates were calculated as  $LT_{50} = 88.44$  hours (3 days 16 hours) and  $LT_{90} = 115$  hours (4 days 19 hours). These results were compared to CrpeNPVpx0 using repeated measures ANOVA to determine whether the data differed significantly over the time intervals, with an interaction plot of the two treatments shown in Figure 3.2.4.8.



**Figure 3.2.4.8.** An interaction plot showing the relationship between CrpeNPVpx0 and CrpeNPVpx12 in time-response bioassays with neonate *T. leucotreta*. The shaded region represents 95% confidence interval. The keys, npv represents CrpeNPVpx0, whereas px12 represents CrpeNPVpx12.

The time series plot (Figure 3.2.4.8) showed an exponential increase in larval mortality for CrpeNPVpx12 between 56 and 72 hours in comparison to CrpeNPVpx0, indicating a potentially enhanced speed of kill of the virus at this point. However, ultimately the estimated  $LT_{50}$  and  $LT_{90}$  values for CrpeNPVpx12 were slightly, but significantly higher than that of CrpeNPVpx0, indicative of a decrease in the speed of kill.

### 3. Whole genome analysis:

A total of 229 544 reads were produced by next generation sequencing (NGS) of CrpeNPVpx12 gDNA. These reads were assembled in Geneious (version R11) (Biomatters Ltd, New Zealand) using the map to reference method. The full genome sequence of CrpeNPV (GenBank accession: MH394321.1) (Marsberg, *et al.*, 2018) was used as the assembly reference to reconstruct the original viral genome, with the assembly results shown in Table 3.2.4.7.

**Table 3.2.4.7.** Assembly results of CrpeNPVpx12 genome against the reference.

Length	Pairwise % identity	Coverage						
		Min	Max	Mean	St Dev	Q20	Q30	Q40
<b>115805</b>	99.8%	53	163	108.4	12.8	99.9%	99.8%	97.4%

As shown in Table 3.2.4.7., the NGS data assembled were of good quality, as indicated by the expected Q-value ( $Q > 97\%$ ). The reads mapped to the reference with a total length of 115805 bp and a 99.8% pairwise identity, and a mean coverage of 108.4. The resulting contig of the assembly was annotated, and SNPs were identified inside and outside of coding regions of the genome (Table 3.2.4.8).

**Table 3.2.4.8.** SNPs with gaps, identified on the mapped data of CrpeNPVpx12 against the reference CrpeNPV.

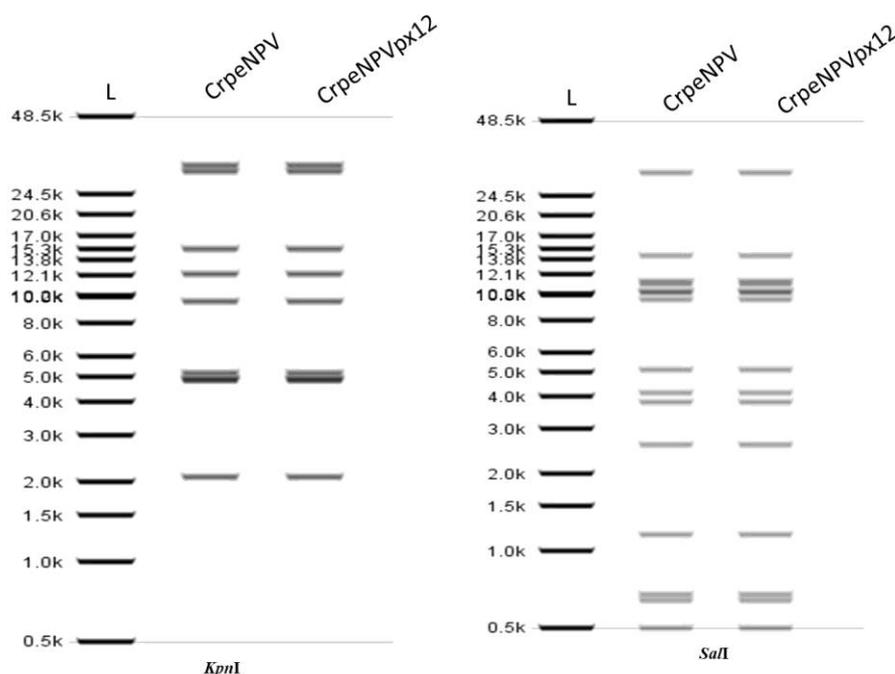
Polymorphism type	Amino acid change	Coding region	Protein effect	Length	Codon change
<b>Deletion</b>		ac26-like protein	Frame Shift	17	
<b>Insertion (tandem repeat)</b>				1	

<b>SNP (transition)</b>	Gly->Asp	dUTPase	Substitution	1	GGT->GAT
<b>SNP (transition)</b>		p87/vp80	None	1	TAC-> TAT
<b>SNP (transversion)</b>	Gly->Cys	p45/p48	Substitution	1	GGC->TGC

From the analysis (Table 3.2.4.8.), a significant change was the 17 bp deletion in the ac26-like protein gene, between 11262 and 11278 bp on the reference sequence, which resulted in a frame shift. This frame shift appeared to shorten the ac26-like ORF from 342 bp to 325 bp resulting in a shortening of the coded protein by 5 amino acids. A single nucleotide was inserted at position 5916 on the reference sequence, which had no change on the protein sequence. Another synonymous SNP occurred at position 77828 on the reference in the p87/vp80 gene. A non-synonymous SNP occurred at position 77206 on the reference sequence in the p45/p48 gene, which resulted in an amino acid substitution (Glycine for Cysteine). Another non-synonymous SNP occurred at position 81065 on the reference in the dUTPase gene, which resulted in an amino acid substitution (Glycine for Asparagine).

#### 4. Restriction endonuclease analysis (REN):

To further investigate potential genetic change in the genome of CrpeNPVpx12, *in silico* digestion of the assembled genome with several restriction enzymes was performed. As seen in representative profiles using *KpnI* and *SaII*, the banding pattern and fragment size were the same as the reference sequence, an indication that the genome had not changed. The results for this analysis are shown in Figure 3.2.4.9.



**Figure 3.2.4.9.** Schematic REN profiles of CrpeNPV and CrpeNPVpx12 genomes with *KpnI* and *SaII* digested *in silico*, with L representing a gene ruler.

## Conclusion

### Baculovirus synergism (D Taylor)

The primary aim of this study was to test for synergistic interactions between CrleGV-SA and CrpeNPV. Both dose-response and time-response surface-dose biological assays were conducted and showed that a mixed infection of the two viruses provided an improvement in the lethal concentration against *T. leucotreta* neonates but resulted in a deterioration in the speed of kill compared to either virus by itself. This provided further insight into the relationship and interactions between the two viruses in a mixed infection. These results may enable future investigation into the interaction between CrleGV-SA and CrpeNPV that could allow for the improvement of baculovirus-based biopesticides for the management of *T. leucotreta* in citrus orchards.

### Improved virulence (P Iita)

The overall aim of this study was to conduct repeated passaging of CrpeNPV through a heterologous host, *T. leucotreta*, to determine the potential for improved virulence or speed of kill against it. The biological assays of the virus recovered after passage, along with the original virus were conducted to evaluate the biological activity against *T. leucotreta* and enable comparison between the two viruses. Results from bioassays showed that the virulence and speed of kill of CrpeNPV did not improve after 12 repeated passages; however, CrpeNPV has demonstrated potential as a biological control agent for *T. leucotreta* management in the field. Potential genetic changes in the genome of the virus population during passage were also investigated, with only minor polymorphisms observed in the genome. These results may be fundamental to continued investigation into the effect of repeated passage on pathogenicity and genetic diversity of CrpeNPV.

### **Future research**

While this study reported that a mixed infection of CrleGV-SA and CrpeNPV in *T. leucotreta* larvae took significantly longer to kill the larvae than either virus alone, it may be possible to exploit the improved lethal concentration of the mixed infection while ensuring that competition between the viruses does not result in a decrease in the speed of kill. Firstly, the mechanism by which the mixed infection results in an improved lethal concentration must be established. This would involve the inspection of the peritrophic membranes of insects infected with both viruses as well as with each virus in isolation, and potentially larvae that have been fed with inactivated OBs of each virus. Similar studies have been done with other viruses and hosts. By using a combination of SDS-PAGE and scanning electron microscopy, Biedma *et al.* (2015) were able to determine that proteins embedded in the OBs of EpapGV were responsible for disrupting the peritrophic membrane of *A. gemmatalis*, causing an improvement in the lethal concentration of AngeMNPV. This would establish whether the improved lethal concentration is due to proteins embedded in the OBs disrupting the peritrophic membrane. If this is the case it would suggest that one of the viruses has a homologue of enhancin or gp37, or a novel protein with a similar function, embedded in the OB. A repeat of this study could be conducted using a mixed infection where one of the viruses is inactivated, thereby potentially supplying proteins to disrupt the peritrophic membrane without allowing competition between the viruses in the later stages of infection.

Similarly, a study should be conducted where a GV that produces enhancin or gp37 for which *T. leucotreta* is not a host is added to a formulation of either CrleGV-SA or CrpeNPV to provide peritrophic membrane-disrupting proteins without there being a possibility of viral competition. A similar study has been conducted on *A. gemmatalis* where the addition of EpapGV improved the efficacy of AngeMNPV without interfering with the reproduction of the virus in the later stages of infection (Biedma *et al.*, 2015).

A study should also be conducted to analyse any potential impacts of the *per os* infectivity factors (PIFs) between the two viruses. These proteins control the entry of the virion into the insect cells. (Boogaard 2020). Should the PIF proteins of the two viruses inhibit the entrance of the other virus into a cell, this could be used as a potential explanation for the slower speed of kill for the mixture over each virus by itself. If the PIFs of each virus do inhibit each other, it is possible that this effect could be mitigated by spraying the different viruses asynchronously to give one virus the time to begin infecting before another is introduced.

Once more is known about the interactions between CrleGV-SA and CrpeNPV in mixed infections and ways to mitigate competition between the two viruses have been investigated, field trials must be conducted to test whether the results obtained in this study are applicable in the field. Initial field trials (unpublished data) seem to indicate that a mixed infection and CrpeNPV alone are less effective than the current commercial CrleGV-SA-based products.

### **Technology transfer**

A one-page summary of results as of March 2020 was submitted to the 2020 Citrus Symposium. It was the intention that these results be presented in a talk, but due to the COVID-19 outbreak the conference was cancelled.

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### 3.2.5 FINAL REPORT: FCM population phenology in the warm northern citrus production regions and implications for management practices

Project 1227 (April 2019 – March 2021) by Hannah Otto, Angelique van der Grijp (InsecTec), Lezel Beetge (Noordchem) and Sean Moore (CRI)

#### Summary

Control measures for FCM have traditionally not been initiated earlier than November, an approach which has generally proved successful. However, the bulk of research conducted to determine the effectiveness of these management practices and the timing of applications, have been conducted in the cooler Cape regions. In these regions, FCM activity generally only starts to pick up to meaningful levels during November, when evening temperatures become sufficiently warm for FCM activity to increase. However, in the northern citrus production regions, particularly Limpopo Province, evening temperatures can reach these levels already in August. Consequently, initiation of control measures only in November is a lot later than ideal. This pertains particularly to the use of area-wide population suppression technologies, such as SIT and mating disruption, which need to be initiated very early in the season, while FCM levels are still very low, to achieve optimal efficacy. Consequently, a study was conducted to determine exactly when evening temperatures in the northern production regions increase to the moth's activity thresholds and when these increases in activity are first detected. Ten orchards were selected in the Letsitele and Hoedspruit regions and were monitored weekly for FCM moth catches in pheromone-baited traps. Simultaneously, temperature and humidity in these orchards were logged. Temperatures began to increase around mid-July, leading to an increase in FCM activity. Consequently, it may be necessary to initiate control measures, particularly semiochemical-based area-wide technologies, such as mating disruption, earlier in these warm northern citrus production regions, to achieve optimal suppression of FCM. A new project will now be initiated to test this.

#### Opsomming

Beheermaatreëls is tradisioneel nie vroeër as November vir VKM ingestel nie, 'n benadering wat oor die algemeen sukses getoon het. Die grootste deel van die navorsing wat gedoen is om die doeltreffendheid van hierdie bestuurspraktyke en die tydsberekening van toedienings te bepaal, is egter in die koeler Kaapse streke gedoen. In hierdie streke begin VKM-aktiwiteit meestal eers in November tot die betekenisvolle vlakke toe te neem, wanneer aandtemperatuur warm genoeg word vir verhoging in VKM-aktiwiteit. In die noordelike sitrusproduksiestreke, veral Limpopo Provinsie, kan die aandtemperatuur egter reeds in Augustus hierdie vlakke bereik. Gevolglik is die inisiëring van beheermaatreëls eers in November heelwat later as ideaal. Dit is veral belangrik vir die gebruik van areawye populasie onderdrukkings tegnologieë, soos SIT en paringsontwrigting, wat baie vroeg in die seisoen geïnisieer moet word, terwyl die VKM-vlakke nog baie laag is, om optimale effektiwiteit te verkry. Gevolglik is 'n studie onderneem om vas te stel presies wanneer die temperatuur in die noordelike produksiestreke tot die aktiwiteitsdrempelwaardes van die mot toeneem en wanneer die toename in aktiwiteit die eerste keer opgespoor kan word. Tien boorde is in die Letsitele- en Hoedspruit-streke gekies en is weekliks gemonitor vir VKM vangstes in feromone-lokvalle. Terselfdertyd is temperatuur en humiditeit in hierdie boorde aangeteken. Temperatuur begin om en by middel-Julie om te styg, wat tot 'n verhoging in VKM aktiwiteit lei. Gevolglik, is dit dalk nodig om beheermaatreëls, veral semiochemie-gebaseerde areawye tegnologieë, soos paringsontwrigting, vroeër in die seisoen te inisieer in hierdie warm noordelike streke, om optimale onderdrukking van VKM te bereik. 'n Nuwe projek sal nou voorgesit word om hierdie te toets.

#### Introduction

Control measures for FCM have traditionally not been initiated earlier than November, an approach which has generally proved successful. However, the bulk of research conducted to determine the effectiveness of these management practices and the timing of applications, have been conducted in the cooler Cape regions. In these regions, FCM activity generally only starts to pick up to meaningful levels during November, when evening temperatures become sufficiently warm for FCM activity to increase. However, in the northern citrus production regions, particularly Limpopo Province, evening temperatures can reach these levels already in August. Consequently, initiation of control measures only in November is a lot later than ideal.

The first and most important step in protecting citrus fruit against FCM attack, is to suppress the FCM population. This can only be effectively achieved, using area-wide technologies such as SIT and mating disruption, by initiating these practices very early in the season, while FCM levels are still very low. Allowing FCM population levels to increase before initiating these measures, will dramatically compromise their efficacy.

Consequently, it is imperative to determine exactly when evening temperatures in the northern production regions increase to the moth's activity thresholds and when these increases in activity are first detected. This will enable us to structure management practices specifically for these warm northern production areas.

This has become even more important than ever before, with the regulation of FCM by the EU and the objective of only exporting FCM-free fruit to the EU and other FCM-sensitive markets.

### Stated objectives

- To record temperatures in citrus orchards in Limpopo throughout the year and relate these to FCM activity thresholds
- To record FCM trap catches in citrus orchards in Limpopo throughout the year and to relate these to temperatures recorded
- To structure FCM management practices that are specifically appropriate for the warm northern citrus production regions.

### Materials and methods

Ten orchards in Limpopo, where mating disruption was not being used, were selected for the trial: seven in Letsitele and three in Hoedspruit. These consisted of Valencias (Turkeys, Bennies and Midnights) and grapefruit (Star Rubys). Temperature-humidity buttons were placed into the orchards in April 2019 at the same time as FCM pheromone traps were hung. If the farmer already had a trap in close proximity, the farmer's trap was removed (and our monitoring data provided to the farmer). The buttons were set to record every 30 min and data were downloaded periodically, at least every three month, as this was the maximum data capacity for the loggers at the chosen setting. Traps were read weekly and pheromone dispensers changed when necessary. Trap monitoring and temperature/humidity measuring were conducted for 24 consecutive months from April 2019 to April 2021.

**Table 3.2.5.1.** Details of trial sites in Letsitele and Hoedspruit.

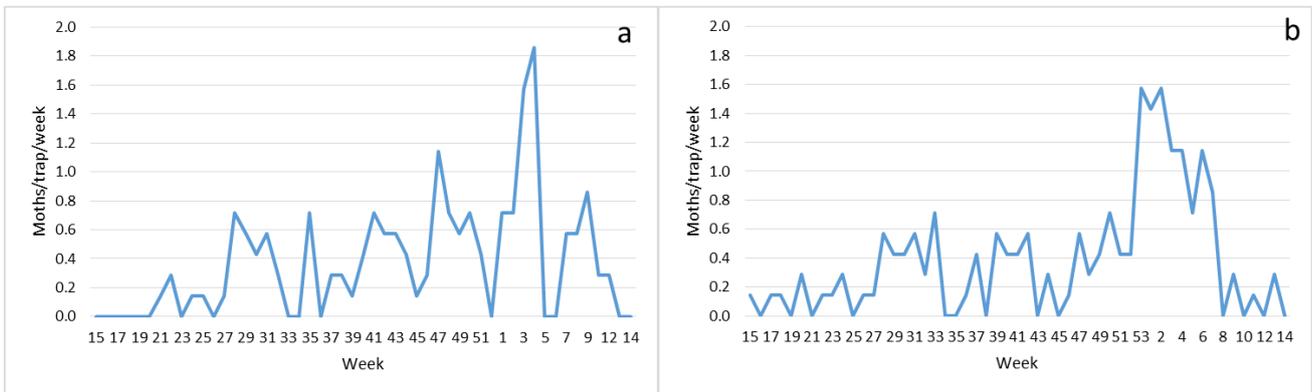
Region	Farm	Orchard number	Cultivar	GPS Coordinates
Letsitele	Muller Boerdery	Laborie 4, Block 7	Turkey	S23°46.219' E030°30.020
	Mahela	Block RU	Midnight	S23°51.655' E030°21.509'
	Min Bdy Nov	Block N	Bennie Valencia	S23°53.175' E030°23.764'
	Min Bdy LQ	Block H	Midnight	S23°51.944' E030°24.967'
	Beli Farm	Block K6	Turkeys	S23°53.627' E029°38.522'
	Muller Bdy 2	Labori 3	Star Ruby	S23.8304' E30.194'
	Bruboer	Block 1	Valencia	S23.8623' E30.4312'
Hoedspruit	T Landman 1	Block TU	Turkey	S24°25.504' E030°49.258'
	T Landman 2	Block R	Delta	S24°23.690' E030°49.939'

	Olifants Estate	River		Star Ruby	S24°22.022' E030°41.958'
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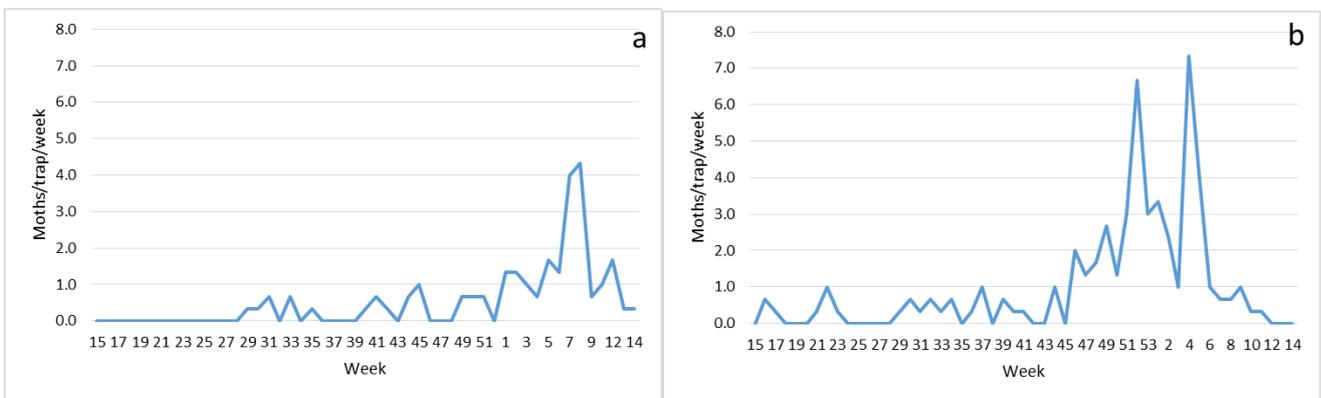
This enabled us to determine how early control measures for FCM should be initiated in these warm northern production regions and will enable us to structure a management programme specific to these regions, aimed at inducing permanent suppression of FCM populations in these regions.

**Results and discussion**

Moth catches were generally relatively low, particularly in the Letsitele area, with average moths caught per trap per week over a two-year period, never exceeding 1.86 (Figure 3.2.5.1). Catches were slightly higher in the Hoedspruit region, averaging 0.77 moths per trap per week over the 24 month period (Figure 3.2.5.2), as opposed to an average of 0.38 moths per trap per week in Letsitele. Catches peaked at an average of 7.33 moths per trap per week in Hoedspruit.

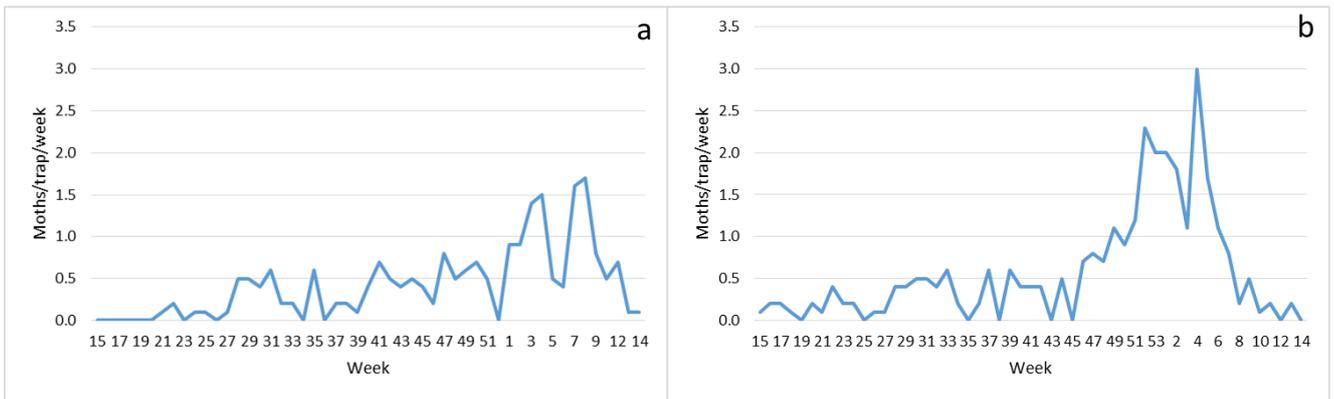


**Figure 3.2.5.1.** FCM caught per trap per week at seven sites in Letsitele from week 15 to week 14 in (a) 2019/2020, and (b) 2020/21.



**Figure 3.2.5.2.** FCM caught per trap per week at three sites in Hoedspruit from week 15 to week 14 in (a) 2019/2020, and (b) 2020/21.

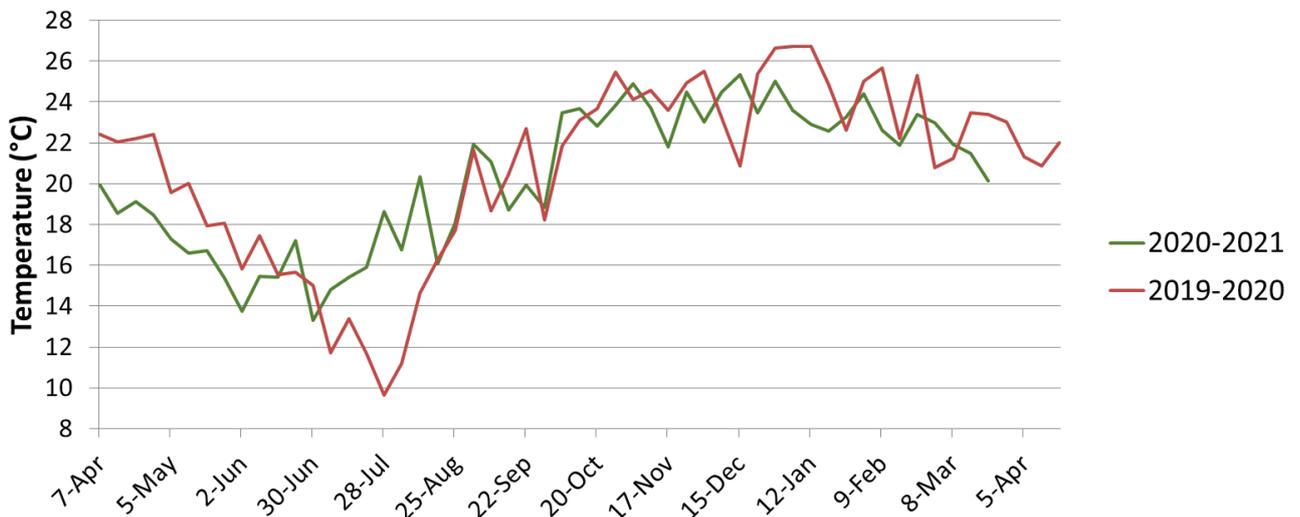
Combining the data from all 10 traps in the two regions, produced an average of 0.5 moths per trap per week over the full 24 month period, peaking at an average of 3 moths per trap per week (Figure 3.2.5.3)



**Figure 3.2.5.3.** FCM caught per trap per week at 10 sites in Letsitele and Hoedspruit from week 15 to week 14 in (a) 2019/2020, and (b) 2020/21.

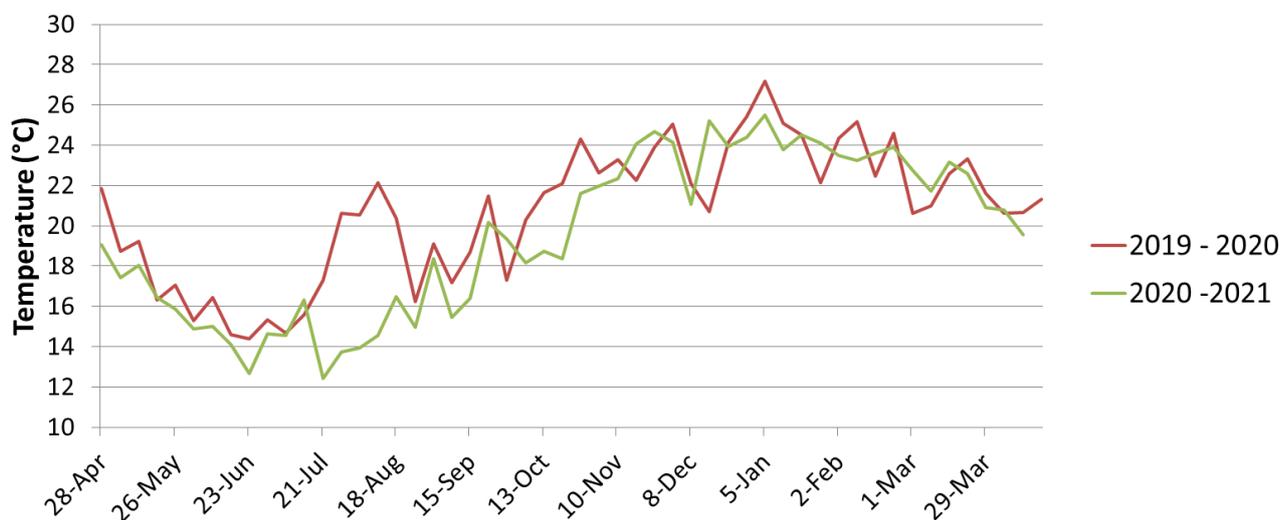
Over the two seasons, moth catches peaked between week 52 and week 8, thus the highest level of FCM activity being in the first two months of the year. Thereafter, there was a relative lull in FCM activity (starting from week 8 to week 13) for several weeks, with activity generally appearing to pick up again from about week 27.

The mean weekly temperature between 18h00 and 01h00, the main activity time of FCM, for April to April for both 2019/20 and 2020/21 for one of the sites in Letsitele (Mahela Block RU) is depicted in Figure 3.2.5.4. The lowest recorded temperature in 2019/20 was the week of 30 June, which is week 26 in the year. However, in 2020/21, the temperature continued to dip after this date, reaching a low four weeks later (28 July, being week 30). During 2019/20, temperatures showed a notable increase from the week of 21 July (week 29 in the year), whereas in 2020/21, this only occurred in the week of 18 August (week 33). The increase in temperature was sustained from these dates in both years.



**Figure 3.2.5.4.** Mean weekly temperatures recorded between 18h00 and 01h00 at one of the trial sites in Letsitele (Mahela Block RU) between 25 April and 24 April of the following year, for 2019-20 and 2020-21.

The mean weekly temperature between 18h00 and 01h00, the main activity time of FCM, for April to April for both 2019/20 and 2020/21 for one of the sites in Hoedspruit (T Landman Block TU) is depicted in Figure 3.2.5.5. The lowest recorded temperature in both years was the week of 23 June, which is week 25 in the year. Temperatures showed a notable increase from the week of 14 July (week 28 in the year). This was sustained in 2019-20, but temperatures dropped off again in 2020-21 and began warming again in mid-August (around week 32).



**Figure 3.2.5.5.** Mean weekly temperatures recorded between 18h00 and 01h00 at one of the trial sites in Hoedspruit (T Landman 1 Block TU) between 25 April and 24 April of the following year, for 2019-20 and 2020-21.

For both Letsitele and Hoedspruit, it is clear that this increase in temperature from about mid-July, coincided exactly with the increase in FCM activity after autumn. Assessments of the minimum temperature for flight in wild FCM, indicate somewhere between 10 and 15°C (Stotter and Terblanche, 2009). Around mid-July, temperatures during the normal flight times for FCM (measured between 18h00 and 01h00) would have begun to exceed 15°C with greater frequency. Consequently, temperatures in Limpopo Province at this time of the year, which we view as mid-winter, are not an impediment to FCM activity.

## Conclusion

Temperatures in the Letsitele and Hoedspruit regions of Limpopo Province begin to increase around mid-July, leading to an increase in FCM activity. Consequently, control measures, particularly semiochemical-based area-wide technologies, such as mating disruption, may historically have been initiated too late for optimal efficacy in the warm northern citrus production regions of the country. Initiation of mating disruption in early July in Limpopo, rather than only in October, as was ascertained as the appropriate timing for the cooler southern regions of the country, may not only suppress the FCM population sufficiently early in the season, but may also serve to protect late Valencias, from FCM attack shortly before harvest, a serious problem that has emerged in the last couple of seasons. A new project will now be initiated to test the efficacy of a mating disruption programme initiated early in July versus the conventional timing of only October.

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### 3.2.6 FINAL REPORT: Improvement of the quality of sterile moths for FCM SIT

Project 1221 (August 2020 – April 2021) by Liana De Araujo (XSIT), Craig Chambers (River Bioscience), Sean Moore (CRI)

#### Summary

The sterile insect technique (SIT) of *Thaumatotibia leucotreta* (false codling moth) was first implemented in South Africa in 2007. The SIT programme was considered successful within the Western and Eastern Cape, but moth quality and performance are factors that need to be regularly monitored to ensure the programme remains successful. Previous research has shown that fertility of irradiated moths is higher than originally determined in 2003. Two methods were tested in this project to try and explain the differences recorded in fertility data. The first experiment compared fertility and larval development between moths mated or not mated prior to irradiation. The second experiment compared the same parameters for moths that are active or inactive during irradiation. The results are not final yet as data analysis is still underway but basic table summaries have been provided in the meantime. Conclusions will be made thereafter.

#### Opsomming

Die steriele insektegniek (SIT) van *Thaumatotibia leucotreta* (valskodlingmot) is in 2007 vir die eerste keer in Suid-Afrika geïmplementeer. In die Wes en Oos-Kaap is die program as suksesvol beskou, alhoewel faktore soos motkwaliteit en vlugvermoë moet gereeld gemonitor en hersien word om te verseker dat die program suksesvol bly. Vorige navorsing het getoon dat die vrugbaarheid van die bestraalde motte hoër was as wat oorspronklik in 2003 bepaal is. Twee verskillende metodes was getoets om die verskille in die resultate te probeer verklaar. Die eerste eksperiment vergelyk vrugbaarheid en larweontwikkeling tussen motte wat voor bestraling gepaar is of nie gepaar is nie. Die tweede eksperiment het dieselfde parameters vergelyk vir motte wat aktief of onaktief was tydens bestraling. Die resultate is nog nie gefinaliseer nie, omrede data-ontleding nog moet plaasvind. Wanneer alle data verwerk is sal 'n gevolgtrekking gemaak word.

#### Introduction

The sterile insect technique (SIT) of *Thaumatotibia leucotreta* (false codling moth) was first implemented in South Africa in 2007 when the rearing facility was built (Hofmeyr *et al.* 2015). The SIT programme was considered successful within the Western and Eastern Cape (Nepgen 2014), but moth quality and performance are factors that need to be regularly monitored to ensure the programme remains successful. At XSIT (X-Sterile Insect Technique), the SIT procedure includes moths exposed to radiation at a given dose or Gray (Gy) to ensure effective sterility (Bloem *et al.* 2003) with minimal loss to moth quality (Boersma and Carpenter 2016). Moths are handled and transported cold to ensure ease of transport and radiation (Dowell *et al.* 2005) but also to eliminate the chance of mating and add to the assurance of high moth quality. The cold chain process includes the radiation step. With regular use, the radiation source weakens over time (half-life=63.26 months) (Hofmeyr *et al.* 2016), resulting in regular adjusted radiation times to acquire the required radiation Gray. Towards the maturation of the source, the radiation times are extensive enough to allow for moths to warm up and become active.

Work conducted within project 1164 indicated that fertility of the parental generation (P1) for irradiated moths, although still low, was higher than originally determined by Bloem *et al.* (2003). Although any F1 generation in the field from a sterile male-wild female crossing will always be far smaller than that of a wild-wild crossing, it is nonetheless important to determine and resolve the cause for potentially elevated fertility of irradiated moths. Whether activity during radiation has any effect on sterility is unknown and was therefore assessed in this project. It was also assessed whether the Gray (Gy) utilised for FCM sterility effectively sterilizes females that have been mated prior to radiation.

#### Stated objective

- To determine why irradiated moths and their F1 generation are not as sterile as previously determined and to find a solution to the problem.

## Materials and methods

The full experimental process was carried out at the XSIT facility. Pupae were removed from pupation boards, sorted by sex, and placed into individual pill vials to ensure no mating takes place. The pill vials were stored in a rearing room at  $26 \pm 2^\circ\text{C}$  with relative humidity (RH) of  $65 \pm 10\%$ , though the humidity within the vials exceeded 80%. The vials were monitored daily and removed from the selection of pill vials. When enough males and females eclosed on a single day, the experiment was initiated. For both experiments three pairings were used: fertile male and fertile female (FM x FF), sterile male and fertile female (SM x FF), and fertile male and sterile female (FM x SF). Fecundity and fertility of each pairing were measured, with 20 moth pairs of each combination.

### *Mating prior to radiation*

Half of the males and females were paired and left to mate for about 17 hours while the other half were kept in their individual vials to ensure no mating took place. After 17 hours, all moths were placed in a cold room at  $6 \pm 1^\circ\text{C}$  for 30 minutes for easy handling. A third of the mated females and males were removed and placed inside separate petri dishes for radiation (one petri dish for females, one petri dish for males). A third of the non-mated males and females were prepared for radiation in the same manner. While radiation was underway, the fertile males and fertile females were paired. After radiation, the SM x FF and FM x SF pairs were completed. All pairs were placed inside pill vials lined with wax paper for oviposition and plugged with damp cotton wool. The egg sheets were changed, and the cotton was dampened daily for 12 days. Eggs were allowed 7 days to hatch; thereafter total oviposition and hatch were counted and recorded. Fifty neonates were collected for each treatment pair and placed inside individual glass vials filled with Xsit's baked artificial diet to track development. These were stored in the same rearing room as the pill vials. The number of larvae to complete development until the adult stage was recorded.

### *Activity during radiation*

Half of the males and females were placed inside a cold room at  $6 \pm 1^\circ\text{C}$  for 1 hour (inactive) while the other half were kept in a rearing room at  $26 \pm 2^\circ\text{C}$  (active). A third of the males and females for both treatments were placed inside separate petri dishes for radiation (one petri dish for females, one petri dish for males). At the time of radiation, the active and inactive moths were handed over to the radiator. While radiation was underway, the fertile males and fertile females were paired. After radiation, the SM x FF and FM x SF pairs were completed. All pairs were placed inside pill vials lined with wax paper for oviposition and plugged with damp cotton wool. The egg sheets were changed, and the cotton was dampened daily for 12 days. Eggs were allowed 7 days to hatch; thereafter total oviposition and hatch were counted and recorded. Fifty neonates were collected for each treatment pair and placed inside individual glass vials filled with Xsit's baked artificial diet to track development. These were stored in the same rearing room as the pill vials. The number of larvae to complete development until the adult stage was recorded.

## Results and discussion

When determining the number of pupae needed to start the trial where irradiation of pre-mated and non-mated moths was compared, pre-trials indicated that one must start with at least 3.5 times more pupae than moths needed for the trial to ensure emergence of sufficient moth pairs on the same day (de Villiers et al 2019). For this trial however, 1000 male and 1000 female pupae were put aside to guarantee enough male and female eclosions on the same day.

For both the pre-mated and non-mated treatment, the FM x SF mating pairs had the lowest fertility across all replicates (Table 3.2.6.1). The highest percentage egg hatch was from the FM x FF pair with an average of 79.6% with the replicates pooled. The pair with the second highest percentage egg hatch for both treatments was SM x FF, with a pooled average of 55.4% for pre-mated and a lower 9.6% for the non-mated, indicating a clear difference in sterility between the treatments. The average percentage hatch for the pre-mated FM x SF pair was 0.91% and 0.49% for the non-mated, which may indicate a significantly reduced effect on sterility for

pre-mated females but would require statistical analysis to confirm. With the very low hatch of the FM x SF pair, it was difficult to find neonates and thus larval development wasn't measured. The average development to adult stage for pre-mated SM x FF was 46.9% and for non-mated, 39%. This data may be skewed due to outbreak of black mould in the vials of replicate 3 thus affecting the development. The completed development of the FM x FF pair was lower (average of 60.5%) than what was found in project 1221 (94%) (de Villiers et al 2019).

**Table 3.2.6.1.** Fecundity and fertility of FCM moths and larval development, comparing moths that were mated prior to irradiation with moths that did not mate.

Pairing	Pre-mated			Non-mated		
	Average number of eggs laid per moth	Percentage egg hatch	Percentage larvae to complete development	Average number of eggs laid per moth	Percentage egg hatch	Percentage larvae to complete development
Repetition 1						
FM x FF	390.65	82.22	52 (n=50)	311.17	80.72	82 (n=50)
SM x FF	403.84	53.78	64 (n=50)	373.95	7.50	46 (n=50)
FM x SF	179.75	0.48	-	227.42	0.49	-
Repetition 2						
FM x FF	462.26	86.17	48.89 (n=45)	484.65	88.90	45 (n=40)
SM x FF	354.05	62.37	38 (n=50)	477	7.86	32 (n=50)
FM x SF	220.65	1.93	-	275.25	0.86	-
Repetition 3						
FM x FF	317.45	85.75	68.57 (n=35)	375.33	88.47	66.67 (n=39)
SM x FF	320.16	50.03	38.64 (n=44)	387.53	13.39	38.89 (n=36)
FM x SF	195.55	0.31	-	184.44	0.11	-

The mating pair with the lowest fecundity for the moths active and inactive during radiation was FM x SF (averaging at 175.8 and 164 respectively) (Table 3.2.6.2). The mating pair with the highest average fecundity of 331.3 for active moths and 331.1 for inactive was SM x FF. The order of highest to lowest percentage egg hatch is the same as the aforementioned trial. The average hatch for active and inactive SM x FF was 24.4% and 20.4% respectively, which may not differ significantly. The average hatch for FM x SF for active and inactive was 0.8% and 0.4% respectively. The low hatch for FM x SF once again made the collection of neonates troublesome thus no larval development was recorded. The complete development for SM x FF was 37.9% for active moths and 39.7% for inactive moths.

**Table 3.2.6.2.** Fecundity and sterility of FCM moths, comparing moths that were inactive during irradiation with moths that were active during irradiation.

Pairing	Active			Inactive		
	Average number of eggs laid per moth	Percentage egg hatch	Percentage larvae to complete development	Average number of eggs laid per moth	Percentage egg hatch	Percentage larvae to complete development
Repetition 1						
FM x FF	243.67	85.85	57.89 (n=38)	226.05	85.23	62 (n=50)
SM x FF	257	23.13	35.71 (n=42)	254.28	13.59	26 (n=50)
FM x SF	141.67	0.46	-	150.74	0.83	-
Repetition 2						
FM x FF	350.68	86.29	70 (n=50)	434.11	89.08	74 (n=50)
SM x FF	399.83	16.97	20 (n=30)	475.11	14.47	37.21 (n=43)
FM x SF	231.95	1.28	-	213.35	0.13	-
Repetition 3						

FM x FF	315.75	90.57	76 (n=50)	284.82	81.99	74 (n=50)
SM x FF	337.11	33.19	58 (n=50)	264	33.15	56 (n=50)
FM x SF	153.71	0.59	-	127.89	0.34	-

## Conclusion

According to the results provided, males and females mated prior to radiation are not sufficiently sterilised. The effect of radiation on the fertility of the pre-mated males is much greater than for the pre-mated females. Activity or lack-there-of during radiation does not appear to affect sterility of the moths. However, all results must still be statistically analysed. It would be of interest to revisit the larval development for these trials due to the black mould contamination that potentially affected the outcome.

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### 3.2.7 FINAL REPORT: Identifying volatile emissions associated with false codling moth infestation of citrus fruit

Project 1090 (April 2014 – March 2021) by Wayne Kirkman, Sean Moore (CRI), Martin Hill, Rui Krause and Roman Tandlich (RU)

## Summary

Previous studies showed that a Solid Phase Micro-extraction (SPME) probe effectively trapped and concentrated headspace volatile compounds surrounding intact citrus fruit. Volatile compound detection was then achieved by using a Gas Chromatography-Mass Spectrometry (GC-MS) system. GC-MS analysis was conducted on five major volatile compounds of citrus: D-limonene, 3,7-dimethyl-1,3,6-octatriene, (E)-4,8-dimethyl-1,3,7-nonatriene, caryophyllene and naphthalene. In trials conducted in 2016 on infested Witkrans Navel oranges, D-limonene levels decreased with time after infestation, while levels of naphthalene increased. The ratio of these compounds was significantly different between healthy and infested fruit for all time periods. In similar trials conducted in 2017 these same trends were not observed, mainly due to variability in D-limonene levels as a result of extremely unusual climatic conditions in the Eastern Cape, which resulted in excessive splitting, fruit drop and scorching of fruit and trees. In the trials conducted in 2018 on infested Washington and Witkrans Navel oranges, as well as Midnight and Delta Valencia oranges, D-Limonene levels decreased significantly and naphthalene levels increased with time after infestation, and the ratio between the two

compounds (D-Limonene/naphthalene) was significantly lower than with healthy fruit. In Clementine mandarins there was a significant increase in beta-Ocimene levels with time after infestation, where levels were undetectable in the control fruit. In 2019 GC-MS trials were repeated on all the same cultivars as the previous year. In all cases, except for Clementine mandarins, D-Limonene levels decreased and naphthalene levels increased with time after infestation, and the ratio between the two compounds (D-Limonene/naphthalene) was significantly lower than with healthy fruit. In Clementine mandarins, as in 2018, there was a significant increase in beta-Ocimene levels with time after infestation. W Kirkman visited the University of California – Davis, to be trained on Differential Mobility Spectrometry (GC-DMS). Trials to detect *Phytophthora* in rhododendron plants were successful. The ability of an electronic nose from RoboScientific in Leeds to detect FCM infested fruit was investigated. Trials were conducted on Washington Navel oranges, infested 2, 6 and 10 days previously. The electronic nose could detect 70, 90 and 90 percent of infested fruit respectively for the three treatments, with 20% false positives. Similar results were recorded on Lane Late Navel oranges. The Selected Ion Flow Tube Mass Spectrometry (Sift-MS) unit situated at the University of Leuven in Belgium showed great promise to detect infested fruit in real time.

## Opsomming

Vorige studies het getoon dat 'n Soliede Fase Mikro-ekstraksie (SPME) kopruimte vlugtige stowwe wat skoon vrugte omring, effektief kan opvang en konsentreer. Opsporing van vlugtige verbindings is met 'n Gas Chromatografie-Massaspektrometrie (GC-MS) sisteem gedoen. GC-MS-analises is op vyf belangrike vlugtige verbindings van sitrus gedoen: D-limonien, 3,7-dimetiel-1,3,6-oktatrieen, (E)-4,8-dimetiel-1,3,7-nonatrieen, cariofeleen en naftaleen. In proewe wat in 2016 op besmette Witkrans Nawellemoene uitgevoer is, het die D-limonien vlakke met tyd na besmetting afgeneem, terwyl vlakke van naftaleen toegeneem het. Daar was ten alle tye 'n beduidende verskil in die verhouding van hierdie verbindings tussen gesonde en besmette vrugte. In soortgelyke proewe in 2017 is hierdie tendense nie waargeneem nie, hoofsaaklik weens die variasie in die D-limonien vlakke, wat veroorsaak is deur uiters ongewone klimaatstoestande wat gelei het tot grootskaalse vrugbars, vrugval en skroei van vrugte en bome. In proewe wat in 2018 op besmette Washington en Witkrans Nawellemoene, asook Midnight en Delta Valencia lemoene uitgevoer is, het D-limonien vlakke met tyd na besmetting betekenisvol afgeneem en naftaleen vlakke het toegeneem. Die verhouding tussen die twee verbindings (D-limonien/naftaleen) was ook betekenisvol laer as in onbesmette vrugte. In Clementine mandaryne was daar 'n betekenisvolle toename in beta-osimeen vlakke met tyd na besmetting met onopspoorbare vlakke in die kontroles. In 2019 is GC-MS proewe herhaal op dieselfde kultivars as die vorige jaar. In alle gevalle, behalwe Clementine mandaryne, het die D-limonien vlakke met tyd na besmetting afgeneem, terwyl vlakke van naftaleen toegeneem het. Daar was ten alle tye 'n beduidende verskil in die verhouding van hierdie verbindings in gesonde en besmette vrugte. In Clementine mandaryne was daar weereens 'n betekenisvolle toename in beta-osimeen vlakke met tyd na besmetting. W Kirkman het die Universiteit van Kalifornia-Davis besoek vir opleiding op hulle Differensiële Mobiliteitsspektrometrie (GC-DMS) eenheid. Proewe om fitofthora in rhododendron plante vanaf blaar vlugstowwe op te spoor was suksesvol. Proewe is gedoen om te kyk of 'n elektroniese neus van RoboScientific in Leeds tussen besmette en gesonde vrugte kon onderskei. Analise is gedoen op Washington Nawellemoene wat 2, 6 en 10 dae vroeër besmet is. Die elektroniese neus kon onderskeidelik 70, 90 en 90 persent van besmette vrugte uitken, met 20% valspositiewes. Soorgelyke resultate is met Lane Late nawellemoene gevind. Die Geselekteerde loon-vloei Buis-massaspektrometrie" (Sift-MS) eenheid van die Universiteit van Leuven in België se vermoë om VKM in sitrusvrugte op te spoor lyk baie belowend.

## Introduction

False codling moth (FCM), *Thaumatotibia leucotreta* (Meyr) (Lepidoptera: Tortricidae), is one of the most important pests on citrus, causing extensive financial losses to the southern African citrus industry (Moore *et al*, 2004; P. Hardman, CGA, personal communication). The European Union (EU), which is by far the largest market for South African citrus, has regulated FCM as a phytosanitary pest, and is increasingly demanding zero interceptions of live FCM larvae. Several effective pre-harvest control measures exist for FCM, but there is no 'silver bullet' which can ensure that no infested fruit reach the packhouse. Such control measures are inadequate for a pest for which there is zero tolerance. There is an urgent necessity to investigate methods for post-harvest detection of FCM. This study concentrated on the use of volatile emissions to differentiate

between FCM-infested and healthy citrus fruit. A Solid phase micro-extraction (SPME) probe has been shown to effectively trap as well as concentrate headspace volatile compounds surrounding intact fruit. Volatile compound detection is achieved by inserting this probe into a Gas Chromatograph-Mass Spectrometry (GC-MS) system (Van der Walt, 2012). SPME detection of volatiles emitted by fruit and differences in emission profiles between healthy and infested fruit have shown that volatile analysis has great potential as a post-harvest screening option (Van der Walt, 2012). Five major volatile compounds of interest were released by the infested oranges. These major volatile compounds are D-limonene, 3,7-dimethyl-1,3,6-octatriene, (E)-4,8-dimethyl-1,3,7-nonatriene, caryophyllene and naphthalene. Limonene was one of the most abundant volatile compounds released by the infested citrus fruit. Naphthalene, which is possibly produced due to larval feeding and development within the fruit, maintained higher concentrations than controls throughout the infestation within the fruit. Naphthalene would be a good indicator of FCM infestation, however, not primarily for early infestation detection. A significantly higher concentration of D-limonene, 3,7-dimethyl-1,3,6-octatriene, (E)-4,8-dimethyl-1,3,7-nonatriene and naphthalene was detected using the SEP over the SPME technique (Van der Walt, 2012). These results need to be verified, and processes refined, to avoid unwanted variables, and to build on the previous study. Attempts must be made to identify compounds unique to infested fruit, which could be detected instantaneously by other technologies, such as near infra-red spectroscopy. The ultimate aim is that this will lead to the development of a detection system for exposing infested citrus fruit post-harvest (packhouse line). In this project we therefore propose to build on the promising foundation laid by van der Walt (2012). The ability to detect FCM infested fruit was also evaluated for an electronic nose, Gas Chromatography – Differential Mobility Spectrometry (GC-DMS) and Selected Ion Flow Tube Mass Spectrometry (SIFT-MS).

## **Materials and methods**

### Gas Chromatography – Mass Spectrometry (GC-MS)

#### *Targeting compounds unique to FCM infested fruit.*

A series of trials were conducted in 2016 with the aim of finding compounds which are unique to FCM infested fruit. In all these trials, the fibre was exposed to the headspace in 250 ml bottles with a Polytetrafluoroethylene (PTFE) septum. Three larvae extracted from fruit harvested in an orchard of Palmer Navel oranges in the Sundays River Valley were analysed. Three larvae extracted from artificial diet, and diet alone were also analysed. These trials were conducted to determine the volatile profile of larvae.

Seemingly infested fruit were collected from the same orchard as in the previous trial. Healthy fruit were also collected. Segments of healthy and infested fruit were evaluated (the segments of infested fruit were cut at the point of infestation, and included some frass. All analyses were conducted using a 30 minute SPME exposure time at 20°C, and these trials were repeated three times.

#### *Analyses of whole fruit.*

In 2016, 25 fruit were labelled and each inoculated with six neonate FCM larvae 21 and 14 days before harvest, in an orchard of Witkrans Navel oranges in the Sundays River Valley. It was intended to inoculate similar amounts of fruit seven and two days before harvest, but this was not possible due to rain. These fruit were harvested, along with 10 seemingly infested fruit from trees in close proximity to the data trees, as well as 10 healthy fruit for a control. The fruit were carefully examined, and six fruit from each treatment were selected. These were placed in 2 L glass jars with a PTFE septum (Figure 3.2.7.1). The SPME fibre was exposed to the headspace for 30 minutes at 20°C for all treatments, and the volatile profile of each fruit was determined by GC-MS. The fruit were then dissected to confirm infestation and larval instars were recorded. The results were statistically analysed using ANOVA and multiple range tests (Statgraphics 2001).



**Figure 3.2.7.1.** Fruit in a 2 L glass jar with a PTFE septum.

In all the subsequent GC-MS trials, similar procedures were followed. In each trial, twenty-five fruit were labelled and each inoculated on the tree with six neonate FCM larvae at weekly intervals from 28 to 7 days before harvest. These fruit were harvested at maturity, along with 10 healthy fruit for a control. The fruit were carefully examined, and six fruit from each treatment were selected. These were placed in 2 L glass jars with a PTFE septum (Figure 3.2.7.1). The SPME fibre was exposed to the headspace for 30 minutes at 20°C for all treatments, and the volatile profile of each fruit was determined by GC-MS. The fruit were then dissected to confirm infestation and larval instars were recorded.

In 2017 GC-MS trials were conducted on Washington Navel oranges, Mor mandarins, as well as Midnight and Delta Valencia oranges.

In 2018, GC-MS trials were conducted on Clementine Mandarins, as well as Washington and Witkrans Navel oranges, and Midnight and Valencia oranges.

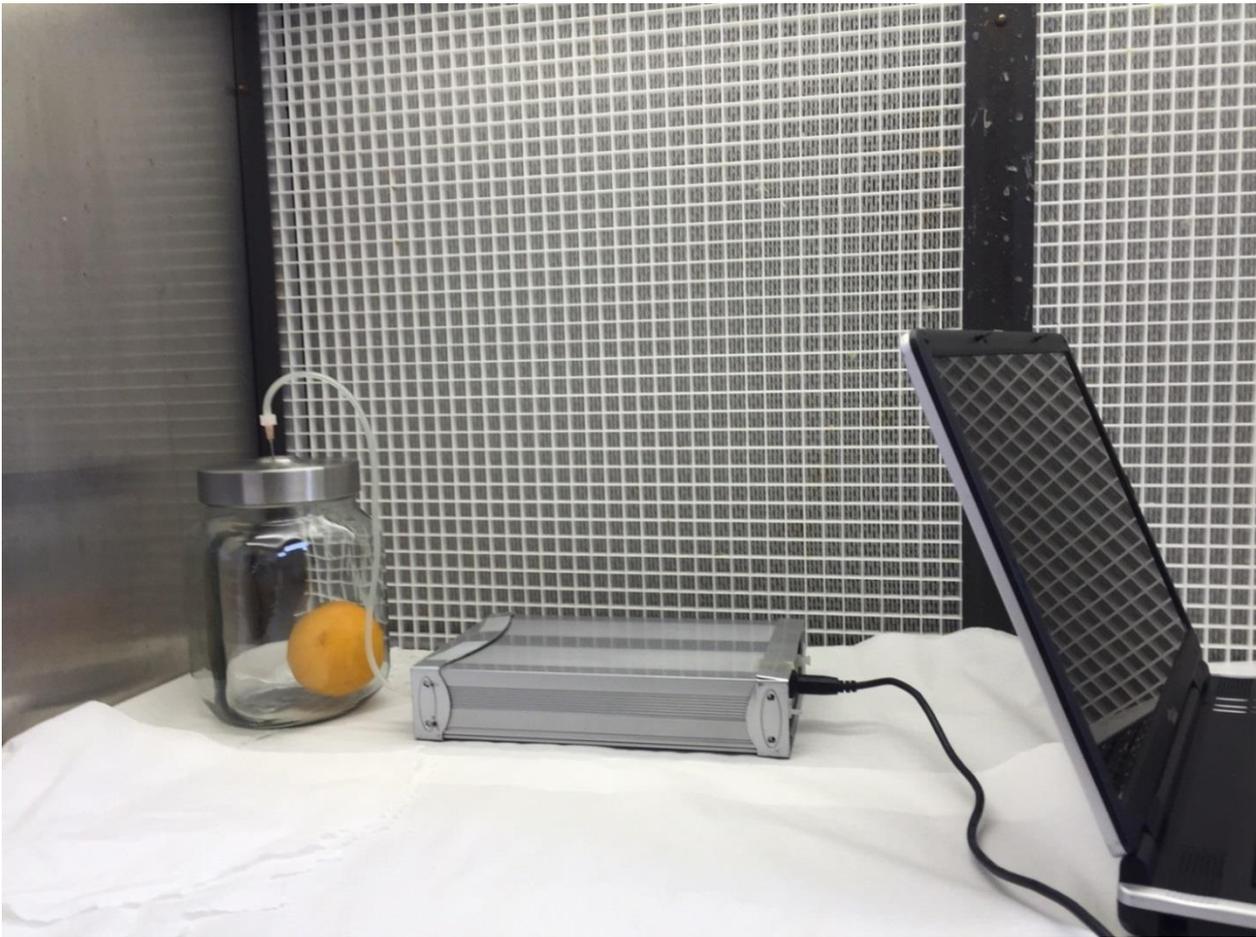
In 2019 GC-MS trials were conducted on Clementine mandarins, Washington Navel oranges, as well as Midnight and Delta Valencia oranges.

#### Electronic Nose

W Kirkman visited RoboScientific at the University of Leeds, and received training on their “Bloodhound” electronic nose. The unit was successful in differentiating between injured and healthy fruit. The unit was brought back to South Africa for trial purposes. Fruit were placed in sealed bags or glass jars, and left for 15 minutes for the headspace to equalise. The inlet needle was then inserted into the container, and the air from the headspace was sucked over the sensors, and results were sent to the attached computer (Figure 3.2.7.2).

In the first trial, Palmer Navel Oranges were harvested from an orchard in the Sundays river Valley. Half of them were inoculated with neonate FCM larvae. Ten days after infestation, 20 infested fruit and 20 healthy fruit were analysed. In the second trial, due to possible air contamination in the first trial, the trial was conducted in a more isolated laboratory, in the same way as the first trial. In a third trial, Lane Late Navel oranges were

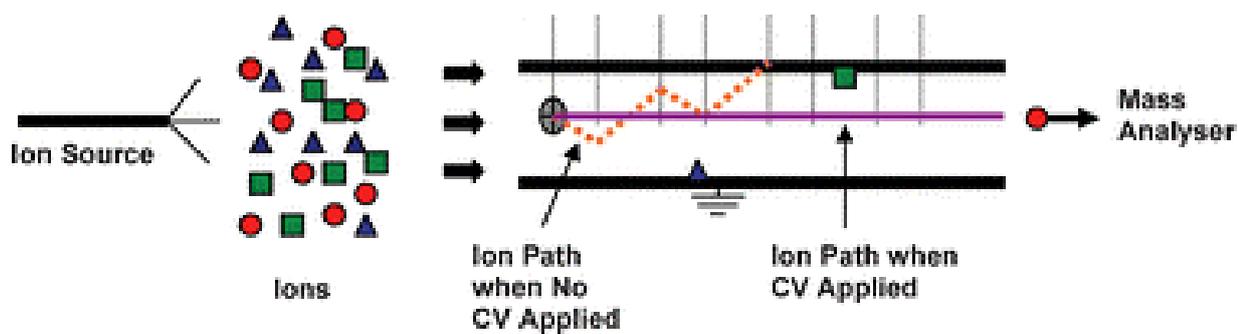
analysed 2, 6, and 10 days after infestation. This time the trials were conducted under a laminar flow cabinet to eliminate air contamination. A fourth trial was conducted similar to the previous one, using Washington Navel oranges.



**Figure 3.2.7.2.** Electronic nose trials conducted in a laminar flow cabinet.

#### Gas Chromatography – Differential Mobility Spectrometry (GC-DMS)

Dr Sean Moore had been in contact with Dr Cristina Davis, of the Department of Mechanical and Aerospace Engineering at the University of California (Davis) (UC-Davis), for a number of years. A collaborative project was initiated with her to investigate the ability of their Gas Chromatography – Differential Mobility Spectrometry (GC-DMS) device to identify FCM-infested fruit. DMS is based on the fact that each chemical species has a unique dependence of its mobility on the electric field strength; therefore, the differences in ion mobilities can be used to identify specific chemicals. An additional direct current (DC) voltage, called a “compensation voltage” (CV), is applied to compensate for ion drift under differential field conditions and to allow a particular chemical species to pass through the device for detection. The CV value is related to the ion structure and mass and is, therefore, particular to a specific ion. The retention time (RT) is the time that the ion takes to move through the detector under certain conditions, and this also unique to each compound (Aksenov *et al*, 2014). Figure 3.2.7.3 shows the DMS process. The ions from the GC are ionised by a radioactive Nickel source, then move through the detector, where CV is applied to avoid ion drift. RT and CV are then used to identify the compounds.



**Figure 3.2.7.3.** DMS procedure for identifying compounds.

Aksenov *et al* (2016) used their portable GC-DMS “suitcase” (Figure 3.2.7.4) to detect trees infected with Huanglongbing disease, a devastating disease of citrus, which has destroyed more than half of citrus trees in Florida. Volatiles were collected from within the canopy of trees.



**Figure 3.2.7.4.** GC-DMS “suitcase”.

W Kirkman visited UC-Davis for a week to be trained on the GC-DMS unit. Part of the training was conducting a trial to see if the unit could identify rhododendron trees whose roots were infected by *Phytophthora* sp. from leaf volatiles (Figure 3.2.7.5).



**Figure 3.2.7.5.** Volatiles extracted from bagged rhododendron leaves in an attempt to identify *Phytophthora* infection using the GC-DMS unit.

#### Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

Trials were conducted with a Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) (Figure 3.2.7.6) at the University of Leuven in Belgium. This unit conducts real-time volatile emission evaluation, with ppt accuracy. It has a multi-position inlet, and can evaluate 16 separate samples simultaneously. These units are currently used commercially to detect contaminants in shipping containers, soil samples and tea consignments. Fruit were injured 24 hours prior and just before evaluation, to simulate FCM infestation. Healthy fruit were evaluated as a control. Multivariate statistics were applied to analyse measured data.



**Figure 3.2.7.6.** SIFT-MS unit used for trials at the University of Leuven.

## Results and discussion

### Gas Chromatography – Mass Spectrometry

#### *Targeting compounds unique to FCM infested fruit.*

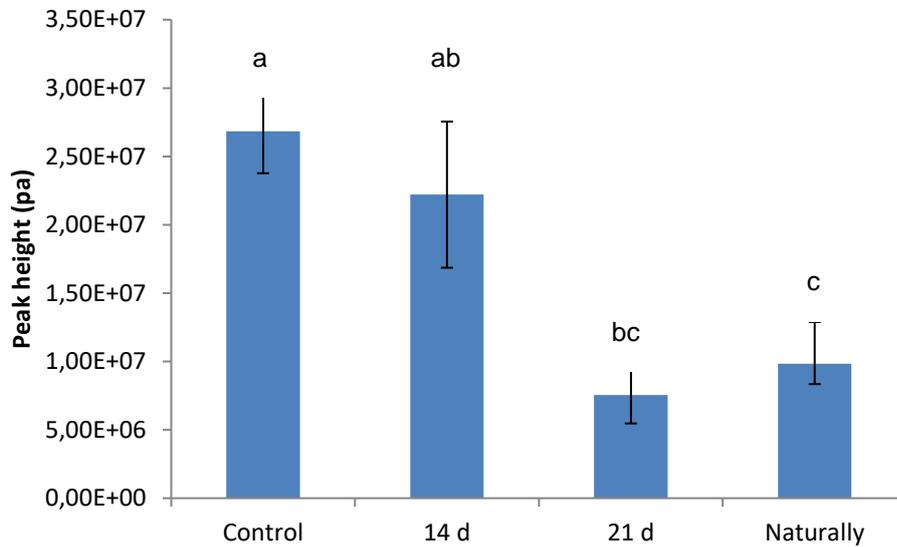
The volatile profiles of larvae extracted from diet, and diet alone, were similar, with no compound unique to larvae being found. This is probably because the larvae had been feeding on the diet, and so would emit similar volatiles as diet. Also, the diet analysed was from a jar containing FCM larvae. Analysis of unused diet will be conducted in the future, to be compared to the profile of larvae extracted from diet. The volatile profiles of larvae extracted from fruit, infested fruit and healthy fruit were similar. However, oxime and methyl eugenol were present in the profiles of larvae extracted from fruit and infested fruit, but not in healthy fruit. It is assumed that these compounds are present due to interaction between the larva and fruit, as they were not present in larvae extracted from diet.

#### *Analyses of whole fruit.*

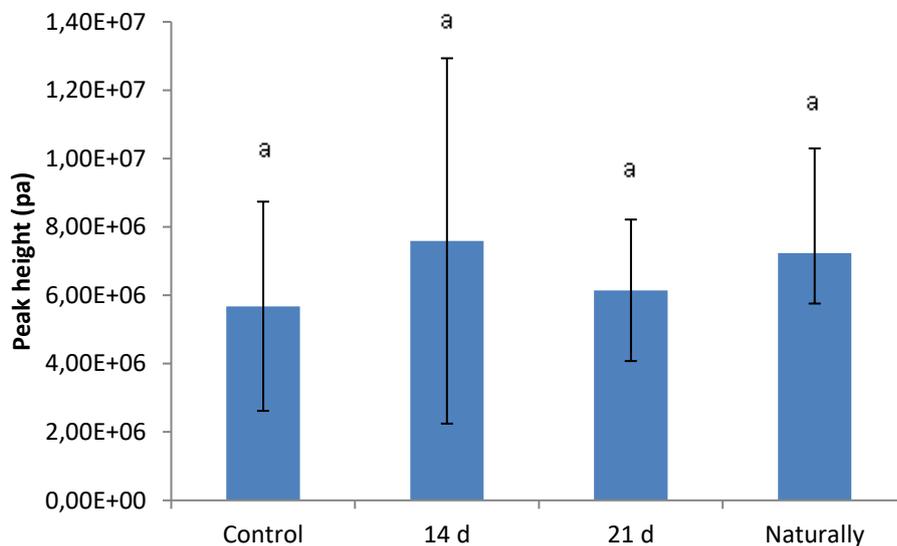
##### 2016

The three major volatiles recorded were D-Limonene, caryophyllene and a derivative of naphthalene, naphthalene,1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethy-7-1-(1-methylethyl)-, (1R-1.alpha.,7.beta.,8a.alpha)), referred to as “naphthalene”. D-Limonene levels, indicated by chromatogram peak heights, were lower than the healthy fruit in all three infested treatments, and significantly so for the 21 day and naturally infested treatments (Figure 3.2.7.7). The levels decreased with time after infestation. Caryophyllene levels did not differ between treatments (Figure 3.2.7.8). “Naphthalene” levels were higher in all three infested treatments,

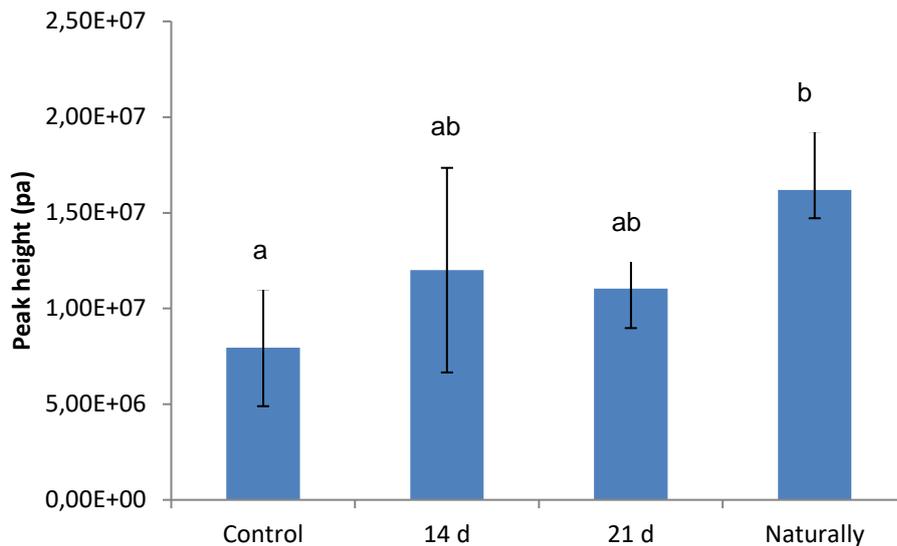
significantly so for the naturally infested treatment (Figure 3.2.7.9). The levels increased with time after infestation. As a result of the decreased levels of D-Limonene and increased levels of “naphthalene” with time after infestation, the ratio between the two compounds (D-Limonene/”naphthalene”) was plotted, and all three infested treatments were significantly different from the healthy fruit control (Figure 3.2.7.10). Larvae recorded in the 14 d treatment were first to second instar, while those in the 21 d treatment were second to third instar. Larvae in the naturally infested treatment were fourth and fifth instar.



**Figure 3.2.7.7.** D-Limonene levels for healthy Witkrans Navel oranges and oranges infested at different periods before harvest. Different letters above bars denote significant differences between values ( $P < 0.05$ , Duncan multiple range test).

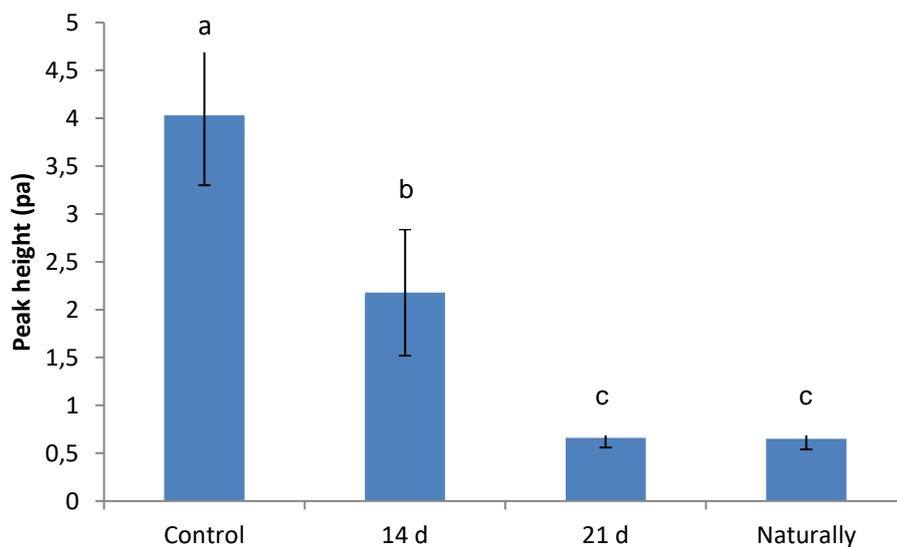


**Figure 3.2.7.8.** Caryophyllene levels for healthy Witkrans Navel oranges and oranges infested at different periods before harvest. Different letters above bars denote significant differences between values ( $P < 0.05$ , Duncan multiple range test).



**Figure 3.2.7.9.** “Naphthalene” levels for healthy Witkrans Navel oranges and oranges infested at different periods before harvest.

\* Different letters above bars denote significant differences between values ( $P < 0.05$ , Duncan multiple range test).

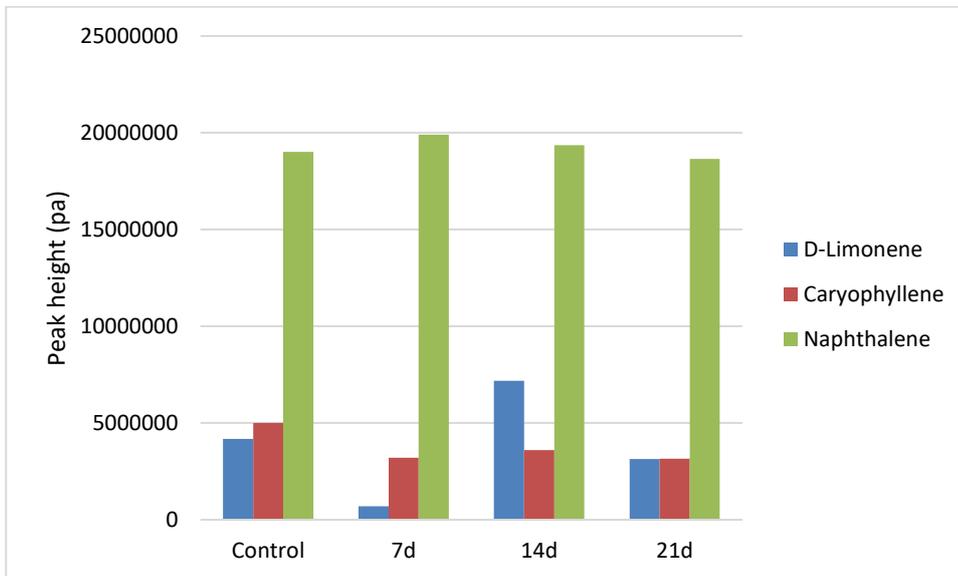


**Figure 3.2.7.10.** Peak heights for the ratio of D-Limonene to “naphthalene” levels, for healthy Witkrans Navel oranges and oranges infested at different periods. Different letters above bars denote significant differences between values ( $P < 0.05$ , Duncan multiple range test).

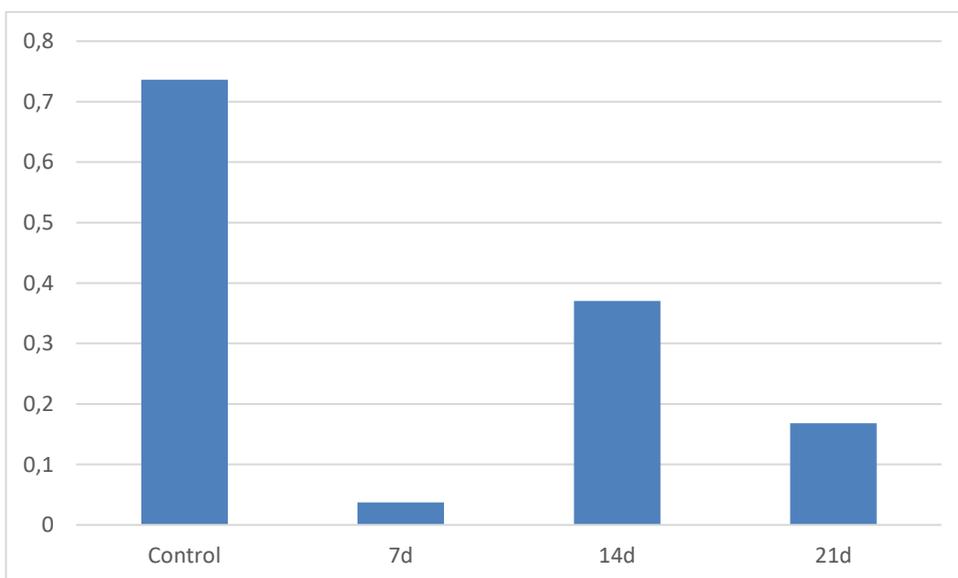
### 2017

The four major volatiles recorded were D-Limonene, beta-ocimene, caryophyllene and naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethy-7-1-(1-methylethyl)-, (1R-1.alpha.,7.beta.,8a.alpha), referred to as “naphthalene”. In previous trials in 2016, D-Limonene levels were lower than the healthy fruit in all three infested treatments, and significantly so for the 21 day and naturally infested treatments. The levels decreased with time after infestation. “Naphthalene” levels were higher in all three infested treatments, significantly so for the naturally infested treatment, the levels increased with time after infestation. As a result of the decreased levels of D-Limonene and increased levels of “naphthalene” with time after infestation, the ratio between the two compounds (D-Limonene/“naphthalene”) was plotted, and all three infested treatments were significantly different from the healthy fruit control (Figure 3.2.7.10). In the 2017 trials conducted with Washington Navel oranges, results were vastly different (Figure 3.2.7.11). D-Limonene levels were much lower, and Naphthalene levels higher, and the differences in D-Limonene/“naphthalene” ratio with time after infestation were not observed (Figure 3.2.7.12). Similar results were observed in the trials conducted in 2017

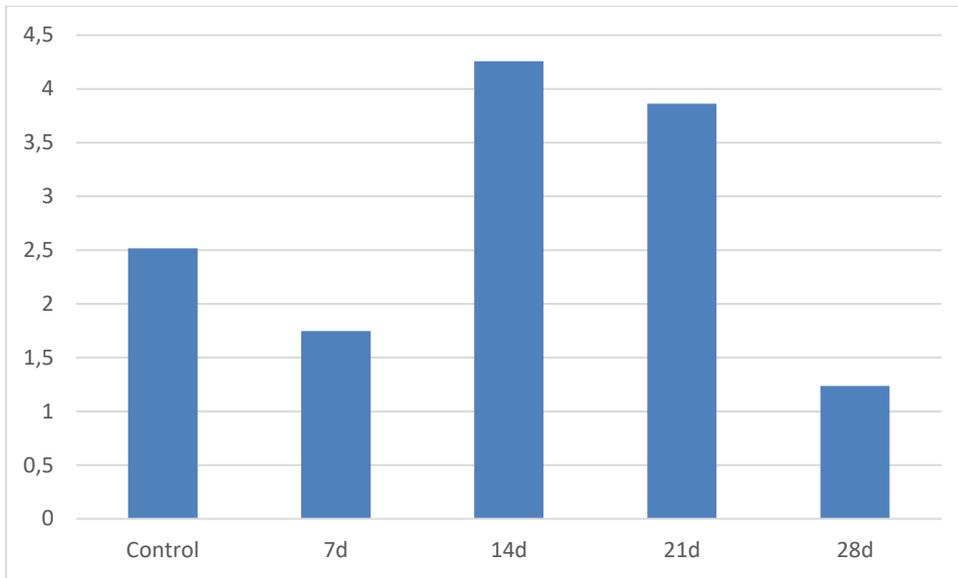
on Mor mandarin (Figure 3.2.7.13), Midnight Valencia (Figure 3.2.7.14) and Delta Valencia (Figure 3.2.7.15) fruit. This was mainly due to variability in D-limonene levels in all cultivars, which can most likely be ascribed to the extremely unusual climactic conditions in the Eastern Cape, which resulted in excessive splitting and fruit drop, as well as scorching of Valencia orchards by uncharacteristic berg winds.



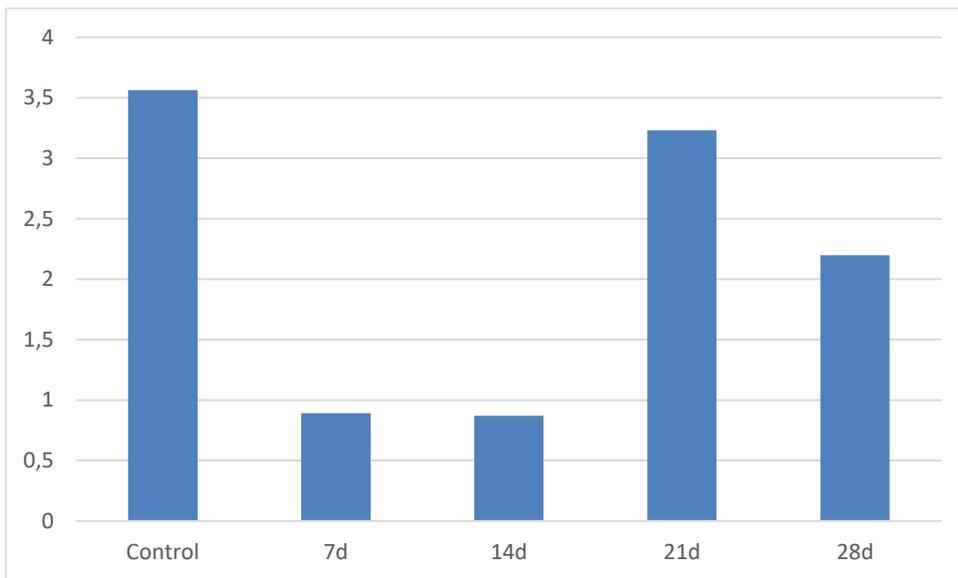
**Figure 3.2.7.11.** D-Limonene, caryophyllene and “naphthalene” levels for healthy Washington Navel orange fruit and fruit infested at different periods in 2017.



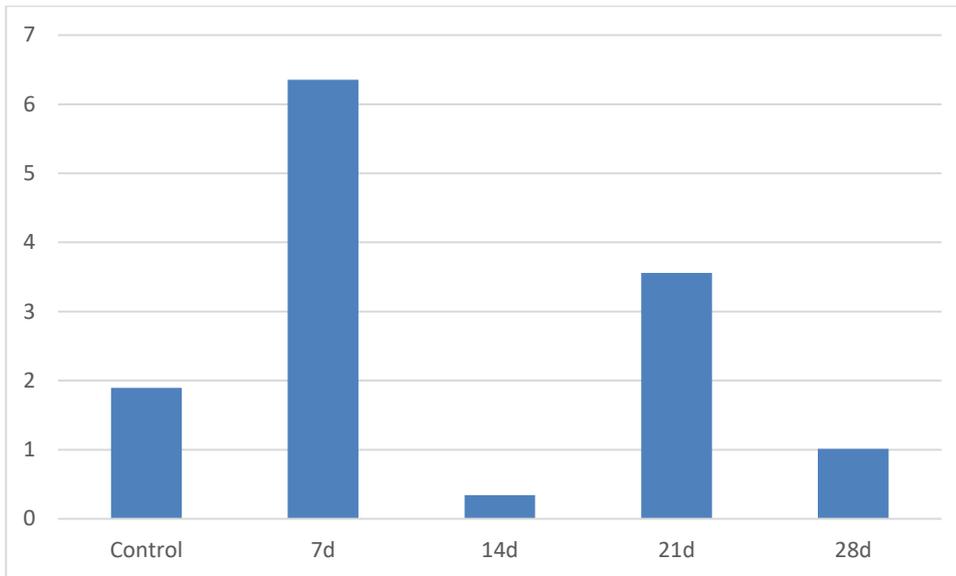
**Figure 3.2.7.12.** The ratio of D-Limonene to “naphthalene” levels, for healthy Washington Navel orange fruit and fruit infested at different periods in 2017.



**Figure 3.2.7.13.** The ratio of D-Limonene to "naphthalene" levels, for healthy Mor mandarin fruit and fruit infested at different periods in 2017.



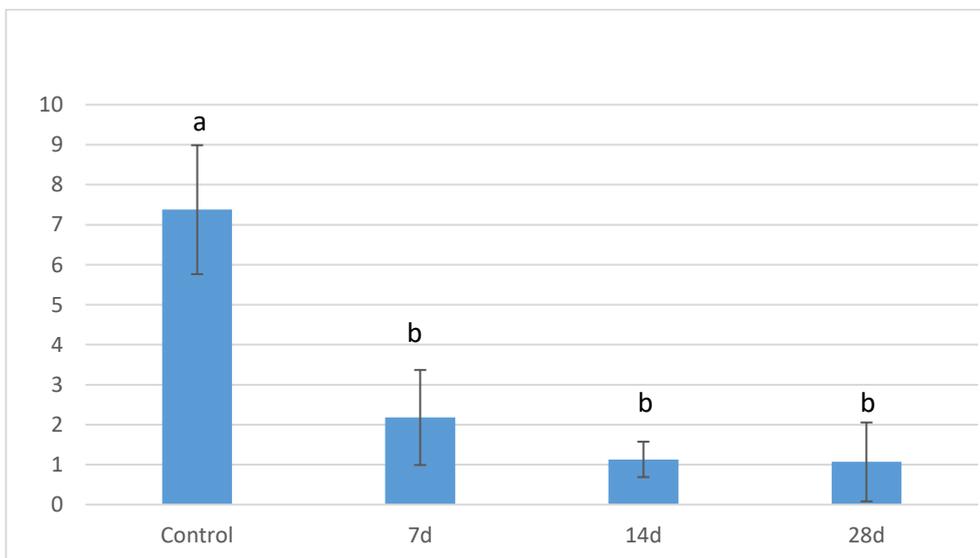
**Figure 3.2.7.14.** The ratio of D-Limonene to "naphthalene" levels, for healthy Midknight Valencia fruit and fruit infested at different periods in 2017.



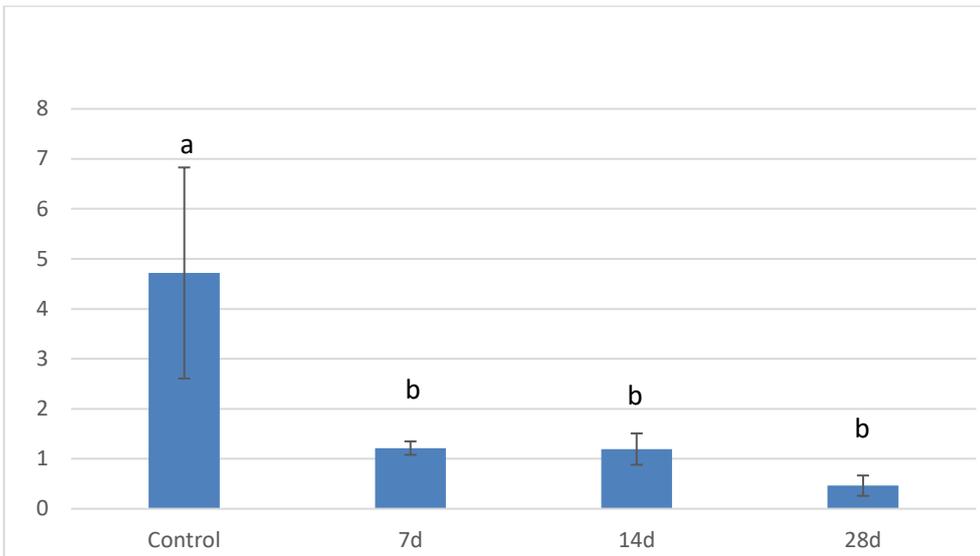
**Figure 3.2.7.15.** The ratio of D-Limonene to “naphthalene” levels, for healthy Delta Valencia fruit and fruit infested at different periods in 2017.

2018

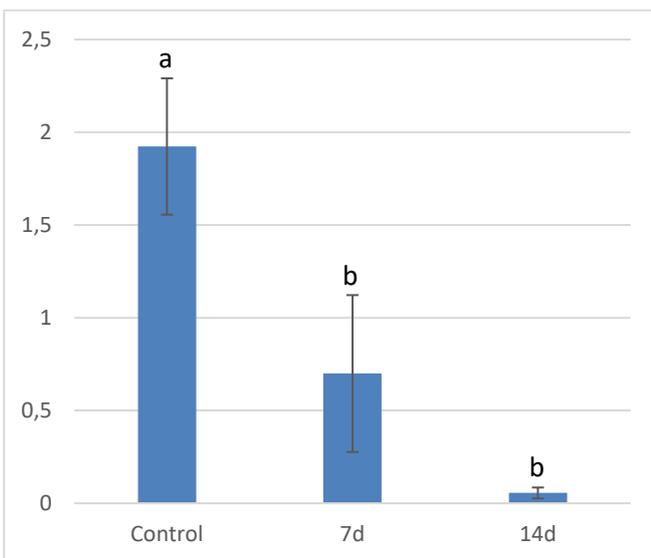
However, in the trials conducted on Washington (Figure 3.2.7.16) and Witkrans (Figure 3.2.7.17) Navel oranges in 2018, as well as Midnight (Figure 3.2.7.18) and Delta (Figure 3.2.7.19) Valencia oranges, the trend of decreased levels of D-Limonene and increased levels of “naphthalene” with time after infestation, and the ratio between the two compounds (D-Limonene/“naphthalene”) was similar to 2016, significantly lower for all infested treatments than the untreated controls. This emphasizes the fact that the results from 2017 were not representative and were affected by climactic conditions. It was interesting to note that with Clementine Mandarins, there was an increase in beta-ocimene levels with time after infestation, where levels were undetectable in the control fruit (Figure 3.2.7.20).



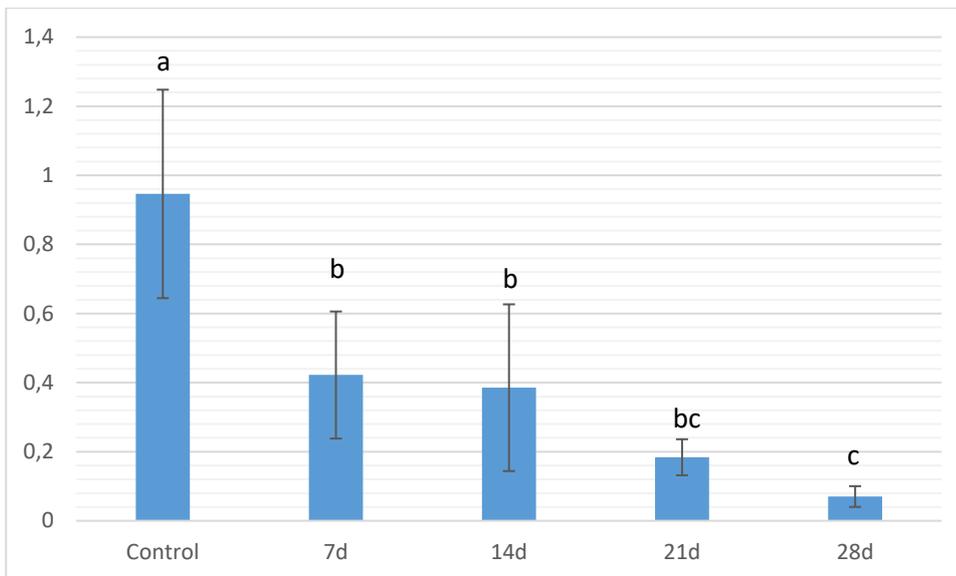
**Figure 3.2.7.16.** The ratio of D-Limonene to “naphthalene” levels, for healthy Washington Navel orange and fruit infested at different periods in 2018. Different letters above bars denote significant differences between values ( $P < 0.05$ , Fisher LSD Test).



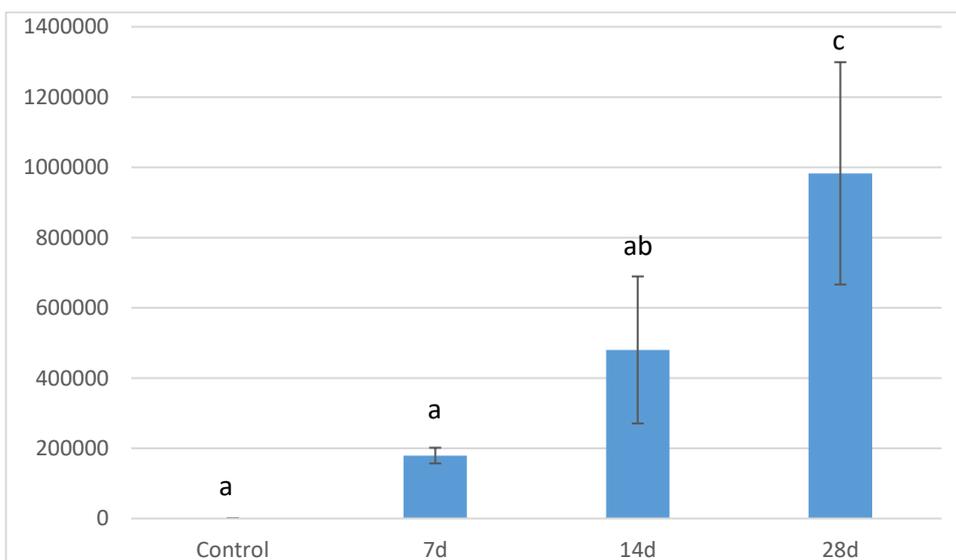
**Figure 3.2.7.17.** The ratio of D-Limonene to “naphthalene” levels, for healthy Witkrans Navel orange and fruit infested at different periods in 2018. Different letters above bars denote significant differences between values ( $P < 0.05$ , Fisher LSD Test).



**Figure 3.2.7.18.** The ratio of D-Limonene to “naphthalene” levels, for healthy Midnight Valencia fruit and fruit infested at different periods in 2018. Different letters above bars denote significant differences between values ( $P < 0.05$ , Fisher LSD Test).



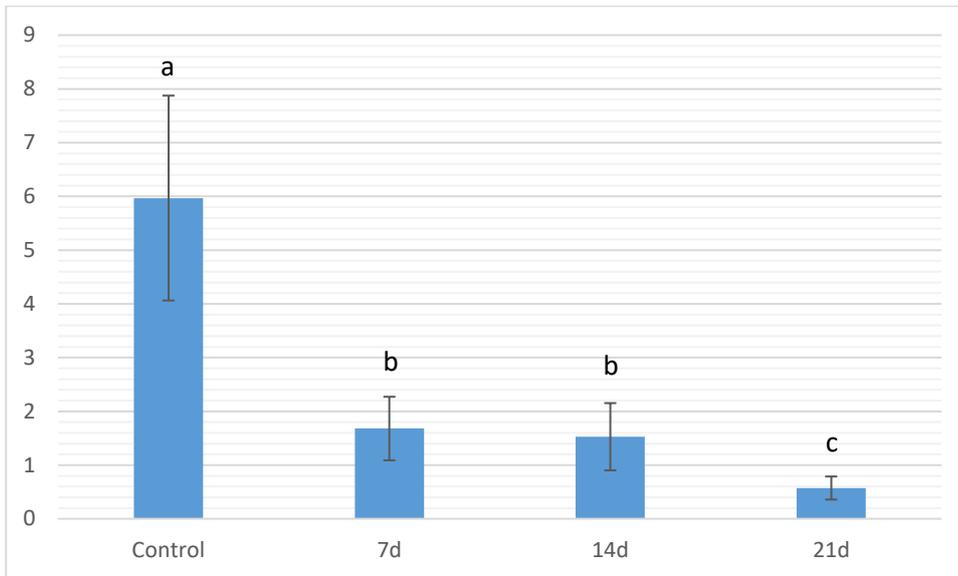
**Figure 3.2.7.19.** The ratio of D-Limonene to "naphthalene" levels, for healthy Delta Valencia fruit and fruit infested at different periods in 2018. Different letters above bars denote significant differences between values ( $P < 0.05$ , Tukey HSD Test).



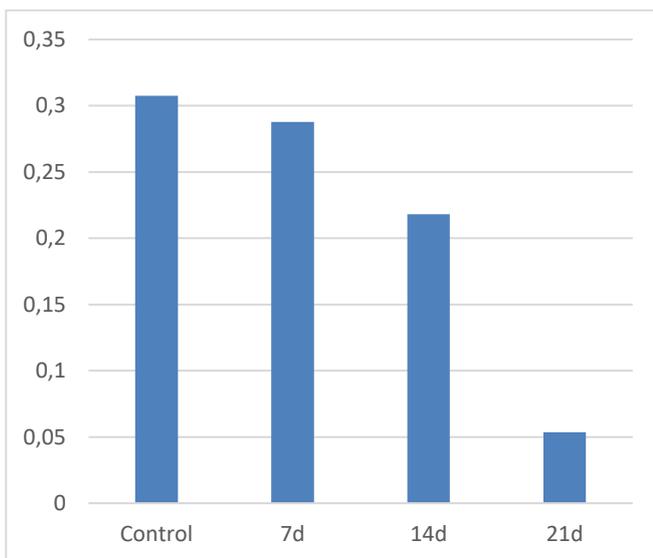
**Figure 3.2.7.20.** Beta-ocimene levels for healthy Clementine Mandarin fruit and fruit infested at different periods in 2018. Different letters above bars denote significant differences between values ( $P < 0.05$ , Fisher LSD Test).

### 2019

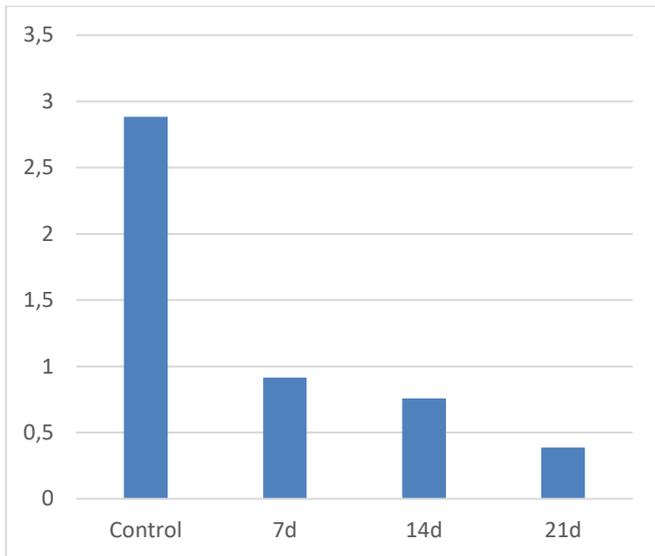
In the trials conducted on Washington Navel oranges in 2019 (Figure 3.2.7.21), as well as Midnight (Figure 3.2.7.22) and Delta Valencia oranges in 2019 (Figure 3.2.7.23), the trend of decreased levels of D-Limonene and increased levels of "naphthalene" with time after infestation, and decrease in the ratio between the two compounds (D-Limonene/"naphthalene") was once again observed. In Clementine Mandarins in 2019, there was an increase in beta-Ocimene levels with time after infestation, where levels were undetectable in the control fruit, which was a similar result to 2018 trials.



**Figure 3.2.7.21.** The ratio of D-Limonene to “naphthalene” levels, for healthy Washington Navel orange fruit and fruit infested at different periods in 2019. Different letters above bars denote significant differences between values ( $P < 0.05$ , Tukey HSD Test).



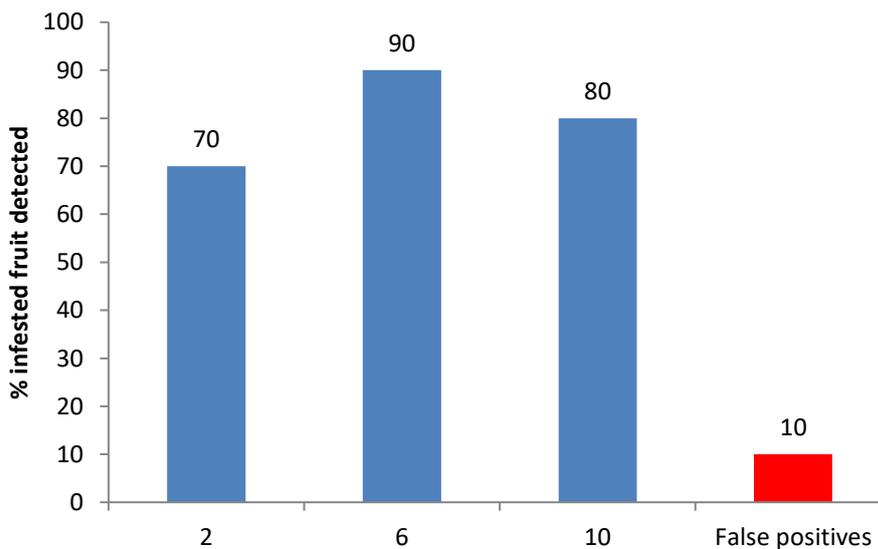
**Figure 3.2.7.22.** The ratio of D-Limonene to “naphthalene” levels, for healthy Midnight Valencia fruit and fruit infested at different periods in 2019.



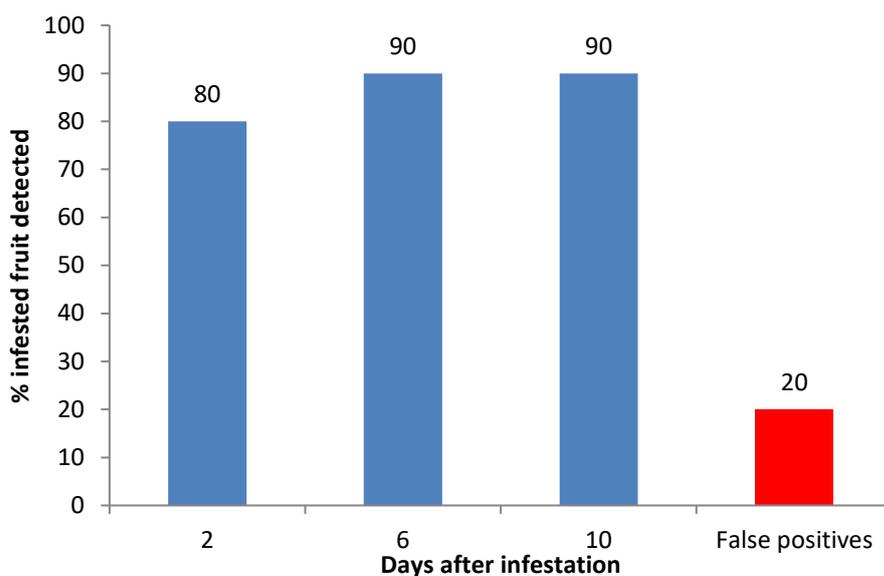
**Figure 3.2.7.23.** The ratio of D-Limonene to “naphthalene” levels, for healthy Delta Valencia fruit and fruit infested at different periods in 2019.

### Electronic Nose

In the first trial, the e-nose correctly identified 60% of the infested fruit, but also classified 35% of the control fruit as infested. These poor results were probably due to air contamination in the laboratory. The second trial was conducted in a separate laboratory where there was less chance of air contamination. In this trial 75% of infested fruit were correctly identified, and 25% of the control fruit were incorrectly classified as infested. The third trial was conducted under a laminar flow cabinet, using Lane Late Navel oranges and the results were more encouraging (Figure 3.2.7.24), with higher levels of detection, and a lower percentage of false positives. In the fourth trial, conducted on Washington Navel oranges, detection of infested fruit was improved, with 80%, 90% and 90% correctly detected for 2, 6 and 10 days after infestation (Figure 3.2.7.25). Twenty percent of the control fruit were incorrectly classed as infested.



**Figure 3.2.7.24.** Percentage of infested Lane Late Navel oranges detected for various number of days after infestation, and percentage of false positives in control fruit, using an electronic nose.



**Figure 3.2.7.25.** Percentage of infested Washington Navel oranges detected for various number of days after infestation, and percentage of false positives in control fruit, using an electronic nose.

Gas Chromatography – Differential Mobility Spectrometry (GC-DMS)

The unit was able to detect *Phytophthora* infested rhododendron plants from leaf volatiles. Unfortunately, the full results cannot be shown as they are due to be published.

Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

The SIFT-MS unit could clearly differentiate between healthy fruit (A), and fruit injured 24 hours prior to evaluation (B) (Figure 3.2.7.26). The presence of D-Limonene could be identified after one second of SIFT-MS analysis following 20 seconds of headspace collection (Figure 3.2.7.27).

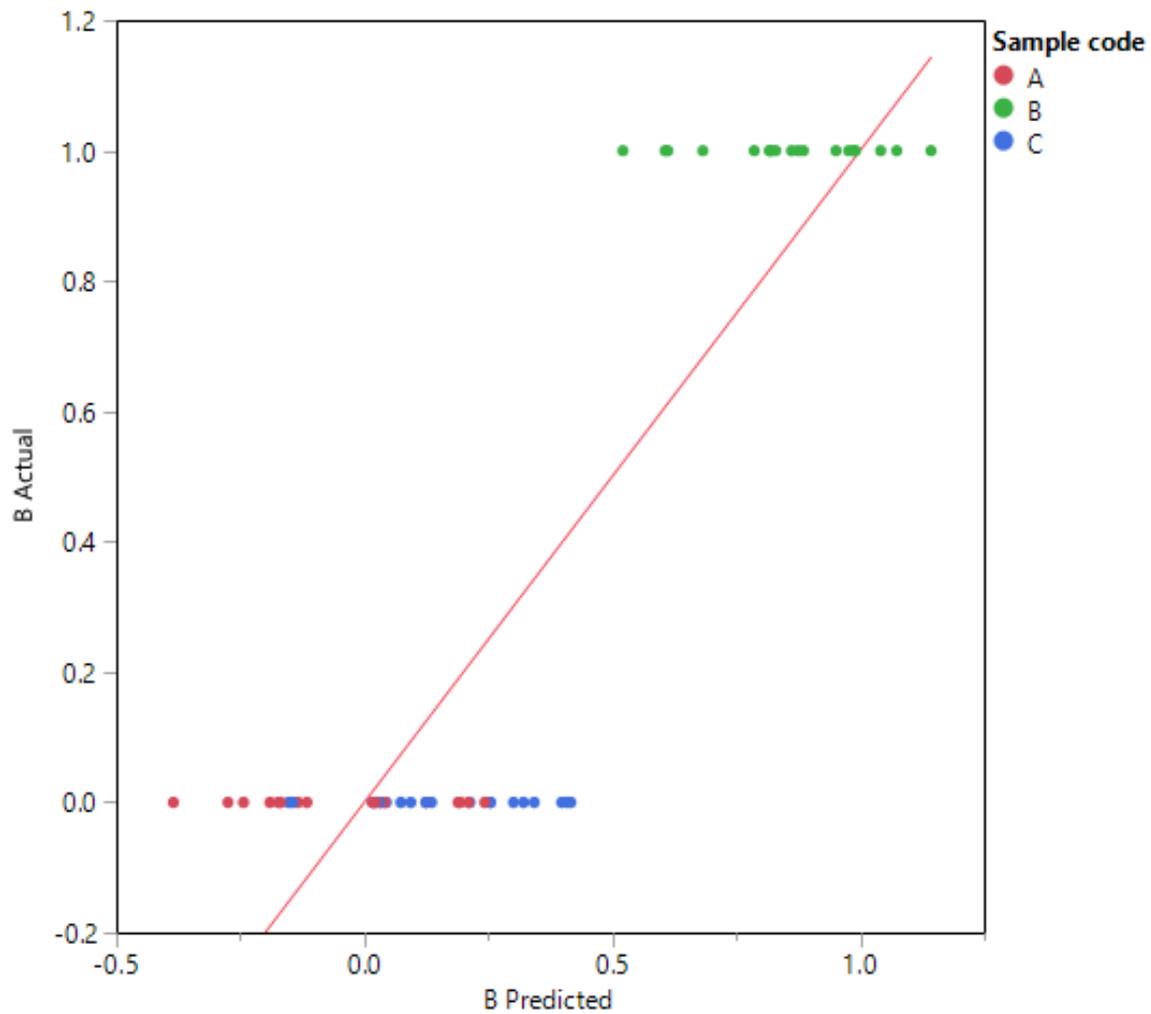


Figure 3.2.7.26. Plot showing separation between healthy fruit and fruit injured 24 hours earlier.

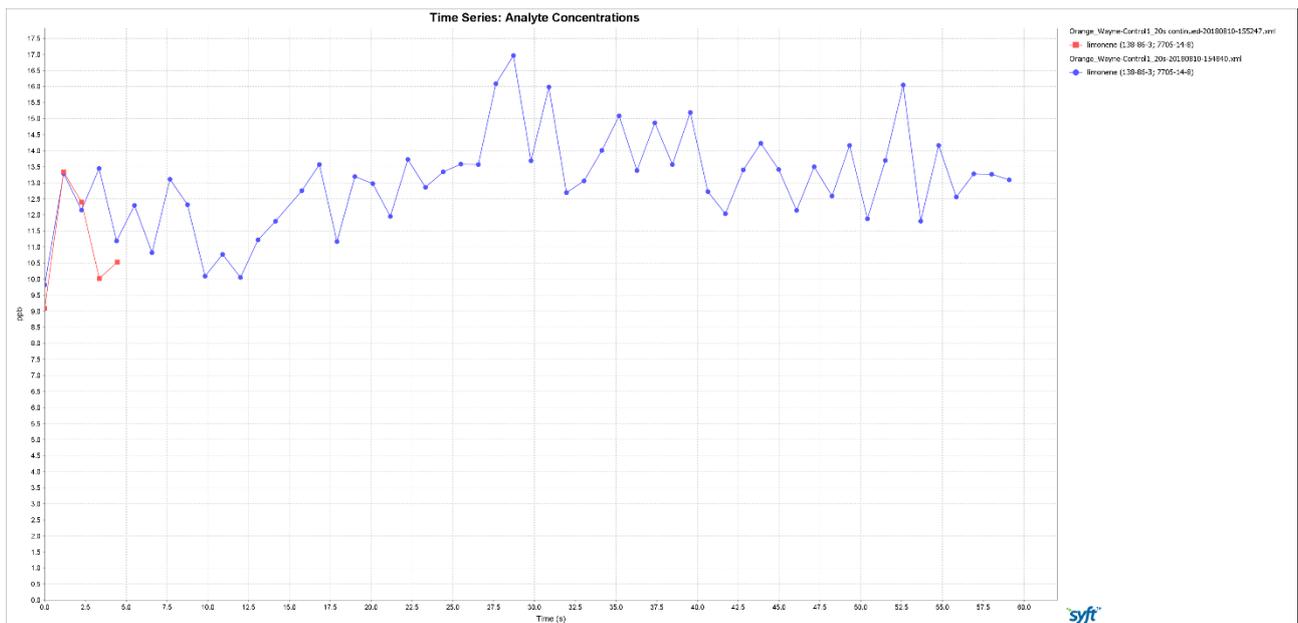


Figure 3.2.7.27. D-Limonene levels after one second of SIFT analysis following 20 seconds of headspace collection.

## Conclusion

Oxime and methyl eugenol have been identified as compounds unique to FCM infested fruit when analysing segments of fruit. D-Limonene and “naphthalene” levels differ between healthy and infested fruit, significantly so at longer periods after infestation. The ratio between these two products differs from the control for all treatments longer than 14 days after infestation, and appears to be definitive of infestation for all cultivars, if one excludes the data from 2017, where climactic conditions affected the results. In Clementine Mandarins the levels of beta-ocimene increase with time after infestation. The RoboScientific electronic nose has the ability to detect FCM infested fruit, but would need to be upgraded with more sophisticated sensors and software to be of use. A Differential Mobility Spectrometry (GC-DMS) unit at the University of California – Davis was able to detect phytophthora in rhododendron plants from leaf volatiles. Unfortunately CRI was not able to secure a GC-DMS unit to evaluate its ability to detect FCM infested citrus fruit. Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) shows great promise to detect volatile emissions within a few seconds.

## Future Research

Discussions have been held with Nukor, who use an online X-ray tomography unit, supplied by Biometric, to detect defects inside logs for the forestry industry. This technology could prove useful in postharvest FCM detection in citrus, and collaborative research is planned. This will be conducted under a new project. Further research on the Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) will be conducted under a new project.

## Technology Transfer

An oral presentation titled “Postharvest detection of false codling moth in citrus fruit: from X-ray to volatiles and beyond” was presented at the CRI Citrus Symposium in August 2016.

An oral presentation titled “Postharvest detection of false codling moth in citrus fruit” was presented at the International Congress of Entomology in Orlando, Florida, September 2016.

An oral presentation titled “Postharvest detection of false codling moth in citrus fruit” was presented at the South African Post Harvest Innovation Conference in Stellenbosch, November 2016.

An oral presentation titled “Postharvest detection of false codling moth in citrus fruit” was presented at the Entomological Society of South Africa Symposium in Pretoria, July 2017.

An oral presentation titled “Postharvest detection of false codling moth in citrus fruit” was presented at the Rhodes University Postgraduate Symposium in Grahamstown, November 2018.

An oral presentation titled “Postharvest detection of false codling moth in citrus fruit” was presented at the Citrus Research Symposium in 2018.

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### 3.2.8 **PROGRESS REPORT: Impact of abbreviated and complete cold-treatment on survival and fitness of FCM larvae**

Project 1039 (April 2012 – March 2020) by Sean Moore, Wayne Kirkman, Peter Stephen, Sean Thackeray, Mellissa Peyper, Kim Stoltz, Luke Cousins, Tim Grout and Vaughan Hattingh (CRI)

#### **Summary**

As of 1 January 2018, FCM was regulated as a phytosanitary organism by the European Union. The regulation requires citrus fruit to be sourced from an FCM-free area or place of production, or to receive a cold treatment or any other treatment that can ensure the exported consignment is free of FCM. Cold disinfestation of citrus fruit from South Africa to Europe is not feasible, due to the relatively short shipping time, particularly from Cape Town port, but more importantly due to the large volumes of fruit exported to Europe and the inadequate infrastructure to facilitate cold treatment of these large volumes. Consequently, a multi-step systems approach has been developed as an alternative treatment to cold sterilisation. The final stage in the systems approach is a time-temperature shipping protocol. Although this is a cold treatment, it is not a complete disinfestation treatment. The precise level of mortality required by this treatment is determined by the measured efficacy of the preceding steps in the systems approach. The mortality of the most cold-tolerant larval stages of FCM, using several time-temperature combinations, have been determined. Since complete mortality for 4°C and above were not achieved in any of the 26 day trial periods, trials at extended durations are being conducted. Complete mortality at 4°C was achieved at 28 days, with the trial evaluated up to 30 days to ensure complete mortality. Extended duration trials for 4.5°C is still underway. A second regional cold trial at 3°C was completed to determine if there was a difference in cold susceptibility of fifth instars between seven cultures of different regional origin. Five of the seven cultures are reared at Rhodes University, being Addo, Nelspruit, Marble Hall, Old and Citrusdal. One culture each is reared at each of River Bioscience in Hermitage and XSit in Citrusdal. There was a lot of variability in mortality between the cultures up to 22 days, where after the mortality seemed to have become less variable, with most cultures reaching complete mortality. An additional trial for 2°C from 16 to 26 days was completed to ensure enough data are collected for this temperature regime. 100% mortality was reached at 18 days. Another trial was completed at 2°C to establish whether 5th instar FCM infested fruit collected from the field has the same cold susceptibility as 5th instar FCM larvae reared in jars. We expected complete mortality for the larvae in the jars and the fruit. Unfortunately one live larva was found in fruit in replicate 1 and two live larvae in replicate 2. This may be attributed to certain probes taking longer to reach temperature or averaging higher than the set temperature and consequently, the trial will be repeated. Lastly, a jar of larvae was removed from each of three replicates after 16 days at both 4°C and 4.5°C. Larvae were removed from the diet and immediately categorised as alive or dead (moving or still) and a range of other characteristics identified e.g. colour, shape, length, turgidity, bruising. Each larva was then kept individually on diet and survival and development monitored. Relating survival to these characteristics has identified turgidity and compactness of the larva to be strongly related to survival. We are now attempting to quantify these parameters more precisely.

#### **Opsomming**

Vanaf 1 Januarie 2018 is VKM as geregleer as 'n fitosanitêre organisme deur die Europese Unie. Die regulasie vereis dat sitrus vrugte vanaf 'n VKM vrye-area of plek van produksie verkry word, of 'n koue behandeling ontvang of enige ander behandeling wat kan verseker dat die versending vry van VKM is. Koue ontsmetting van sitrus vrugte vanaf Suid-Afrika na Europa is nie haalbaar nie, as gevolg van die relatiewe kort verskepingstydperk, veral vanaf die Kaapstad hawe, maar meer belangrik as gevolg van die hoë volumes vrugte wat uitgevoer word na Europa en die onvoldoende infrastruktuur om die koue behandelings te fasiliteer van hierdie groot volumes. Gevolglik is 'n multi-stap stelselsbenadering as 'n alternatiewe behandeling vir koue sterilisasie ontwikkel. Die finale stap in die stelselsbenadering is 'n tyd-temperatuur verskepings protokol. Al is hierdie wel 'n koue behandeling, is dit nie 'n volledige ontsmettings behandeling nie. Die presiese vlak van mortaliteit wat deur hierdie stap benodig word, word bepaal deur die gemete doeltreffendheid van die vorige stappe in die stelselsbenadering. Tot op hede is die mortaliteit van die mees kouetolerante lewensstadiums

van VKM deur gebruik van verskeie tyd-temperatuur kombinasies bepaal. Aangesien volledige mortaliteit vir 4°C of hoër nie bereik is in enige van die 26 dag proef periodes nie, is proewe by verlengde periodes gedoen. Volledige mortaliteit by 4°C is na 28 dae verkry, met die proef geevalueer tot op 30 dae om te verseker dat volledige mortaliteit bereik is. Verlengde tydperk proewe vir 4.5°C is steeds aangaande. 'n Tweede streeks koue proef by 3°C is voltooi om vas te stel of daar 'n verskil is in die koue vatbaarheid van vyfde instars tussen die sewe streeks kulture. Vyf van die sewe kulture word gekweek by Rhodes Universiteit, dit is Addo, Nelspruit, Marble Hall, Old en Citrusdal. Een kultuur elk word gekweek by River Bioscience in Hermitage en XSite in Citrusdal. Daar was baie wisselvalligheid in die mortaliteit tussen die kulture tot met 22 dae, waarna die mortaliteit minder veranderlik geword het, met meeste van die kulture wat volledige mortaliteit behaal het. 'n Addisionele proef vir 2°C van 16 tot 26 dae is voltooi om te verseker dat genoeg inligting versamel is vir die temperatuur regime. 100% mortaliteit is verkry teen 18 dae. Nog 'n proef is voltooi teen 2°C om vas te stel of 5de instar VKM geïnvesteerde vrugte verkry vanaf boorde dieselfde koue vatbaarheid het as 5de instar larwes in houers geteel. Ons het volledige mortaliteit van alle larwes verwag in beide die vrugte en die houers. Ongelukkig is een lewendige larwe in die replikaat 1 en twee lewendige larwes in replikaat 2 gekry. Dit kan toegeskryf word aan sekere peile wat langer geneem om die temperatuur te bereik, of 'n hoër gemiddeld as die ingestelde temperatuur gehad het, gevolglik sal die proef herhaal word. Laastens is 'n houer larwes van elk van die drie herhalings verwyder teen 16 dae vir beide 4°C en 4.5°C. Larwes is verwyder vanaf die dieet en dadelik gekategoriseer as lewend of dood (bewegend of stil) en 'n reeks ander eienskappe bv. kleur, vorm, lengte, turgiditeit, en kneusing. Elke larwe was individueel op dieet gehou en die oorlewing en ontwikkeling gemonitor. Deur oorlewing met hierdie eienskappe in verband te bring, word geïllustreer dat turgiditeit en kompaktheid van die larwe sterk verband hou met oorlewing. Ons probeer nou om hierdie parameters meer presies te kwantifiseer.

### 3.2.9 **PROGRESS REPORT: Evaluating hot air treatments for postharvest FCM control** Project 1060 (2013/4, 2015/6-2021/2) by T G Grout, P R Stephen and K C Stoltz (CRI)

#### **Summary**

Although the USDA-APHIS has treatment schedules for vapour heat on citrus and several papers were published in the 1980s and 90s that said it could be done safely, only Thailand is risking this treatment on a commercial basis. Their treatment is at 43°C and 50-65% relative humidity (RH) and the fruit is held at 5-10°C afterwards. Previous research showed that the third instar of false codling moth (FCM) was more tolerant to heat than younger instars or the egg stage, and that when fruit was placed in 46°C vapour heat for 6 h, 100% mortality of 681 larvae was obtained. At 44°C for 6 h, there were survivors so this temperature and period was used in 2016/7 to compare the susceptibility of third, fourth and fifth instars. Third instars were more susceptible than the other two instars, which were similar in their susceptibility, although mortalities of the latter instars ranged from 37 to 58%. Susceptibility to heat did seem to vary between egg batches. In 2020/1, further treatments were conducted with fifth instar FCM in oranges reaching pulp temperatures of 45°C and 47°C. Some replicates were discarded due to faults with the equipment but nine replicates at 45°C caused a mean corrected mortality of 83.9% in 4 572 larvae. Ten replicates at 47°C using 6 049 larvae gave a corrected mean mortality of 88.0%. Experiments are now being conducted where the dwell time at the maximum temperature is extended before cooling.

#### **Opsomming**

Hoewel die USDA-APHIS behandelingskedules vir damphitte op sitrus het, en verskeie artikels in die 1980s en 90s gepubliseer is wat aandui dat dit veilig gedoen kan word, is dit slegs Thailand wat hierdie behandeling op 'n kommersiële basis waag. Hulle behandeling is by 43°C en 50-65% relatiewe humiditeit (RH) en die vrugte word daarna by 5-10°C gehou. Vorige navorsing het getoon dat die derde instar van valskodlingmot (VKM) meer bestand teen hitte as jonger instars of die eier fase was, en wanneer vrugte in 46°C damphitte vir 6 h geplaas is, is 100% mortaliteit van 681 larwes verkry. Daar was oorlewendes by 44°C vir 6 h, so hierdie temperatuur en periode is in 2016/7 gebruik om vatbaarheid van derde, vierde en vyfde instars te vergelyk. Derde instars was meer vatbaar as die ander twee instars, wat dieselfde in hul vatbaarheid was, hoewel mortaliteite van vierde en vyfde instars van 37 tot 58% gevarieer het. Vatbaarheid vir hitte blyk tussen eier bondels te varieer. In 2020/1 is verdere behandelings met vyfde instar VKM in lemoene uitgevoer, en

pulptemperature van 45°C en 47°C is bereik. Sommige herhalings is weggegooi weens foute met die toerusting, maar nege herhalings by 45°C het tot 'n gemiddelde gekorrigeerde mortaliteit van 83.9% in 4 572 larwes gelei. Tien herhalings by 47°C, deur gebruik te maak van 6 049 larwes, het 'n gemiddelde gekorrigeerde mortaliteit van 88.0% gegee. Proewe word nou uitgevoer waar die wagperiode by die maksimum temperatuur verleng word, vóór verkoeling.

### 3.2.10 **PROGRESS REPORT: Identification and development of an attractant for monitoring FCM adult females**

Project 1162 (April 2017 – March 2020) by Sean Moore, Wayne Kirkman, Mellissa Peyper (CRI) and Tamryn Marsberg (RU)

#### **Summary**

FCM is currently monitored in the field using traps baited with synthesised female moth sex pheromone, thus attracting only male moths. However, such a system can never be adequately accurate, as it is the females (not the males) that lay eggs on the fruit, leading to the larvae that do the damage. Additionally, male moths are believed to fly greater distances than females and are therefore not necessarily representative of the female population in the area. Identification of an attractant for females would enable a far more accurate monitoring technique. Previous work identified a few volatile compounds emitted by citrus fruit, and blends of compounds, that may have potential for attracting adult female FCM. A field net trial conducted to test the attractiveness of these compounds resulted in only one trap catching one female. A wind tunnel was erected, and trials with all the volatiles will be repeated with virgin females. These trials will start soon.

#### **Opsomming**

VKM word tans in die veld gemonitor deur gebruik van lokvalle met 'n lokaas van gesintetiseerde wyfie mot seksferomoon, en lok dus net mannetjie motte. So 'n stelsel kan egter nooit akkuraat genoeg wees nie, want dit is die wyfies (nie mannetjies nie) wat eiers op die vrugte lê, wat lei tot die larwes wat die skade doen. Daarbenewens, blyk dit dat mannetjie motte groter afstande as wyfie motte vlieg en is daarom nie noodwendig verteenwoordigend van die wyfie populasie in die area nie. Identifikasie van 'n lokmiddel vir wyfies sal 'n meer akkurate moniterings tegniek moontlik maak. Vorige werk het 'n paar vlugtige stowwe en mengsels van verbindings geïdentifiseer wat belofte inhou vir aanlokking van volwasse wyfie VKM. 'n Veldnetproef uitgevoer om die aanloklikheid van hierdie verbindings te toets het gelei tot die vangs van slegs een wyfie VKM. 'n Windtonnel is daarna opgerig en proewe met al die verbindings gaan met ongepaarde wyfies herhaal word. Hierdie proewe gaan eersdaags begin.

### 3.2.11 **PROGRESS REPORT: Synergism between insecticides for improved control of FCM**

Project 1226: (April 2020 – March 2021) by Tamryn Marsberg and Sean Moore (CRI)

#### **Summary**

Synergism was investigated between *Cryptophlebia leucotreta* granulovirus (CrLeGV) and an insect growth regulator (IGR). The IGR was selected for synergism as it binds to the ecdysone receptor complex in lepidoteran larvae and mimics insect moulting, resulting in early death. This is similar to that achieved by deletion of the ecdysteroid glycosyl transferase (*egt*) gene in baculoviruses i.e. moulting, suppressed by the *egt* gene, is restored, resulting in a more rapid death. Synergism between CrLeGV and the IGR was examined using surface dose bioassays against neonate *Thaumatotibia leucotreta* (false codling moth) larvae. Previous results with the selected IGR showed an antagonistic relationship for the majority of the treatments. Alternative insecticides were then selected, azadirachtin (NeemOil®, Biogrow) and emamectin benzoate (Warlock®, ADAMA). Bioassay results with Warlock® and CrLeGV showed mostly antagonistic results. Results with various low concentration of NeemOil® and CrLeGV were extremely promising, with the majority of the treatments showing a synergistic relationship. Further studies will focus on laboratory fruit bioassays and eventually testing various treatment of azadirachtin and CrLeGV in the field. Various other chemical and natural insecticides will also be identified and tested for synergism with CrLeGV.

## Opsomming

Sinergisme is ondersoek tussen *Cryptophlebia leucotreta* granulovirus (CrleGV) en 'n insekgroeireguleerder (IGR). Die IGR is gekies vir sinergisme, aangesien dit bind aan die ecdisoon-reseptorkompleks in Lepidoptera larwes en die insek vervelling naboots, wat lei tot vroeë dood. Dit is dieselfde as wat bereik word deur die verwydering van die ecdisteroïed glikosiel transferase (egt) geen in bakulovirusse, d.w.s. vervelling, onderdruk deur die egt geen, word herstel, wat lei tot 'n vinninger dood. Sinergisme tussen CrleGV en die IGR is ondersoek met behulp van oppervlak dosis biotoetse teen pasuitgeboreide *Thaumatotibia leucotreta* (valskodlingmot) larwes. Vorige resultate met die geselekteerde IGR het 'n antagonistiese verhouding vir die meeste behandelings getoon. Alternatiewe insekdoders is toe gekies, azadirachtin (NeemOil®, Biogrow) and emamectin benzoate (Warlock®, ADAMA). Biotoets resultate met Warlock® en CrleGV het die mees antagonistiese resultate getoon. Resultate met verskillende lae konsentrasies van NeemOil® en CrleGV was baie belowend, en die meeste behandelings het 'n sinergistiese verhouding getoon. Verdere studies sal op laboratoriumvrugte biotoetse fokus en uiteindelik die toediening van azadirachtin en CrleGV in veldproewe. Verskeie ander chemiese en natuurlike insekdoders sal ook geïdentifiseer en getoets word vir sinergisme met CrleGV.

### 3.2.12 **PROGRESS REPORT: Identification and evaluation of male false codling moth pheromones and an investigation of the usefulness for monitoring of female moths**

Project 1256 (April 2020 – Dec 2022) by Adam Shuttleworth, Steve Johnson (UKZN), Sean Moore (CRI), Ally Harari and Victoria Soroker (ARO, VC)

#### Summary

It has been previously determined that the male false codling moth produces pheromone from three androconia and that at least one of these is involved in mating. If these pheromones can be isolated and their role in mating identified, they might be useful as female attractants for monitoring FCM in the field. Currently, trap monitoring is conducted using the female pheromone for attracting male moths. However, this system is not adequately accurate in predicting the threat that FCM poses to the crop, as the male tends to disperse more widely than the female and it is not the male that causes the damage. Isolation and identification of the male pheromones would open the door for their use in the management of FCM, including development of a more accurate monitoring technique and possibly also enhancement of the efficacy of the sterile insect technique. Initiation of this study was unfortunately not possible during 2020/21, due to the Covid pandemic lockdown. However, the study is proceeding during the 2021/22 year.

#### Opsomming

Dit is voorheen vasgestel dat die mannetjie valskodlingmot feromoon produseer van drie androkonië en dat minstens een hiervan betrokke is met paring. As hierdie feromone geïsoleer kan word en hulle rol in paring geïdentifiseer kan word, kan hulle waardevol wees as wyfie lokmiddels vir monitering van VKM in die veld. Tans word monitering gedoen met gebruik van die wyfie feromoon vir aanlokking van mannetjie motte. Hierdie stelsel is egter nie voldoende akkuraat om die dreiging te bepaal wat VKM vir die oes skep nie, omrede die mannetjie is geneig om wyer te versprei as die wyfie mot, en dit is nie die mannetjie wat die skade veroorsaak nie. Isolering en identifisering van die mannetjie feromone sal die deur oopmaak vir sy gebruik in die bestuur van VKM, insluitend die ontwikkeling van 'n meer akkurate monitering tegniek en moontlik ook verbetering van die doeltreffendheid van die steriele insek tegniek. Dit was nie moontlik om hierdie studie gedurende die 2020/21 te begin nie as gevolg van die Covid pandemie inperkings. Die studie word egter gedurende 2021/22 voortgesit.

### 3.2.13 **PROGRESS REPORT: Using the antennal response of the FCM larval parasitoid, *Agathis bishopi*, for identifying key volatiles indicative of FCM fruit infestation**

Project 1260 (April 2020 – March 2022) by Adam Shuttleworth, Steve Johnson (UKZN), Wayne Kirkman, Sean Moore (CRI) and Luke Cousins (RU)

#### Summary

FCM has an effective larval parasitoid, *Agathis bishopi*, which has the ability to locate and parasitise first and second instar FCM within a short period after the larva has penetrated the fruit. If we can identify the volatile cue leading the parasitoid to the precise point in the fruit infested by the larva, we may be able to exploit this for non-destructive identification of FCM infested fruit at a very early stage of infestation. In this study, we aim to use Coupled Gas Chromatography-Electroantennographic Detection (GC-EAD) to identify the key volatile/s indicative of infestation, by recording the antennal response of *A. bishopi* to infested fruit. Ultimately, a device such as an electronic nose or a differential mobility spectrometry (DMS) device could be programmed to detect the identified volatile, rather than depending on the live parasitoid to do this. Due to the Covid pandemic lockdown, it was not possible to initiate this study during 2020/21. However, the study is proceeding during 2021/22.

## Opsomming

VKM het 'n doeltreffende larwe parasiet, *Agathis bishopi*, wat die vermoë het om eerste en tweede instar VKM te vind en te parasiteer binne 'n kort interval na die larwe die vrug gepenetreer het. As ons die vlugtige stof leidraad kan identifiseer wat die parasiet tot die presiese punt in die vrug lei waar die besmetting plaasvind, sal ons hierdie dalk kan gebruik vir nie-vernieteginde identifikasie van VKM besmette vrugte teen 'n baie vroeë stadium van besmetting. In hierdie studie wil ons Gepaarde Gas Kromotografie-Electroantennografiese Opsporing (GC-EAD) gebruik om die sleutel vlugtige stowwe, aanduidend van besmetting te identifiseer, deur om die antenna respons van *A. bishopi* teenoor besmette vrugte aan te teken. Uiteindelik, kan 'n toestel soos 'n elektroniese neus of 'n differensiële mobiliteit spektrometrie (DMS) toestel geprogrammeer word om die geïdentifiseerde vlugtige stowwe op te spoor, eerder as om die lewendige parasiet te gebruik. As gevolg van die Covid pandemie inperking, was dit nie moontlik om die studie gedurende 202/21 te begin nie. Die studie word egter gedurende 2021/22 voortgesit.

### 3.2.14 PROGRESS REPORT: Improving understanding of mating disruption

Project 1262 (April 2020 – March 2022) by Adam Shuttleworth, Steve Johnson (UKZN) and Sean Moore (CRI)

## Summary

Mating disruption for FCM was successfully introduced in South Africa in the late 1990s. Currently, there are four mating disruption products registered and commercially available for FCM. Data on the relative release rates and hence relative pheromone densities of the four products is absent. Additionally, other than for Isomate, adequate data on the influence of temperature on release rate are not available. This is important, as we need to know when, during the season, mating disruption loses its efficacy. Lastly, the optimal (or minimal) density of pheromone in the environment to induce mating disruption and the relationship between pheromone density and reduction in mating events is unknown. This study aims to investigate these important aspects in the successful application of mating disruption for FCM. Unfortunately, initiation of the study was not possible during 2020/21, due to the Covid pandemic lockdown. However, the study is indeed proceeding during 2021/22.

## Opsomming

Paringsontwrigting vir VKM is in die laat 1990s met sukses in Suid-Afrika ingebring. Tans is daar vier paringsontwrigting produkte geregistreer en kommersieel beskikbaar vir VKM. Data op die relatiewe loslatings tempo's van die vier produkte is nie beskikbaar nie. Daarbenewens, behalwe vir Isomate, is voldoende data op die invloed van temperatuur op die loslatings tempo ook nie beskikbaar nie. Hierdie is belangrik omrede ons moet weet op watter stadium in die seisoen verloor paringsontwrigting sy doeltreffendheid. Laastens, is dit onbekend wat die optimale (of minimale) digtheid van feromoon in die omgewing is om ontwrigting van paring te veroorsaak sowel as die verhouding tussen feromoon digtheid en vermindering in parings gevalle. Die doel van hierdie studie is om hierdie belangrike aspekte vir die suksessvolle toepassing van parings ontwrigting vir VKM te ondersoek. Ongelukkig was dit nie moontlik om die studie gedurende 2020/21 voort te sit nie, as gevolg van die Covid panemie afsluiting. Die studie word egeter in 2021/22 voortgesit.

### 3.2.15 **PROGRESS REPORT: Sterile Insect Technique and Mating Disruption in the control of FCM** Project 1282 (2020/21 – 2021/22) by MJ Gilbert, Claire Love and Courtney Morris (CRI)

#### **Summary**

Blocks of citrus, each of approximately 4 ha in size formed the basis for the following treatments at Denau (Vriesland) farm, De Doorns: a) Checkmate as registered: no SIT releases, b) Isomate as registered: no FCM SIT releases, c) Control: no FCM SIT releases, d) Checkmate as registered: with weekly FCM SIT releases, e) Isomate as registered: with weekly FCM SIT releases, f) Control: with weekly FCM SIT releases. Unfortunately, in practice it was not possible to maintain a site where a control block with no sterile FCM releases was acceptable to the grower so treatment “c)” had to be dropped. Within each block 3 yellow delta FCM traps containing Chempac FCM lure were placed at head height on the southern side exterior of the tree canopy. The traps are examined weekly for trapped FCM. Sterile and wild FCM were recorded separately. A row of five data trees were marked at each trap site for the collection and examination of fallen/infested fruit. Application of mating disruption products was carried out along with monitoring of wild and sterile FCM. To date numbers of FCM have been very low with no fruit fall due to this pest. However, numbers of wild and sterile FCM males were highest where sterile FCM were released with no additional mating disruption products being applied. Blocks where Checkmate and Isomate were applied in addition to sterile FCM releases showed a 75% reduction in wild males caught. Sterile FCM male trap catches were suppressed by 94% when comparing the Isomate treated block to the control block. Regarding Checkmate, sterile FCM male catches were suppressed by 90% in treated blocks compared to the control.

#### **Opsomming**

Sitrus blokke, elkeen om en by 4 ha groot het die basis vir die volgende behandelings by Denau (Vriesland) plaas, De Doorns gevorm. a) Checkmate soos geregistreer: geen SIT vrylaatings, b) Isomate soos geregistreer: geen SIT vrylaatings, c) Kontrole: geen SIT vrylaatings d) Checkmate soos geregistreer: met weeklikse SIT vrylaatings, e) Isomate soos geregistreer: met weeklikse SIT vrylaatings, f) Kontrole: met weeklikse SIT vrylaatings. Ongelukkig, in die praktyk, is dit onmoontlik om 'n perseel te kry waar 'n kontrole blok met geen SIT vrylaatings aanvaarbaar was vir 'n sitrusboer nie. As gevolg daarvan is behandeling c) uitgelos. Binne elke blok is 3 geel delta VKM valle met Chempac VKM lokmiddel teen kop-hoogte op die suidelike kant buitekant van die boom geplaas. Die valletjies is weekliks vir VKM gemonitor. Steriele en wilde VKM is afsonderlik aangeteken. 'n Ry van vyf bome is by naby elke valletjie gemerk vir die optel en ondersoek van vrugte wat geval het of tekens van VKM infestasië gewys het. Toediening van paringsontwrigting produkte en moniteering van wilde en steriele VKM is uitgevoer. Tot op datum is getalle van VKM baie laag met geen besmette vrugval nie. Nietemin is getalle wilde en steriele VKM die hoogste waar steriele VKM losgelaat is sonder die toediening van enige addisionele paringsontwrigting produkte. Blokke waar Checkmate en Isomate toegedien is, sowel as steriele VKM loslaatings, het 'n 75% vermindering in wilde mot vangstes gewys. Steriele VKM vangstes in die Isomate-behandelde blok was met 94% onderdruk in vergelyking met die kontrole blok. Wat Checkmate aanbetref, is steriele VKM vangstes met 90% in behandelde blokke in vergelyking met kontroles onderdruk.

### 3.2.16 **PROGRESS REPORT: Comparing the performance of mating disruption for false codling moth control in netted and open orchards**

Project 1283 (2020/21 – 2021/22) by M Gilbert, Claire Love and Courtney Morris (CRI)

#### **Summary**

Many citrus growers are enclosing orchards under netting. Covering an orchard brings benefits in reduced sunburn, hail- and wind damage. Nevertheless, the short and/or long term effect on pest populations have not been fully investigated. In the case of the important phytosanitary pest, false codling moth (FCM) *Thaumatotibia leucotreta* (Meyrick), mating disruption (MD) under netting needs to be compared with MD in open orchards in order to gauge any effect of orchard netting on pest populations. Suitable sites were identified at Denau (Rheebokskloof) and Denau (Vriesland) near De Doorns in the Western Cape. Blocks of citrus, under netting and open, each of approximately 4 ha in size, formed the basis of the following treatments: a)

Checkmate: applied as registered, b) Isomate: applied as registered, c) Control: no applications of chemical mating disruption products. In each block, three yellow delta FCM traps loaded with Chempac FCM lure were hung at head height and evenly spaced throughout the orchard. Traps were monitored weekly with wild and sterile FCM being separately recorded. In addition, at each trap site a row of 5 trees was marked for the collection and evaluation of any fallen/infested fruit that occurs during the season. Isomate suppressed FCM activity equally in both netted and open blocks, giving the lowest counts of both wild and sterile FCM. With Checkmate, higher numbers of wild FCM were caught under nets than in the open. In control blocks, more wild and sterile FCM were under nets than in the open. Three times as many wild moths were caught in the control blocks under nets than in the open. At this initial stage it does not seem as if the presence or absence of nets has a significant effect on moth numbers caught in traps. Unfortunately, moth numbers were low and no fruit infestation was traced thus far.

## Opsomming

Heelwat sitruskwekers maak boorde toe onder nette. Om 'n boord toe te maak bring voordele soos verminderde sonbrand, haelskade en windmerke. Nogtans, die kort en/of lang termyn gevolge op plaag populasies is nog nie volledig ondersoek nie. In die geval van die belangrike fitosanitêre plaag, valskodlingmot (VKM) *Thaumatotibia leucotreta* (Meyrick), paringsontwrigting (PO) onder nette moet vergelyk word met PO in oop boorde ten einde enige verskil in effektiwiteit te bepaal. Geskikte persele op Denau (Rheebokskloof) en Denau (Vriesland) is naby De Doorns in die Wes Kaap geïdentifiseer. Blokke sagtesitrus, onder net en oop, van ongeveer 4 ha grootte, is behandel soos volg: a) Checkmate toegedien soos geregistreer, b) Isomate toegedien soos geregistreer, c) Kontrole: geen toediening van PO produkte nie. In elke blok, is drie geel delta VKM valletjies gelaai met Chempac VKM lokmiddel teen kophoogte gehang en eweredig deur die boord versprei. Daarby, by elke valletjie is daar 'n ry vyf bome gemerk vir die versameling en evaluasie van enige vrugte wat gedurende die seisoen geval het. Isomate het VKM aktiwiteit tot dieselfde mate onderdruk in albei oop en toe boorde en het die laagste tellings van albei wilde en steriele motte getoon. Met Checkmate, is daar hoër getalle wilde motte onder net as in die oop gevang. In kontrole blokke, is drie keer soveel wilde motte onder net gevang as in die oop boorde. Op hierdie vroeë stadium, blyk die asof die aanwesigheid of afwesigheid van nette nie 'n betekenisvolle effek het op mot vangstes waar paringsontwrigting uitgevoer word nie. Ongelukkig is wilde mot getalle baie laag en geen vruginfestasie is opgemerk nie.

### 3.2.17 PROGRESS REPORT: An assessment of the reasons for lower FCM infestation in organic versus conventional citrus orchards

Project 1253 (2019/8-2021/9) by Luke Cousins, Sean Moore (CRI), Martin Hill (Rhodes University), Mellissa Peyper, Candice Coombes (CRI) and Antoinette Malan (Stellenbosch University)

## Summary

Significantly lower levels of FCM infestation are recorded in fruit from organic than fruit from conventional orchards, in packhouse assessments in the Eastern Cape. Conventional wisdom might conclude that naturally occurring biological control would be greater on an organic farm. However, this cannot simply be assumed. Consequently, this study is evaluating all of the possible factors that could be contributing to this difference, namely above and below ground biocontrol, fruit biochemistry and farming practices. The progress of the project remains on schedule and the second and final citrus season is currently underway. Analysis of FCM ecology began in October 2019. Numbers of wild FCM caught within study sites have remained fairly low, but total catches on conventional farms are higher than organic farms. Similarly to the previous season, egg counts have remained low with no clear differences. Significantly higher magnesium content in conventional fruit has been revealed ( $P = 0.02$ ). Recent data from early season fruit analysis appears to support this finding and possibly highlight a few other nutritional differences. Oviposition preference trials show a slight conventional fruit preference, with the final oviposition trials for fruit late in the second season still to be conducted. Eight soil sample sets (over 450 individual soils samples) have been collected and baited, however genetic identification of EPF and EPN isolates is still underway. Differences in abundance between EPFs and EPNs in organic and conventional soil remain unclear, however, the prevalent genera of EPF appear to differ between farming practices, with *Metarhizium* EPF more commonly found in the organic farms (55% of isolates), while *Beauveria* has been more commonly found on conventional farms (67% of isolates). Numbers of rove

beetles and ants, known predators of FCM pupae, were higher on organic farms based on preliminary examination of pitfall traps.

## Opsomming

Aansienlike laer vlakke VKM-besmetting word in vrugte van organiese boorde as vrugte van konvensionele boorde aangeteken, in die pakhuisbeoordelings in die Oos-Kaap. Konvensionele wysheid kan die gevolgtrekking maak dat 'n hoër vlak van biologiese bestryding op 'n organiese plaas voorkom. Hierdie kan egter nie net aanvaar word nie. Gevolglik word in hierdie studie alle moontlike faktore wat tot hierdie verskil kan bydra, geëvalueer, naamlik bogrondse en ondergrondse biologiese beheer, vrug biochemie en boerderypraktyke. Die vordering van die projek bly volgens skedule en die tweede en laaste sitrus seisoen is tans aan die gang. Die ontleding van die VKM-ekologie is in Oktober 2019 begin. Die aantal wilde VKM wat op studiegebiede gevang is, het redelik laag gebly, maar die totale vangs op konvensionele plase is hoër as organiese boerderye. Soortgelyk aan die vorige seisoen het die eiertelling laag gebly sonder duidelike verskille. Aansienlike hoër magnesiuminhoud in konvensionele vrugte is onthul ( $P = 0.02$ ). Onlangse gegewens van vrugte-analise in die vroeë seisoen ondersteun hierdie bevinding en beklemtoon moontlik enkele ander voedingsverskille. Eierleggings-voorkeurproewe toon 'n effense konvensionele vrugvoorkeur, en die finale eierleggingsproewe vir vrugte laat in die tweede seisoen moet nog uitgevoer word. Agt grondmonsterstelle (meer as 450 individuele grondmonsters) is versamel en met larwes gelok, maar genetiese identifikasie van EPF- en EPN-isolate is nogsteeds aan die gang. Verskille in volheid tussen EPF en EPN in organiese en konvensionele grond bly onduidelik, maar die algemene EPF-genusse blyk te verskil tussen boerderypraktyk, met *Metarhizium* EPF wat meer algemeen in die organiese boerderye voorkom (55% van die isolate), terwyl *Beauveria* meer algemeen voorkom op konvensionele plase (67% van die isolate). Die aantal rooikewers en miere, bekende roofdiere van VKM-papies, was hoër op organiese plase, gebaseer op die voorlopige ondersoek na slagatstrikke.

### 3.2.18 PROGRESS REPORT: Field trials for control of FCM

Project 1225 (April 2019 – March 2020) by Sean Moore, Wayne Kirkman, Mellissa Peyper, Tammy Marsberg (CRI), Luke Cousins, Marcel van der Merwe, David Taylor (RU)

## Summary

*Thaumatotibia leucotreta* false codling moth (FCM)) has been declared a regulated pest by the EU. This regulation has called for the development of new control options for FCM. These control options include new and experimental chemical and biological insecticides. Trials were conducted as semi-field bioassays, using two orchards in the Sundays River Valley: Pennyholme and Skinner, to test various products. The same treatments were used at both Pennyholme and Skinner: untreated control, Cryptogran, Cryptogran + molasses, Cryptogran + yeast 1, Cryptogran + yeast 1 + molasses, Cryptogran + yeast 2, Cryptogran + yeast 2 + molasses, Cryptogran + CrpeNPV + molasses and CrpeNPV + molasses. The Pennyholme trial was initiated in May and the highest percentage reduction was obtained from the Cryptogran only treatment. Results were variable and unexpected, this was due to a less than optimal infestation rate of the larvae, as fruit may still have not been ripe enough. The trial was then repeated later on in the season at Skinner. The Skinner spray trial took place in July when fruit had coloured up. The treatment showing the highest percentage reduction in infestation was Cryptogran + yeast 2, with Cryptogran and molasses showing the lowest percentage reduction. A normal spray trial was conducted at Boerboon farm, to examine the effect of molasses in overcoming the difficulty to achieve good spray coverage late in the season, using the following treatments: untreated control, molasses, methoxyfenozide, methoxyfenozide + molasses, Cryptogran and Cryptogran + molasses. The Cryptogran treatment showed the highest percentage reduction in infestation, with the molasses treatment having the highest infestation level. However, efficacy of all treatments was poor, possibly as spray coverage was purposefully sub-optimal. These trials will be repeated in 2021 with an improved semi-field bioassay technique.

## Opsomming

*Thaumatotibia leucotreta* (valskodlingmot (VKM)) is deur die EU tot 'n gereguleerde plaag verklaar. Hierdie regulasie het die ontwikkeling van nuwe beheeropsies vir VKM genoodsaak. Hierdie beheeropsies sluit nuwe en eksperimentele chemiese en biologiese insekdoders in. Twee proewe is as semi-veld biotoets in boorde in die Sondagsriviervallei uitgevoer: Pennyholme en Skinner, om verskillende produkte te toets. Dieselfde behandelings is by Pennyholme en Skinner toegedien, en is as volg: onbehandelde kontrole, Cryptogran, Cryptogran + melasse, Cryptogran + gis 1, Cryptogran + gis 1 + melasse, Cryptogran + gis 2, Cryptogran + gis 2 + melasse, Cryptogran + CrpeNPV + melasse en CrpeNPV + melasse. Die Pennyholme proef is in Mei begin en die hoogste persentasie afname is verkry deur die Cryptogran behandeling. Die resultate was wisselvallig en onverwags; dit was te wyte aan 'n minder as optimale infestasië syfer van die larwes, aangesien vrugte moontlik nog nie ryp genoeg was nie. Die proef is toe later in die seisoen by Skinner herhaal. Die Skinner-spuitproef het in Julie plaasgevind toe vrugte opgekleur was. Die behandeling wat die hoogste persentasie afname in besmetting getoon het, was die van Cryptogran + gis 2, met Cryptogran en melasse wat die laagste persentasie afname het. 'n Gewone spuitproef is by Boerboon Plaas uitgevoer, met die volgende behandelings: onbehandelde kontrole, melasse, metoksiefenosied, metoksiefenosied + melasse, Cryptogran en Cryptogran + melasse. Die Cryptogran behandeling het die hoogste persentasie afname in besmetting getoon, met die melassebehandeling wat die hoogste infestasië vlak getoon het. Werking met alle behandelings was swak, waarskynlik omrede die bedekking van alle behandelings doelgerig suboptimaal was. Hierdie proewe sal in 2021 herhaal word met 'n geoptimaliseerde semi-veld biotoets tegniek.

### 3.2.19 **PROGRESS REPORT: Genetic analysis and field application of a UV-resistant strain of CrleGV for improved control of *Thaumatotibia leucotreta***

Project 1263 (2020 – 2021/22) by T T Bennett (RU), M Hill (RU), S D Moore (CRI), M Jukes and C Knox (RU)

#### **Summary**

*Thaumatotibia leucotreta* is a serious pest of the citrus industry in South Africa and is controlled using an integrated pest management (IPM) programme. One of the components in the IPM programme is *Cryptophlebia leucotreta* granulovirus (CrleGV-SA), the active ingredient of Cryptogran® (River Bioscience (Pty) Ltd, South Africa), which has been used successfully in the field for many years. One of the main factors influencing baculovirus insecticides such as Cryptogran® and its application in the field is UV irradiation. The DNA of the virus is damaged by exposure to UV light and this decreases the efficiency of the biopesticide. Thus there is a need to improve resistance of the OBs to UV. In a recent study, a UV-resistant virus strain of CrleGV-SA, which differed genetically and biologically from the wildtype virus, was selected after repeated exposure of viral OBs to UV-irradiation. This UV-resistant strain has potential as a biocontrol agent for improving the control of *T. leucotreta* in the field. It is important to determine whether the genetic and biological stability of this strain is maintained when passaged through the host for commercial purposes, which is the aim of this study.

#### **Opsomming**

*Thaumatotibia leucotreta* is 'n ernstige plaag in die sitrusbedryf in Suid-Afrika en word beheer met behulp van 'n geïntegreerde plaagbestuursprogram (IPM). Een van die komponente in die IPM-program is *Cryptophlebia leucotreta* granulovirus (CrleGV-SA), die aktiewe bestanddeel van Cryptogran® (River Bioscience (Pty) Ltd, Suid-Afrika) wat jare lank suksesvol in die veld gebruik is. Een van die belangrikste faktore wat bakulovirus-insekdoders beïnvloed, soos Cryptogran®, en die toepassing daarvan in die veld, is UV-bestraling. Die DNA van die virus word beskadig deur blootstelling aan UV-lig en dit verminder die doeltreffendheid van die biologiese plaagdoder. Daar is dus 'n behoefte om die weerstand van die OBs teen UV te verbeter. In 'n onlangse studie is 'n UV-weerstandige virusisolaat van CrleGV-SA wat geneties en biologies van die wildtipevirus verskil, gekies na herhaalde blootstelling van virale OBs aan UV-bestraling. Hierdie UV-weerstandige isolaat het potensiaal as 'n biobeheermiddel om die beheer van *T. leucotreta* in die veld te verbeter. Dit is belangrik om vas te stel of die genetiese en biologiese stabiliteit van hierdie isolaat gehandhaaf word wanneer dit vir kommersiële doeleindes deur die gasheer gevoer word, wat die doel van hierdie studie is.

### 3.3 PROGRAMME: FRUIT FLY

Programme coordinator: Aruna Manrakhan (CRI)

#### 3.3.1 Programme summary

The aim of the fruit fly research programme is to collate information on the biology, ecology and control of fruit fly pests in order to effectively mitigate the risk from these pests in citrus from southern Africa. There is a zero tolerance of fruit fly in export citrus. Three fruit fly species were listed as pests of citrus: *Ceratitis capitata*, *Ceratitis rosa* and *Bactrocera dorsalis*. There have however been records of other fruit fly species present in South Africa on citrus. *Ceratitis cosyra* was recorded on citrus from South Africa in Europe and *Ceratitis quilicii* was reared from citrus in Reunion Island. Not all citrus types are susceptible to fruit flies in South Africa. Commercial lemons and limes are non-hosts.

A project on the efficacy of attract and kill products in suppressing fruit fly populations in orchards was completed (3.3.2). All attract and kill products effectively suppressed fruit fly populations. A project on determination of equivalence of a *C. capitata* cold treatment for *C. cosyra* was completed (3.3.3). Based on the results of this project, all cold treatments for *C. capitata* should be equally effective against *C. cosyra*.

Six other projects are still ongoing from previous years. The fruit fly rearing project continues (3.3.4) and supports other projects within the programme. The efficacy of a cold treatment at 3.5°C for fruit flies was quantified (3.3.5.). The effect of cold treatment interruptions on fruit fly larval survival is being determined (3.3.6). The role of variables such as temperature and relative humidity in influencing lure emission and trapping outcomes is being investigated (3.3.7). The distribution, host ranges and demography of *C. quilicii* are being studied (3.3.8). Under a project funded by the European Union Horizon 2020 programme, new technologies such as E-traps and detection system models for *B. dorsalis* are being investigated (3.3.9).

#### Programopsomming

Die doel van die vrugtevlug navorsingsprogram is om inligting oor die biologie, ekologie en beheer van vrugtevlugplae te versamel, ten einde die risiko van hierdie plae in sitrus vanaf suidelike Afrika effektief te beperk. Daar is 'n nul-toleransie vir vrugtevlug in uitvoe sitrus. Drie vrugtevlug spesies is as plae van sitrus gelys: *Ceratitis capitata*, *Ceratitis rosa* en *Bactrocera dorsalis*. Daar was egter al rekords van ander vrugtevlug spesies teenwoordig in Suid-Afrika op sitrus. *Ceratitis cosyra* is op sitrus vanaf Suid-Afrika in Europa aangeteken en *Ceratitis quilicii* is vanaf sitrus in Reunion Eiland geteel. Nie alle sitrus tipes is vatbaar vir vrugtevlug in Suid-Afrika nie. Kommersiële suurlemoene en lemmetjies is nie-gashere.

'n Projek op die doeltreffendheid van lokmiddel en uitwis produkte in die onderdrukking van vrugtevlug populasies in boorde is voltooi (3.3.2). Alle lokmiddel en uitwis produkte het vrugtevlug populasies effektief onderdruk. 'n Projek oor die vasstel van 'n ekwivalent van 'n *C. capitata* koue-behandeling vir *C. cosyra* is voltooi (3.3.3). Gebaseer op die resultate van hierdie projek, behoort alle koue-behandelings vir *C. capitata* ewe effektief teen *C. cosyra* te wees.

Ses ander projekte word steeds vanaf vorige jare voortgesit. Die vrugtevlug teelprojek gaan voort (3.3.4) en ondersteun ander projekte binne die program. Die doeltreffendheid van 'n koue-behandeling by 3.5°C vir vrugtevlug is gekwantifiseer (3.3.5.). Die effek van koue-behandeling onderbrekings op vrugtevlug larwe oorlewing, word vasgestel (3.3.6). Die rol van veranderlikes soos temperatuur en relatiewe humiditeit in die beïnvloed van lokmiddel vrystelling en lokval uitkomste, word ondersoek (3.3.7). Die verspreiding, gasheerreeks en demografie van *C. quilicii* word bestudeer (3.3.8). Nuwe tegnologieë soos E-traps en opsporing stelselmodelle vir *B. dorsalis* word onder 'n projek wat deur die European Union Horizon 2020 program befonds word, ondersoek (3.3.9).

#### 3.3.2 FINAL REPORT: Attract and kill methods for fruit flies: efficacy and application of new and registered products

Project 1211 (April 2018- March 2021) by Aruna Manrakhan, John-Henry Daneel, Leani Serfontein, Rooikie Beck, Wayne Kirkman, Mellissa Peyper, Tamryn Marsberg and Sean Moore (CRI)

## Summary

The aims of this project were to quantify the efficacy and optimise the application of attract and kill products for control of fruit flies in citrus. Field trials were conducted between 2018 and 2020 in citrus orchards to evaluate attract and kill devices. In 2018 and 2019, M3 and Magnet Med bait stations were evaluated at densities at each end of their recommended ranges. In 2020, M3 and Magnet Med were evaluated at densities at the higher end of and above their recommended ranges. Two new products (Cera Trap and Beta P1) were included in field trials in 2020. The effect of weathering on the efficacy of M3 and Magnet Med was then quantified. The phytotoxicity of bait sprays on mandarin fruit was determined. In laboratory and semi field trials, the effectiveness of baits to different fruit fly species were compared. *Ceratitis capitata* was the dominant fruit fly species recorded in orchards during the field trials. While catches of *C. capitata* did not differ significantly between treatments, irrespective of bait station densities, low incidences of fruit infestation by *C. capitata* were recorded close to harvest in single replicate blocks treated with M3 and Beta P1 bait stations. Responses of *C. capitata* females to M3 bait stations decreased after four weeks of aging in the field while for Magnet Med, there was no effect of weathering on fly responses. All bait spray mixtures left phytotoxic marks on mandarin fruit when applied before colour break. All attract and kill devices tested were effective against all fruit fly species in semi field studies. Attract and kill devices within their recommended ranges would suppress fruit fly adult populations in citrus orchards. When M3 bait stations are used, shorter replacement intervals (currently at 12 weeks) may be considered.

## Opsomming

Die doelwitte van hierdie projek was om die doeltreffendheid van lokmiddel en uitwis produkte vir die beheer van vrugtevlieë in sitrus te evalueer, en die toediening daarvan te optimaliseer. Veldproewe is tussen 2018 en 2020 in sitrusboorde uitgevoer ten einde lokmiddel en uitwis toestelle te evalueer. In 2018 en 2019 is M3 en Magnet Med lok-aasstasies by digthede op elke end van hul aanbevole reekse geëvalueer. In 2020, is M3 en Magnet Med by digthede op die hoër end van en bó hul aanbevole reekse geëvalueer. Twee nuwe produkte (Cera Trap en Beta P1) is in die veldproewe in 2020 ingesluit. Die effek van verwerking op die doeltreffendheid van M3 en Magnet Med is toe gekwantifiseer. Die fitotoksiteit van lok-aasspuitte op mandarynvrugte is bepaal. In laboratorium- en semi-veldproewe, is die doeltreffendheid van lok-aasmiddels teen verskillende vrugtevlieg spesies vergelyk. *Ceratitis capitata* was die dominante vrugtevlieg spesie wat in boorde gedurende die veldproewe aangeteken is. Terwyl vangste van *C. capitata* nie betekenisvol tussen behandelings verskil het nie, ongeag lok-aasstasie digthede, is lae voorkomste van vrugbesmetting deur *C. capitata* naby oes in enkelherhaling blokke wat met M3 en Beta P1 lok-aasstasies behandel is, aangeteken. Reaksies van *C. capitata* wyfies teenoor M3 lok-aasstasies het ná vier weke van veroudering in die veld afgeneem, terwyl daar vir Magnet Med geen effek van verwerking op vlieg reaksies was nie. Alle lok-aasspuitmengsels het fitotoksiese merke op mandarynvrugte gelaat wanneer vóór kleurbreek toegedien is. Alle lokmiddel en uitwis toestelle wat getoets is, was teen alle vrugtevlieg spesies in semi-veldstudies doeltreffend. Lokmiddel en uitwis toestelle binne hul aanbevole reekse, sal vrugtevlieg volwasse populasies in sitrusboorde onderdruk. Wanneer M3 lok-aasstasies gebruik word, kan korter vervangings-intervalle (tans op 12 weke) oorweeg word.

## Introduction

Fruit fly pests in commercial fruit orchards across the world are mainly controlled using attract and kill methods. Attract and kill products for fruit flies contain fruit fly attractants which are either food-based (often protein-based) or male lures. The killing methods in attract and kill products can be either through the use of insecticides or by retention (for example drowning in liquid based traps). Depending on the type of attract and kill product, methods of deployment can be either as sprays or stations/traps.

Currently in South Africa, there is still a limited set of attract and kill products which are registered for fruit fly control in citrus orchards (Manrakhan, 2020) Among the registered attract and kill products, the efficacy of some products, in particular the protein based fruit fly bait stations, is often questioned especially in commercial orchards under high fruit fly prevalence. The number of applications of currently registered products might also in the future be more limited as part of insecticide resistance management. As such during a season, two to

three different fruit fly control products may need to be applied. Finally for the increased mandarin cultivation, phytotoxicity due to insecticidal bait sprays is a concern (Manrakhan *et al.*, 2015). For mandarin orchards, alternatives to bait sprays may need to be sought.

At the same time, a number of new fruit fly attract and kill methods such as the protein based Biotrap Fruit Fly Attractant Gel (Biotrap Australia Pty Ltd, Australia), the protein based Fruit fly Mania (Kenya Biologics Ltd, Kenya), the protein based Cera trap (Bioiberica, Spain), the male lure based MEGO Kit mass trapping product for *Ceratitis* and *Bactrocera dorsalis* males (Insect Science Pty Ltd, South Africa) were recently developed with some already being commercialised and used in other parts of the world.

The aims of this project were to optimise the application of currently registered attract and kill methods and determine the efficacy of new attract and kill products.

### **Stated objectives**

- A. To determine the best density of registered protein-based bait stations in areas under high fruit fly prevalence
- B. To determine the effects of weathering on attract and kill efficacy of registered protein-based bait stations
- C. To determine phytotoxicity of ground-based and aerial protein bait spray mixtures on mandarin
- D. To determine attractiveness and toxicity of new attract and kill methods under laboratory and semi field conditions

### **Materials and methods**

#### Application of registered protein-based fruit fly bait stations under high fruit fly prevalence

Registered protein-based fruit fly bait stations were evaluated in citrus orchards over three seasons: 2018, 2019 and 2020. In 2018 and 2019, two application rates of two bait stations: M3 bait station and Magnet Med within their recommended ranges were compared in citrus farms which were at different levels of fruit fly prevalence (low and high) based on historical counts. In 2018, trials were carried out in Midnight Valencia orchards and in 2019, trials were carried out in Nadorcott mandarin orchards. In 2020, M3 bait station and Magnet Med were each tested at their highest recommended application rate and at a rate above the recommended range in Nadorcott mandarin orchards in two citrus farms under two practices: one under net and one in the open. Two newly registered attract and kill products: CeraTrap and Beta P1 were included in the 2020 trial. In 2018 and 2019, trials were carried out over 9-10 weeks, starting between March and June. In 2020, trials were carried out for 26-28 weeks starting between February and March. In each year and on each farm, there were three replicate blocks of each treatment. The details on the study sites, treatments evaluated and conditions during the trial are provided in Table 3.3.2.1.

**Table 3.3.2.1.** Details of study sites used in the evaluation of bait products and climatic conditions prevailing during trials conducted between 2018 and 2020

Year	GPS Co-ordinates	Province	Farm	Treatments evaluated	No. of replicate blocks and sizes/ha	Climatic conditions prevailing during trial		
						Mean ( $\pm$ SE) temperature	Mean ( $\pm$ SE) relative humidity	Total rainfall (mm)
2018	S33.459488° E25.550635°	Eastern Cape	Riverside	M3 at 300 units per ha	3 (1,1,1)	12.4 $\pm$ 0.2	73.1 $\pm$ 0.7	43.9
				M3 at 400 units per ha	3 (1,1,1)			
				Magnet Med at 50 units per ha	3 (1,1,1)			
				Magnet Med at 75 units per ha	3 (1,1,1)			
	S25.685661° E31.190521°	Mpumalanga	Siyalima	M3 at 300 units per ha	3 (1,1,1)	14.0 $\pm$ 1.1	58.8 $\pm$ 0.6	6.1
				M3 at 400 units per ha	3 (1,1,1)			
				Magnet Med at 50 units per ha	3 (1,1,1)			
				Magnet Med at 75 units per ha	3 (1,1,1)			
2019	S25.403013° E31.088977°	La Visagie	M3 at 300 units per ha	3 (1,1,1)	18.5 $\pm$ 0.1	64.4 $\pm$ 0.4	98.8	
			M3 at 400 units per ha	3 (1,1,1)				
			Magnet Med at 50 units per ha	3 (1,1,1)				
			Magnet Med at 75 units per ha	3 (1,1,1)				
	S25.566113° E31.319189°	Greenstone	M3 at 300 units per ha	3 (1,2,1)	16.4 $\pm$ 0.1	64.4 $\pm$ 0.5	63.9	
			M3 at 400 units per ha	3 (1,1,1)				
			Magnet Med at 50 units per ha	3 (1,1,1)				
			Magnet Med at 75 units per ha	3 (1,1,1)				
2020	S25.403013° E31.088977°	La Visagie	M3 at 400 units per ha	3 (1,1,1)	18.2 $\pm$ 0.1	66.3 $\pm$ 0.3	343.9	
			M3 at 600 units per ha	3 (1,1,1)				
			Magnet Med at 75 units per ha	3 (1,1,1)				
			Magnet Med at 100 units per ha	3 (1,1,1)				
			CeraTrap at 100 units per ha	3 (1,1,1)				
			Beta P1 at 400 units per ha	3 (1,1,1)				
	S25.362031° E30.867698°	Louranza	M3 at 400 units per ha	3 (1,1,1)	17.7 $\pm$ 0.1	63.0 $\pm$ 0.4	283.8	
			M3 at 600 units per ha	3 (1,1,1)				
			Magnet Med at 75 units per ha	3 (1,1,1)				
			Magnet Med at 100 units per ha	3 (1,1,1)				
			CeraTrap at 100 units per ha	3 (1,1,1)				
			Beta P1 at 400 units per ha	3 (1,1,1)				
M3 at 400 units per ha	3 (1,1,1)							

The efficacy of treatments was assessed by monitoring of adult fruit fly populations using trapping and by doing a fruit damage assessment close to harvest in each replicate block. Adult fruit flies were monitored using four attractant-trap combinations: Capilure baited Sensus trap, Questlure baited Sensus trap, 3-component Biolure baited Chempac Bucket trap and ME baited Lynfield trap. There were two traps of each attractant-trap combination in each replicate block of each treatment. Traps were positioned in the middle rows of each replicate block and were spaced at least 30 m from each other. Traps were checked and emptied weekly except between March and May 2020 where trap intervals in each study side were higher due to restricted movement during the national lockdown. Attractants and insecticides inside traps were replaced after 6 weeks. Flies collected in traps were emptied in vials and brought back to the laboratory for identification to species and sex. Fruit in each replicate block were assessed for fruit fly damage either one week prior to harvest or at harvest. In each replicate block, 500 fruit were selected at random from trees. A total of 10 fruit from each of 50 trees in the middle rows of each replicate block were selected at random. During the assessment, each fruit was checked for visible fruit fly damage symptoms. The symptoms checked were the presence of fruit fly punctures and discolouration of peel resulting from fruit fly punctures. Fruit showing damage symptoms were picked, brought to the lab, checked under the microscope, weighed and incubated individually at ambient room temperature over a layer of sand for a period of eight weeks to confirm fruit fly infestation, determine the fruit fly species and degree of fruit fly infestation (number of flies per kg of infested fruit). Additionally, about 3 kg of fruit (about 10 fruit) were collected from the ground from each replicate block, brought back to the lab, weighed and incubated in bulk in aerated plastic containers over a sand layer at ambient room temperature for eight weeks to determine fruit fly infestation and degree of infestation. Fruit selected from the ground were those that had visible insect damage symptoms.

For each replicate block in each trial site, male and female numbers of the targeted fruit fly pests (*C. capitata*, *C. rosa* and *B. dorsalis*) were processed as number of flies per trap per day. This was the total number of flies over the number of traps of the attractant-trap combination over the average number of trap days. Capilure baited traps targeted *C. capitata* and *C. rosa* males. Questlure baited traps and 3-component Biolure baited traps targeted females of all three species. ME baited Lynfield traps targeted males of *B. dorsalis*. The percentage fruit fly damage was calculated for each replicate block of each treatment for fruit assessed on the trees at or near harvest. The degree of infestation was processed as total number of flies over total weight of damaged fruit within the replicate block for fruit sampled from the tree. For fruit sampled from the ground, degree of infestation was processed as the total number of flies over total weight of sampled fruit within the replicate block.

Differences in captures of targeted fruit fly pests between treatments and farms were determined using Mixed Models with time as a repeated factor and replicate plot as subject factor. Interactions between treatment and farm as well as between treatment and time were also included in the model.

#### Effects of weathering on attract and kill efficacy of registered protein-based bait stations

The effects of weathering of M3 bait station and Magnet Med on responses of *C. capitata* to these products were determined in nylon screen field cages at Citrus Research Centre (CRC), Citrus Research International, Nelspruit. Six ages of M3 bait stations and Magnet Med were evaluated in no choice and choice tests. In no choice tests, the tested bait station was evaluated on its own. In choice tests, a test bait station was paired with a fresh bait station for the evaluation. The bait stations were tested at the following ages: fresh (0 week), 4 weeks, 8 weeks, 12 weeks, 16 weeks, 20 weeks and 24 weeks. M3 bait stations and Magnet Med were aged in a late Valencia orange orchard at Crocodile Valley between June and December 2018.

For the tests, *C. capitata* adult flies were obtained from laboratory reared colonies maintained at CRC. Upon eclosion, flies were fed with only sugar and water and were therefore completely deprived of protein. Flies used were 7-14 days old. One hundred males and 100 females were used per test.

Flies were released into a cage one hour before start of the test. Two cages were used concurrently. Each cage contained 10 potted orange trees. Two water dispensers were placed inside each cage to provide a water source for flies. M3 bait stations and Magnet Med were tested in separate cages. Cages were rotated across the replicates for each type of bait station. Choice and no choice tests were carried out in alternate weeks.

In the no choice test, each cage contained one tested bait station. In the choice tests, each cage contained a pair of bait stations: one weathered and one fresh.

Each bait station was placed inside a black painted Chempac Bucket trap. A Dichlorvos strip (1 g) was placed inside each trap to kill any attracted flies. In no choice tests, traps were hung in the middle of the cage. In choice tests, traps were hung at equal distances in the centre of each cage. In choice tests, initial positions of the traps containing bait stations were allocated at random. Traps were rotated four times in each cage following the first placement.

Baits were exposed for 23 hours inside the cages (from 14:00 until 13:00 the following day) after which they were removed, brought to the lab and emptied. Specimens were separated by sex and counted. In each test, there were four replicates conducted for each type of bait station and age.

The mean number of *C. capitata* females and males captured in traps with each age of bait station were calculated. For the no choice assays, effects of bait stations, age of bait station, fly sex and interactions between bait station and age were determined using ANOVA. For the choice assays, a multi-way ANOVA was used to compare catches between aged and fresh stations. The fixed effects were treatment group (bait station & age), test (aged versus fresh station) and fly sex. The interactions between treatment group, test and age were also determined. Prior to the statistical analysis, all data were log (x+1) transformed to stabilise variances.

#### Phytotoxicity of ground-based and aerial protein bait spray mixtures on mandarin

##### *Phytotoxicity of Hymlure malathion treatment on mandarin*

An experiment was carried out in a Nadorcott mandarin orchard near Nelspruit, Mpumalanga in June 2018 to determine the phytotoxicity of HymLure malathion mixture when applied at or before the colour break stage. Five trees were selected in the orchard for evaluation of treatments. Fruit on each tree was selected at 0.5-1.5 m above ground. Fruit selected were free of any phytotoxic or damage marks. On each tree, four fruit at the colour break stage were selected. One treatment was tested per fruit. Treatments were applied between 9:00 am and 12:00 pm. Four circles were drawn at the stylar end of each fruit. Four 10 µl droplets of the treatment were placed, one in the middle of each of the four marked circles. Treatments were applied using an autopipette. Separate tips were used for each treatment.

The treatments evaluated were as follows:

1. GF-120 at 10% (dilution with water)
2. HymLure and malathion mixture (4 ml HymLure and 1.75 ml malathion in 1 L of water)
3. GF-120 at 10% (dilution with water) with delayed bait drying
4. HymLure and malathion mixture (4 ml HymLure and 1.75 ml malathion in 1 L of water) with delayed bait drying
5. Water (control)

For delayed bait drying, fruit were covered with a transparent polyethylene bag lined at the bottom with a wet paper towel after bait application. The bag was tied around the branch in such a way that the fruit was held in the middle of the plastic container with no part of the fruit touching the bag. The bag was removed after 24 hours.

Each treatment was applied on each of the five trees selected. There were therefore five replicates per treatment. All treated fruit were picked at seven days after treatment. Immediately after picking, the fruit were washed by immersing in tap water for 5 minutes. During immersion, dried bait droplets were removed using a fine cloth. The fruit were dried. After one week, the fruit were dipped in an Ethephon solution (25 ml/5 L) for 30 seconds to induce a colour change. Fruit were dried at ambient room temperature with the stylar end facing up. Two weeks after the Ethephon dip, fruit were examined for presence of phytotoxicity in each marked area. Marks were first checked with the naked eye to determine symptoms visible with the naked eye. Thereafter fruit were examined under the microscope to record phytotoxic symptoms which could only be detected when viewed under the microscope. Presence of symptoms was recorded as 1 and absence as 0. Data were

summarised as average number of droplets with phytotoxic symptoms for each treatment. A logistic regression was used to determine the effects of treatment on presence of symptoms either visible to the naked eye or under the microscope.

#### *Phytotoxicity of ground based bait spray mixtures on mandarin at the colour break stage*

In June 2019, another trial was conducted to assess whether ground based bait sprays (with GF-120 used as a control) could leave phytotoxic symptoms on mandarin fruit when applied at colour break. This time, bait sprays were applied on the entire tree using a knapsack sprayer.

The study was carried out in a Nadorcott mandarin farm near Kaapmuiden, Mpumalanga Province. Trees were selected over three rows in a block which had a once-off aerial treatment of HymLure and malathion when the fruit were at colour break. The following treatments were then applied using a knapsack sprayer when the fruit were still at the colour break stage:

1. GF-120 (10% dilution with water)
2. HymLure and Exirel SE (4 ml of HymLure and 1 ml of Exirel in 0.5 L of water)
3. HymLure and malathion EC (4 ml of HymLure and 1.75 ml of malathion EC in 1 L of water)

For GF-120, 45 ml of the mixture was applied per tree. For the other baits, about 100 ml of mixture was applied per tree. Each treatment was applied on six trees. Trees with no ground-based sprays were selected as a control. At harvest, 10 fruit were selected and picked from each of three canopy layers: top, middle and bottom on each tree (30 fruit per tree). Fruit were assessed for presence/absence of stippling marks (burns on stomata around oil glands) which were visible to the naked eye. Presence was denoted as 1 and absence as 0. Effects of treatment and canopy layer on presence/absence of stippling marks were analysed using a logistic regression.

#### Attractiveness and toxicity of new attract and kill methods under laboratory and semi-field conditions

##### *Laboratory trials to determine attractiveness of new products*

One of the newly registered attract and kill products for fruit fly control in citrus orchards in South Africa is CeraTrap. The product is used as a mass trapping system. CeraTrap is a hydrolysed protein bait. The CeraTrap bait (600 ml undiluted bait) is contained in a CeraTrap trap and placed at 50 – 100 units per ha in citrus orchards. The attractiveness of this new bait was compared to a standard bait HymLure in olfactometer bioassays.

Three fruit fly species: *Ceratitis capitata*, *Ceratitis rosa* and *Bactrocera dorsalis* were used in the tests. Flies used originated from laboratory colonies maintained at Citrus Research International, Nelspruit. Virgin females which were 7-10 days old were used in the tests. These females were completely protein deprived (fed on only sugar and water since emergence).

The two baits tested were: 1. HymLure at 0.8% (currently used concentration in the citrus industry) and 2. CeraTrap bait (undiluted). Each bait type was absorbed on a cotton roll. A cotton roll contained 2 ml of each bait type.

A custom-made four-arm olfactometer was used for the tests. The olfactometer consisted of four clear plexiglass cylindrical tubes each divided into three adjoining chambers: release chamber, catch chamber and bait chamber with the latter ending with a fan. The four cylindrical tubes were connected to an extractor fan, each at the end of the release chamber, which together with the fan would create an air current of up to 197 feet/min. The air current in each arm was measured before the start of each test. Four light tubes were installed above each cylindrical tube to provide uniform light over the tubes. The prevailing light above each chamber was measured using a light meter.

Ten minutes before the introduction of the bait in the bait chamber, 20-30 females previously chilled for 30 minutes in a refrigerator were placed in each chamber. No choice tests were carried out whereby one bait type was compared to a control (blank cotton roll). In the olfactometer, two tubes contained the controls and two tubes contained the test bait. Once the bait was introduced in the chamber, the fans were activated. Each test was carried out for one hour, at the end of which flies in the catch chamber were counted. Dead flies in the release chamber were also counted in order to determine the net flies tested. Tests were carried out on the three species on one test day. The order of tests among the species was allocated at random. Tests on the two baits were carried out over two days on the same fly cohort of each species. The order of test of the baits was also at random. There were eight replicates in total for each bait test and each species using eight fly cohorts of each species.

Data were summarized as percentage of females caught (number of flies in catch chamber divided by net number of tested flies multiplied by 100). An ANOVA was used to determine effects of bait and species on catches. Data were arcsine square root transformed to stabilize variances before analysis.

#### *Field cage trials to determine attractiveness of new products*

Attraction responses of fruit flies to CeraTrap and three protein attractants including HymLure (tested in the laboratory) were further determined in field cage assays on laboratory reared *B. dorsalis*, *C. capitata* and *C. rosa*. Males and females of all three species were used for the tests and were 7-11 days old on test day. Flies were completely protein deprived (fed on only sugar and water since emergence).

Treatments evaluated were:

1. HymLure at 0.8% (currently used concentration in the citrus industry)
2. CeraTrap bait (undiluted)
3. Three-component Biolure
4. Questlure

The four attractants were tested in choice and no choice assays. Tests were carried out in two nylon screen field cages. Ten potted orange trees were present inside each cage. For each test, 100 males and 100 females were used for each species. Flies were released into the cage one hour before the start of the test. Two water dispensers were placed inside each cage to provide a water source for flies. Each attractant was placed inside a black painted Chempac Bucket trap. For Cera Trap and HymLure, two hundred ml of each liquid attractant was poured inside a trap. For the dry attractants: three-component Biolure and Questlure, the dispenser (membrane dispenser or capsule) was suspended from the top of the trap and two hundred ml of water were poured in the trap in order for them to be exposed as wet traps. For all attractants, a Dichlorvos strip (1 g) was additionally placed inside each trap to kill any attracted flies that did not drown. The Dichlorvos strip was suspended in a basket glued to the top of the trap. Traps were hung in the middle of each cage. The baited traps were exposed for 23 hours inside the cages (from 14:00 on test day until 13:00 the following day) after which they were removed. Following trap removal, the liquid was sieved. Captured flies were removed from the sieve and placed in alcohol. Specimens captured were identified to species and sex.

In choice tests, there were five traps in total in each cage: four traps with the tested attractants and one control trap as described above with water only. Initial positions of the traps containing the attractants were allocated at random. The traps were then rotated four times in each cage following the first placement (at 16:00, 08:00, 10:00 and 12:00) such that trap positions were occupied equally. There were six replicates of the choice test conducted over six weeks.

In a no-choice test, only one selected attractant was placed per cage. In each cage, there was also a black painted Chempac Bucket trap filled with water only (control trap). The control trap also contained a DDVP strip. Cages used for the four attractants were rotated across the replicates and attractants were tested over three days due to a limited number of cages. Initial positions of traps within a cage were allocated at random. The traps were then rotated once (at 08:00) on the day following the first trap placement in each cage such that trap positions were occupied equally. There were six replicates conducted over six weeks for each type of attractant.

Choice and no-choice tests were alternated across the 12 test weeks.

#### *Toxicity of attract and kill products for fruit flies*

In a final experiment, the toxicity of three attract and kill products was compared to a standard protein bait spray product in field cages. Four fly species were tested: *B. dorsalis*, *C. capitata*, *C. cosyra* and *C. rosa*. The flies were obtained from laboratory colonies maintained at CRC, Nelspruit. Upon eclosion, flies were supplied with water and sugar while being completely deprived of protein. Fly species used in the trials were between 7 – 10 days old. Approximately 10 males and 10 females from each species were used for each treatment. The products evaluated were: CeraTrap, M3 and Magnet Med.

Four ages of these products were tested: Fresh (just removed from pack), 4 weeks, 8 weeks and 12 weeks. The products were aged by placing them under a shed (roof with three open sides). The evaluation was carried out using paired tests with toxicities of fresh and aged attract and kill products compared concurrently. All treatments were measured against a registered bait spray, GF120 (Corteva Agriscience™). GF-120 was diluted with water (10% dilution) as per the recommendations on its label. Each tree had a standard of 120-130 leaves. GF120 was applied using a hand held spray bottle on a tree and a volume of 50 ml was used for each tree. The spray bottle was held 5-10 cm from the tree while spraying and the GF-120 bait spray was always applied fresh. There was a control where no attract and kill product was used.

Trials were carried out in nylon screen cages (Length=109 cm; Breadth=60 cm; Height=184 cm) that were placed inside two field cages. The bottom of each cage was lined with a white canvas sheet. Each cage held one young Empress mandarin seedling that was placed inside the centre of each cage. All trees were grown in pots under the same conditions. The same trees were used for the same treatments during the course of the trial. A petri dish containing sugar and an inverted jar containing water were suspended inside the cage to ensure presence of food in the cage. Precautions were taken to ensure that no ants came into the cages during the trials. The legs of the cages were placed over bowls filled with soap water. Ant glue was smeared around the bowls. Each attract and kill product was suspended on the tree inside the nylon screen cage. The different fly species were released into the cage concurrently. The number of dead flies in the trap and on the floor was recorded up until 72 h after release. The dead flies were removed from the trap and the sex and species were identified. All the remaining flies were removed from the cage using an aspirator at the end of the trial. These flies were killed by freezing, and the sex and species were also recorded to account for the number of flies released. There were four replicates for each age of the attract and kill product tested.

Data were summarised as cumulative percentage fly mortality at 72 hours after exposure. This was calculated as the number of flies dead after 72 hours over the number of flies released. Mortality for each bait tested was corrected from control (natural mortality). Effects of species, bait and bait age were analysed using Mixed Models.

## **Results and discussion**

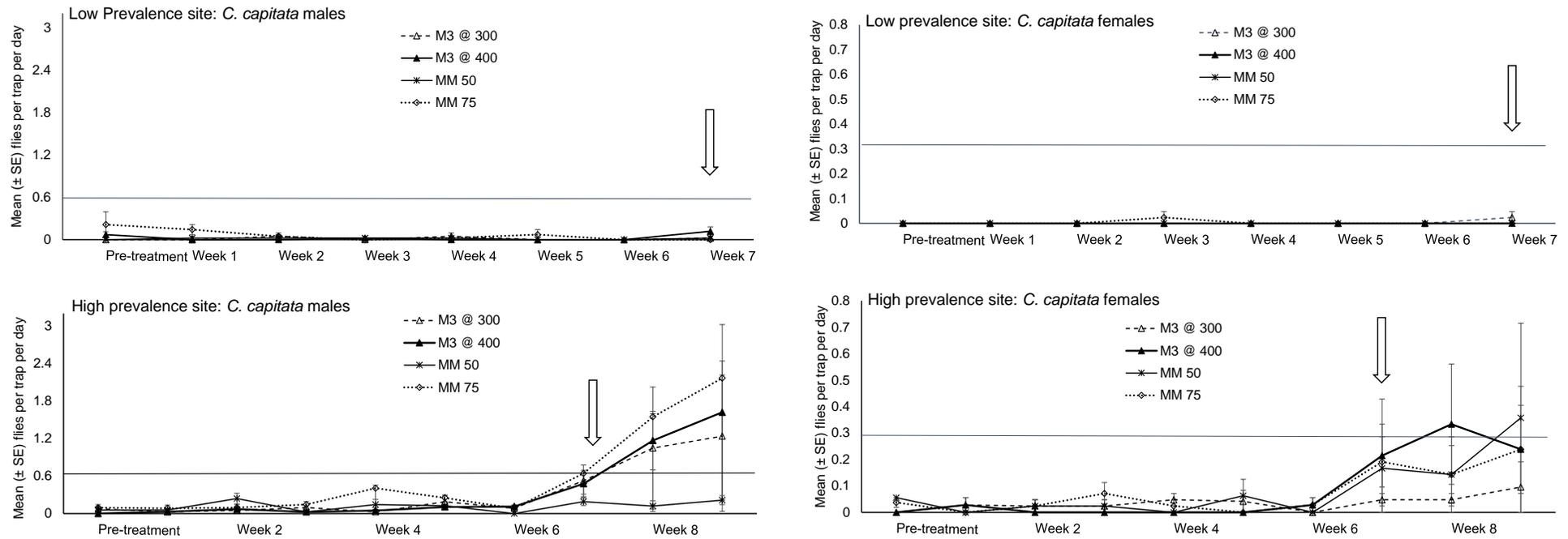
### Application of registered protein-based fruit fly bait stations under high fruit fly prevalence

In all trial sites over the three years, *C. capitata* was the dominant fruit fly pest recorded in traps. All trapping results were therefore presented on this species. There were variations in catches of *C. capitata* males across the weeks in each trial site over the three years and between trial sites in 2019 and 2020 (Table 3.3.2.2). Results are therefore presented separately for the different trial sites and presented across time for each site (Figs. 3.3.2.1-3.3.2.3). Catches of *C. capitata* females were higher in Biolure baited Chempac traps compared to Questlure baited Sensus traps in all farms and years. Therefore for *C. capitata* females, only catches obtained in the Biolure baited Chempac traps in the different treatments were presented (Figs. 3.3.2.1-3.3.2.3).

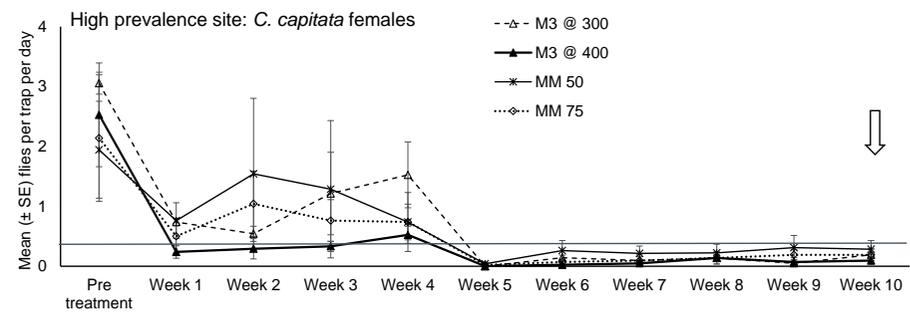
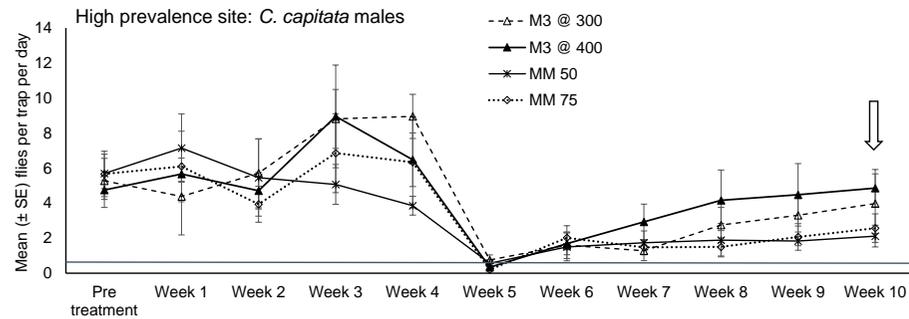
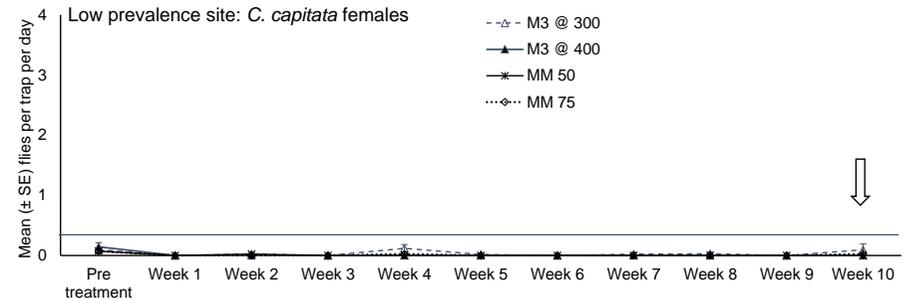
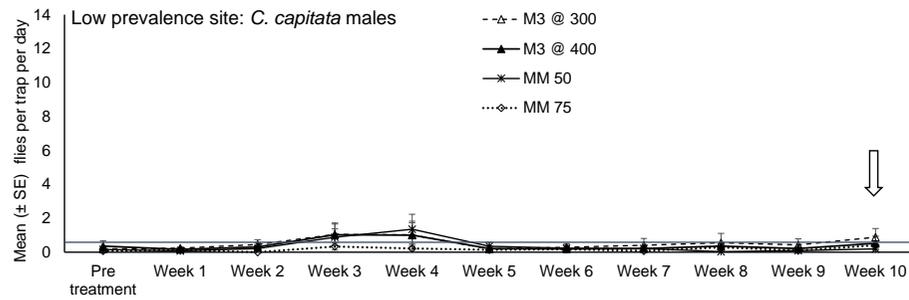
In 2018 (in Valencia orchards) and 2019 (in mandarin orchards), there were no differences in catches of *C. capitata* males and females between M3 and Magnet Med, irrespective of the densities they were tested within their recommended ranges (Table 3.3.2.2). In 2020, when M3 and Magnet Med were evaluated along with two

new treatments – CeraTrap and BetaP1 in a farm under net and in a farm with open orchards, there was a significant treatment and farm interaction (Table 3.3.2.2). This implied that there were differences between treatments and these were specific to farms. In orchards under net, catches of *C. capitata* males and females in CeraTrap treated blocks were significantly higher compared to catches in the other treatments (Fig. 3.3.2.3). In 2018, no fruit fly infestation was recorded from fruit sampled from the tree at harvest and from the ground in any of the treated blocks (Table 3.3.2.3). In 2019, fruit fly infestation was recorded on mandarin fruit sampled from the tree at harvest in one replicate block treated with M3 at 300 units per ha in the low prevalence area (Table 3.3.2.4). Despite higher fruit fly catches in the high prevalence area in 2019, no fruit fly infestation was recorded on any of the fruit sampled from the tree at harvest in any of the treated blocks (Table 3.3.2.4). However, patterns of fruit fly infestation on fruit sampled from the ground in the two farms in 2019 (Table 3.3.2.4) indicated that degree of fruit fly infestation was higher in blocks treated with the lower density of M3 (300 units per ha) compared to the higher density of this treatment (400 units per ha). M3 is recommended at 400 units per ha in soft citrus orchards due to their higher susceptibility to fruit flies. It will be important for this recommendation to be followed should M3 be selected as a fruit fly control measure in mandarin orchards. In 2020, fruit fly infestation was recorded on fruit sampled from trees in open orchard blocks treated with M3 at 600 units per ha (density higher than the recommended rate) and those treated with Beta P1 at 400 units per ha (Table 3.3.2.5).

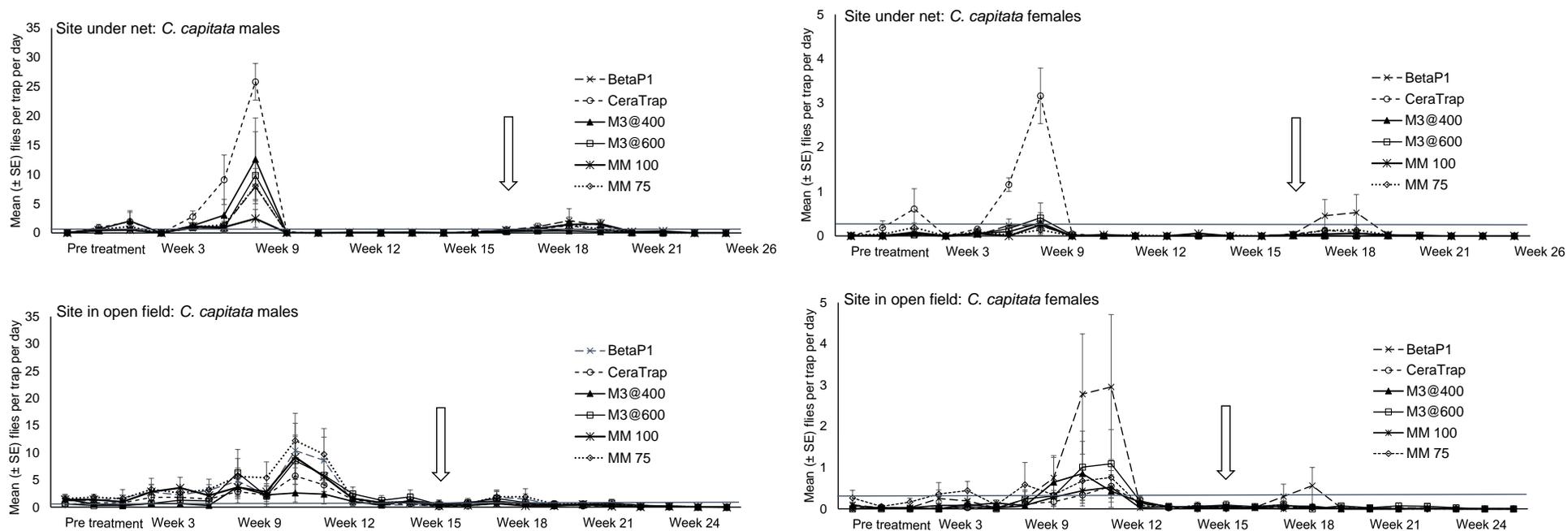
The efficacy of M3 (known as Ceranock in other parts of the world), Magnet Med and CeraTrap in reducing damage and suppressing *C. capitata* adult populations has been previously documented (Hafsi *et al.*, 2016; Navarro-Llopis *et al.*, 2013). Given that Magnet Med prevented fruit fly damage on tree at harvest throughout the trial years, it might be the more effective attract and kill product among those evaluated in citrus orchards with regard to reducing fruit fly risk in citrus. There was no fruit fly damage to fruit at harvest in the CeraTrap treated block but this product has only been evaluated for a year and further field trials may have to be conducted to confirm its field efficacy, particularly in orchards under net.



**Figure 3.3.2.1.** Catches of *C. capitata* males and *C. capitata* females in Midnight Valencia orchard blocks treated with M3 at 300 units per ha, M3 at 400 units per ha, Magnet Med (MM) at 50 units per ha and MM at 75 units per ha across weeks between May and August 2018 in two farms at difference pest prevalence levels. The *C. capitata* male catches were in Capilure baited Sensus traps and the *C. capitata* female catches were in Biolure baited Chempac Bucket traps. The arrows indicate harvest weeks in the farms and the grey solid lines indicate threshold levels for *C. capitata* in the two trapping systems. The threshold levels in Capilure baited trap is 4 males per trap per week (or 0.6 flies per trap per day) and those in Biolure baited Chempac bucket trap is 2 females per trap per week (or 0.3 flies per trap per day) for *C. capitata*.



**Figure 3.3.2.2.** Catches of *C. capitata* males and *C. capitata* females in Nadorcott mandarin orchard blocks treated with M3 at 300 units per ha, M3 at 400 units per ha, Magnet Med (MM) at 50 units per ha and MM at 75 units per ha across weeks between March and June 2019 in two farms at difference pest prevalence levels. The *C. capitata* male catches were in Capilure baited Sensus traps and the *C. capitata* female catches were in Biolure baited Chempac Bucket traps. The arrows indicate harvest weeks in the farms and the grey solid lines indicate threshold levels for *C. capitata* in the two trapping systems. The threshold levels in Capilure baited trap is 4 males per trap per week (or 0.6 flies per trap per day) and those in Biolure baited Chempac bucket trap is 2 females per trap per week (or 0.3 flies per trap per day) for *C. capitata*.



**Figure 3.3.2.3.** Catches of *C. capitata* males and *C. capitata* females in Nadorcott mandarin orchard blocks treated with BetaP1 at 400 units per ha, CeraTrap at 100 units per ha, M3 at 400 units per ha, M3 at 600 units per ha, Magnet Med (MM) at 75 units per ha and MM at 100 units per ha across weeks between February and September 2020 in two farms: one of which had cultivation under net and the other had cultivation in open fields. The *C. capitata* male catches were in Capilure baited Sensus traps and the *C. capitata* female catches were in Biolure baited Chempac Bucket traps. The arrows indicate harvest weeks in the farms and the grey solid lines indicate threshold levels for *C. capitata* in the two trapping systems. The threshold levels in Capilure baited trap is 4 males per trap per week (or 0.6 flies per trap per day) and those in Biolure baited Chempac bucket trap is 2 females per trap per week (or 0.3 flies per trap per day) for *C. capitata*.

**Table 3.3.2.2.** Results of the Mixed models on the effects of treatment, farm, time and interactions thereof on catches of *C. capitata* males and *C. capitata* females in field trials conducted between 2018 and 2020. The degrees of freedom (df), F values and P values are provided for each effect. Significant effects (P values less than 0.05) are indicated in bold.

Trial year	Target fly group	Effects	Df	F	P
2018	<i>C. capitata</i> males	Farm	1,232	1.65	0.20
		Treatment	3,232	1.45	0.23
		<b>Treatment time</b>	<b>8,232</b>	<b>5.64</b>	<b>&lt;0.0001</b>
		Treatment * Farm	3,232	0.06	0.98
		Treatment * Treatment time	24,232	0.65	0.98
	<i>C. capitata</i> females	Farm	1,232	3.50	0.06
		Treatment	3,232	0.48	0.70
		<b>Treatment time</b>	<b>8,232</b>	<b>4.52</b>	<b>&lt;0.0001</b>
		Treatment * Farm	3,232	0.04	0.99
		Treatment * Treatment time	24,232	0.55	0.96
2019	<i>C. capitata</i> males	<b>Farm</b>	<b>1,173</b>	<b>177.72</b>	<b>&lt;0.0001</b>
		Treatment	3,173	2.18	0.09
		<b>Treatment time</b>	<b>9,173</b>	<b>9.57</b>	<b>&lt;0.0001</b>
		Treatment * Farm	3,173	1.17	0.32
		Treatment * Treatment time	27,173	0.47	0.99
	<i>C. capitata</i> females	<b>Farm</b>	<b>1,173</b>	<b>45.35</b>	<b>&lt;0.0001</b>
		Treatment	3,173	2.22	0.087
		<b>Treatment time</b>	<b>9,173</b>	<b>4.08</b>	<b>&lt;0.0001</b>
		Treatment * Farm	3,173	1.98	0.12
		Treatment * Treatment time	27,173	0.48	0.99
2020	<i>C. capitata</i> males	<b>Farm</b>	<b>1,954</b>	<b>19.40</b>	<b>&lt;0.0001</b>
		Treatment	5,954	1.36	0.24
		<b>Treatment time</b>	<b>25,954</b>	<b>12.86</b>	<b>&lt;0.0001</b>
		<b>Treatment * Farm</b>	<b>5,954</b>	<b>3.04</b>	<b>0.01</b>
		Treatment * Treatment time	125,954	0.75	0.98
	<i>C. capitata</i> females	<b>Farm</b>	<b>1,954</b>	<b>13.46</b>	<b>0.00</b>
		<b>Treatment</b>	<b>5,954</b>	<b>3.09</b>	<b>0.00</b>
		<b>Treatment time</b>	<b>25,954</b>	<b>5.22</b>	<b>&lt;0.0001</b>
		<b>Treatment * Farm</b>	<b>5,954</b>	<b>4.54</b>	<b>0.00</b>
		Treatment * Treatment time	125,954	1.20	0.08

**Table 3.3.2.3.** Results of fruit fly damage assessment of fruit sampled at or near harvest from the trees and from the ground in Valencia orange orchards under different bait treatments in farms under low and high fruit fly prevalence in 2018.

Trial site	Treatment	Fruit sampled from the tree				Fruit sampled from the ground			
		Total number of fruit sampled	Total number of infested fruit (replicate block numbers)	Degree of fruit infestation (number of larvae/pupae per kg of infested fruit)	Fruit fly species recorded	Total weight of fruit sampled (kg)	Degree of fruit fly infestation (number of flies per kg of sampled fruit)	Fruit fly species recorded	
Low prevalence site	M3 @ 300 units per ha	1500	0	0.00	-	na*			
	M3 @ 400 units per ha	1500	0	0.00	-	na			
	Magnet Med @ 50 units per ha	1500	0	0.00	-	na			
	Magnet Med @ 75 units per ha	1500	0	0.00	-	na			
High prevalence site	M3 @ 300 units per ha	1510	0	0.00	-	8.90	0.00	-	
	M3 @ 400 units per ha	1500	0	0.00	-	7.77	0.00	-	
	Magnet Med @ 50 units per ha	1500	0	0.00	-	8.32	0.00	-	
	Magnet Med @ 75 units per ha	1500	0	0.00	-	7.95	0.00	-	

\*na- Not available due to no fruit found on the ground

**Table 3.3.2.4.** Results of fruit fly damage assessment of fruit sampled at or near harvest from the trees and from the ground in mandarin orchards under different bait treatments in farms under low and high fruit fly prevalence in 2019.

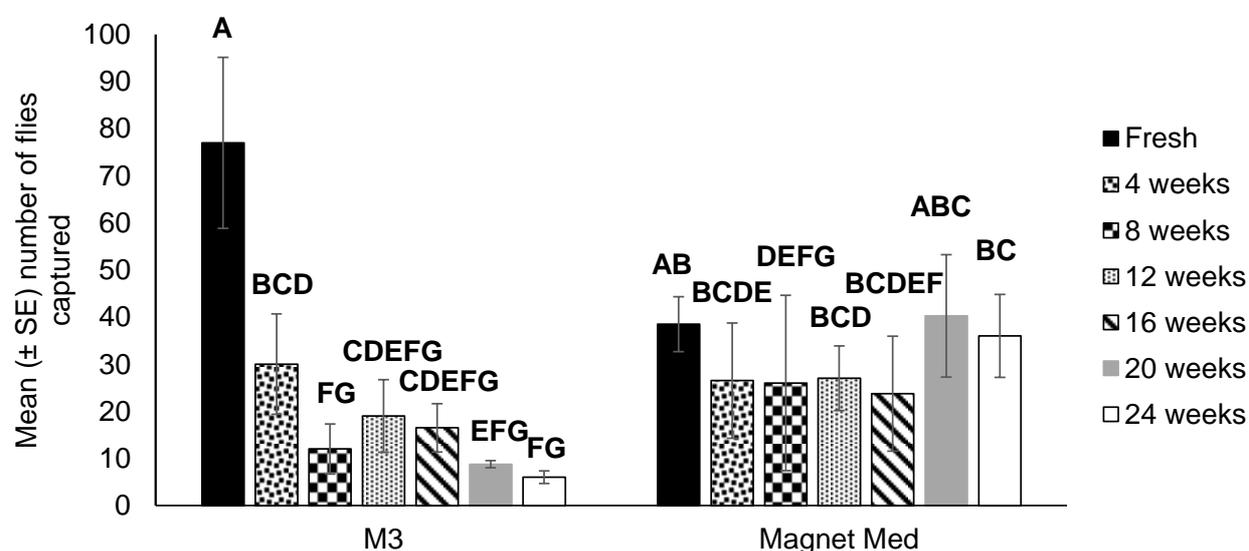
Trial site	Treatment	Fruit sampled from the tree				Fruit sampled from the ground			
		Total number of fruit sampled	Total number of infested fruit (replicate block numbers)	Degree of fruit infestation (number of larvae/pupae per kg of infested fruit)	Fruit fly species recorded	Total weight of fruit sampled (kg)	Degree of fruit fly infestation (number of flies per kg of sampled fruit)	Fruit fly species recorded	
Low prevalence site	M3 @ 300 units per ha	1500	3 (1)	16.80	<i>C. capitata</i>	8.24	1.94	<i>C. capitata</i>	
	M3 @ 400 units per ha	1500	0	0.00	-	6.66	0.45	<i>C. capitata</i>	
	Magnet Med @ 50 units per ha	1500	0	0.00	-	7.82	0.13	<i>C. cosyra</i>	
	Magnet Med @ 75 units per ha	1500	0	0.00	-	6.43	0.16	<i>C. capitata</i>	
High prevalence site	M3 @ 300 units per ha	1497	0	0.00	-	9.92	0.71	<i>C. capitata</i> & <i>B. dorsalis</i>	
	M3 @ 400 units per ha	1499	0	0.00	-	8.66	0.58	<i>C. capitata</i> & <i>B. dorsalis</i>	
	Magnet Med @ 50 units per ha	1488	0	0.00	-	10.42	1.06	<i>C. capitata</i> & <i>C. cosyra</i>	
	Magnet Med @ 75 units per ha	1489	0	0.00	-	10.10	0.79	<i>C. capitata</i> & <i>B. dorsalis</i>	

**Table 3.3.2.5** Results of fruit fly damage assessment of fruit sampled at or near harvest from the trees and from the ground in mandarin orchards under different bait treatments in a farm under net and one in the open in 2020.

Trial site	Treatment	Fruit sampled from the tree				Fruit sampled from the ground			
		Total number of fruit sampled	Total number of infested fruit	Degree of fruit infestation (number of larvae/pupae per kg of infested fruit)	Fruit fly species recorded	Total weight of fruit sampled (kg)	Degree of fruit infestation (number of pupae per kg of sampled fruit)	Fruit fly species recorded	
Under net	M3 @ 400 units per ha	1500	0	0.00	-	12.54	0.00		
	M3 @ 600 units per ha	1500	0	0.00	-	10.01	0.00		
	Magnet Med @ 75 units per ha	1500	0	0.00	-	12.57	0.00		
	Magnet Med @ 100 units per ha	1500	0	0.00	-	9.38	0.00		
	Cera Trap @ 100 units per ha	1500	0	0.00	-	12.04	0.00		
	BetaP1 @ 400 units per ha	1500	0	0.00	-	9.13	0.00		
Open	M3 @ 400 units per ha	1500	0	0.00	-	9.37	0.43	<i>C. capitata</i>	
	M3 @ 600 units per ha	1500	2	10.58	<i>C. capitata</i>	12.55	0.16	<i>C. capitata</i>	
	Magnet Med @ 75 units per ha	1500	0	0.00	-	13.63	0.15	<i>C. capitata</i>	
	Magnet Med @ 100 units per ha	1500	0	0.00	-	10.36	0.39	<i>C. capitata</i>	
	Cera Trap @ 100 units per ha	1500	0	0.00	-	12.28	0.00		
	BetaP1 @ 400 units per ha	1500	1	16.81	<i>C. capitata</i>	11.51	0.00		

Effects of weathering on attract and kill efficacy of registered protein-based bait stations

In no choice semi field cage assays, Magnet Med was found to be, in general, significantly more attractive than M3 bait stations for *C. capitata* (Table 3.3.2.6 and Fig. 3.3.2.4). There was also a significant effect of bait station age on responses of flies (Table 3.3.2.6) as well as a significant interaction between age and bait station (Table 3.3.2.6 and Fig. 3.3.2.4). When fresh, M3 attracted a higher number of *C. capitata* adults compared to Magnet Med but differences between the two were not statistically significant. There was a general decrease in fly response as the M3 bait station aged. In contrast, there was no consistent decrease in fly response to Magnet Med as the bait station aged. Responses to the bait station were not influenced by sex of the fly (Table 3.3.2.6).



**Figure 3.3.2.4.** Responses of *C. capitata* adults (males and females) to M3 and Magnet Med bait stations at different ages in no choice semi field assays.

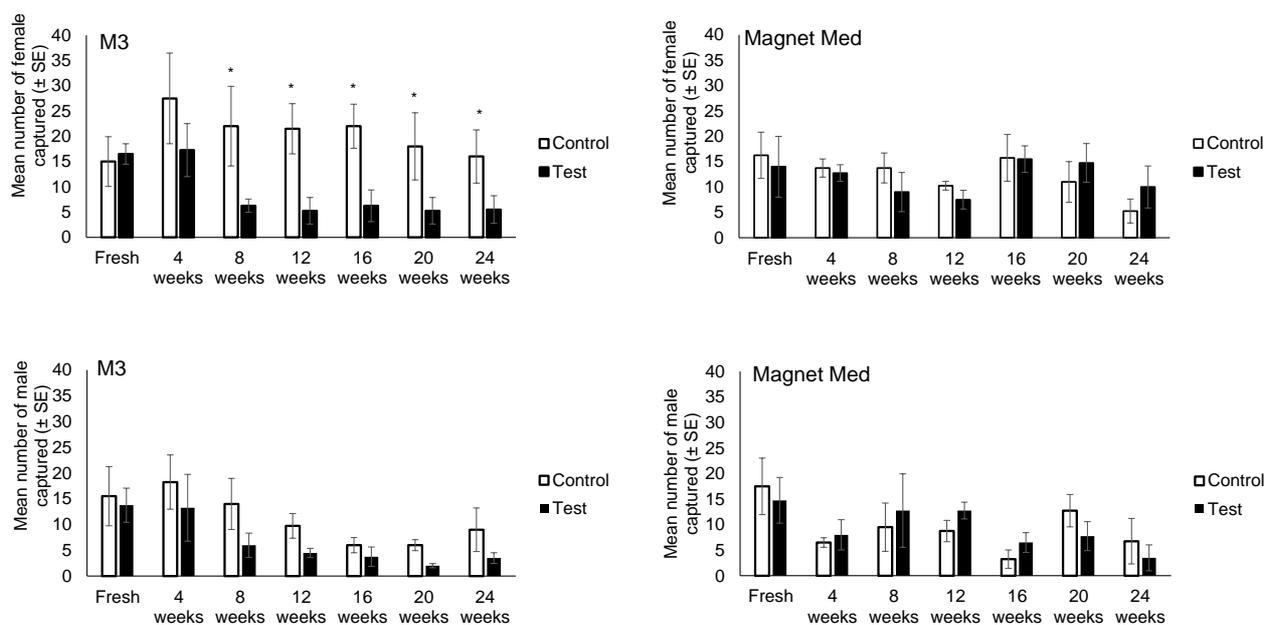
**Table 3.3.2.6.** Results of the Mixed models on the effects of bait station, bait station age, replicate, fly sex and interactions thereof on catches of *C. capitata* adults in no choice and no choice field cage trials. In the choice assays the treatment type was bait station and age pooled together and the test was the choice between fresh/aged bait and fresh control. The degrees of freedom (df), F values and P values are provided for each effect. Significant effects (P values less than 0.05) are indicated in bold.

Assays	Source	Type	DF	Sum of squares	Mean squares	F	Pr > F
No choice	Bait station	Fixed	1.00	0.87	0.87	6.30	<b>0.01</b>
	Replicate	Random	3.00	0.42	0.14	1.02	0.39
	Age	Fixed	6.00	4.94	0.82	5.96	<b>&lt;0.0001</b>
	Sex	Fixed	1.00	0.39	0.39	2.85	0.10
	Bait station*Age	Fixed	6.00	2.27	0.38	2.74	<b>0.02</b>
	Error			94.00	12.96	0.14	
Choice	Treatment type (Bait station plus age)	Fixed	13.00	4.45	0.34	3.61	<b>&lt;0.0001</b>
	Replicate	Random	3.00	1.25	0.42	4.40	<b>0.01</b>
	Test (Fresh/aged versus control fresh)	Fixed	1.00	1.48	1.48	15.65	<b>0.00</b>
	Sex	Fixed	1.00	2.55	2.55	26.85	<b>&lt;0.0001</b>
	Error						

Treatment type*Test	Fixed	13.00	2.76	0.21	2.24	<b>0.01</b>
Treatment type*Test*Sex	Fixed	13.00	1.44	0.11	1.17	0.31
Error		175.00	16.60	0.09		

In choice assays, the treatment type (bait station and age), test (choice between fresh/aged and fresh control), fly sex and interaction between treatment type and fly sex were all significant in determining fly captures (Table 3.3.2.6). Responses of females to M3 bait stations which were 8 weeks or older were lower compared to those elicited by fresh stations (Fig. 3.3.2.5). For males on the other hand, there were no significant differences in responses to fresh and aged M3 bait stations. Fresh and aged Magnet Med were equally attractive to females and males. This implies that M3 bait stations would be effective for up to 8 weeks while Magnet Med would be effective for up to six months.

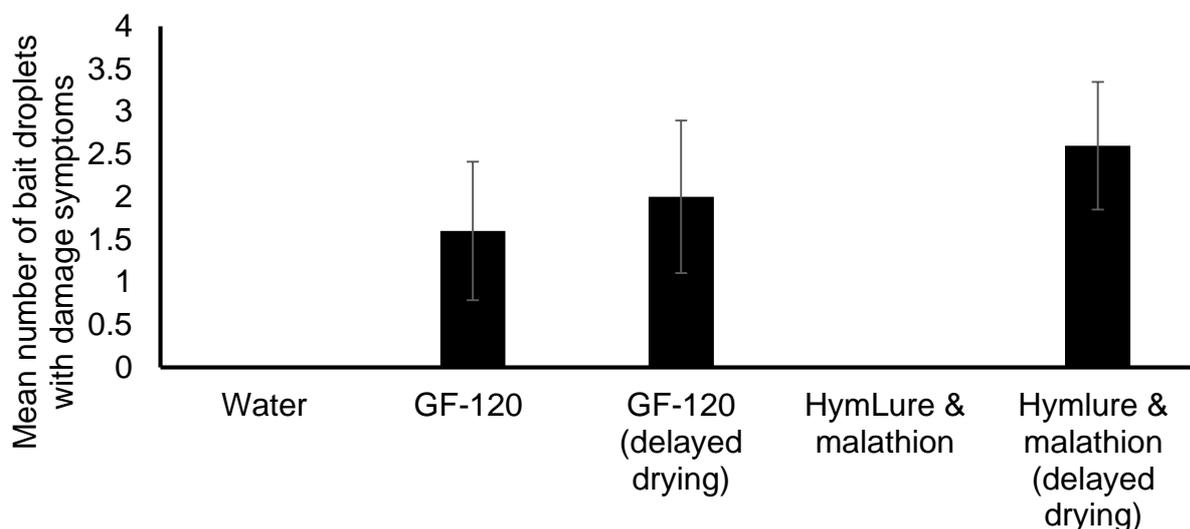
In a study by Navarro-Llopis *et al.* (Navarro-Llopis *et al.*, 2008), Biolure which is similar to the attractant used in Magnet Med was found to attract flies for up to 3 months (12 weeks). Changes in the efficacy of the attractant in time were linked to changes in the amount of attractant components particularly ammonium acetate and trimethylamine from the dispenser. It is possible that the Biolure dispenser used in Magnet Med is more effective in conserving the attractant components for up to 24 weeks.



**Figure 3.3.2.5.** Responses of *C. capitata* females (top graph) and males (bottom graphs) to M3 and Magnet Med bait stations which were aged up to 24 weeks (test) and compared at each age with a fresh station (control) of the specific bait station type. The \* symbol indicates significant differences in mean counts between control and test ( $P < 0.05$ ) for a bait station age.

#### Phytotoxicity of ground-based and aerial protein bait spray mixtures on mandarin

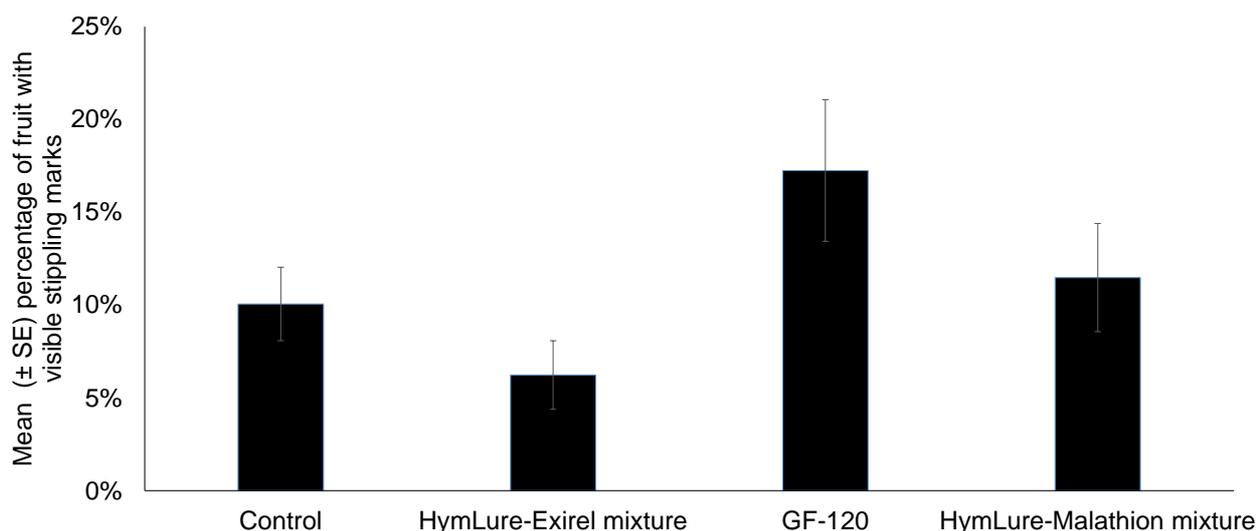
No visible phytotoxic symptoms could be detected on fruit treated with the HymLure malathion mixture. There was also no visible phytotoxic symptoms on fruit treated with GF-120 droplets with and without delayed bait drying. The GF-120 treatments were the control treatments as they were previously found to cause phytotoxic symptoms on mandarin fruit when applied at or before colour break (Manrakhan *et al.* 2015). Phytotoxic symptoms on these control treatments could only be seen when viewed under the microscope (10 x magnification). When viewed under the microscope, phytotoxic symptoms were also present in the HymLure malathion treatment which had delayed bait drying (Fig. 3.3.2.6). There were significant differences in occurrence of phytotoxic symptoms between treatments ( $\chi^2 = 175.33$ ,  $P < 0.0001$ ).



**Figure 3.3.2.6.** Mean ( $\pm$ SE) number of marked areas with phytotoxic symptoms following water and bait application. These symptoms were only visible under the microscope.

*Phytotoxicity of ground based bait spray mixtures on mandarin at the colour break stage*

In the 2019 field trial, visible phytotoxic symptoms could be seen on mandarin fruit treated with the three baits: GF-120, HymLure plus Exirel and HymLure plus malathion at colour break (Fig. 3.3.2.7). Visible phytotoxic symptoms however were also recorded on fruit that were not treated with three ground applied bait treatments (control). Nonetheless there were significant differences in presence of phytotoxic symptoms between the treatments, with the highest incidence recorded for GF-120 and the least incidence recorded for the HymLure and Exirel mixture as well as the control ( $\chi^2=10.23$ ,  $P=0.02$ ). There was no significant effect of canopy layer on incidence of phytotoxic marks ( $\chi^2=3.09$ ,  $P=0.21$ ).

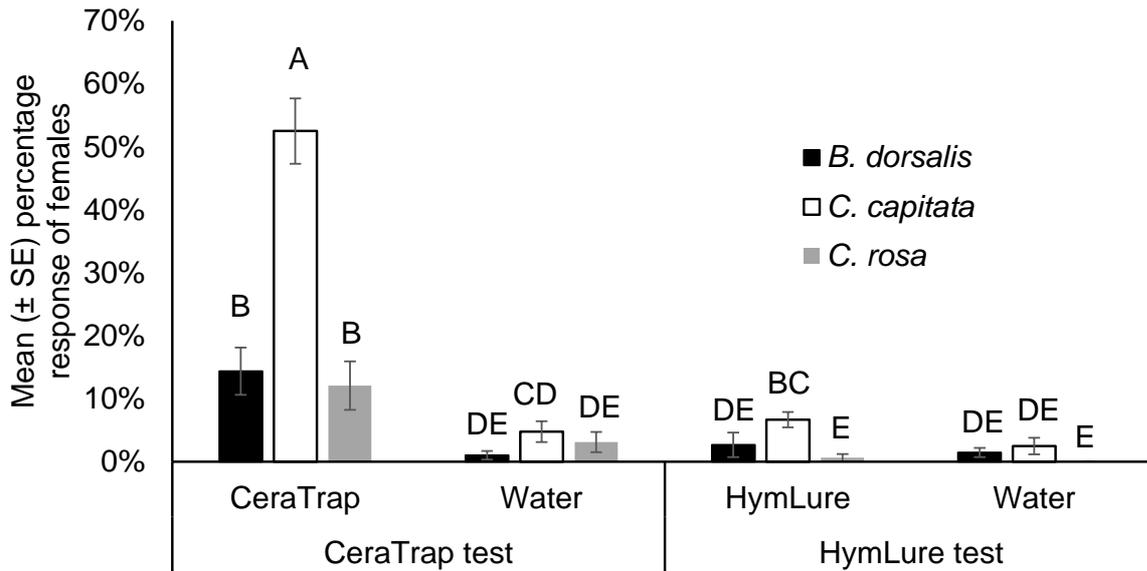


**Figure 3.3.2.7.** Effects of three ground-based bait spray treatments on incidence of stippling marks on mandarin fruit when applied at the colour break stage.

*Laboratory trials to determine attractiveness of new products*

In the olfactometer trial, CeraTrap was more attractive than HymLure for all three fruit fly species tested (Fig. 3.3.2.8) (Test material:  $F_{3,84}= 60.97$ ,  $P<0.0001$ ). *Ceratitis capitata* was the most responsive to baits out of the three fruit fly species (Fig. 3.3.2.8) (Species:  $F_{2,84}= 27.78$ ,  $P<0.0001$ ). There was a significant interaction between material tested in the olfactometer and species (Test material\* Species:  $F_{6,84}= 6.01$ ,  $P<0.0001$ ).

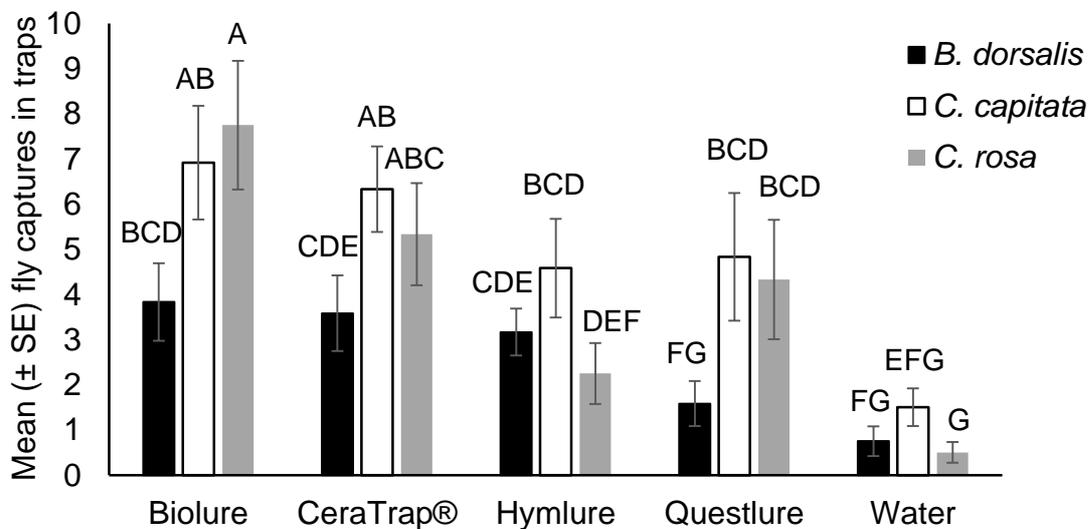
CeraTrap would therefore be a promising attractant for use in the control of all three fruit fly species and would possibly provide greater efficacy of control than HymLure malathion bait sprays.



**Figure 3.3.2.8.** Responses of *B. dorsalis*, *C. capitata* and *C. rosa* females to CeraTrap bait and HymLure in laboratory olfactometer assays.

*Field cage trials to determine attractiveness of new products*

Generally, in choice tests, CeraTrap and Biolure were more attractive than HymLure and Questlure for all three fruit fly species ( $F_{4,164}=18.82$ ,  $P<0.0001$ ) (Fig. 3.3.2.9). *Ceratitis capitata* and *C. rosa* responded more to baits than *B. dorsalis* ( $F_{2,164}=6.03$ ,  $P=0.00$ ) (Fig. 3.3.2.9). There was no difference in responses to treatments between males and females ( $F_{1,164}=0.7$ ,  $P=0.40$ ). There was also no significant interaction between attractant and species ( $F_{8,164}=1.30$ ,  $P=0.25$ ).

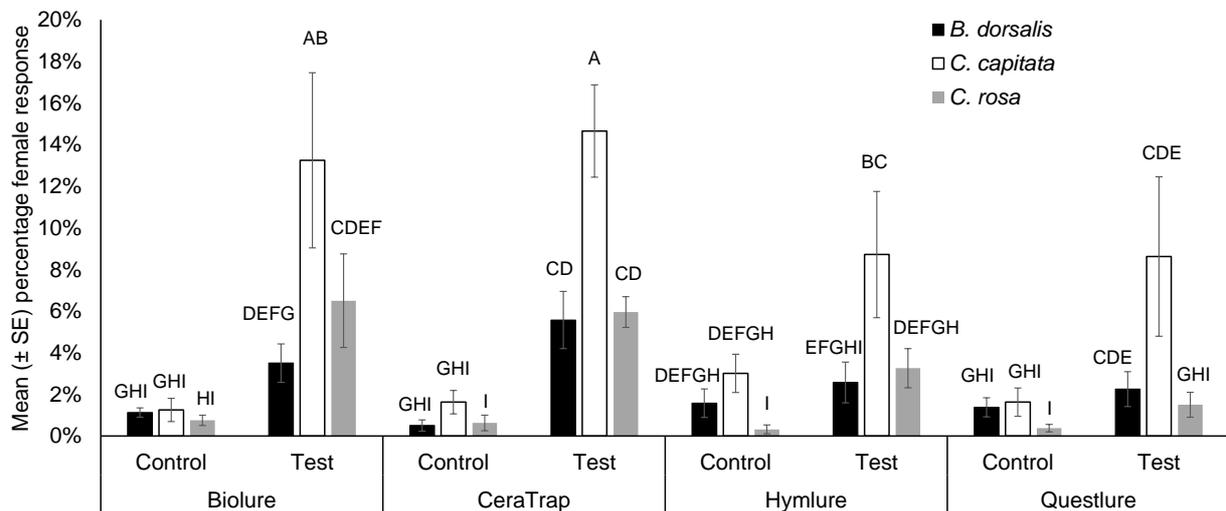


**Figure 3.3.2.9.** Responses of *B. dorsalis*, *C. capitata* and *C. rosa* to four protein based attractants used for control in choice field cage tests.

In no choice tests, there were significant effects of treatments ( $F_{7,167}=16.84$ ,  $P<0.0001$ ) and species ( $F_{2,167}=17.05$ ,  $P<0.0001$ ) on fly responses. There were no differences between males and females of all species in their responses to the baits ( $F_{1,167}=1.61$ ,  $P=0.20$ ). There was also no significant interaction between

treatment and species ( $F_{14,167}=1.37$ ,  $P=0.18$ ). Similar to the findings in choice tests, Biolure and CeraTrap were generally more attractive than the other baits (Fig. 3.3.2.10). *Ceratitis capitata* was the most responsive to protein baits compared to *B. dorsalis* and *C. rosa* (Fig. 3.3.2.10).

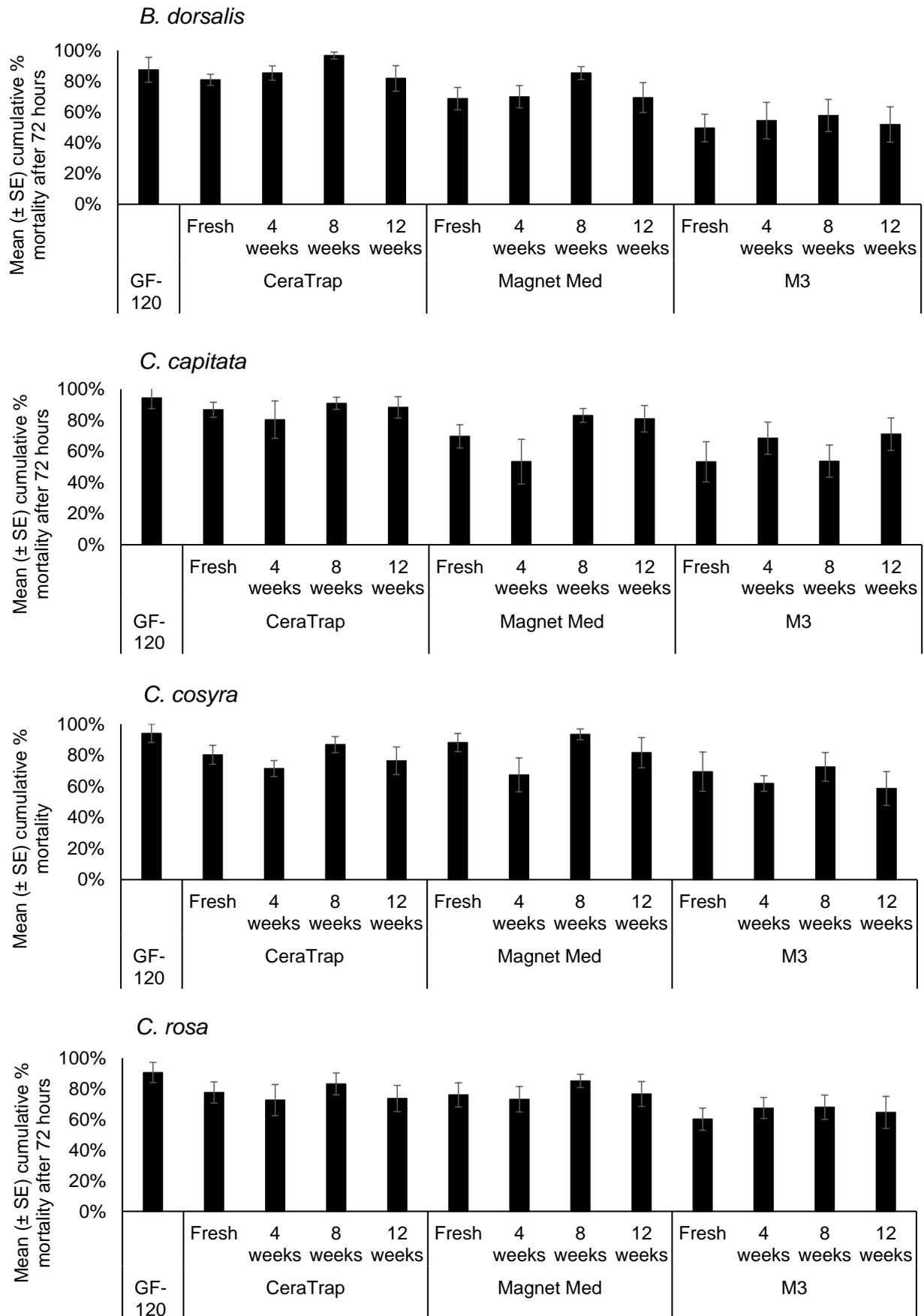
Attract and kill products with Biolure and CeraTrap bait would possibly outperform products using HymLure and Questlure. Biolure is the attractant in Magnet Med. Questlure is the attractant in M3 bait stations. Based on the field results in this project, no infestation of citrus was ever recorded in any of the blocks treated with Magnet Med while there were a few infestations of fruit on the tree in blocks treated with M3 bait stations. All attract and kill products using proteinaceous baits would potentially be better at suppressing *C. capitata* populations compared to populations of the other two pests. For the latter, control tools other than proteinaceous attract and kill products would be required particularly earlier in the season when their populations are higher.



**Figure 3.3.2.10.** Responses of *B. dorsalis*, *C. capitata* and *C. rosa* to four protein based attractants used for control in no choice field cage tests. Control for each bait was a trap baited with only water to measure random movement of flies.

#### Toxicity of attract and kill products for fruit flies

The results on the toxicity of attract and kill products matched the results of fly responses to baits. There was a significant effect of species, bait and bait age in fly mortality (Species:  $F_{3,27}=5.05$ ,  $P=0.00$ , Bait:  $F_{3,27}=72.88$ ,  $P<0.0001$ ; Bait age:  $F_{3,27}=3.63$ ,  $P=0.01$ ). *C. capitata* was the most susceptible species possibly because of its higher response to the baits compared to the other species. CeraTrap and Magnet Med were more effective than M3 in effecting fly kill (Fig. 3.3.2.11). In the field cage studies, CeraTrap and Biolure (attractant in Magnet Med) were also the more attractive. There were no differences in mortality between females and males ( $F_{1,27}=0.55$ ,  $P=0.46$ ). For all attract and kill products, there were variations in mortality across ages. There was however no clear trends of decline in efficacy as the products progressively aged.



**Figure 3.3.2.11.** Cumulative percentage fly mortality at 72 hours after exposure to three attract and kill products of four ages and fresh standard GF-120 spray for four fruit fly species.

## Conclusion

Currently registered attract and kill methods within their recommended ranges were found to be effective in controlling fruit fly pests. Some products provided better control than the others. Magnet Med and CeraTrap were more effective than M3 bait station. All bait stations would be better alternatives than bait spray mixtures on mandarin fruit at colour break as they would avoid stippling marks on fruit at harvest. The M3 bait stations, if used, should be renewed before its current recommended lifetime of 12 weeks (preferably before 8 weeks).

## Future research

Further research could be conducted on the placement of attract and kill methods within the control area. Although there are recommendations on the proper placement of attract and kill products, these are not always followed. Attract and kill products are often set up either on the fencing lines of citrus orchards or in full sun on the citrus trees. Such deployment could compromise efficacy of the products.

## Technology transfer

- Manrakhan A. 2018. Fruit fly control. CRI Integrated Pest Management and Disease Management workshops and symposium feedback, September 2018.
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- 3.3.3 **FINAL REPORT: Development of new cold disinfestation treatments for fruit fly pests on citrus**  
Project 1245 (2019/20 – 2020/21) by A Manrakhan, J-H Daneel, R Beck, G Shongwe and V Hattingh (CRI)

## Summary

The equivalence of an existing cold disinfestation treatment targeting *Ceratitidis capitata* was sought for the marula fly, *Ceratitidis cosyra*, which is of potential phytosanitary concern in citrus from South Africa. The marula fly was previously recorded on citrus from South Africa in online databases and in fruit surveys in East and West Africa. The cold tolerance of *C. capitata* and *C. cosyra* was first determined at 3.5°C for 18 days. The conditions required for complete mortality of the third larval stage of *C. cosyra* under an existing *C. capitata*

cold treatment at 1°C for 14 days were determined. The efficacy of this existing *C. capitata* treatment for disinfestation of *C. cosyra* was thereafter confirmed in large scale trials. *C. capitata* was found to be more cold tolerant than *C. cosyra*. The period to achieve 99% mortality at 3.5°C was estimated to be 14.8 days for *C. capitata* and 9.6 days for *C. cosyra*. No survivors of third instar larvae of *C. cosyra* were recorded beyond 11 days of cold treatment. In large scale trials at the lowest mean temperature of 1.25°C for 14 days, there were no survivors from a total estimate of 18 437 treated *C. cosyra* third instar larvae. Cold disinfestation treatments for *C. capitata* should be equally effective against *C. cosyra*.

## Opsomming

Die ekwivalent van 'n bestaande koue-ontsmettingsbehandeling wat *Ceratitits capitata* teiken, is vir die maroela vlieg, *Ceratitits cosyra*, gesoek, wat potensieel van fitosanitêre belang in sitrus vanaf Suid-Afrika is. Die maroela vlieg is voorheen op sitrus vanaf Suid-Afrika in aanlyn databasisse aangeteken, en in vrug-opnames in Oos- en Wes-Afrika. Die koue-bestandheid van *C. capitata* en *C. cosyra* is eerstens by 3.5 °C vir 18 dae vasgestel. Die vereiste toestande vir volledige mortaliteit van die derde larwe stadium van *C. cosyra*, onder 'n bestaande *C. capitata* koue-behandeling by 1°C vir 14 dae, is bepaal. Die effektiwiteit van hierdie bestaande *C. capitata* behandeling vir ontsmetting van *C. cosyra* is daarna in grootskaalse proewe bevestig. Daar is gevind dat *C. capitata* meer koue-bestand as *C. cosyra* was. Die periode nodig om 99% mortaliteit by 3.5°C te bereik, is op 14.8 dae vir *C. capitata* en 9.6 dae vir *C. cosyra* geraam. Geen oorlewendes van derde instar larwes van *C. cosyra* is na 11 dae van koue-behandeling aangeteken nie. In grootskaalse proewe by die laagste gemiddelde temperatuur van 1.25°C vir 14 dae, was daar geen oorlewendes uit 'n totaal geskatte 18437 behandelde *C. cosyra* derde instar larwes nie. Koue-ontsmettingsbehandelings vir *C. capitata* behoort net so effektief teen *C. cosyra* te wees.

## Introduction

Some of the export markets of citrus from South Africa require disinfestation treatments to ensure that fruit being traded are fruit fly free. Disinfestation treatments that are developed are then adopted through bilateral agreement between exporting and importing countries. The disinfestation treatments mostly used for exported citrus from South Africa are cold treatments at temperatures of 1°C or below for 16 to 22 days (Grout *et al.* 2011, Moore *et al.* 2016). These treatments are specific to fruit fly species which are considered of quarantine importance in the importing countries.

There are three fruit fly species that are considered pests of commercial citrus in southern Africa: *Ceratitits capitata* (Wiedemann) (Mediterranean fruit fly or Medfly), *Ceratitits rosa* Karsch (Natal fly) and *Bactrocera dorsalis* (Hendel) (Oriental fruit fly) (Grout and Moore 2015). Trials recently conducted at Citrus Research International have demonstrated that out of these three fruit fly species, Medfly was the more cold tolerant one. These findings are also supported by previous published information that Medfly is as or more cold tolerant than *B. dorsalis* (Hallman *et al.* 2011, Hallman *et al.* 2013) and more cold tolerant than Natal fly (Ware and du Toit 2017). Disinfestation cold treatments that are then effective against Medfly should be equally effective against the two other fruit fly pests of citrus.

Composition of fruit fly pests on citrus in southern Africa can however change particularly in cases when fruit flies are introduced in an area, host shifts occur and revisions are made in taxa. One recent taxonomic change that occurred was the split of Natal fly into two species *Ceratitits rosa* Karsch and the Cape fly *Ceratitits quilicii* De Meyer, Mwatawala & Virgilio (De Meyer *et al.* 2016). There are no records of the Cape fly on citrus in South Africa but there are now new records of this species on some citrus types (fruit conditions not specified) in Reunion island (Moquet *et al.* 2021). *Ceratitits quilicii* was found to be more abundant than *C. rosa* in citrus orchards in South Africa (Daneel 2020) and therefore remains a potential threat for citrus in the country. *Ceratitits cosyra* (Walker) is not considered a pest of commercial citrus in southern Africa although there are records of the species associating with some citrus types in East and West Africa (Mwatawala *et al.* 2009, Ndiaye *et al.* 2012). However, these records did not provide details on the origin (ground or tree; commercial or non-commercial) and conditions (damaged, not damaged) of the citrus fruit from which *C. cosyra* was reared (Mwatawala *et al.* 2009, Ndiaye *et al.* 2012). *Ceratitits cosyra* has been recorded as an intercepted fruit fly in citrus from South Africa (Europhyt 2006, 2018) although these interceptions could not be cross verified. This

species was also listed as a pest that requires mitigation measures for export of citrus from eSwatini to USA. There is therefore a phytosanitary concern also on this species in citrus fruit exported from South Africa. Such a concern could put at risk markets that require cold disinfestation treatments for fruit flies should equivalence of existing cold disinfestation treatments for those potentially problematic species not be sufficiently demonstrated.

This study aimed at determining the equivalence of an existing cold disinfestation treatment for mitigation of the risk of a fruit fly species that is of recent concern on citrus from South Africa. An existing *C. capitata* cold disinfestation schedule of at or below 1.11°C for 14 days was selected (USDA 2016). *Ceratitis quilicii* was the species initially targeted in this project but a colony of this species could not be grown to a level required for cold disinfestation trials. In March 2020, the target species was shifted to another potential pest: *C. cosyra* for which a full grown colony was available.

## **Stated objectives**

### Original objectives

- A. Determination of treatment conditions at 1°C for complete mortality of the most cold tolerant immature stage of *C. quilicii*
- B. Confirmation of treatment schedule at 1°C for 14 days for disinfestation of *C. quilicii*

### Modified objectives in March 2020

- A. Comparison of the in vitro cold tolerance of *C. capitata* and *C. cosyra*.
- B. Determination of treatment conditions for complete mortality of the third larval stage of *C. cosyra* at 1°C for 14 days
- C. Confirmation of treatment schedule at 1°C for 14 days for disinfestation of *C. cosyra*.

## **Materials and methods**

### *A. Comparison of the in vitro cold tolerance of C. capitata and C. cosyra*

The in vitro cold tolerance of the third larval stages of *C. capitata* and *C. cosyra* was compared at 3.5°C. This temperature was selected as it is representative of the fruit temperature during shipping of the bulk of citrus exported from southern Africa. This temperature is also at the upper end of the scale of temperatures used in fruit fly cold treatments, thereby potentially providing opportunity to detect small differences between temperature sensitivity of the species.

The two fruit fly species tested were reared at the Citrus Research Centre of Citrus Research International (CRI) in Nelspruit, Mpumalanga, South Africa. The third larval stages of the two species were used in the tests.

### Artificial larval diet

The larval diet used for the two species in the cold tolerance study was a carrot based diet with carrot powder (Amidor (Pty) Ltd., Johannesburg, South Africa) and brewer's yeast (Organic World, Randburg, South Africa) in the ratio of 2:1. The carrot powder and brewer's yeast mixture constituted 29.3% of the diet. The remaining ingredients were water (70.4%) and the preservatives, methyl 4-hydroxybenzoate and sorbic acid (both from Sigma-Aldrich Pty. Ltd., Kempton Park, South Africa) at 0.2% and 0.1% of the diet mixture respectively.

### Preparation of immature stages

To obtain the third larval stages, eggs of the two species were collected from the colonies over a 24 hour period. Egg-water mixtures for the two species were prepared by placing one ml of eggs of *C. capitata* and one ml of eggs of *C. cosyra* in 17 and 14 ml of deionized filtered water respectively in order to get ~25 eggs of each species per 0.025 ml aliquot of the egg-water mixture.

Aliquots (0.025 ml each) of egg-water mixture were placed on moist blue blotting squares (~1 cm x ~1 cm). Eggs on moist blotting squares were counted until a total count of 100 was reached. Once a count of 100 eggs was reached, the moist blotting squares were transferred onto the surface of 100 g of the larval diet contained in a plastic dish (12.5 cm diameter and 2.5 cm height). Two holes (4 mm in diameter) were pierced on opposite sides of the plastic dish to allow aeration and to avoid water condensation inside the dish. The diet dish containing 100 eggs was covered with a plastic lid. The diet dish was then placed in a transparent flip-top container (12.5 cm x 12.5 cm x 6 cm) with about 50 holes (< 2 mm in diameter) pierced in the top.

For development of each species, diet containers with eggs as described above were placed in a temperature controlled room. The temperature setting in the room was adjusted to achieve a target temperature of 26°C. For each species, diet containers were incubated for 6 days in order to obtain third instar larvae.

For each species and replicate, there were five diet containers prepared for each of ten cold treatment exposure periods and an untreated control. There were therefore 55 diet containers in total for each species and replicate. The diet containers were labelled according to the species, day of exposure and replicate number. Additionally for species and replicate, there were two diet containers with eggs (200 eggs in total) which were prepared to verify the composition of the immature stages on the day of the exposure to the cold treatment.

Lights were switched off in the chamber throughout incubation. A humidifier was placed in the environmental chamber in order to achieve a relative humidity of 60%.

#### Cold treatment

Two built-in cold rooms (cold room 1: 4.40 m length x 3.45 m width x 2.14 m height; cold room 2: 3.78 m length x 2.92 m width x 2.43 m height) located at the Citrus Research Centre were used for the tests. Refrigeration in each cold room was supplied by an air cooled condensing unit, a compressor using R22 as a refrigerant and an induced draft evaporator, fitted with three five bladed fans. The average air flow rate of room 1 was 5 860 m<sup>3</sup>/h and that of room 2 was 4 634 m<sup>3</sup>/h. Defrost cycles in each room were set at four hourly intervals.

The target temperature tested was 3.5°C for a total period of 18 days. The susceptibility of the third larval stages of *C. capitata* and *C. cosyra* to the cold treatment was compared concurrently at ten exposure periods: one, two, four, six, eight, 10, 12, 14, 16 and 18 days after the start of the cold treatment.

There were four replicates in this trial. Each replicate of the cold treatment was conducted separately in the two cold rooms such that the treatment from precooling through to the final exposure period was also separately repeated.

For each replicate, the diet containers of the two species were packed in 10 aerated plastic crates (50 cm x 32 cm x 29 cm), one for each exposure period. The crates for each exposure period had a total of 10 diet containers for the two species. Each crate also contained two extra diet containers with no eggs for temperature monitoring during storage after the cold treatment. The 10 crates containing diet containers were placed in two rows in the cold room over a platform of empty crates which were in turn placed on top of a wooden pallet. The crates were randomized for each replicate. At the end of each exposure period of each replicate, the crate with containers labelled for that period was removed and replaced with an empty crate containing empty flip top containers. This replacement was done in order to maintain the layout of the treated crates and thus similar airflow within the arena. At the start of the cold treatment, all crates were covered with a layer of blanket in order to stabilize temperature in the treated arena.

Inside each cold room, air temperatures at two points: one at air delivery and one at the air return and temperatures inside five diet containers, each with 100 g of larval diet and no eggs, were recorded every 5 minutes using a logger (either a Grant Squirrel 2020 2F8, Monitoring and control Laboratories, Johannesburg, South Africa or a Grant Squirrel 2040 4F16, Monitoring and control Laboratories, Johannesburg, South Africa) with temperature probes of the type-T thermocouple system. Diet containers with no eggs used for temperature measurements were prepared a day before the cold treatment and kept in the incubation room until placement in the cold room. The diet containers for temperature measurements in the cold rooms were also each placed

in a flip-top container. Temperature probes in diet containers were distributed in the two rows of crates inside the cold chamber.

Prior to the start of temperature measurement in the cold chamber for each replicate, temperature probes were calibrated by immersion in ice-water as follows. Ice made from purified water was first crushed into particles of less than 20mm diameter and then placed in a 2.2 L insulated flask with about 400 ml purified water. The ice/water mixture was left for at least ten minutes for the temperature to stabilise and then a glass mercury thermometer (Brannan, England) was inserted to confirm a stable reading of exactly 0°C. When temperature readings of the probes were stabilised, calibration runs were started. At least three calibration runs with logging intervals of three minutes were conducted. After at least 10 readings, each run was stopped, the ice mixture was agitated and then the next run was started. The readings of each probe were first averaged for each run and then averaged over the three runs. A calibration factor was derived for each probe: Calibration factor = True temperature of ice/water (0°C) ± average probe reading. Calibration factors were all within ± 0.3°C from 0°C.

The start point of the cold treatment was considered when three of the probes reached 3.5°C or below.

At the end of each exposure period, the cold treated diet containers were moved to the temperature controlled room which was used for the incubation and set at a target temperature of 26°C. The containers for the third larval stages were held for two days before being dissected to determine mortality.

The untreated diet containers (control) were kept in the incubation room until the day of the cold treatment when they were then dissected to determine the untreated control mortality.

#### Verification of immature stage composition

For each species and replicate, two dishes were examined separately. In each dish, unhatched and hatched eggs were first determined. Live and dead larvae were counted. All live larvae found were removed and killed in water just off the boil following the methods of White & Elson-Harris (1994). The killed larvae were then preserved in 100% ethanol for larval instar determination based on the characteristics of their mouthparts (White & Elson-Harris, 1994). Third instars of both species were characterised by the presence of sclerotized mandibles. For *C. capitata* additionally, the third instar larva was characterised by the absence of a pre-apical tooth.

Additionally, the body length (from mandible to end of last body segment) of each larva from a sample of 10 larvae from each dish was measured using a Vernier calliper.

#### Mortality assessment

In the determination of mortality, live and dead larvae in each dish were examined and counted under a stereomicroscope. Live and dead larvae were first checked and counted on the surface of the diet in a dish. The diet was then washed in water onto a fine stainless steel sieve of 180 µm aperture (Madison test sieve, Johannesburg, South Africa) in order to loosen the diet for easier retrieval and identification of larvae. A larva was considered dead if there was no visible movement even when prodded. Fully formed pupae found were considered as live.

#### Data analysis

Data were summarized as average percentage mortality for each species at each cold exposure period. For each species and each exposure period Abbott's formula (Abbott, 1925) was used to correct for untreated control mortality. Effects of species and exposure period on mortality were analysed using logistic regression (Logit model) (Addinsoft 2020). Estimates of cold exposure periods to achieve 50%, 90%, 95% and 99% mortality levels for each species were derived using the Probit model (Addinsoft 2020).

#### *B. Determination of treatment conditions at 1°C*

*Citrus sinensis* (L.) Osbeck cv Valencia C100 sourced directly from a commercial citrus orchard: Crocodile Valley, Golden Frontiers Citrus, Ehlanzeni district, Mpumalanga Province, were used for the tests. Fruit were

stored in a cold room set at 4°C prior to the start of inoculations. Before each test (replicate), the weight, diameter and internal characteristics (sugar, acidity and pH) of 12 randomly selected fruit were measured. Fruit were prepared a day before the tests by first removing calyces and second dipping in a fungicide mixture of Imazalil as sulphate and Guazatine (0.67 g of Imazalil and 4.8 ml of Guazatine in 1 L of water) for 1 minute in a plastic tub. The temperature of the water was adjusted to be between 35°C and 40°C.

Third instar larvae of *C. cosyra* eggs were used for the tests. They were prepared by inoculation of at least 40 eggs per fruit. Prior to inoculation, a 6 mm-diameter hole was bored at about 30 mm deep in the fruit beneath the calyx using a cork borer (of 5 mm-diameter). A brewer's yeast:water mixture (1:2) was placed into the hole (between 0.2 and 0.5 ml). After inoculation of eggs, the hole was plugged with cotton wool before being sealed with molten wax. Following sealing of hole, fruit were dipped in a similar fungicide mixture as described above. Each inoculated fruit will be placed in a brown paper bag. Fruit which were inoculated were then incubated for nine days in a temperature controlled room set to achieve 26°C inside the fruit. Between 272 and 300 fruit with eggs were prepared for use in each of six cold treatment exposures and an untreated control.

#### Cold treatment tested

One cold room equipped with a refrigeration unit and located at CRI Nelspruit was used for the tests. The treatment tested was at 1°C for 14 days. The susceptibility of third instar larvae of *C. cosyra* to the cold treatment was determined at six exposure periods: 5, 7, 9, 11, 12 and 14 days after the start of the cold treatment. Fruit in the cold room were divided by exposure day.

Temperature measurements inside the cold room were carried out as described above. The start point of the treatment was when 3 of the probes reached 1°C. At the end of each exposure period, fruit containing larvae were removed from the cold room. Fruit were placed in the same temperature controlled room as for the incubation. After the cold treatment, the fruit were held for two days before being dissected to determine mortality.

The untreated control was dissected on the day of the cold treatment (nine days after egg inoculation) to determine mortality. In the determination of mortality, dead and live larvae were counted. A larva was considered dead if there was no visible movement even when prodded.

There were three replicates of these tests using three fly cohorts. Each replicate was conducted at a time such that the treatment from precooling through to the final exposure period was separately repeated.

Data were summarized as observed percentage mortality. Observed mortality was calculated from the number of surviving third instar larvae over the estimated number of treated larvae. The number of treated larvae for each replicate was the product of infestation rate and the number of treated fruit. The infestation rate was calculated from the number of surviving third instar larvae over the number of fruit in the control. The cold exposure period to achieve 99% mortality of *C. cosyra* in Valencia oranges as estimated using the Probit model (Addinsoft 2020).

#### *C. Confirmation of treatment schedule at 1°C for 14 days for disinfestation of C. cosyra*

Large scale trials to confirm the efficacy of an existing *C. capitata* cold treatment schedule of at or below 1.11°C for 14 days for *C. cosyra* were carried out in *Citrus sinensis* (L.) Osbeck cv Late Valencia sourced directly from a commercial packhouse (prior to being placed on the packing line) in Karino, Ehlanzeni District, Mpumalanga Province and in C100 Valencia oranges from Crocodile Valley (same orchard as described above). Fruit were processed in the same way as described above except for the exclusion of brewer's yeast and water mixture as additional larval food in the C100 Valencia oranges prior to inoculation. The exclusion of yeast in C100 Valencia oranges was found to increase infestation rate by *C. cosyra* in small scale parallel trials.

Fruit with third instar larvae of *C. cosyra* were placed in a cold room at a target temperature of 1°C for a total period of 14 days. Temperature measurements inside the cold room and after cold treatment were carried out as described above. Mortality was also determined as described above. There were four replicates of the large scale tests.

The number of estimated treated larvae was calculated as the product of infestation rate and the number of fruit treated. Infestation rate was calculated as the number of survivors in the control over the number of fruit used in the control. Observed mortality rates were calculated as the number of survivors from the treatment over the number of estimated treated larvae. True mortality (3 survivors over 100 000) at 95% confidence interval was then calculated as follows:  $1 - [(3 + \sqrt{\text{number of observed survivors} \times 100000}) / \text{number of tested individuals} / 100000]$ .

## Results and discussion

### A. Comparison of the *in vitro* cold tolerance of *C. capitata* and *C. cosyra*

The mean egg hatch ( $\pm$ SE) for *C. capitata* and *C. cosyra* was 93.38%  $\pm$  1.06% and 95.13%  $\pm$  1.03% respectively. Larvae of the two species were mostly at their third instar stage (Table 3.3.3.1).

**Table 3.3.3.1.** Larval characteristics of diet dishes of *C. capitata* and *C. cosyra* which were treated as third instars in the four replicates of the cold tolerance study.

Replicate	Species	Mean larval length $\pm$ SE (mm)	Total number of larvae found	% Instar composition		
				First	Second	Third
1	<i>C. capitata</i>	7.88 $\pm$ 0.03	177	0	0	100
	<i>C. cosyra</i>	7.77 $\pm$ 0.04	172	0	0	100
2	<i>C. capitata</i>	7.99 $\pm$ 0.03	169	0	0	100
	<i>C. cosyra</i>	7.69 $\pm$ 0.05	172	0	0	100
3	<i>C. capitata</i>	6.07 $\pm$ 0.10	82	0	7	93
	<i>C. cosyra</i>	6.10 $\pm$ 0.08	140	0	1	99
4	<i>C. capitata</i>	6.17 $\pm$ 0.13	170	0	4	96
	<i>C. cosyra</i>	7.09 $\pm$ 0.15	172	0	17	83

In this study, the time taken from start of cooling until at least three probes in the diet containers were at  $\leq$  3.5°C was 4.08, 4.25, 4.83 and 4.17 hours in the first, second, third and fourth replicate respectively. After the start of the cold treatment, the mean ( $\pm$  SE) diet temperatures for 18 consecutive days in the cold room was 3.52°C  $\pm$  0.00°C, 3.54°C  $\pm$  0.00°C, 3.58°C  $\pm$  0.00°C and 3.52°C  $\pm$  0.00°C for the first, second, third and fourth replicate respectively.

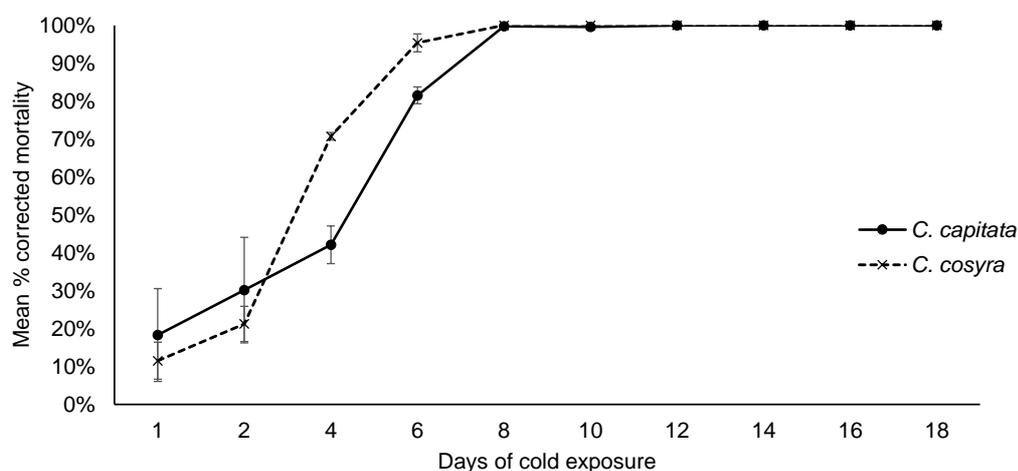
#### Mortality of *C. capitata* and *C. cosyra*

Mortality rates of *C. cosyra* were significantly higher than those of *C. capitata* following exposure to the cold treatment ( $\chi^2 = 111.57$ ,  $df = 1$ ,  $P < 0.0001$ ) (Fig 3.3.3.1). There were no survivors of *C. cosyra* beyond six days of cold treatment whilst for *C. capitata* survivorship extended up until ten days of cold exposure (Fig. 3.3.3.1).

There was also a significant effect of exposure period on mortality (Exposure period:  $\chi^2 = 4794.83$ ,  $df = 9$ ,  $P < 0.0001$ ).

Results from the Probit analysis to estimate the number of days of cold exposure to achieve 50%, 90%, 95% and 99% mortality are presented in Table 3.3.3.2. The estimated cold exposure periods to achieve 50%, 90%, 95% and 99% mortality levels were higher for *C. capitata* than for *C. cosyra*.

The lower mortality rates of *C. capitata* compared to *C. cosyra* due to cold treatment found in this study corroborate with results obtained in the study by Grout and Stoltz (2007) on temperature related development of the two species, where larval survival rates of *C. cosyra* were lower than those of *C. capitata*, particularly at temperatures below 20°C.



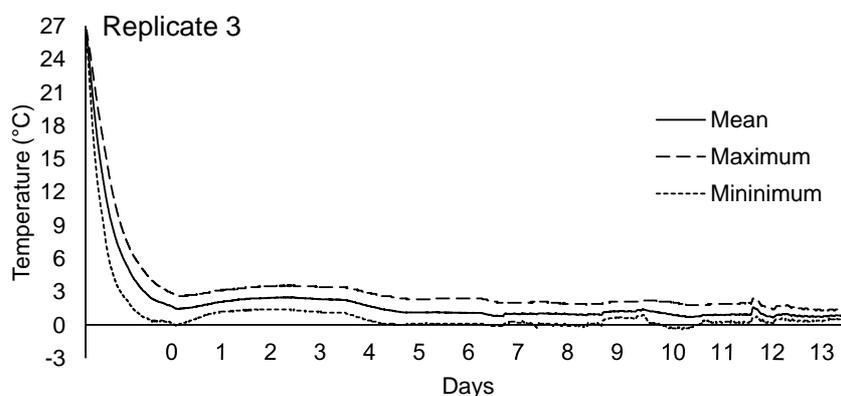
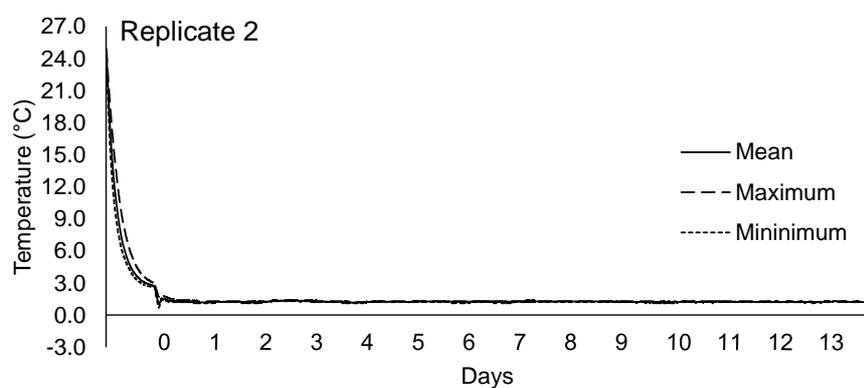
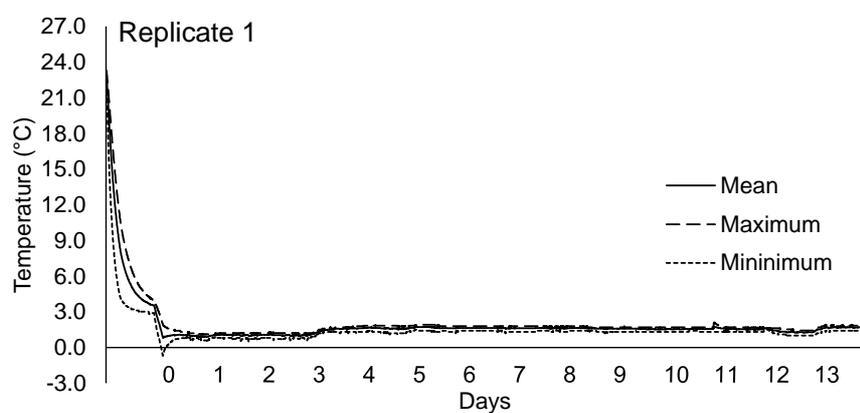
**Figure 3.3.3.1.** Mortality of third instar larvae of *C. capitata* and *C. cosyra* at a mean ( $\pm$ SE) temperature of  $3.54^{\circ}\text{C} \pm 0.00^{\circ}\text{C}$  at ten exposure periods over 18 consecutive days

**Table 3.3.3.2.** Estimated exposure periods at  $3.5^{\circ}\text{C}$  required for 50%, 90%, 95% and 99% mortality of third instar larvae of *C. capitata* and *C. cosyra* in a carrot-based medium.

Species	Estimated exposure days (95% CI) for four mortality levels			
	50%	90%	95%	99%
<i>C. capitata</i>	2.79 (2.74 - 2.85)	7.00 (6.84 - 7.17)	9.08 (8.84 - 9.34)	14.80 (14.27 - 15.38)
<i>C. cosyra</i>	2.60 (2.55 - 2.65)	5.34 (5.22 - 5.46)	6.54 (6.38 - 6.72)	9.59 (9.27 - 9.93)

#### B. Determination of treatment conditions at $1^{\circ}\text{C}$

The durations from start of cooling until at least three probes in the fruit were at  $1^{\circ}\text{C}$  were 26.92, 24.42 and 38.42 hours in the first, second, and third replicate respectively (Fig. 3.3.3.2). After the start of the cold treatment, the mean ( $\pm$  SE) fruit temperatures in the cold room were  $1.46^{\circ}\text{C} \pm 0.00^{\circ}\text{C}$ ,  $1.25^{\circ}\text{C} \pm 0.00^{\circ}\text{C}$  and  $1.35^{\circ}\text{C} \pm 0.00^{\circ}\text{C}$  for the first, second and third replicate respectively (Fig. 3.3.3.2). There were no major differences in external and internal characteristics of the fruit used in the three replicates (Table 3.3.3.3). No survivors of third instar larvae of *C. cosyra* were recorded beyond 11 days of cold treatment (Table 3.3.3.4). Based on observed mortality rates of *C. cosyra* in Valencia C100 at the lowest mean temperature tested  $1.25^{\circ}\text{C}$  for 14 days, the number of days of cold exposure (95% CI) estimated to achieve 99% mortality for this species was 11.27 (11.17, 11.36). This implies that the treatment schedule for *C. capitata* of at or below  $1.11^{\circ}\text{C}$  for 14 days (USDA 2016) should adequately mitigate the risk of *C. cosyra* in citrus.



**Figure 3.3.3.3.** Mean, maximum and minimum temperature profiles during three replicates of the determination of cold conditions at a target temperature of 1°C for 14 days.

**Table 3.3.3.3.** External and internal characteristics of Valencia C100 oranges used in the three replicates of the determination of cold conditions at a target temperature of 1°C

Replicate	Weight/g	Diameter/mm	Acid (%)	Brix °	pH
1	183.6 ± 7.7	71.1 ± 1.1	1.3	11.0	3.0
2	190.9 ± 4.8	74.0 ± 0.8	1.3	12.1	3.1
3	226.7 ± 11.1	75.3 ± 1.4	1.1	13.8	3.8

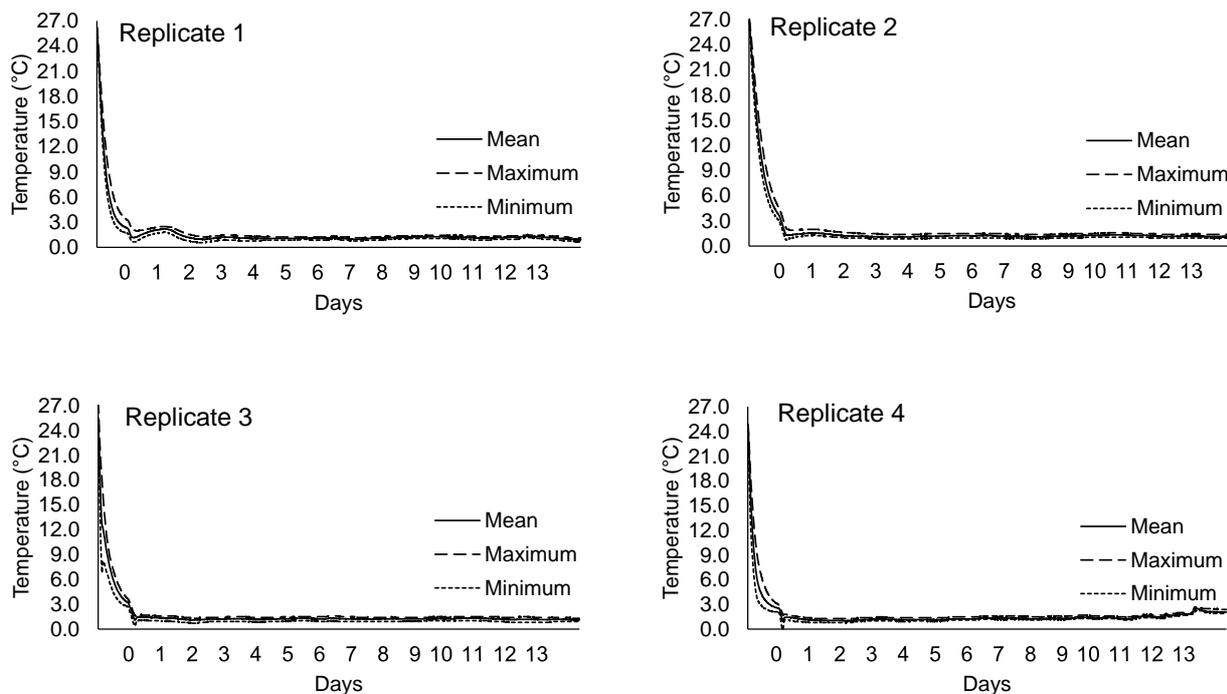
**Table 3.3.3.4.** Mortality of third instar larvae of *C. cosyra* in fruit treated at a temperature of 1°C for a range of exposure periods

Replicate	Exposure period	Number of fruit	Estimated number of treated larvae	Total number of survivors	Observed mortality (%)
1	5	272	1025	140	86.34%
	7	280	1025	84	91.80%
	9	280	1025	0	100.00%
	11	280	1025	2	99.80%
	12	280	1025	0	100.00%
	14	280	1025	0	100.00%
	Control		279		1025
2	5	300	3080	831	73.02%
	7	300	3080	485	84.25%
	9	299	3080	198	93.57%
	11	296	3080	25	99.19%
	12	300	3080	0	100.00%
	14	300	3080	0	100.00%
	Control		300		3080
3	5	300	1081	259	76.04%
	7	300	1081	132	87.79%
	9	300	1081	79	92.69%
	11	300	1081	13	98.80%
	12	300	1081	0	100.00%
	14	300	1081	0	100.00%
	Control		300		1081

*C. Confirmation of treatment schedule at 1°C for 14 days for disinfestation of C. cosyra*

Temperature profiles and summaries of the large scale tests are shown in Figure 3.3.3.4 and Table 3.3.3.5. The external and internal characteristics of Valencia oranges used in the tests are provided in Table 3.3.3.6. Since infestation rate was low in Late Valencia oranges in replicate 1 (Table 3.3.3.7), Valencia C100 oranges were used in the other three replicates.

There were no survivors from a total estimate of 18437 third instar larvae of *C. cosyra* which were treated at the lowest mean temperature of 1.25°C for 14 days (Table 3.3.3.7). The number of test individuals would however have to be increased for approval of a treatment of at or below 1.11°C for 14 days for disinfestation of *C. cosyra* by export fruit markets. Some of these markets require treatments that would result in either no survivors in over 29 000 treated larvae or no survivors in over 93 613 treated larvae (Follett and Neven 2006).



**Figure 3.3.3.4.** Mean, maximum and minimum temperature profiles during four replicates of large scale trials of a cold treatment at a target temperature of 1°C for 14 days

**Table 3.3.3.5.** Summary of temperatures in the cold room during large scale tests on mortality of third instar larvae of *C. cosyra* at 1°C for 14 days

Replicate	Fruit loaded into chamber (Date and Time)	Start of treatment (Date and time)	End of treatment (Date and time)	Total Duration (days)	Average of fruit temperatures (°C)
1	11/02/2021 08:47	12/02/2021 09:52	26/02/2021 09:42	14	1.20 ± 0.00
2	04/03/2021 09:00	05/03/2021 11:55	19/03/2021 11:25	14	1.21 ± 0.00
3	25/03/2021 09:21	26/03/2021 12:41	09/04/2021 12:11	14	1.24 ± 0.00
4	15/04/2021 08:30	16/04/2021 10:25	30/04/2021 09:55	14	1.36 ± 0.00

**Table 3.3.3.6.** External and internal characteristics of Valencia oranges used in the four replicates of the large scale tests of the cold treatment at a target temperature of 1.11°C.

Replicate	Weight/g	Diameter/mm	Acid (%)	Brix °	pH
1*	79.64 ± 3.35	53.40 ± 0.86	1.35	15.2	3.38
2**	217.36 ± 12.10	74.40 ± 1.36	1.00	13.8	3.56
3**	175.23 ± 12.49	69.00 ± 1.83	1.10	14.5	3.51
4**	140.98 ± 5.01	64.80 ± 0.85	0.80	14.1	3.78

\* Late Valencia oranges

\*\* Valencia C100 oranges

**Table 3.3.3.7.** Mortality of third instar larvae of *C. cosyra* in large scale tests of fruit treated at between 1.2°C and 1.4°C for 14 days.

Replicate	Date	Control		Treated			Observed Mortality	True Mortality (95% CI)
		No. of Fruit	No. of live insects	No. of Fruit	Estimated No. of treated insects*	Total No. of survivors		
1	February 11, 2021	124	64	500	258	0	100.00%	98.84%
2	March 04, 2021	100	2275	300	6825	0	100.00%	99.96%
3	March 25, 2021	50	1963	149	5850	0	100.00%	99.95%
4	April 15, 2021	30	1635	101	5505	0	100.00%	99.95%
Total		304	5937	1050	18437		100.00%	99.98%

\* Estimated number of treated larvae = Infestation rate (No. of live insects in control/No. of fruit) X No. of treated fruits

### Conclusion

Cold disinfestation treatments for *C. capitata* should be equally effective against *C. cosyra*.

### Future research

Additional replicates of the exploratory and large scale tests should be carried out for full acceptance of a cold disinfestation treatment of at or below 1.2°C for 14 days for *C. cosyra*.

### Technology transfer

None.

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### 3.3.4 PROGRESS/PROPOSAL REPORT: Fruit fly rearing

Project 407 (April 1999 – March 2022) by Aruna Manrakhan, John-Henry Daneel, Glorious Shongwe and Rooikie Beck (CRI)

#### Summary

Colonies of five fruit fly species: *Ceratitidis capitata*, *Ceratitidis rosa*, *Ceratitidis quilicii*, *Ceratitidis cosyra* and *Bactrocera dorsalis* continue to be maintained at Citrus Research International (CRI), Nelspruit. Fruit flies from these colonies were used in CRI Projects 1171, 1211, 1245 and 1254. Pupae of Medfly were supplied to Stellenbosch University for trials under FF-IPM Horizon 2020 project. Fruit fly larval diet mixtures were supplied to University of Pretoria and Agribiotech Research Consultancies for research on biology and post-harvest fruit fly management respectively. Volumes of pupae produced per week for all colonies except *C. quilicii* were above 400 ml (between 10, 000 and 20, 000 pupae). For all colonies except *C. quilicii*, percentages of egg hatch and adult emergence were on average above 70 and 60 respectively. All colonies were refreshed by using males and females reared from fruit. Between November 2020 and March 2021, new *C. rosa* and *C. cosyra* colonies were founded from a total of 513 and 1715 flies respectively. For *C. rosa*, most of the flies were reared from *Syzygium jambos* collected from Nelspruit whilst for *C. cosyra*, most of the flies were reared from *Walburgia salutaris* also collected from Nelspruit. Between January and March 2021, new *B. dorsalis* and *C. capitata* colonies were founded from a total of 141 and 535 wild flies respectively. The wild *B. dorsalis* flies were reared from *Mangifera indica* collected in Nelspruit and Deerpark. The wild *C. capitata* flies were reared from *Coffea arabica* collected from Burgershall. The colony of *C. quilicii* was refreshed by addition of wild flies to the existing colony. Between January and March 2021, 1459 *C. quilicii* adults reared mostly from *Prunus persica* collected from Bourke's Luck, Mpumalanga Province were added to the colony.

#### Opsomming

Kolonies van vyf vrugtevlieg spesies: *Ceratitidis capitata*, *Ceratitidis rosa*, *Ceratitidis quilicii*, *Ceratitidis cosyra* en *Bactrocera dorsalis* word steeds by Citrus Research International (CRI), Nelspruit, in stand gehou. Vrugtevlieë

vanaf hierdie kolonies is in CRI Projekte 1171, 1211, 1245 en 1254 gebruik. Papies van Medvlieg is aan die Universiteit van Stellenbosch verskaf vir proewe onder die FF-IPM Horizon 2020 projek. Vrugtevlieg larwe voedingsmengsels is aan die Universiteit van Pretoria en Agribiotech Research Consultancies vir navorsing op biologie en na-oes vrugtevlieg bestuur, onderskeidelik, verskaf. Volumes van papies wat per week vir alle kolonies geproduseer is, behalwe vir *C. quilicii*, was b6 400 ml (tussen 10, 000 en 20, 000 papies). Vir alle kolonies, behalwe *C. quilicii*, was persentasies van eiers wat uitgebroei het en volwasse opkoms op 'n gemiddelde van b6 70 en 60, onderskeidelik. Alle kolonies is vernuwe deur mannetjies en wyfies te gebruik wat vanaf vrugte geteel is. Tussen November 2020 en Maart 2021, is nuwe *C. rosa* en *C. cosyra* kolonies vanaf 'n totaal van onderskeidelik 513 en 1715 vlie6 gestig. Vir *C. rosa* is die meeste vlie6 vanaf *Syzygium jambos* geteel wat vanaf Nelspruit versamel is, terwyl vir *C. cosyra*, meeste van die vlie6 vanaf *Walburgia salutaris* geteel is, wat ook vanaf Nelspruit versamel is. Tussen Januarie en Maart 2021, is nuwe *B. dorsalis* en *C. capitata* kolonies vanaf 'n totaal van onderskeidelik 141 en 535 wilde vlie6 gestig. Die wilde *B. dorsalis* vlie6 is vanaf *Mangifera indica* geteel wat in Nelspruit en Deerpark versamel is. Die wilde *C. capitata* vlie6 is vanaf *Coffea arabica* geteel wat vanaf Burgershall versamel is. Die kolonie van *C. quilicii* is vernuwe deur die byvoeg van wilde vlie6 tot die bestaande kolonie. Tussen Januarie en Maart 2021, is 1459 *C. quilicii* volwassenes, wat meestal vanaf *Prunus persica* geteel is wat vanaf Bourke's Luck, Mpumalanga-provinsie, versamel is, tot die kolonie gevoeg.

### 3.3.5 PROGRESS REPORT: Efficacy of FCM partial cold treatments for fruit fly pests of citrus

Project 1171 (April 2017- March 2022) by Aruna Manrakhan, John-Henry Daneel, Rooikie Beck, Glorious Shongwe, Sean Moore, Vaughan Hattingh (CRI)

#### Summary

Cold shipping forms part of post-harvest measures for mitigation of risk of fruit fly pests in citrus other than lemons and limes from South Africa under a Fruit Fly Systems Approach. The shipping temperatures used are at set points that vary between -1°C and 4°C with estimated pulp temperatures of between 0°C and 5°C. There are internationally approved cold treatments for fruit flies at temperatures which are at or below 3°C. This study aims at determining the efficacy of a treatment at 3.5°C on fruit fly larval mortality. The relative cold tolerances of third instar larvae of three fruit fly pests of citrus in southern Africa: *Ceratitis capitata*, *Ceratitis rosa* and *Bactrocera dorsalis* were first determined at 3.5°C for 18 days. Tests were then continued with the most cold tolerant fruit fly species. Fruit fly mortality at 3.5°C was determined over different exposure periods. The efficacy of a treatment at 3.5°C for 24 days was finally confirmed in large scale tests. *Ceratitis capitata* was found to be more cold tolerant than *C. rosa* and *B. dorsalis*. No survivor of *C. capitata* in Valencia oranges, was recorded beyond 16 continuous days of treatment at 3.5°C in exploratory tests. Large scale tests conducted at 3.5°C for 24 days with a total of more than 26 000 third instar larvae of *C. capitata* resulted in no survivors. A treatment at or below 3.5°C for 24 days can effectively mitigate the risk of fruit flies in export citrus from South Africa.

#### Opsomming

Koue-verskeping vorm deel van na-oes maatre6s ten einde die risiko verbonde aan vrugtevliegplae in sitrus, behalwe vir suurlemoene en lemmetjies vanaf Suid-Afrika, onder 'n Vrugtevlieg Stelselbenadering, te beperk. Die verskepingstemperatuur wat gebruik word, is op vasgestelde punte wat tussen -1°C en 4°C varieer, met geskatte pulptemperatuur van tussen 0°C en 5°C. Daar is internasionaal-goedgekeurde koue-behandelings vir vrugtevlie6 by temperatuur wat by of laer as 3°C is. Hierdie studie het ten doel om die effektiwiteit van 'n behandeling by 3.5°C op vrugtevlieglarwe mortaliteit te bepaal. Die relatiewe koue-bestandheid van derde instar larwes van drie vrugtevliegplae van sitrus in suidelike Afrika: *Ceratitis capitata*, *Ceratitis rosa* en *Bactrocera dorsalis* is eers by 3.5°C vir 18 dae bepaal. Toetse is dan voortgesit met die mees koue-bestande vrugtevlieg spesies. Vrugtevlieg mortaliteit by 3.5°C is oor verskillende blootstellingsperiodes bepaal. Die effektiwiteit van 'n behandeling by 3.5°C vir 24 dae is finaal in grootskaalse toetse bevestig. Daar is gevind dat *Ceratitis capitata* meer koue-bestand as *C. rosa* en *B. dorsalis* was. Geen oorlewende *C. capitata* is in Valencia lemoene aangeteken na meer as 16 aaneenlopende dae van behandeling by 3.5°C in verkennende toetse nie. Grootskaalse toetse wat by 3.5°C vir 24 dae, met 'n totaal van meer as 26 000 derde instar larwes van *C.*

*capitata*, uitgevoer is, het tot geen oorlewendes gelei nie. 'n Behandeling by of onder 3.5°C vir 24 dae, kan effektief die risiko van vrugtevlieë in uitvoersitrus vanaf Suid-Afrika beperk.

### 3.3.6 **PROGRESS REPORT: The impact of interruptions on Medfly cold treatment efficacy**

Project 1204 (2018/9 – 2021/2) by T G Grout, P R Stephen, K C Stoltz and V Hattingh (CRI)

#### **Summary**

Progress was slow in 2019 due to a shortage of cold rooms but five replicates were conducted in 2020 where a single interruption after three days of cold treatment was compared with two interruptions after two and four days of cold treatment against third instar Medfly. A mean fruit pulp temperature of 0.9°C for 6 days was used to ensure some survivors and interruptions caused the pulp temperature to reach 3.1°C before being allowed to cool. The mean corrected mortality obtained for five replicates without interruptions was 95.72%. The mean corrected mortality after a single interruption in five replicates was 94.68% and for two interruptions, 94.61%. More than 6000 larvae were used per treatment. Further evaluations will now be conducted where infested fruit is left in the cold room and the chilling unit turned off until the internal fruit temperature has increased the desired amount.

#### **Opsomming**

Vordering in 2019 was stadig weens 'n tekort aan koelkamers, maar vyf herhalings is in 2020 uitgevoer waar 'n enkele onderbreking ná drie dae van koue-behandeling, met twee onderbrekings ná twee en vier dae van koue-behandeling, teen derde instar Medvlieg vergelyk is. 'n Gemiddelde vrugpulptemperatuur van 0.9°C vir 6 dae is gebruik om 'n paar oorlewendes te verseker, en onderbrekings het veroorsaak dat die pulptemperatuur 3.1°C bereik het voordat dit toegelaat is om af te koel. Die gemiddelde gekorrigeerde mortaliteit wat vir vyf herhalings sonder onderbrekings verkry is, was 95.72%. Die gemiddelde gekorrigeerde mortaliteit ná 'n enkele onderbreking in vyf herhalings was 94.68%, en vir twee onderbrekings, 94.61%. Meer as 6000 larwes is per behandeling gebruik. Verdere evaluasies sal nou uitgevoer word waar besmette vrugte in die koelkamer gehou word en die verkoelingseenheid afgestel word totdat die interne vrugtemperatuur die verlangde hoeveelheid toegeneem het.

### 3.3.7 **PROGRESS REPORT: Understanding fruit fly trap efficiency: the role of physical and biotic variables**

Project 1229 (2019/20 – 2020/1) by Prof. Christopher Weldon (UP)

#### **Summary**

A systems approach for quarantine pests is a phytosanitary risk mitigation measure that can be used to achieve quarantine security. At least two independent risk mitigating measures should be implemented in a fruit fly systems approach. These measures can include areas of low fruit fly prevalence, fruit fly control and monitoring measures in the field, inspection, and cold storage. In the citrus industry, risk of fruit fly infestation in the field is measured by trapping. This project set out to establish the role of temperature, relative humidity and fly physiology on the efficiency of fruit fly lures. Milestones indicated in the project proposal have been delayed due to new climate rooms at the University of Pretoria not being fit for purpose and late receipt of custom-made field cages required to pursue semi-field trials. University and then South African national government implemented shutdowns to prevent the spread of COVID-19 also led to further delays. No progress could be made until May 2020, when the University of Pretoria received approval from the Department of Higher Education and Training to recommence agricultural research. Despite these delays, we have collected data in the field that shows weight loss by BioLure, EGOLure and Methyl Eugenol Lure increases as weekly average temperature increases. Weekly average relative humidity affects only BioLure, which can gain weight when relative humidity is high and temperatures are low. These results suggest a role for weather in the attractiveness of lures due to increased volatilisation of lure components, with lure concentration known to affect responsiveness of some fruit fly species. This may necessitate that management actions triggered by trap captures be adjusted depending on prevailing environmental conditions.

## Opsomming

'n Stelselbenadering vir kwarantynplae is 'n fitosanitêre risiko-beperkingsmaatreël wat gebruik kan word om kwarantynsekuriteit te bewerkstellig. Ten minste twee onafhanklike risiko-beperkende maatreëls moet in 'n vrugtevlug stelselbenadering geïmplementeer word. Hierdie maatreëls kan gebiede met lae voorkoms van vrugtevlug, vrugtevlugbeheer en moniteringsmaatreëls in die veld, inspeksie en verkoeling insluit. In die sitrusbedryf word die risiko van vrugtevlugbesmetting in die veld deur vangste gemeet. Hierdie projek het ten doel om die rol van temperatuur, relatiewe humiditeit en vlieg fisiologie op die doeltreffendheid van vrugtevlug lokmiddels vas te stel. Mylpale, soos aangedui in die projekvoorstel, is vertraag weens nuwe klimaatkamers by die Universiteit van Pretoria wat nie vir die doel geskik was nie, en láát ontvangs van pasgemaakte veldhokke wat benodig word om semi-veldproewe uit te voer. Die Universiteit, en daarna die Suid-Afrikaanse nasionale regering, se implementering van toemaak-aksies ten einde die verspreiding van COVID-19 te voorkom, het tot verdere vertraging gelei. Geen vordering kon tot Mei 2020 gemaak word nie, toe die Universiteit van Pretoria goedkeuring vanaf die Departement van Hoër Onderwys en Opleiding ontvang het om weer met landboukundige navorsing voort te gaan. Ten spyte van hierdie vertraging, is data in die veld versamel, wat aandui dat gewigsverlies deur BioLure, EGOlure en Metiel Eugenol Lokmiddel toeneem namate weeklikse gemiddelde temperatuur toeneem. Weeklikse gemiddelde relatiewe humiditeit affekteer slegs BioLure, wat gewig kan optel wanneer relatiewe humiditeit hoog en temperature laag is. Hierdie resultate dui op 'n rol vir die weer in die aantrekkingskrag van lokmiddels weens verhoogde vlugtigheid van lokmiddel komponente, met die bekende feit dat lokmiddel konsentrasie die reaksie van sommige vrugtevlugspesies affekteer. Dit kan noodsaak dat bestuurs-aksies wat deur lokvalvangste aan die gang gesit word, aangepas word afhangende van heersende omgewingstoestande.

### 3.3.8 PROGRESS REPORT: Redefining dispersal potential for adequate fruit fly pest management (Diptera, Tephritidae)

Project 1254 (2019/20 – 2021/22) by A. Manrakhan (CRI), S. K. Tsatsu (Stellenbosch University), L. Serfontein (CRI), M. Karsten (Stellenbosch University), P. Addison (Stellenbosch University), M. De Meyer (Royal Museum for Central Africa) and M. Virgilio (Royal Museum for Central Africa).

## Summary

The dispersal potential of two cryptic fruit fly pest species in South Africa: *Ceratitits rosa* and *C. quilicii* are being quantified by determination of their distributions and host ranges in the country as well as by an assessment of their demography on different fruit types. The distributions and host ranges of the two species were determined by trapping and fruit sampling. Traps baited with EGO lure, an attractant known for males of the two species, were set up in selected sites in seven provinces in South Africa where citrus is produced. Traps were set up for a period of four weeks during each of three periods: autumn-winter (May-August) of 2020, spring-summer (October-December) of 2020 and late summer (March) of 2021. *Ceratitits quilicii* was found in all provinces except Northern Cape. *Ceratitits rosa* was recorded only in Limpopo, Mpumalanga and KwaZulu-Natal provinces. *Ceratitits quilicii* was more abundant than *C. rosa* in these three provinces. Higher numbers of both species were recorded in spring-summer and late summer periods. *Ceratitits quilicii* was reared from *Psidium guajava*, guava, collected near Stellenbosch, *Acca sellowiana*, feijoa and *Casimiroa edulis*, white sapote, collected in the Mpumalanga province. No *C. quilicii* nor *C. rosa* was reared from a total of approximately 21 kg of citrus sampled (all citrus types pooled) across South Africa. Demographic studies of the two species were completed on two fruit types: guava and *Prunus persica* (peach). The two species developed better in peach compared to guava. There were no major differences in developmental rates, immature survival and pupal characteristics between the two species on guava. However, when placed in groups (groups of five adult pairs), *C. quilicii* had lower adult survival rates compared to *C. rosa*.

## Opsomming

Die verspreidingspotensiaal van twee kriptiese vrugtevlug plaagspesies in Suid-Afrika: *Ceratitits rosa* en *C. quilicii* word gekwantifiseer deur bepaling van hul verspreiding en gasheerreeks in die land, asook deur die vasstelling van hul demografie op verskillende vrug tipes. Die verspreiding en gasheerreeks van die twee spesies is vasgestel deur lokvalle en vrug monsterneming. Lokvalle met EGO lokmiddel, 'n lokmiddel wat vir

manneljies van die twee spesies bekend is, is in geselekteerde persele in sewe provinsies in Suid-Afrika waar sitrus verbou word, opgestel. Lokvalle is vir 'n periode van vier weke gedurende elk van drie periodes opgestel: herfs-winter (Mei-Augustus) van 2020, lente-somer (Oktober-Desember) van 2020 en láát somer (Maart) van 2021. *Ceratitis quilicii* is in alle provinsies behalwe in die Noord-Kaap gevind. *Ceratitis rosa* is slegs in Limpopo-, Mpumalanga- en KwaZulu Natal-provinsie aangeteken. *Ceratitis quilicii* was meer volop as *C. rosa* in hierdie drie provinsies. Hoër getalle van beide spesies is in lente-somer en láát somer periodes aangeteken. *Ceratitis quilicii* is geteel vanaf *Psidium guajava*, koejawel, wat naby Stellenbosch versamel is, *Acca sellowiana*, feijoa, en *Casimiroa edulis*, wit sapote, wat in die Mpumalanga-provinsie versamel is. Geen *C. quilicii* óf *C. rosa* is vanaf 'n totaal van ongeveer 21 kg sitrus (alle sitrus tipes saamgevoeg) wat regoor Suid-Afrika versamel is, geteel nie. Demografiese studies van die twee spesies is op twee vrug tipes voltooi: koejawel en *Prunus persica* (perske). Die twee spesies het beter in perske as in koejawel ontwikkel. Daar was geen groot verskille in ontwikkelingstempo's, onvolwasse oorlewing, en papie kenmerke tussen die twee spesies op koejawel nie. Wanneer egter in groepe geplaas is (groepe van vyf volwasse pare) het *C. quilicii* laer volwasse oorlewingstempo's gehad in vergelyking met *C. rosa*.

### 3.3.9 PROGRESS REPORT: In-silico boosted, pest prevention and off-season focussed IPM against new and emerging fruit flies

Project 1261 (April 2019-March 2022) by A. Manrakhan, L. Serfontein, R. Beck (CRI), D. Nestel (Agricultural Research Organisation, Israel) & D. Kriticos (CSIRO, Australia)

#### Summary

The aim of this study is to develop a detection system for the Oriental fruit fly, *Bactrocera dorsalis*, which is optimised both spatially (detection area) and temporally (detection time). A climatic model will be developed to determine optimal trapping time and site for early pest detection. A preliminary electronic trap (E-trap) that enabled real time detection of *B. dorsalis* was evaluated. Baseline data on *B. dorsalis* populations in Mpumalanga Province was collected as from October 2020 for the construct of the climatic model. Three grids with methyl eugenol baited traps and Biolure baited traps were set up in three sites: Crocodile Valley, Schoemanskloof and Ermelo, which were at different altitudes (low to high). Prevalence of *B. dorsalis* became lower with increasing altitude. Males of *B. dorsalis* were first detected in the high altitude site (Ermelo) in January 2021. Whilst at the medium and low altitude sites, *B. dorsalis* male catches were already recorded at the start of the study. The performance of the E-trap baited with methyl eugenol was determined in two commercial citrus farms in Mpumalanga Province between October 2020 and January 2021. In each farm, three electronic traps baited with methyl eugenol were compared with three conventional traps, Chempac bucket traps, each baited with the same methyl eugenol dispenser. Images of the E-trap were downloaded daily. Conventional traps were checked every fortnight in the field. The E-trap was found to be as effective as the conventional trap in terms of total *B. dorsalis* male catches. The E-trap however generally enabled an earlier detection of *B. dorsalis* since images could be downloaded daily. In upcoming trials, E-traps will be placed in one of the current study sites, with timing and location being based on the climatic model.

#### Opsomming

Die doel van hierdie studie is om 'n opsporingstelsel vir die Oosterse vrugtevlug, *Bactrocera dorsalis*, te ontwikkel, wat beide ruimtelik (opsporinggebied) en tydelik (opsporingstyd) geoptimaliseer is. 'n Klimaat model sal ontwikkel word om die optimale vangtyd en plek, vir vroeë plaag-opsporing, te bepaal. 'n Voorlopige elektroniese lokval (E-trap) wat intydse opsporing van *B. dorsalis* moontlik gemaak het, is geëvalueer. Basiese data oor *B. dorsalis* populasies in Mpumalanga-provinsie is vanaf Oktober 2020 versamel vir die konstruksie van die klimaat model. Drie roosters met lokvalle met metiel-eugenol en Biolure lok-aas is op drie persele opgestel: Crocodile Valley, Schoemanskloof en Ermelo, wat by verskillende hoogtes bo seevlak (laag tot hoog) was. Die voorkoms van *B. dorsalis* het laer geword met toename in hoogte bo seevlak. Manneljies van *B. dorsalis* is die eerste keer in die hoë hoogte perseel (Ermelo) in Januarie 2021 opgespoor, terwyl vangste van *B. dorsalis* mannetjies by die medium en lae hoogte persele reeds aan die begin van die studie aangeteken is. Die doeltreffendheid van die E-trap lokval met metiel-eugenol is in twee kommersiële sitrusplase in Mpumalanga-provinsie tussen Oktober 2020 en Januarie 2021 bepaal. In elke plaas is drie elektroniese lokvalle met metiel-eugenol, met drie konvensionele lokvalle, Chempac emmer lokvalle, elk met dieselfde

metiel-eugenol dispenser, vergelyk. Beelde van die E-trap is daagliks afgelaai. Konvensionele lokvalle is elke twee weke in die veld nagegaan. Daar is gevind dat die E-trap in terme van totale *B. dorsalis* mannetjie vangste net so effektief as die konvensionele lokval was. Die E-trap het egter oor die algemeen 'n vroeër opsporing van *B. dorsalis* moontlik gemaak aangesien beelde daagliks afgelaai kon word. In opkomende proewe sal E-traps in een van die huidige studiepersele geplaas word, met tydsberekening en ligging gebaseer op die klimaat model.

### 3.4 PROGRAMME: OTHER PESTS

Programme coordinator: Tim G Grout (CRI)

#### 3.4.1 Programme summary

The Other pests programme covers a broad range of pests and includes some projects that are relevant to more than one pest, some of which may be of phytosanitary importance. With increasing pressure on acceptable Maximum Residue Limits on fruit in our export markets it is important to improve our reliance on IPM and reduce the likelihood of creating pest repercussions by using plant protection products that are detrimental to important natural enemies. Biorational control methods such as microbial control are receiving attention in this programme to see whether their efficacy can be improved against red scale and mealybug (3.4.2) or whether synergism between entomopathogenic fungi and entomopathogenic nematodes can be maximised (3.4.4). Periodically, research is required to show that a pest that has been recorded on citrus in South Africa is not a threat to export markets on commercial citrus. This has been the case with oleander scale and two years of surveys in commercial citrus in different production regions in the country has shown that it is not present on fruit or in the canopy (3.4.3). Research against future biosecurity pests has also been conducted to reduce the likelihood of foreign mites being introduced on imported citrus budwood (3.4.8) and improve our ability to control the Asian citrus psyllid *Diaphorina citri* on young trees when it arrives in the country (3.4.6). Growing different types of citrus under netting is becoming increasingly popular and research on changes to the status of various pests under these conditions is continuing (3.4.7, 3.4.10, 3.4.11). The results are sometimes variable between sites but mealybug and red scale populations appear to increase under the nets while damage from thrips under nets often declines. Commercial insectaries offer various natural enemies for use in biocontrol augmentation. Releases of *Aphytis melinus* for the control of red scale are being evaluated but after one season the naturally-occurring *Aphytis africanus* seems to have had more impact where populations were conserved (3.4.9). Key parasitoids of mealybug differ in different production regions and hyperparasitoids may compromise their efficacy to different degrees. Releases of *Anagyrus vladimiri* are being evaluated at different times of the season in Limpopo province (3.4.12). Woolly whitefly became problematic a few years ago throughout the country but the importation and release of the parasitoid *Cales noacki* in some northern citrus production regions helped contribute to this pest's suppression. However, *C. noacki* could not be established in the Eastern Cape province at that time. The parasitoid has now been recovered at various locations in the Western Cape and the objective was to collect sufficient parasitoids to release in the Eastern Cape, but populations in the Western Cape were insufficient to achieve this (3.4.5). Research in this programme will continue to address both existing and future challenges to sustainable citrus IPM.

#### Programopsomming

Die Ander Plae program dek 'n breë reeks van plae, en sluit sommige projekte in wat relevant tot meer as een plaag is, waarvan sommige van fitosanitêre belang is. Met toenemende druk op aanvaarbare Maksimum Residu Limiete op vrugte in ons uitvoermarkte, is dit belangrik om ons afhanklikheid van IPM te verhoog, en die waarskynlikheid te verminder dat plaag gevolge geskep word deur die gebruik van plantbeskermingsprodukte wat skadelik vir belangrike natuurlike vyande is. Bio-rasionele beheermetodes soos mikrobiese beheer ontvang aandag in hierdie program ten einde te sien of hul doeltreffendheid teen rooi dopluis en witluis kan verbeter (3.4.2), of sinergisme tussen entomopatogeniese swamme en entomopatogeniese nematodes gemaksimeer kan word (3.4.4). Navorsing is van tyd tot tyd nodig om aan te toon dat 'n plaag wat op sitrus in Suid-Afrika aangeteken is, nie 'n bedreiging op kommersiële sitrus vir uitvoermarkte is nie. Dit was die geval met oleander dopluis, en twee jaar van opnames in kommersiële sitrus in verskillende produksiestreke in die land, het getoon dat dit nie op vrugte of in die lower teenwoordig is nie

(3.4.3). Navorsing teen toekomstige bio-sekuriteitsplae is ook uitgevoer ten einde die waarskynlikheid te verminder dat vreemde myte op ingevoerde sitrus okuleerhout ingebring word (3.4.8) en verbeter ons vermoë om Asiatiese sitrus bladvlou, *Diaphorina citri*, op jong bome te beheer wanneer dit in die land aankom (3.4.6). Die produksie van verskillende tipes sitrus onder net word al hoe meer gewild, en navorsing op veranderinge aan die status van verskeie plae onder hierdie toestande gaan voort (3.4.7, 3.4.10, 3.4.11). Die resultate is soms variërend tussen persele, maar dit blyk dat witluis en rooi dopluis populasies onder die nette toeneem, terwyl skade weens blaaspootjies onder nette dikwels afneem. Kommersiële insektariums bied verskeie natuurlike vyande vir gebruik in bio-beheer aanvulling. Die vrystellings van *Aphytis melinus* vir die beheer van rooi dopluis word geëvalueer, maar ná een seisoen blyk dit dat *Aphytis africanus* wat natuurlik voorkom, meer impak gehad het waar populasies bewaar is (3.4.9). Belangrike parasitoëde van witluis verskil in verskillende produksiestreke en hiperparasitoëde kan hul doeltreffendheid in verskillende mates beïnvloed. Vrystellings van *Anagyrus vladimiri* word by verskillende tye van die seisoen in Limpopo-provinsie geëvalueer (3.4.12). Wollerige witvlieg het 'n paar jaar terug regoor die land problematies geword, maar die invoer en vrystel van die parasitoëde, *Cales noacki*, in sommige noordelike sitrus produksiestreke het bygedra om die plaag te help onderdruk. *C. noacki* kon egter nie op daardie stadium in die Oos-Kaap-provinsie gevestig word nie. Die parasitoëde is nou by verskeie liggings in die Wes-Kaap herwin, en die doelwit was om genoeg parasitoëde te versamel om in die Oos-Kaap vry te stel, maar populasies in die Wes-Kaap was onvoldoende om dit te bereik (3.4.5). Navorsing in hierdie program sal voortgaan om beide bestaande en toekomstige uitdagings vir volhoubare sitrus IPM aan te spreek.

#### 3.4.2 FINAL REPORT: The efficacy of commercial entomopathogenic fungi products for control of citrus pests

Project 1174 (April 2017 – April 2020) by Sean Moore, Wayne Kirkman, Mellissa Peyper, Tammy Marsberg (CRI) and Luke Cousins (RU)

##### Summary

Currently at least three products based on entomopathogenic fungi (EPF) are actively marketed and sold for control of citrus pests in South Africa. Several other EPF products are known to be produced and sold in niche markets by much smaller companies. Although these products are not always registered for use against the pests being targeted, their usage in the industry is increasing. Claims of good control of a range of citrus pests are becoming more commonplace. However, these claims often conflict with the recorded experiences of scientists. This project aims to clarify the truth on the efficacy and usability of these products. During the first season of the project, no significant efficacy could be recorded against red scale, mealybug or thrips with a preventative programme using both *Beauveria bassiana* and *Metarhizium anisopliae* based products. Subsequently, a corrective trial was applied with three products at a site heavily infested with red scale and mealybug. Two of the products reduced mealybug infestation by 22.7% and 31.8%, compared to the untreated control. No efficacy against red scale was recorded. This project was to be terminated; however, a new *Isaria fumosorosea* product with reported efficacy against the Asian citrus psyllid (ACP), *Diaphorina citri*, was obtained for testing. The intention was to test it against a pest such as psylla, aphids or woolly whitefly, which could be indicative of its efficacy against ACP. However, unfortunately no reasonable level of infestation of any of these pests could be found in the Eastern Cape. Consequently, the product was tested in an orchard with an extremely high mealybug infestation. Unfortunately, no significant efficacy was recorded. During the next season several trials were conducted with three different rates (1x, 2x, and 5x) of *Isaria fumosorosea* on aphids and psylla. The trial on aphids was evaluated one and two weeks after application, where only the 5x rate treatment showed a significant reduction. The trial in an orchard with psylla in Swellendam showed no significant difference between these treatments.

##### Opsomming

Tans word minstens drie produkte gebaseer op entomopatogeniese swamme (EPS) aktief bemark en verkoop vir beheer van sitrusplae in Suid-Afrika. Dit is bekend dat verskeie ander EPS produkte deur kleiner maatskappye vervaardig word en in nis-markte verkoop word. Al is hierdie produkte nie altyd teen die teikenplae geregistreer nie, is hulle gebruik in die bedryf besig om toe te neem. Bewerings van goeie beheer van 'n reeks sitrusplae word al hoe meer algemeen, maar hierdie bewerings bots gereeld met die

ondervindinge van wetenskaplikes in die bedryf. Gedurende die eerste seisoen van die projek kon geen beduidende doeltreffendheid aangeteken word teen rooidopluis, witluis of blaaspootjie met 'n voorkomende program deur beide *Beauveria bassiana* en *Metarhizium anisopliae* gebaseerde produkte te gebruik. Gevolglik is 'n korrektiewe proef toegedien met drie produkte by 'n perseel waar dopluis en witluis besmetting hoog was. Twee van die produkte het die witluis besmetting met 22.7% en 31.8% verlaag in vergelyking met die onbehandelde kontrole. Geen doeltreffendheid is vir dopluis waargeneem nie. Die projek moes daarna beëindig word, maar 'n nuwe *Isaria fumosorosea* produk met berigte van doeltreffendheid teen Asiatiese sitrusbladvlooi (ABV), *Diaphorina citri*, is verkry vir proewe. Die doel was om dit te toets teen plaë soos sitrusbladvlooi, plantluise of wollerige witvlieg, wat kan dui op die doeltreffendheid daarvan teen ABV. Ongelukkig kon geen toepaslike vlak van besmetting van enige van hierdie plaë in die Oos-Kaap verkry word nie. Gevolglik is die produk in 'n boord met 'n uiters hoë witluis besmetting getoets. Ongelukkig is geen betekenisvolle doeltreffendheid waargeneem nie. Gedurende die volgende seisoen is verskeie proewe uitgevoer met drie verskillende dosisse (1x, 2x en 5x) van *Isaria fumosorosea* op plantluise en sitrusbladvlooi. Die proef op plantluise is een en twee weke na toediening geevalueer, en slegs die 5x dosis behandeling het 'n betekenisvolle reduksie getoon. Die proef in 'n boord met sitrusbladvlooi in Swellendam het geen betekenisvolle verskille getoon tussen die behandelings

## Introduction

Currently at least three products based on entomopathogenic fungi (EPF) are actively marketed and sold for control of citrus pests in South Africa. Several other EPF products are known to be produced and sold in niche markets by much smaller (cottage industry) companies. Although these products are not always registered for use against the pests being targeted, their usage in the industry is increasing. Claims of good control of a range of citrus pests are becoming more commonplace. These claims are made both by manufacturers/suppliers and growers, but these claims often conflict with the recorded experiences of scientists (e.g. Grout *et al.* 2012; Moore and Kirkman, 2013; Grout *et al.* 2016). In order to be able to correctly advise the industry, it is considered important for CRI to clarify the truth on the efficacy and usability of these products.

More recently, a new EPF product with reported good efficacy against Asian citrus psyllid (ACP) was obtained, which will be tested against a representative species in citrus in South Africa.

## Stated objectives

- To test the efficacy of at least three commercial EPF products against red scale, mealybug and woolly whitefly (WWF).
- To conduct these tests both in open orchard environments and under nets.
- If possible, to simultaneously test the efficacy of the same EPF products against FCM.
- To test an *Isaria fumosorosea* product against aphids and mealybug.

## Materials and methods

### Preventative application trial against red scale, mealybug and thrips

One trial site was selected in the Eastern Cape (Lane Late Navel orchard at Riverside Farm) and one in the Western Cape (Midnight Valencia orchard at Houdconstant Farm). At the Eastern Cape trial site trees were treated both outside and under shade netting.

Trials were laid out in a single-tree randomised block design and replicated 10 times for each of the netted and open orchard citrus. The following treatments were used (rates determined as per product labels):

- Untreated control
- Broadband (4 x 10<sup>9</sup> spores/ml) - (50 ml/100 L)
- Eco-Bb (2 x 10<sup>9</sup> spores/g) - (equivalent of at least 900 g/ha)

- Real IPM *Metarhizium* ( $1 \times 10^9$  cfu/ml) - (equivalent of at least 200 ml/ha)

Each product was applied four times during the course of the season. The first application was applied on 18 October 2017 to coincide with the first red scale crawler movement. The second and third applications were on 23 November 2017 and 19 December 2017 respectively. The final application of the four was done on 2 February 2018 to coincide with a peak in infestation of the pests on the trees. Treatments were applied as full cover film sprays. Five ml of a wetter (BreakThru) was added to Broadband and Real IPM treatments.

Evaluations of treatment efficacy against red scale and mealybug were done by inspecting 10 fruit on each trial tree and recording the number of infested and clean fruit. This was conducted three times, initiated a week before the final of the four applications had been completed i.e. 26 January, 28 March and 15 May 2018.

### **Corrective application trial against red scale and mealybug**

A further trial site for a corrective spray trial against a high infestation of red scale and mealybug was conducted. This trial was conducted on a 12-year-old Midnight orchard (5.5 m x 2.5 m) on Duikerslaagte (Tibshraeny), in Kirkwood.

Trials were conducted in a single-tree randomised block design, with 10 replicates per treatment. A single application of the following EPF products was made close to sunset on 25 April 2019 as a full cover spray:

- Untreated control
- Eco-Bb (10 g/100 L)                      16 L/tree
- RealMet69 (5 ml/100 L)                  17.5 L/tree
- Broadband (50 ml/100 L)                18.5 L/tree

A pre-spray evaluation was conducted to get comparable results, and the site evaluated on 4 May 2019 (9 days post application) and on 22 May 2019 (27 days post application). Mealybug evaluations were done by checking five fruit on each side of the tree for the presence or absence of mealybug. Ten fruit on each side of the tree were checked for red scale, and then recorded as no red scale, less than 11, and more than 10 red scale. Two red scale infested fruit were collected per tree and 10 red scale per fruit were microscopically inspected to determine whether the scale were alive or dead. Parasitism was also recorded.

### ***Isaria fumosorosea* (Challenger, 2.50E-09 viable conidia)**

Before the project could be terminated a new product was obtained to be tested against indicator species to gauge potential efficacy against ACP, such as aphids. Aphid infested sites were hard to find, and therefore the product was initially tested in an orchard heavily infested with mealybug, and only subsequently in orchards infested with aphids and psylla.

The orchard details of the three trial sites are listed in Table 3.4.2.1.

**Table 3.4.2.1.** Details of the three sites used to test Challenger (*Isaria fumosorosea*) against three different sucking pests

Farm	Region	Co-ordinated	Cultivar	Date sprayed	Insect
Bernol Citrus	Sundays River Valley, Eastern Cape	33°28'26.05"S 25°36'31.50"E	Delta Valencia	14/02/2019	Mealybug, <i>Planococcus citri</i>
Silverton Citrus	Sundays River Valley, Eastern Cape	33°31'55.89"S 25°41'14.73"E	Orri, Clementine	16/09/2019	Black citrus aphid, <i>Aphis citricidus</i>
Thornhill Citrus	Swellendam Western Cape	34° 2'5.29"S 20°31'58.49"E	Tango Mandarin	18/09/2019	African citrus psyllid, <i>Trioza erytreae</i>

The trials were laid out in a single-tree randomised block format and replicated 10 times for each treatment. Before application of the treatments, a pre-spray evaluation was conducted by inspecting 10 fruit (mealybug), 10 branches (aphids) and 10 leaf clusters (citrus psyllid) per data tree and recording the presence or absence of the pest. Data trees were then evaluated in the same way at various intervals after application.

The product was applied at three concentrations of the registered rate of 1L/ha, that is:

- Untreated control
- 1X rate (10.6 ml/100 L)            17 L/tree
- 2X rate (21.2 ml/100 L)            13 L/tree
- 5X rate (53 ml/100 L)                13 L/tree

## Results and discussion

### Preventative application trial against red scale, mealybug and thrips

#### *Eastern Cape evaluations 1, 2 and 3*

No significant differences in red scale or mealybug infestation were recorded between treatments (Table 3.4.2.2) for the evaluation done on 26 January. Significantly higher red scale was recorded inside the net compared to outside for the evaluation done on 28 March. For the third evaluation done on 15 May, significantly higher mealybug infestation and thrips damage was recorded outside nets than inside, while red scale infestation was significantly higher outside nets than inside. It is important to point out that the netting had no sides and was therefore only a net roof. No significant differences in mealybug and red scale infestation or thrips damage were recorded between treatments (Tables 3.4.2.3 and 3.4.2.4).

**Table 3.4.2.2.** Percentage fruit infested with mealybug and red scale under a net roof and outside, at three evaluations.

		26-Jan		28-Mar		15-May	
		Under net (%)	Outside (%)	Under net (%)	Outside (%)	Under net (%)	Outside (%)
1	<b>Mealybug</b>	0.44, a	0.46, a	0.5, c	0.12, c	0.07, e	0.19, f
2	<b>Red scale</b>	0.25, a	0.25, a	0.08, c	0.0, d	0.15, f	0.08, e
3	<b>Thrips</b>	-	-	-	-	0.03 e	0.6 f

\*Significant differences shown by different letters for each date. 26 January – a and b, 28 March – c and d, 15 May – e and f.

**Table 3.4.2.3.** Mealybug and red scale infestation under a net roof for the different treatments at three evaluation dates.

		26-Jan		28-Mar		15-May		
		Mealy-bug	Red scale	Mealy-bug	Red scale	Mealy-bug	Red scale	Thrips
1	<b>Untreated control</b>	0, a*	0.3, a	0, c	1.05, c	0.18, e	2.1, e	0.05, e
2	<b>Eco-Bb (10 g/100L)</b>	0.2, a	0.15, a	0.15, c	0.45, c	0.25, e	1.4, e	0.05, e
3	<b>RealMet69 (5 ml/100L) + Breakthru (5 ml/100L)</b>	0.5, a	0.25, a	0.2, c	0.7, c	0.1, e	0.9, e	0.15, e
4	<b>Broadband (50 ml/100L) + Breakthru (5 ml/100L)</b>	0.7, a	0.2, a	0.65, c	0.95, c	0.13, e	1.5, e	0, e

\*Significant differences shown by different letters for each date. 26 January – a and b, 28 March c and d, 15 May e and f.

**Table 3.4.2.4.** Mealybug and red scale infestation outside for the different treatments at three evaluation dates.

		26-Jan		28-Mar		15-May		
		Mealy-bug	Red scale	Mealy-bug	Red scale	Mealy-bug	Red scale	Thrips
1	Untreated control	0.8, a	0.1, a	0.15, c	0.1, c	0.38, e	0.75, e	0.35, e
2	Eco-Bb (10 g/100L)	0.7, a	0.1, a	0.35, c	0.45, c	0.5, e	1.2, e	0.25, e
3	RealMet69 (5 ml/100L) + Breakthru (5 ml/100L)	0.4, a	0.05, a	0.3, c	0.2, c	0.45, e	0.65, e	0.3, e
4	Broadband (50 ml/100L) + Breakthru (5 ml/100L)	0.5, a	0.3, a	0.35, c	0.3, c	0.5, e	0.55, e	0.3, e

\*Significant differences shown by different letters for each date. 26 January – a and b, 28 March c and d, 15 May e and f.

#### *Western Cape evaluations 1 and 2*

At the first evaluation, fruit treated with Eco-Bb and Real IPM treatments had the lowest red scale infestation of 51% for both treatments, followed by Broadband with 61% infestation and the control with 69% infestation. Trees treated with Broadband showed the highest amount of more severe red scale infestation (more than 5 red scale per fruit) (25%), followed by the control (15%), with Eco-Bb (7%) and Real IPM (4%) being the lowest.

Trees treated with Broadband had the highest mealybug infestation at 28%, followed by the control with 24%, Eco-Bb with 22% and the lowest being for Real IPM at 16%.

For the second evaluation trees treated with Broadband had the lowest red scale infestation of 53%, followed by Real IPM with 58%, the control with 65% and the highest infestation for Eco-Bb with 67%. The control had the highest amount of more severe red scale infestation (more than 5 red scale per fruit) (53%), followed by Eco-Bb (35%), and Real IPM (33%) and Broadband (26%).

No notable differences in mealybug infestation were recorded between treatments with 8%, 6%, 5% and 3% mealybug infestation recorded for the control, Eco-Bb, Broadband and Real IPM respectively.

Trees treated with Real IPM had the lowest FCM infestation of 0.07% followed by Eco-Bb with 0.11% and Broadband and the control with 0.13% infestation each.

#### **Corrective application trial against red scale and mealybug**

No meaningful results were obtained against red scale, as none of the products caused any notable reduction in pest infestation at all (Table 3.4.2.5 and 3.4.2.6). No meaningful results were obtained from the microscopic evaluations done on the red scale infested fruit (Table 3.4.2.7).

**Table 3.4.2.5.** Percentage of fruit infested with red scale and percentage reduction in red scale (RS) infestation relative to the control.

	04/05/2018		22/05/2018		04/05/2018		22/05/2018	
	<11 scale/fruit (%)	Reduction (%)	<11 scale/fruit (%)	Reduction (%)	>10 scale/fruit (%)	Reduction (%)	>10 scale/fruit (%)	Reduction (%)
Untreated control	39	-	28.5	-	26.5	-	25.5	-

<b>Eco-Bb (10 g/100L)</b>	37.5	3.8	25.5	10.5	41.5	-56.6	35.5	-39.2
<b>RealMet69 (5 ml/100L)</b>	38	2.6	25.5	10.5	34.5	-30.2	29	-13.7
<b>Broadband (50 ml/100L)</b>	32	17.9	27	5.3	26	1.9	25	2.0

**Table 3.4.2.6.** Percentage fruit infested with mealybug and percentage reduction in mealybug (MB) infestation against the control.

	<b>04/05/2018</b>		<b>22/05/2018</b>	
	<b>Fruit infested (%)</b>	<b>Reduction (%)</b>	<b>Fruit infested (%)</b>	<b>Reduction (%)</b>
<b>Untreated control</b>	16	-	11	-
<b>Eco-Bb (10 g/100L)</b>	12	25.0	7.5	31.8
<b>RealMet69 (5 ml/100L)</b>	6.5	59.4	8.5	22.7
<b>Broadband (50 ml/100L)</b>	16	0.0	18.5	-68.2

**Table 3.4.2.7.** Percentage alive, dead and parasitized red scale for each treatment.

	<b>% alive</b>		<b>% dead</b>		<b>% parasitized</b>	
	<b>04/05/18</b>	<b>22/05/18</b>	<b>04/05/18</b>	<b>22/05/18</b>	<b>04/05/18</b>	<b>22/05/18</b>
<b>Untreated control</b>	38.5	17.5	49	65	12.5	13
<b>Eco-Bb (10 g/100L)</b>	40.5	23.5	46.5	53.5	13	18.5
<b>RealMet69 (5 ml/100L) + Breakthru (5 ml/100L)</b>	43	16	51.5	74.5	5.5	14.5
<b>Broadband (50 ml/100L) + Breakthru (5 ml/100L)</b>	34	14.5	58	73.5	8.5	15

### **Isaria fumosorosea**

#### *Mealybug trial at Bernol Farm, Sundays River Valley*

None of the treatments provided any reduction in infestation relative to the untreated control at 5, 7 and 11 days after application (Table 3.4.2.8). This is not surprising, as mealybug was not an intended target pest and there was never any assertion that Challenger® would be effective against mealybug. After 40 days, mealybug infestation was lowest in the 5X treatment, but this difference was not significant. Mealybug infestation had declined in all treatments due to the activity of parasitoids and predators.

**Table 3.4.2.8.** Citrus mealybug infestation at four evaluations, where Challenger® was applied at three different rates at Bernol Farm.

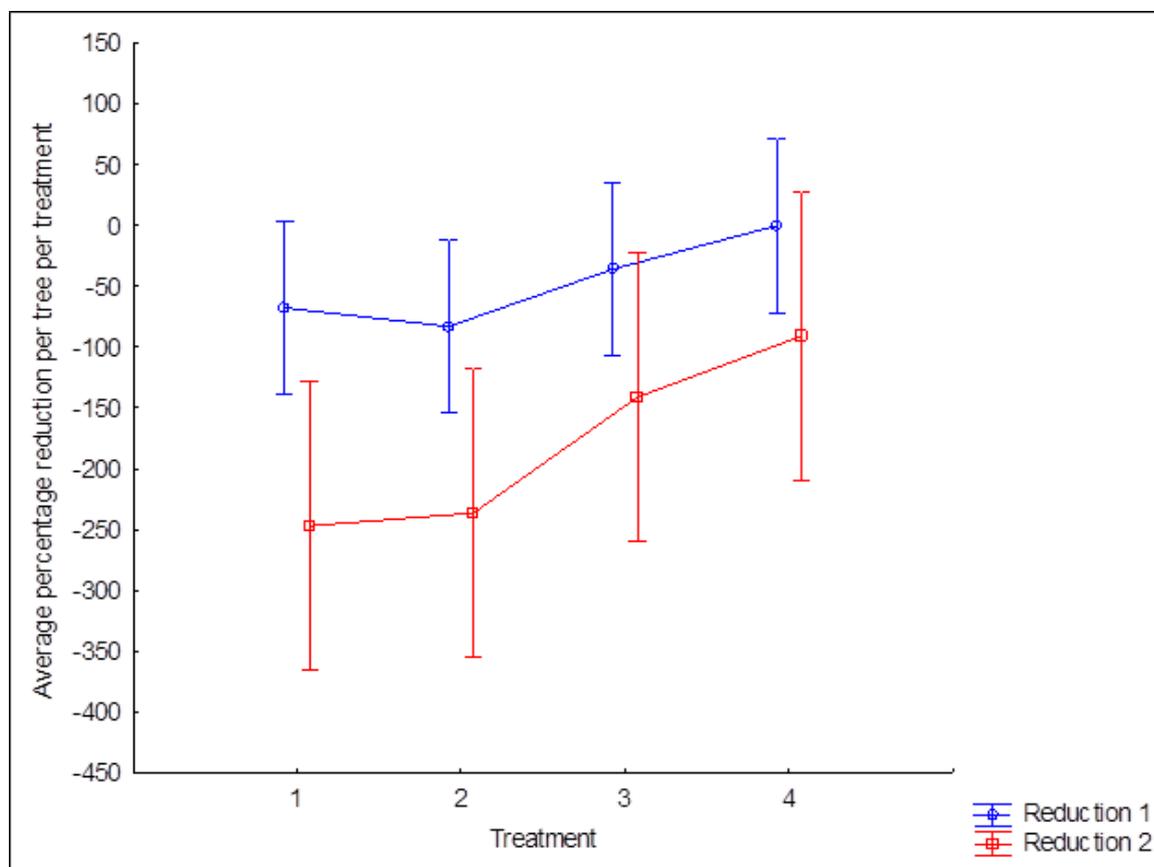
<b>Treatment</b>	<b>Infestation (%)</b>				
	<b>Pre-Spray</b>	<b>Days after spraying</b>			
		<b>5 Days</b>	<b>7 Days</b>	<b>11 Days</b>	<b>40 Days</b>
<b>Control</b>	100	100	100	100	55
<b>1 X</b>	99	100	100	99	52
<b>2 X</b>	100	99	100	100	57
<b>5 X</b>	100	100	99	100	46

*Aphid trial at Silverton Farm, Sundays River Valley*

Relative to the pre-infestation analysis, aphid infestation increased in all treatments. However, increase in infestation four days after spraying was 10.3%, 58.6% and 92.3% less for the three treatments, 1X, 2X and 5X, respectively, than for the untreated control (Table 3.4.2.9). At 9 days after application, only the 2X and 5X treatments still showed a reduced growth in infestation relative to the control, of 28.8% and 57.8%.

**Table 3.4.2.9.** Aphid infestation at two evaluations, where Challenger® was applied at three different rates at Silverton Farm.

Treatment	Days after spraying					
	4 days			9 days		
	Increase in infestation relative to pre-treatment (%)	Decrease in infestation relative to control (%)	Increase in infestation relative to pre-treatment (%)	Decrease in infestation relative to control (%)	Increase in infestation relative to pre-treatment (%)	Decrease in infestation relative to control (%)
<b>Control</b>	68.4		191.8			
<b>1X</b>	61.3	10.3	203.2	-5.9		
<b>2X</b>	28.3	58.6	136.7	28.8		
<b>5X</b>	5.1	92.6	81.0	57.8		



**Figure 3.4.2.1.** Mean “reduction” in aphid infestation per tree for all four treatments at 4 days relative to pre-spray (Reduction 1) and at 9 days relative to pre-spray (Reduction 2). (Wilks lambda = 0.81438, F(6, 70) = 1.2614, p = 0.28645).

A Dunnett's LSD test was used to determine which treatments significantly reduced the number of aphids compared to the control. For none of the three treatments was a decrease in infestation relative to the control significant (Table 3.4.2.10). This was clearly a result of the high degree of variance within treatments.

**Table 3.4.2.10.** Dunnett's LSD test to determine the significance in reduction of aphids compared to the control at Silverton Farm.

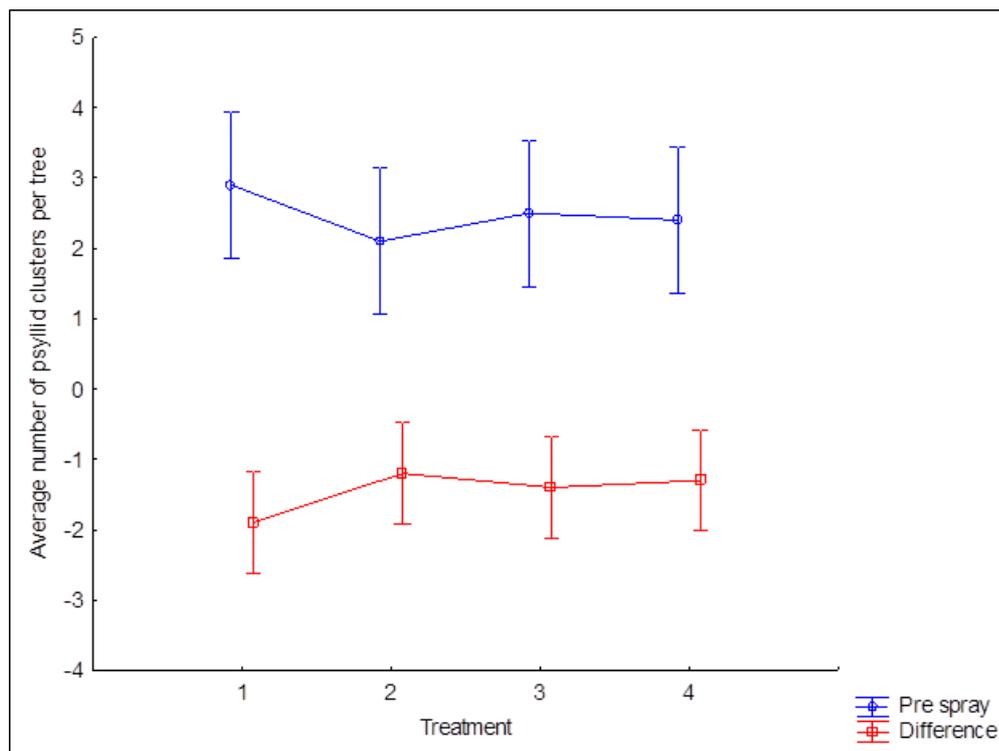
Treatment	4 Days After	9 Days After
Control	-	-
1 X	0.9801	0.9984
2 X	0.8584	0.4417
5 X	0.3975	0.1651

*Psylla trial at Thornhill Citrus, Swellendam*

One evaluation was conducted 7 days after application, at which time there was a decline in infestation for all treatments. However, there was no difference in infestation between any of the treatments and thus no indication that Challenger® had worked (Table 3.4.2.11). However, infestation was very patchy and we are thus not confident that these results are truly representative of the potential efficacy of Challenger®.

**Table 3.4.2.11.** Psylla infestation at one evaluation, where Challenger® was applied at three different rates at Thornhill Citrus.

Treatment	Decrease in infestation relative to pre-treatment (%)	Decrease in infestation relative to control (%)
Control	65.52	-
1 X	57.14	12.78
2 X	56.00	14.53
5 X	54.17	17.32



**Figure 3.4.2.2.** Average number of citrus psyllid clustered branches per tree for all four treatments at pre-spray and 14 days (Difference). (Wilks lambda = 0.93431,  $F(6, 70) = 0.40185$ ,  $p = 0.87541$ ).

A Dunnett's LSD test was used to determine which treatments significantly reduced the number of citrus psyllid compared to the control. No treatments significantly reduced the number of citrus psyllid clusters compared to the control treatment (Table 3.4.2.12).

**Table 3.4.2.12.** Dunnett's LSD test to determine the significance in reduction of citrus psyllid compared to the control at Buffelsjag Citrus

Treatment	Pre Spray	7 Days After
Control	-	-
1 X	0.55886	0.373252
2 X	0.902613	0.629068
5 X	0.831309	0.494588

## Conclusion

Results from the preventative spray trial showed that thrips and mealybug infestation was reduced by covering orchards with a net roof, while red scale infestation was higher. However, as these data contradict a subsequent more extensive study we conducted (Moore *et al.* 2020) and as there were no sides to the net covering in this case, these results must be interpreted cautiously. In contrast to what was expected, covering orchards with nets did not improve efficacy of EPF treatments. Two EPF products applied correctively against high levels of mealybug and red scale infestation reduced mealybug infestation by 31.8% and 22.7% but gave no control of red scale.

No reduction in mealybug infestation was recorded after application of *Isaria fumorosea* at three different rates. No notable efficacy was recorded against psylla; however, infestation was low and very patchy and this may well have compromised the reliability of the results. Efficacy against black citrus aphid was promising. Although aphid infestation increased rapidly after application, the level of increase in infestation at four days after application was 59% and 93% lower than the untreated control, for the two highest concentrations. A meaningful difference persisted for nine days, at least with the highest concentration.

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- Grout, T.G., Moore, S.D., Stephen, P.R. and Kirkman, W. 2016. Evaluation of entomopathogenic fungi and new chemicals against thrips and mealybug. In: CRI Annual Research Report.
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### 3.4.3 FINAL REPORT: Surveying for Oleander scale, *Aspidiotus nerii*

Project 1243 by Sean Moore, Tim Grout, Martin Gilbert, Aruna Manrakhian, Wayne Kirkman, Peter Stephen, John-Henry Daneel, Kim Stoltz, Mellissa Peyper, Elma Carstens, Vaughan Hattings, Tammy Marsberg (CRI)

## Summary

Oleander scale, *Aspidiotus nerii*, is listed as a pest on citrus, including in South Africa. However, on citrus in South Africa, *A. nerii* has only been collected on four occasions. Additionally, this infestation was recorded on branches, not on fruit. None of these detections were in commercial citrus orchards, all being from lemons in home gardens. Although this insect has a relatively cosmopolitan distribution, Philippines and Vietnam list *A. nerii* as a quarantine pest. Consequently, this listing serves as an impediment to the export of South African citrus to these countries. Both countries have stated a requirement for a scientific survey in South African citrus

orchards in order to demonstrate low phytosanitary risk and to remove this hurdle to continued exports to these countries. Consequently, surveys for *A. nerii* were conducted in several citrus orchards, including lemons, throughout South Africa, in which any other CRI IPM field trials were being conducted. These surveys were conducted for two seasons in a total of 61 orchards, 16 of which were monitored for two successive seasons. Ten lemon orchards were included. No infestation of *A. nerii* was recorded. These findings will be published in a peer-reviewed scientific journal.

## Opsomming

Oleander dopluis, *Aspidiotus nerii*, is as 'n plaag op sitrus gelys, insluitend in Suid-Afrika. Op sitrus in Suid-Afrika is *A. nerii* egter net op vier geleenthede versamel. Daarbenewens is hierdie besmetting net op takke aangeteken en nie op vrugte nie. Geen van hierdie onderskeppings is in kommersiële sitrusboorde nie; almal is van suurlemoen bome in huistuine. Alhoewel hierdie insek 'n relatief kosmopolitaanse verspreiding het, word dit deur die Filippyne en Viëtnam gelys as 'n kwarantyn plaag. Gevolglik dien hierdie notering as 'n struikelblok tot die uitvoer van Suid-Afrikaanse sitrus tot hierdie lande. Albei lande vereis 'n wetenskaplike opname in Suid-Afrikaanse sitrusboorde om te demonstreer dat daar 'n lae fitosanitêre risiko bestaan en om hierdie struikelblok tot uitvoere in die toekoms te verwyder. Gevolglik is opnames vir *A. nerii* in verskeie sitrusboorde, insluitend suurlemoene, deur Suid-Afrika gedoen, waar enige ander CRI IPM veldproewe gedoen is. Hierdie opnames is vir twee agtereenvolgende seisoene in 61 boorde gedoen, waar 16 boorde gedurende die twee seisoene ondersoek is. Tien suurlemoen boorde is ingesluit. Geen besmetting van *A. nerii* is egter opgelet nie. Hierdie bevindinge sal in 'n wetenskaplike joernaal gepubliseer word.

## Introduction

Oleander scale, *Aspidiotus nerii*, is listed as a pest on citrus, including in South Africa (Bedford, 1998; Grout and Moore, 2015). However, on citrus in South Africa, *A. nerii* has apparently only been collected on four occasions. Additionally, this infestation was recorded on branches, not on fruit (CPC, 2016). The first collection was in 1966 and the last collection was in 1997. None of these detections were in commercial citrus orchards, all being from lemons in home gardens (JH Giliomee, South Africa, correspondence to CABI, 2016). Lemon (*Citrus limon*) is reportedly the only citrus species that hosts *A. nerii* (Anonymous, 2004). Although this insect has a relatively cosmopolitan distribution, there are certain Asian countries to which South African citrus is exported, where *A. nerii* is not present, and which list *A. nerii* as a quarantine pest.

For example, in 2015 a revised draft PRA was received from the Philippines. Nineteen pests that cannot spread via the fruit pathway or are not recorded as pests of Citrus in South Africa, were listed as quarantine pests. Technical/scientific information was submitted to the Philippines in 2016 to reconsider the quarantine status of the 19 pests on the quarantine list. The information provided scientific evidence that these pests (including *Aspidiotus nerii*) are either not associated with the pathway (fresh citrus fruit) or are not recorded as pests of citrus in South Africa.

In the July 2016 feedback from the Philippines only nine of the 19 pests were removed from the quarantine list – *A. nerii* was not removed from the list. Further additional scientific evidence to remove these pests was provided to the Philippines. In the 2017 response, five of the 10 pests were removed. The other five, including *A. nerii*, were retained on the list, despite the fact that South Africa repeatedly provided the latest scientific/technical information as evidence that fresh citrus fruit from South Africa is not a pathway for spreading any of these listed pests. The Philippines however kept on referring to “old scientific evidence” (Bedford, 1998) for keeping these five pests on the list. Further information was provided to them in December 2017 to demonstrate that four of the five pests are not associated with citrus fruit and/or are not recorded pests of citrus and /or citrus in South Africa. Although the latest scientific information available indicated that one of the pests, *A. nerii*, has never been found on citrus fruit in commercial South African citrus orchards (last record of the pest in South Africa was in 1997 on branches of lemon trees in a home garden) (Grout and Moore, 2015; CPC, 2016), South Africa indicated that the listing of this pest as a quarantine pest with a low risk rating will be accepted, until such time as the results of a survey may provide further evidence for removal from the quarantine list. In their feedback in 2018, the Philippines accepted the listing of *A. nerii* as a low risk pest pending the result of the scientific survey to be conducted in commercial orchards.

Furthermore, the first draft PRA from Vietnam (received in 2014) erroneously listed four pests as being of quarantine importance, namely *Ceratitis quinaria*, *Aspidiotus nerii*, *Chrysomphalus pinnulifer* and *Pseudomonas syringae pv syringae*. They referenced the CABI Crop Protection Compendium (CPC) for justification of their decision to keep *C. quinaria* and *A. nerii* on the quarantine list. CRI and SA-DAFF agreed to engage with CABI to correct the errors in the CPC. CRI submitted scientific information in 2015 to the editor of the CPC to update the datasheets for *C. quinaria* and *A. nerii*. The updated datasheets for *A. nerii* and *C. quinaria* were received from the editor of the CPC and submitted to Vietnam in 2016. The updated datasheet on *A. nerii* stated that although citrus is a host of this pest, fresh citrus fruit from South Africa is not a pathway for this pest. The updated datasheet on *C. quinaria* stated that *Citrus* spp is not a host for this fruit fly. In feedback from Vietnam *C. quinaria* was removed from the list but not *A. nerii*. Further information to support the removal of *A. nerii* was submitted to Vietnam in 2016 and 2017 but they did not remove *A. nerii* from the quarantine list.

In 2018 feedback was submitted to Vietnam, who indicated that the listing of *A. nerii* as a quarantine pest with a low risk rating will be accepted, pending a scientific survey to provide further support that the pest is not present in commercial citrus orchards.

Consequently, such surveys had to be conducted. We therefore conducted two seasons of surveying for *A. nerii* in all citrus orchards in South Africa in which any other CRI research trials were being conducted. These surveys were conducted in as many orchards as possible and for the full duration of each trial. The intention was to ultimately collect sufficient information to demonstrate that in South Africa, commercially produced citrus fruit do not host *A. nerii*. The results of this study will be published in a peer-reviewed scientific journal and will thus hopefully be accepted by our export markets.

### Stated objective

- To conduct thorough surveys for *A. nerii* in many commercial citrus orchards in all citrus production regions of South Africa over two seasons.

### Materials and methods

Scouting for *A. nerii* was conducted in all orchards throughout South Africa in which any other research trials were being conducted within the IPM research portfolio. This included orchards in three provinces: Eastern Cape, Western Cape and Mpumalanga. This monitoring was done as regularly as possible (but not more regularly than once per week) for the full duration of each trial, by inspecting 10 fruit and supporting twigs on each of 10 trees, positioned diagonally or in a V-formation or a W-formation (depending on the orchard size (Grout, 2003)), distributed through the full extent of the orchard. Any scale insects found which resembled *A. nerii* or whose identity was uncertain, were collected for identification, either morphologically or molecularly (Marsberg *et al.*, 2012). Monitoring was conducted during two consecutive citrus growing seasons: 2019/20 and 2020/21.

### Results and discussion

Task table

Objective / Milestone	Achievement
A. Surveys of <i>A. nerii</i> in all citrus production areas where CRI trials are being conducted	Two full seasons completed – no <i>A. nerii</i> recorded.

In 2018/19 surveys were conducted in 27 orchards in the Eastern Cape (Table 3.4.3.1), four orchards in the Western Cape (Table 3.4.3.2) and 12 orchards in Mpumalanga (Table 3.4.3.3). In 2019/20 surveys were conducted in 17 orchards in the Eastern Cape (Table 3.4.3.4), five orchards in the Western Cape (Table 3.4.3.5) and 12 orchards in Mpumalanga (Table 3.4.3.6). In the Western Cape and Mpumalanga, the same

orchards were used for two consecutive seasons, whereas in the Eastern Cape, different orchards were used in each season.

In the Eastern Cape, each orchard was inspected between once to seven times. In the Western Cape, inspections were conducted weekly from January to July in the first season and up to September in the second season, thus up to 25 times each. In Mpumalanga, inspections were conducted monthly, making up eight inspections per orchard.

Included in the survey were 10 lemon orchards, five of which were monitored for two successive seasons. Lemon is the only citrus type previously recorded as being infested by *A. nerii* (Anonymous, 2004).

No oleander scale was recorded in any of the orchards during the two successive seasons.

## **Conclusion**

No oleander scale was recorded in any of the surveys conducted. This will hopefully provide adequate evidence to export markets that list oleander scale as a phytosanitary organism that commercially produced citrus in South Africa will not act as a pathway for the insect. The results of this study will be published in a peer-reviewed scientific journal.

**Table 3.4.3.1.** Details of orchards scouted for oleander scale infestation in the Eastern Cape during the 2018/19 season.

Farm	Orchard no.	Cultivar	GPS Coordinates	Inspection date (2019)						
				10 Jan	23 Jan	14 Mar	10 Apr	7 May		
Sur-le-Sun	A	Fukumoto Navel	33°26'9.13"S 25°29'18.70"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May		
	B	Palmer Navel	33°26'21.48"S 25°29'35.03"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May		
		Eureka Lemon	33°26'24.10"S 25°29'20.04"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May		
	22A	Newhall Navel	33°26'25.53"S 25°29'33.89"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May		
	22B	Newhall Navel	33°26'26.35"S 25°29'41.05"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May		
	23 A	Newhall Navel	33°26'30.94"S 25°29'31.77"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May	21 Aug	
	23 B	Newhall Navel	33°26'32.13"S 25°29'38.96"E	10 Jan	23 Jan	14 Mar	10 Apr	7 May	21 Aug	
Olifantsbos	1	Cambria Navel	33°37'35.16"S 25°41'02.17"E	14 Mar	23 Jan	14 Mar	10 Apr	7 May		
	2	Cambria Navel	33°37'35.67"S 25°41'01.81"E	10 Apr	23 Jan	14 Mar	10 Apr	7 May		
	3	Cambria Navel	33°37'39.70"S 25°41'09.65"E	10 Apr	23 Jan	14 Mar	10 Apr	7 May		
	18	Cambria Navel	33°37'25.77"S 25°21'37.17"E	10 Apr	23 Jan	14 Mar	10 Apr	7 May		
	22	Cambria Navel	33°37'31.48"S 25°41'22.65"E	14 Mar	23 Jan	14 Mar	10 Apr	7 May		
	24	Cambria Navel	33°37'33.15"S 25°41'17.74"E	7 May	23 Jan	14 Mar	10 Apr	7 May		
Mistkraal	46	Eureka Lemon	33°26'22.20"S 25°30'23.91"E	17 Jan						
	47	Washington Navel	33°26'19.43"S 25°30'20.33"E	17 Jan						

	50	Eureka Lemon	33°26'18.17"S 25°30'25.49"E	24 Jan						
	54		33°26'11.37"S 25°30'19.45"E	24 Jan						
	56	Palmer Navel	33°26'16.65"S 25°30'13.20"E	3 Jan	10 Jan	17 Jan	24 Jan	30 Jan		
	57	Palmer Navel	33°26'18.55"S 25°30'14.47"E	17 Jan 2019						
	58	Eureka Lemon	33°26'20.40"S 25°30'12.30"E	17 Jan 2019						
Bernol	35	M7	33°28'26.29"S 25°36'31.54"E	25 Feb 2019						
	36	Valley Gold	33°28'28.28"S 25°36'33.14"E	14 Feb 2019						
	37	Delta Valencia	33°28'32.39"S 25°36'38.78"E	14 Feb 2019	19 Feb	21 Feb	25 Feb	26 Mar	3 Apr	25 Apr
Douglasdale	47	Washington Navel	33°26'21.13"S 25°31'45.79"E	10 Jan 2019	23 Jan	14 Mar	10 Apr	7 May		
	52	Washington Navel	33°26'24.84"S 25°31'40.34"E	10 Jan 2019	23 Jan	14 Mar 2019	10 Apr	7 May		
	54	Palmer Navel	33°26'22.94"S 25°31'38.57"E	10 Jan 2019	23 Jan	14 Mar	10 Apr	7 May		
Elim	54	Valencia	33°29'12.79"S 25°39'55.77"E	21 Aug 2019						

**Table 3.4.3.2.** Details of orchards scouted for oleander scale infestation in the Western Cape during the 2018/19 season.

Farm	Orchard no.	Cultivar	GPS Coordinates	Inspection date (2019)
Das Bosch	5B	Eureka lemon	33°08'08.23"S 01°21.07"E	Weekly, 30 Jan - 25 Jul
	7B	Eureka lemon	33°08'03.01"S 19°02'01.06"E	Weekly, 30 Jan - 18 Jun

Denau	3	Nule Clementine	33°32'12.70"S 19°32'05.99"E	Weekly, 30 Jan - 25 Jul
	5	Nule Clementine	33°32'27.04"S 19°32'01.71"E	Weekly, 30 Jan - 25 Jul

**Table 3.4.3.3.** Details of orchards scouted for oleander scale infestation in Mpumalanga Province during the 2018/19 season.

Farm	Orchard no	Cultivar	GPS Coordinates	Inspection date							
				15 Jan	15 Feb	13 Mar	11 Apr	14 May	12 Jun	23 Jul	23 Aug
Siyalima	6	Eureka lemon	25°41'10.8"S 31°11'22.5"E	15 Jan	15 Feb	13 Mar	11 Apr	14 May	12 Jun	23 Jul	23 Aug
	4	Limoneira lemon	25°41'30.2"S 31°11'16.7"E	16 Jan	16 Feb	14 Mar	12 Apr	15 May	13 Jun	24 Jul	24 Aug
	2	Bahianina Navel	25°40'44.8"S 31°10'44.4"E	17 Jan	17 Feb	15 Mar	13 Apr	16 May	14 Jun	25 Jul	25 Aug
	5	Newhall Navel	25°41'22.2"S 31°11'27.8"E	18 Jan	18 Feb	16 Mar	14 Apr	17 May	15 Jun	26 Jul	26 Aug
	1	Turkey Valencia	25°40'04.3"S 31°10'48.2"E	19 Jan	19 Feb	17 Mar	15 Apr	18 May	16 Jun	27 Jul	27 Aug
	3	Midnight Valencia	25°41'09.1"S 31°11'25.6"E	20 Jan	20 Feb	18 Mar	16 Apr	19 May	17 Jun	28 Jul	28 Aug
Crocodile Valley	3	Witkrans Navel	25°27'32.9"S 31°01'40.7"E	18 Jan	13 Feb	13 Mar	8 Apr	15 May	11 Jun	23 Jul	22 Aug
	4	Witkrans Navel	25°27'41.1"S 31°05'54.4"E	19 Jan	14 Feb	14 Mar	9 Apr	16 May	12 Jun	24 Jul	23 Aug
	2	Palmer Navel	25°28'02.2"S 31°01'04.8"E	20 Jan	15 Feb	15 Mar	10 Apr	17 May	13 Jun	25 Jul	24 Aug
	1	C100 Valencia	25°28'21.1"S 31°00'33.7"E	21 Jan	16 Feb	16 Mar	11 Apr	18 May	14 Jun	26 Jul	25 Aug
	6	Delta Valencia	25°28'00.8"S 31°02'55.9"E	22 Jan	17 Feb	17 Mar	12 Apr	19 May	15 Jun	27 Jul	26 Aug
	5	Late Valencia	25°27'51.1"S 31°02'31.4"E	23 Jan	18 Feb	18 Mar	13 Apr	20 May	16 Jun	28 Jul	27 Aug

**Table 3.4.3.4.** Details of orchards scouted for oleander scale infestation in the Eastern Cape during the 2019/20 season.

Farm	Orchard no.	Cultivar	GPS Coordinates	Inspection date					
Huguenot	23	Palmer Navel	33°36'24.35"S 25°40'05.34"E	12 Sep 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
	38	Delta Valencia	33°40'5.34"S 25°39'39.17"E	12 Sep 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
	39	Delta Valencia	33°36'49.58"S 25°39'38.79"E	12 Sep 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
Olifantskop	12	Delta Valencia	33°37'17.77"S 25°40'31.47"E	10 Oct 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
	13	Palmer Navel	33°37'21.30"S 25°40'28.78"E	10 Oct 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
	18	Delta Valencia	33°37'22.11"S 25°40'39.25"E	10 Oct 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
Hippo Pools	11	Palmer Navel	33°24'34.60"S 25°24'36.12"E	24 Oct 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
	14	Palmer Navel	33°24'37.71"S 25°24'29.29"E	24 Oct 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
Westhaven	38	Palmer Navel	33°24'17.45"S 25°24'41.13"E	14 Nov 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
	43	Palmer Navel	33°24'15.43"S 25°24'58.35"E	14 Nov 2019	15 Jan 2020	29 Jan 2020	12 Feb 2020	26 Feb 2020	
Allandale	27	Autumn Gold Navel	33°26'33.56"S 25°32'00.27"E	16 Jan 2020	23 Jan 2020	30 Jan 2020	13 Feb 2020	26 Feb 2020	12 Mar 2020
Boerboon	15B	Lane Late Navel	33°27'48.98"S 25°34'08.09"E	20 Feb 2020	27 Feb 2020	6 Mar 2020	20 Mar 2020	3 Apr 2020	17 Apr 2020
Cormic	10	M7	33°45'24.55"S 24°49'20.24"E	3 Mar 2020	17 Mar 2020				
Dunbrody	2A	Eureka Lemon	33°27'59.74"S 25°33'46.48"E	19 Mar 2020					
	3A	Eureka Lemon	33°28'02.87"S 25°33'43.32"E	2 Apr 2020					

Pennyholme	9	Witkrans Navel	33°29'50.81"S 25°40'25.37"E	7 May 2020	14 May 2020	21 May 2020	4 June 2020		
Skinner	35	Midnight Valencia	33°25'02.43"S 25°27'18.70"E	1 July 2020	8 July 2020	15 July 2020	29 Jul 2020		

**Table 3.4.3.5.** Details of orchards scouted for oleander scale infestation in the Western Cape during the 2018/19 season.

Farm	Orchard no.	Cultivar	GPS Coordinates	Inspection date
Thornlands	TAB 2	Tango Mandarin	34°01'56.42"S 20°31'49.90"E	25 Sep 2019
Das Bosch	5B	Eureka lemon	33°08'08.23"S 19°01'21.07"E	Weekly, 17 Dec 2019 - 21 Sep 2020
	7B	Eureka lemon	33°08'03.01"S 19°02'01.06"E	Weekly, 17 Dec 2019 - 21 Sep 2020
Denau	3	Nule Clementines	33°32'12.70"S 19°32'05.99"E	Weekly, 16 Dec 2019 - 3 June 2020
	5	Nule Clementines	33°32'27.04"S 19°32'01.71"E	Weekly, 16 Dec 2019 - 3 June 2020

**Table 3.4.3.6.** Details of orchards scouted for oleander scale infestation in Mpumalanga Province during the 2018/19 season.

Farm	Orchard no	Cultivar	GPS Coordinates	Inspection date (2020)							
				15 Jan	15 Feb	13 Mar	11 Apr	14 May	12 Jun	23 Jul	23 Aug
Siyalima	6	Eureka lemon	25°41'10.8"S 31°11'22.5"E	15 Jan	15 Feb	13 Mar	11 Apr	14 May	12 Jun	23 Jul	23 Aug
	4	Limoneira lemon	25°41'30.2"S 31°11'16.7"E	16 Jan	16 Feb	14 Mar	12 Apr	15 May	13 Jun	24 Jul	24 Aug
	2	Bahianina Navel	25°40'44.8"S 31°10'44.4"E	17 Jan	17 Feb	15 Mar	13 Apr	16 May	14 Jun	25 Jul	25 Aug
	5	Newhall Navel	25°41'22.2"S 31°11'27.8"E	18 Jan	18 Feb	16 Mar	14 Apr	17 May	15 Jun	26 Jul	26 Aug
	1	Turkey Valencia	25°40'04.3"S 31°10'48.2"E	19 Jan	19 Feb	17 Mar	15 Apr	18 May	16 Jun	27 Jul	27 Aug
	3	Midnight Valencia	25°41'09.1"S 31°11'25.6"E	20 Jan	20 Feb	18 Mar	16 Apr	19 May	17 Jun	28 Jul	28 Aug

Crocodile Valley	3	Witkrans Navel	25°27'32.9"S 31°01'40.7"E	18 Jan	13 Feb	13 Mar	8 Apr 2020	15 May	11 Jun	23 Jul	22 Aug
	4	Witkrans Navel	25°27'41.1"S 31°05'54.4"E	19 Jan	14 Feb	14 Mar	9 Apr 2020	16 May	12 Jun	24 Jul	23 Aug
	2	Palmer Navel	25°28'02.2"S 31°01'04.8"E	20 Jan	15 Feb	15 Mar	10 Apr	17 May	13 Jun	25 Jul	24 Aug
	1	C100 Valencia	25°28'21.1"S 31°00'33.7"E	21 Jan	16 Feb	16 Mar	11 Apr	18 May	14 Jun	26 Jul	25 Aug
	6	Delta Valencia	25°28'00.8"S 31°02'55.9"E	22 Jan	17 Feb	17 Mar	12 Apr	19 May	15 Jun	27 Jul	26 Aug
	5	Late Valencia	25°27'51.1"S 31°02'31.4"E	23 Jan	18 Feb	18 Mar	13 Apr	20 May	16 Jun	28 Jul	27 Aug 20 20

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- 3.4.4 **FINAL REPORT: Synergism and formulation of entomopathogenic fungi for foliar control of various citrus pests**  
**REVISED TITLE: Potential Synergism between Entomopathogenic Fungi and Entomopathogenic Nematodes for the control of false codling moth (*Thaumatotibia leucotreta*)**  
Project 1188 (February 2019 – December 2020) by Samantha Prinsloo (RU – MSc), Candice Coombes (RU – Entomology), Martin Hill (RU - Entomology), Sean Moore (CRI)

## Summary

False codling moth (FCM), *Thaumatotibia leucotreta*, is a major phytosanitary pest of citrus in South Africa. Sufficient control measures for the soil-dwelling life stages of FCM have yet to be identified and owing to restrictions on the use of insecticides, non-chemical control options have been investigated including the use of entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN). This study conducted laboratory bioassays to assess the virulence of these four control agents on fifth instar FCM, in 24-well plates. These results reaffirmed the virulence of the four microbial control agents at their recommended doses of 50 IJs (EPN) and  $1 \times 10^7$  conidia/ml (EPF) against fifth instar FCM with 80 to 96% larval mortality recorded. The EPF isolate exhibited the lowest mortality whilst *S. yirgalemense* induced the greatest mortality. The lethal concentration (LC) values for each isolate were also determined using dose response bioassays. The LC<sub>50</sub> results in order from lowest to highest EPN IJ concentration requirements were 4.38 IJs (*S. yirgalemense*), 4.47 IJs (*S. jeffreyense*) and 7.11 IJs (*H. noenieputensis*). The EPF isolate exhibited an LC<sub>50</sub> of  $3.42 \times 10^5$  conidia/ml. Lastly, research has shown that the combination of two control agents may increase control of late instar lepidopteran and coleopteran larvae, through synergistic interactions. This study found that when all three EPN species were combined simultaneously and sequentially with the EPF isolate *M. anisopliae* FCM AR 23 B3, additive interactions took place, with exception of the simultaneous application of *S. yirgalemense* and *H. noenieputensis* with the EPF and *S. jeffreyense* applied 24 h post EPF application. For the former, a synergistic interaction was found, whilst for the latter two, an antagonistic interaction was recorded. Although no strongly synergistic interactions were observed, additive interactions have been shown to reach a synergistic level when certain parameters are changed. Thus, it is recommended that due to the additive

interactions observed in this study, laboratory soil-bioassays and field trials should be carried out for all three EPN species in combination with the EPF isolate. This research will inevitably facilitate the constant knowledge into management strategies for the phytosanitary pest, FCM in South African citrus.

## Opsomming

Valskodlingmot (VKM), *Thaumatotibia leucotreta*, is 'n belangrike fitosanitêre plaag van sitrus in Suid-Afrika. Voldoende beheermaatreëls vir die lewensfases van FCM in die grond moet nog geïdentifiseer word en weens beperkings op die gebruik van insekdoders is nie-chemiese bestrydingsopsies ondersoek, insluitend die gebruik van entomopatogene swamme (EPS) en entomopatogene aalwurms (EPN). Hierdie studie het laboratorium-bio-toetse uitgevoer om die virulensie van hierdie vier beheermiddels op die vyfde instansie VKM in 24-put plate te bepaal. Hierdie resultate bevestig die virulensie van die vier mikrobiële beheermiddels teen hul aanbevole dosisse van 50 IJ's (EPN) en  $1 \times 10^7$  conidia / ml (EPS) teen die vyfde stadium FCM met 80 tot 96% larwes mortaliteit aangeteken. Die EPF-isolaat vertoon die laagste mortaliteit, terwyl *S. yirgalemense* die grootste sterftesyfer het. Daarbenewens is die dodelike konsentrasie (LC) waardes vir elke isolaat bepaal met behulp van dosisrespons bioassays. Die LC<sub>50</sub>-resultate in die volgorde van die laagste tot die hoogste EPN IJ-konsentrasievereistes was 4.38 IJ's (*S. yirgalemense*), 4.47 IJs (*S. jeffreyense*) en 7.11 IJ's (*H. noenieputensis*). Die EPF-isolaat vertoon 'n LC<sub>50</sub> van  $3,42 \times 10^5$  konidia/ml. Laastens het navorsing getoon dat die kombinasie van twee beheermiddels die beheer van laat-instar lepidopteran- en coleopteranlarwes kan verhoog deur sinergistiese interaksies. Hierdie studie het bevind dat toe al drie EPN-spesies gelyktydig en opeenvolgend met die EPS-isolaat *M. anisopliae* FCM AR 23 B3 gekombineer is, additiewe interaksies plaasgevind het, met uitsondering van die gelyktydige toediening van *S. yirgalemense* en *H. noenieputensis*, met die EPS en *S. jeffreyense* het 24 uur na die EPS-aansoek aansoek gedoen. Vir eersgenoemde is 'n sinergistiese interaksie gevind, terwyl vir laasgenoemde 'n antagonistiese interaksie was gevind. Alhoewel geen sterk sinergistiese interaksies waargeneem is nie, is daar getoon dat additiewe interaksies 'n sinergistiese vlak bereik wanneer sekere parameters verander word. Vanweë die additiewe interaksies wat in hierdie studie waargeneem word, word aanbeveel dat laboratoriumgrond-bio-toetse en veldproewe vir al drie EPN-spesies in kombinasie met die EPS-isolaat uitgevoer moet word. Hierdie navorsing sal onvermydelik die voortdurende kennis van bestuurstrategieë vir die fitosanitêre plaag, VKM in Suid-Afrikaanse sitrus, vergemaklik.

## Introduction

South Africa is the 14th largest citrus producer in the world and the 2nd largest citrus exporter worldwide (CGA 2018a). The citrus industry in South Africa currently has close to 100 insect pests with ten different insect species that are classified as economically important pests (Smith & Peña 2002; Grout & Moore 2015; De Meyer *et al.* 2016; Manrakhan *et al.* 2018). *Thaumatotibia leucotreta* Meyrick (1912) (Lepidoptera: Tortricidae) (FCM), is the most important pest in South Africa due to its phytosanitary status (Venette *et al.* 2003; Moore 2021). In addition to the phytosanitary status of this pest, the pre- and post-harvest damage caused by FCM results in financial losses to farmers and the industry at large, with an (outdated) estimated ZAR 100 million lost as a result of FCM infestations (Kirkman & Moore 2007).

From oviposition to the fourth instar, the FCM life cycle takes place in the foliar region of citrus trees, within the fruit (Daiber 1979a, b, 1980; Newton 1998, Love *et al.* 2014). The fifth and final instar will leave the fruit once they near pupation and drop into the soil below the host tree, which initiates the subterranean FCM life stage (Daiber 1979b). Current control strategies primarily target the foliar life stages using a combination of IPM strategies, which include biological control using insect pathogens. However, the soil dwelling life stages still lack sufficient control methods.

Microbial control agents (MCA) can be used to fill this gap in FCM control programmes because certain agents also originate in the soil. These MCA are entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN). Currently only three EPF products are registered in South Africa, BroadBand™ (BASF, South Africa), Eco-Bb® (Plant Health Products, South Africa), both incorporating *Beauveria bassiana* strains as their active ingredient, and Real Metarhizium 69 (Real IPM, Nairobi, Kenya), produced from a *M. anisopliae* strain (Goble *et al.* 2011; Coombes *et al.* 2013). However, Goble *et al.* (2010, 2011) identified three promising fungal isolates

for the control of FCM in South Africa, *M. anisopliae* sensu lato Metchnikoff (Sorokin) G 11 3 L6, *M. anisopliae* sensu stricto (s. str) FCM Ar 23 B3 and *B. bassiana* s. str Balsamo (Vuillemin) G Ar 17 B3 which required further testing (Coombes *et al.* 2013). The three isolates were found to successfully induce mortality in soil-dwelling life stages, wandering fifth instar larvae (Coombes *et al.* 2013, 2016).

South African EPN species are understudied with minimal surveys conducted, and it remains the same for the rest of Africa (Malan *et al.* 2006; Hatting *et al.* 2019). In South Africa, a total of eight *Steinernema* species and four *Heterorhabditis* species have been recorded (Malan & Moore 2016). Despite these discoveries only one commercially available EPN product was available in South Africa, Cryptonem™ (River Bioscience, South Africa) which comprised *Heterorhabditis bacteriophora* Poinar (Malan *et al.* 2006). However, its registration had been suspended due to the exotic strain used (produced in Germany (e-nema) (S.D. Moore, pers. comm.)). More recently, both Cryptonem™ and a new *S. feltiae*-based product have been approved for registration, but are not yet commercially available (Moore 2021). Six of the native EPN species have been found to be virulent against FCM larvae/pupae (*S. khoisanae*, *S. citrae*, *S. yirgalemense*, *S. jeffreyense*, *H. zealandica*, *H. bacteriophora* and *H. noenieputensis*) and adults (*S. yirgalemense* and *S. khoisanae*) (Malan *et al.* 2011, 2016; Steyn *et al.* 2019a, b). This research focused on three of these EPN species: *S. yirgalemense*, *S. jeffreyense* and *H. noenieputensis*, as these species have been considered as the most promising candidates against FCM (Malan *et al.* 2011; Steyn *et al.* 2017; Malan & Moore 2016; Manrakhan *et al.* 2013; Steyn *et al.* 2019a, b).

Synergism between predators can occur and can be manipulated to improve IPM programmes for pest population control. The term 'synergism' is used when the combined effect of multiple organisms is greater than the sum of the single organisms (Piggott *et al.* 2015). These interactions require a 'stressor', which causes the sum of the combined interaction effects to be greater than the individual effects. A stressor in this context could be products released by the primary infector, which weakens or compromises the host's immune system, allowing for a rapid secondary infection to occur because the host is now more susceptible, (Piggott *et al.* 2015; Wakil *et al.* 2017). Hence, synergistic interactions can increase the control efficacy of IPM programmes. Other benefits of using combinations of control agents are that a variety of modes of action can be used to combat the insect host and its defence/immune system (Devi 2019). In addition, multiple life stages could be targeted at a time when combined control agents are released (Devi 2019).

IPM programmes involve the combination of multiple control strategies to target pest populations. Understanding the interactions between multiple control products is key to improving and efficiently implementing IPM strategies. If interactions between two control agents can be more effective (synergistic) than the products alone, an increase in pest population control can be achieved. The interactions between combinations of different EPN and EPF have been investigated and potential synergistic relationships have been found in both greenhouse and field trials (Ansari *et al.* 2004, Choo *et al.* 2002; Gaugler *et al.* 1994; McCoy *et al.* 1988; Mehdi *et al.* 2018, Vey *et al.* 2001; Wang *et al.* 1995).

## **Stated objectives**

- A – Determine the ability of adjuvants to improve the ability of EPF to infect various arboreal citrus pests.
- B – Evaluate possible synergism of CrleGV, EPN and chemical and botanical pesticides when applied with EPF
- C – The use of adjuvants to improve foliar control of citrus pests
- D – Synergism between EPF and other control agents

## **Materials and methods**

### Insect cultures

Fifth instar FCM were obtained from a laboratory-reared colony established at Rhodes University. Larvae were reared on artificial diet according to Moore *et al.* (2014), using 350 ml glass jars plugged with cotton wool, in which pupation occurs. The upward movement of larvae into the cotton wool is indicative of fifth instars.

### EPN cultures

*Steinernema yirgalemense* 157-C, GenBank accession number EU625295 (Malan *et al.* 2011) and *S. jeffreyense* J194, GenBank accession number KC897093 (Stokwe 2016) IJs were obtained from in vitro cultures maintained at Stellenbosch University. *Heterorhabditis noenieputensis* 158-C, GenBank accession number JN620538 (Stokwe 2016), IJs were also obtained from Stellenbosch University, but were cultured in vivo, using wax moth larvae (*Galleria mellonella*). Fresh IJ cultures of all three EPN species were maintained by in vivo culturing using fifth instar FCM larvae obtained from Rhodes University. The EPN were passed through these insects every two weeks to ensure viability or, if cultures were older than two weeks, fresh nematodes were ordered from Stellenbosch University. Infected larvae were kept at room temperature in modified White traps for collection of fresh IJs. The harvested IJs were then placed in sterile vented culture flasks and stored at room temperature. The flasks were agitated weekly to aerate the IJs (A.P. Malan, pers. comm.).

The desired concentration was then calculated using the formula (A.P. Malan, pers. comm.):

$$\left(\frac{x}{y} - 1\right) \times V = z$$

Where:

x = average number of IJs counted

y = desired number of IJs

z = necessary adjustments to original stock (removal or addition of sterile dH<sub>2</sub>O)

V = stock volume

#### EPF cultures

The PPRI Fungal Accession number for *M. anisopliae* FCM Ar 23 B3 is PPRI 9561, the Rhodes code is FCM Ar 23 B3 (Goble *et al.* 2011) and the GenBank accession number is KF83418 (Chartier Fitzgerald *et al.* 2016). The isolate was originally obtained from Arundel (33°30'57" S; 25°39'11" E), from loamy soil within a cultivated citrus orchard in April 2008 (Goble *et al.* 2011). *Metarhizium anisopliae* FCM Ar 23 B3 was obtained from laboratory cultures stored at Rhodes University. The fungal isolate was cultured on Sabouraud Dextrose Agar (SDA) supplemented with 50 mg/L chloramphenicol in a controlled environment (CE) room at 26 ± 1°C on a 12 h photoperiod. For all experiments, 14 day old plate cultures were used. The isolate was passaged through fifth instar FCM at the beginning of a new experiment/replicate to maintain fungal viability and virulence. Plates that had been sub-cultured three times were discarded. Fungal suspensions were prepared with 0.01% Tween® 20 by gently scraping the surface of growth petri dishes to release the conidia and the concentration determined using a Helber bacteria counting chamber with Thoma ruling as viewed under a light microscope (400X),

#### Efficacy of EPN mortality alone on FCM fifth instars

The three EPN species have been shown to be virulent against FCM. However, a single study that incorporates all three species using fifth instar FCM larvae has yet to be completed. Therefore, this study aimed at identifying the virulence of the three EPN species through bioassays in 24-well plates using a uniform concentration rate of 50 IJs/larvae for initial assessment and 50 IJs, 25 IJs, 12.5 IJs, 6.25 IJs and 3.125 IJs, for the subsequent dose-response bioassays. The dose response bioassays were necessary to determine further variations between the three EPN species' virulence against fifth instar FCM, with the LC<sub>50</sub>, LC<sub>70</sub>, and LC<sub>90</sub> values needed for use in the EPN and EPF interaction experiments. Both the individual EPN species and the dose response bioassays follow the same experimental design, barring the differences in IJ concentrations (specifically for the dose response bioassays). Sterile filter paper discs (12.7 mm) were placed in every alternate well to which 50 µl of the recommended dose was pipetted onto each disc. Fifth instar FCM were then added to each treated well. A glass lid was placed over the wells to prevent larval movement between wells. Five plates were used per EPN treatment (dose), with a total sample size of 60 larvae per treatment (dose). Each treatment was then grouped together with their respective five plates, using elastic bands, which were then placed into 2 L plastic containers lined with moist tissue paper and placed in a CE room at 26 ± 1°C on a 12 h photoperiod for 48 h. A control treatment was included for both the individual and dose response bioassays whereby 50 µl of distilled water was applied to the wells. Thereafter, the same procedure as described above was followed.

After 48 h the nematode treatments (including the control) were individually assessed and the dead or living larvae were separated and surface-sterilised by washing the larvae in a tea sieve with distilled water over a glass beaker. The dead larvae were placed onto moist filter paper inside petri dishes for a further 48 h; the same was done for the living larvae. The additional 48 h (96 h after inoculation) was included for two reasons. Firstly, surviving larvae may be infected with EPN towards the end of the initial 48 h period, and an additional 48 h is required to allow for death as a result of EPN infection (A.P. Malan, pers. comm.). Secondly, the additional 48 h gives the EPN time to replicate within the host and allows the larva to become heavily infected, ensuring that the cause of death as a result of EPN infection, is accurately recorded (A.P. Malan, pers. comm.). Once the 96 h had passed, the dead larvae were first examined visually for a bacteria-induced colour change (Griffin 2012). EPN infection was then confirmed by assessing the presence or absence of EPN within the larval cavity. An individual larva was placed in a petri dish under a dissection microscope (4 – 8X magnification) and, using fine forceps, the larva was pulled apart and the gut contents squeezed out. Distilled water was added to submerge the gut contents in liquid, which was then examined for the presence or absence of nematodes and recorded. The percentage mortality for each EPN species was then calculated.

Any larvae that had not succumbed to EPN infection 96 h post-treatment, were placed into glass vials that were plugged with cotton wool and left to pupate. Ten days after the first moth eclosed, the experiment ended. The experiment was replicated three times for the individual dose and dose response bioassays.

#### Efficacy of EPF mortality alone on FCM fifth instars

Although the LC<sub>50</sub> of this isolate has been established (Coombes *et al.* 2015), the bioassay procedure is different to that needed for conducting combined EPN and EPF experiments. Furthermore, individual bioassays using a recommended application rate ( $1 \times 10^7$  conidia/ml) and dose response bioassays needed to be conducted for comparative purposes between the individual agents and to obtain the LC<sub>50</sub>, LC<sub>70</sub> and LC<sub>90</sub> values for the combined bioassays. For the dose response bioassays, a three-fold serial dilution was used. This used seven doses:  $1.4 \times 10^4$ ,  $4.1 \times 10^4$ ,  $1.23 \times 10^5$ ,  $3.7 \times 10^5$ ,  $1.11 \times 10^6$ ,  $3.33 \times 10^6$  and  $1 \times 10^7$  conidia/ml (Coombes *et al.* 2015). The experimental protocol was the same for the individual dose EPF isolate and dose-response bioassays.

Using 24-well bioassay plates, sterile filter paper discs (12.7 mm diameter) were placed in every alternate well. Each fifth instar FCM larva was dipped into the fungal suspension and a single larva was placed into a well. A control was included whereby larvae were dipped into 0.01% Tween® 20 solution only. The wells were covered with a piece of glass and the plates were sealed with their corresponding plastic lids. Each treatment contained five bioassay plates, with a total of 60 larvae per treatment. The bioassay plates were grouped per treatment using an elastic band and placed into a 2 L plastic container, which was lined with moist paper towel. The plastic containers were placed in a CE room at  $26 \pm 1^\circ\text{C}$  and in the dark for 7 days.

On day 7, the larvae were examined and were separated into dead or alive. The dead larvae were surface sterilised using 70% ethanol (Coombes *et al.* 2013), placed onto moist filter paper in petri dishes and left to sporulate in a CE room to confirm fungal death. The surviving larvae were placed individually into glass vials that were plugged with cotton wool. The larvae were left to pupate, and the experiment was terminated 10 days after first eclosion was noted. The experiment was replicated three times for both the individual and dose response bioassays.

#### EPF and EPN interactions

Combined EPF and EPN bioassays were conducted to determine the type of interaction that occurs between the EPF *Metarhizium anisopliae* FCM Ar 23 B3 and the three EPN species *Steinernema yirgalemense*, *Steinernema jeffreyense* and *Heterorhabditis noenieputensis*. Based on the findings from the pilot study it was determined that a lower concentration was required to accurately determine the type of interaction. Hence, the dose response bioassays provided these lower concentrations in the form of the LC<sub>50</sub> and LC<sub>70</sub> for the EPN and EPF, respectively. It was also decided that all time intervals tested in the pilot study would be used with the lower concentrations, because they might play a role in influencing the results.

The experimental protocol followed the pilot study outlined above, with the only differences being the concentrations used. In this case, the EPF LC<sub>70</sub> value was used,  $1.4 \times 10^6$  conidia/ml, and the LC<sub>50</sub> values for

all three EPN species were used: *Steinernema yirgalemense*, (4.38 IJs/larva) *S. jeffreyense* (4.47 IJs/larva) and *Heterorhabditis noenieputensis* (7.11 IJs/larva). The controls were the same as described in the pilot study as well as the number of plates and larvae per time interval. The assessment of the larvae once the experiments were completed were also assessed as described above.

## Results and discussion

Objective / Milestone	Achievement
A. Determine the ability of adjuvants to improve the ability of EPF to infect various arboreal citrus pests	Not achieved. Although this project originally outlined several objectives, the scope needed to be reduced to accommodate the two years required for an MSc degree. Therefore, the foliar control of citrus pests' portion of this project was not included.
B. Evaluate possible synergism of CrleGV, EPN and chemical and botanical pesticides when applied with EPF	Partially achieved. This study focused on the interaction between three indigenous EPN strains and one indigenous EPF strain, all of which had previously shown a high level of promise for use against FCM fifth instar larvae.
C. The use of adjuvants to improve foliar control of citrus pests	Not achieved. Prior to the Covid lockdown a proposed methodology for investigating the compatibility of adjuvants and the EPF isolate had been established but was terminated due to lockdown laboratory restrictions and time constraints. Research thus prioritised determining interaction effects between indigenous EPN and EPF strains.
D. Synergism between EPF and other control agents	Achieved in the context of EPN/EPF interactions

This research has been presented as an MSc thesis and any further results or in-depth discussion therefore may be found in this thesis. The key results and findings of this research are however, presented below.

### Objectives: B and D

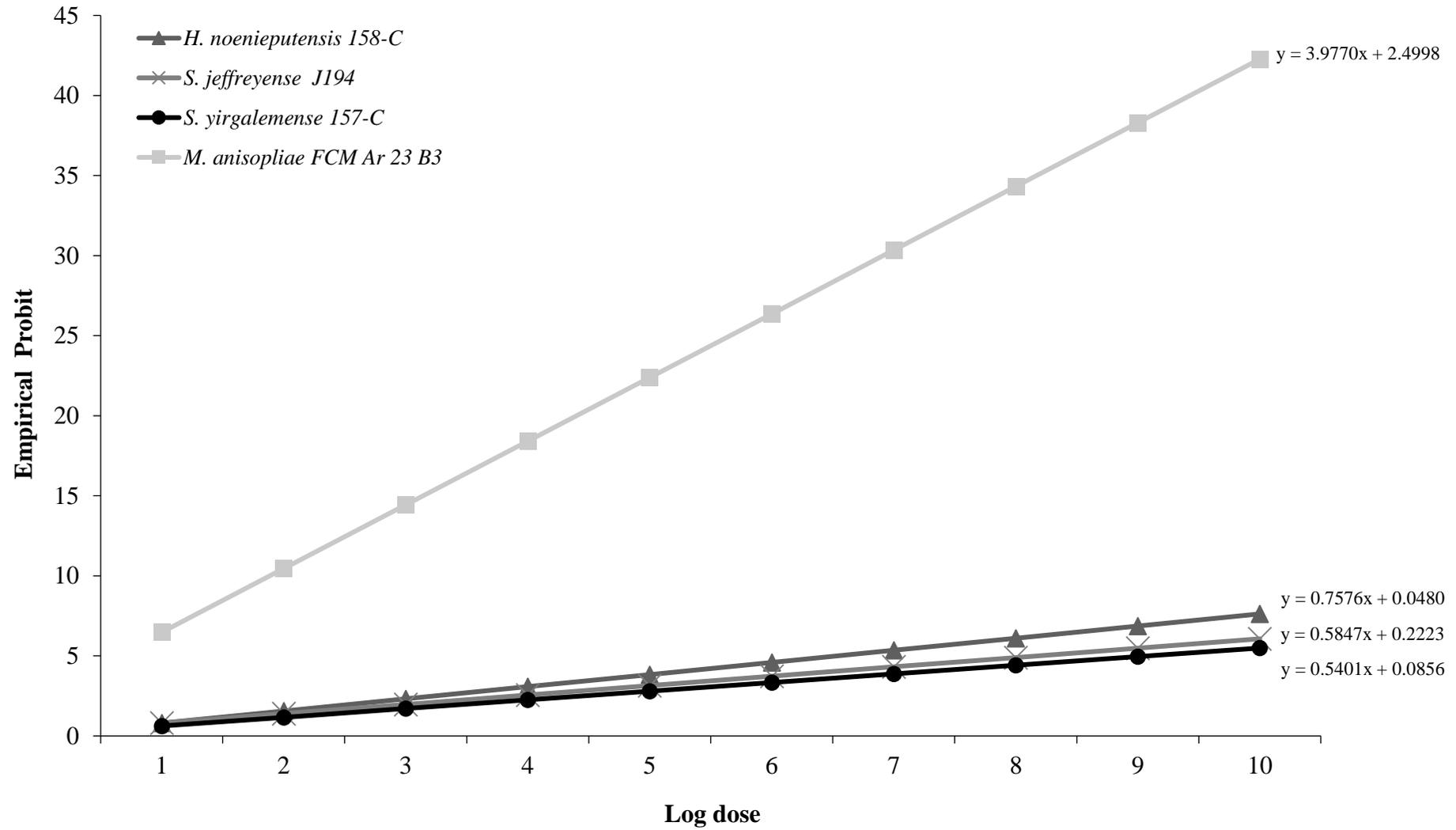
EPF and EPN were the selected control agents used to investigate possible synergism, for the control of the soil dwelling life stages of FCM. Firstly, a uniform method was followed to determine a baseline mortality percentage for each MCA when applied to 5<sup>th</sup> instar FCM. The results from this study were as follows:

#### Screening of EPF and EPN control agents against FCM

The average percentage mortality of FCM fifth instars ranged between 91 and 96% for all EPN species with *S. yirgalemense* causing the highest percentage mortality. The EPF isolate, *Metarhizium anisopliae* FCM Ar 23 B3, induced an average of 80% mortality of FCM fifth instars. Statistical differences amongst treatments were found ( $F_{(3,8)} = 6.433$ ;  $P = 0.01587$ ), with the EPF isolate recording a significantly lower percentage mortality compared to two of the EPN species tested. Control mortality was always a result of handling error, never EPN nor EPF infection.

#### EPN and EPF Dose response bioassays

Dose response bioassays were conducted for the three EPN species and *M. anisopliae* FCM Ar 23 B3 against fifth instar FCM in 24-well plates. Regression lines for each control agent were fitted (Figure. 3.4.4.1). *Steinernema yirgalemense* 157-C:  $y = 0.5401$  (SE of slope = 0.05)  $x + 0.0856$ , *S. jeffreyense* J194:  $y = 0.5847$  (SE of slope = 0.05)  $x + 0.2223$ , *H. noenieputensis* 158-C:  $y = 0.7576$  (SE of slope = 0.05)  $x + 0.0480$  and *M. anisopliae* FCM Ar 23 B3:  $y = 3.9770$  (SE of slope = 0.21)  $x + 2.4998$  (Figure 3.4.4.1).



**Figure 3.4.4.1.** Dose-mortality Probit regression lines for fifth instar FCM treated with three EPN species *Steinernema yirgalemense* 157-C, *Steinernema jeffreyense* J194 and *Heterorhabditis noenieputensis* 158-C, and the EPF isolate *Metarhizium anisopliae* FCM Ar 23 B3.

The LC<sub>50</sub> values for the three EPN species ranged between 4.38 IJs and 7.11 IJs, with *S. yirgalemense* and *S. jeffreyense* being the most and similarly virulent (Table 3.4.4.1). *Metarhizium anisopliae* FCM Ar 23 B3 recorded a LC<sub>50</sub> of 3.42×10<sup>5</sup> conidia/ml. As expected, an increase in concentration was coupled with an increase in FCM mortality for all the tested agents (Figure 3.4.4.1).

**Table 3.4.4.1.** Lethal concentrations (LC<sub>50</sub>, LC<sub>70</sub> and LC<sub>90</sub>) of three EPN species: *Steinernema yirgalemense* 157-C, *Steinernema jeffreyense* J194 and *Heterorhabditis noenieputensis* 158-C, and the EPF isolate *Metarhizium anisopliae* FCM Ar 23 B3, when applied to fifth instar FCM, obtained from a Probit analysis of dose response bioassays.

Control Agents	LC <sub>50</sub> (± SE)	LC <sub>70</sub> (± SE)	LC <sub>90</sub> (± SE)
<i>H. noenieputensis</i> *	7.11 (± 0.5)	14.02 (± 1)	38.6 (± 4.4)
<i>S. yirgalemense</i> *	4.38 (± 0.5)	11.55 (± 1)	46.9 (± 7.7)
<i>S. jeffreyense</i> *	4.47 (± 0.5)	10.96 (± 0.9)	40.05 (± 5.8)
<i>M. anisopliae</i> **	3.42 (± 0.41)	14 (± 1.98)	121 (± 25.0)

\* EPN units = IJs/larva

\*\* EPF units = (×10<sup>5</sup>) conidia/ml

#### EPF and EPN interactions

All three of the possible interactions that could have occurred were present (Table 3.4.4.2), one synergistic, twelve additive and two antagonistic. Specifically considering the individual EPN and the level of interaction that occurred when added to EPF-infected FCM fifth instars, *Steinernema yirgalemense* was the only EPN species that showed a synergistic interaction at 0 h (i.e. EPF and EPN applied on the same day) (Table 3.4.4.2). Additive interactions were observed from 0 – 96 h post EPN application for all EPN species. The exceptions to this trend were two antagonistic interactions observed when *S. jeffreyense* was applied at 24 h post EPF application and when *H. noenieputensis* was applied simultaneously with the EPF (Table 3.4.4.2).

**Table 3.4.4.2.** Interactions recorded when combining entomopathogenic nematodes with *Metarhizium anisopliae* FCM Ar 23 B3 for the control of fifth instar FCM.

	Application rate IJs/larva <sup>b</sup>	Intervals (h)	Observed mortality <sup>c</sup>	Expected mortality <sup>d</sup>	$\chi^2$ <sup>e</sup>	Type of interaction <sup>f</sup>
Sy	4.47	-	43.33	-	-	-
Sj	4.38	-	49.45	-	-	-
Hn	7.11	-	52.17	-	-	-
Ma	1.4×10 <sup>6</sup>	-	65.83	-	-	-
Sy + Ma	4.47	0	100.00	80.64	4.6	Synergistic
Sj + Ma	4.38	0	70.00	82.73	2.0	Additive
Hn + Ma	7.11	0	65.00	83.66	4.2	Antagonistic
Sy + Ma	4.47	24	83.33	80.64	0.1	Additive
Sj + Ma	4.47	24	46.67	82.73	15.9	Antagonistic
Hn + Ma	7.11	24	85.00	83.66	0.0	Additive
Sy + Ma	4.47	48	95.00	80.64	2.6	Additive
Sj + Ma	4.38	48	70.00	82.73	2.0	Additive
Hn + Ma	7.11	48	73.33	83.66	1.3	Additive
Sy + Ma	4.47	72	96.67	80.64	3.2	Additive
Sj + Ma	4.38	72	75.00	82.73	0.8	Additive
Hn + Ma	7.11	72	88.33	83.66	0.3	Additive
Sy + Ma	4.47	96	81.67	80.64	0.0	Additive
Sj + Ma	4.38	96	80.00	82.73	0.1	Additive
Hn + Ma	7.11	96	90.00	83.66	0.5	Additive

<sup>a</sup>Sy = *Steinernema yirgalemense*, Sj = *Steinernema jeffreyense*, Hn = *Heterorhabditis noenieputensis*, + Ma = *Metarhizium anisopliae* FCM Ar 23 B3; <sup>b</sup>*Metarhizium anisopliae* FCM Ar 23 B3 was applied at the LC<sub>70</sub> first thereafter, EPN were applied in daily intervals. ; <sup>c</sup> Percentage observed mortality of 60 FCM fifth instars; <sup>d</sup>Expected mortality (Me) = Mn + Mm (1-Mn). Where Mn and Mm = proportion of observed mortality by EPN or EPF respectively when applied alone; <sup>e</sup>Chi-squared value =  $\chi^2 = \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$ ; <sup>f</sup>Interaction determined as follows: If (Observed – Expected) < 3.84 then Additive. If Observed > Expected, then Synergistic. If Observed < Expected, then antagonistic (Ansari et al. 2004; Pelizza *et al.* 2015).

This information can be used to improve IPM programmes for the control of soil-dwelling FCM life stages, which still lack commercially available control agents for field use. It should be noted that although a synergistic interaction was originally viewed as the most beneficial type of interaction for IPM programmes, antagonistic interactions can also provide useful information for successful implementation of an IPM programme. Specifically, antagonistic interactions can offer information on when not to apply agents simultaneously in the orchards.

The combined EPF and EPN application studies were able to determine the types of interactions that occurred between the MCA at both simultaneous and sequential EPN application. Additive interactions were the most dominant when *S. yirgalemense* and *H. noenieputensis* were applied to FCM larvae that had previously been exposed to *M. anisopliae* 24-96 h prior to EPN application. *Steinernema yirgalemense* and *H. noenieputensis* exhibited synergistic and antagonistic interactions, respectively, when applied simultaneously with the EPF. *Steinernema jeffreyense* also exhibited additive interactions when applied to FCM fifth instars that had previously been exposed to *M. anisopliae* at 0 and 48-96 h post fungal application. Fungal application 24 h before EPN application resulted in an antagonistic interaction. However, it should be noted that this interaction was brought about by a substantial drop in EPN related deaths. This may have possibly skewed the results. An additive interaction is most likely the 'correct' interaction for this time period, based on the additive trend for all other time intervals.

## Conclusion

The combined application of the EPF isolate *M. anisopliae* FCM Ar 23 B3 and three EPN isolates: *S. yirgalemense* 157-C, *S. jeffreyense* J194 and *H. noenieputensis* 158-C, against fifth instar FCM resulted in mostly additive interactions when the EPN agents were applied 24 h post EPF application. Although no strongly synergistic interactions were observed, additive interactions have been shown to reach a synergistic level when certain parameters are changed (e.g. timing between application, soil types), and moving from laboratory to field trials. Hence, following the compatibility methodology outlined above, all three EPN species should be considered for laboratory soil bioassays, with the intention to conduct field trials. It is also highly recommended, due to the variability with the types of interactions observed between different isolates from the same species in other studies, that the symbiotic bacteria and the EPF isolate should be plated together to confirm that neither agent is causing significant inhibition of the other's growth. Regardless of the types of interactions that are obtained from field trials, all four MCA have exhibited high virulence against fifth instar FCM. Thus, these pathogens should continue to be considered as potential control agents for the soil-dwelling life stages of FCM, which still lack adequate control measures within citrus orchards in South Africa.

## Future research

Moving forward, the manner in which we investigate the types of interactions between EPF and EPN needs to be conducted uniformly. The ideal method for doing this would be to conduct a series of laboratory and field combination experiments. The first round of experiments should act as an initial screening test, whereby the type of interaction between the EPF isolate and the EPN symbiotic bacteria should be determined. Ansari *et al.* (2005), along with other interaction studies, have outlined a method for conducting fungal/bacterial combination trials (Tarasco *et al.* 2011; Lalramchuan *et al.* 2020). These methods have also been followed to investigate new methods for controlling plant fungal pathogens, by investigating possible antagonistic interactions between EPN symbiotic bacteria through their production of antifungal properties (Wang *et al.* 2011; Hazir *et al.* 2016; Bock *et al.* 2014; Chacón-Orozco *et al.* 2020). Although these studies have focused on obtaining fungal control agents, as opposed to combined agents for insect control, the principal aim is the same: predicting the type of interaction between two microorganisms, by plating the two agents on the same agar source and assessing whether inhibition of the growth parameters arise. Lalramchuan *et al.* (2020) cite a formula from Balouiri (2016), whereby antifungal activity is assessed by measuring the diameter of the fungal growth when combined with the bacterial isolate against the control plate (fungus only). Once the initial screening of the bacterial and fungal isolates has been completed, promising isolate combinations (non-antagonistic) should be tested further, this time incorporating the EPN and the host insect.

This study chose to investigate the types of interactions between the EPF isolate and the three EPN isolates, using only 24-well bioassay plates. Hence, this interaction was purely an assessment of the compatibility of the agents and not a true representation of how these agents will act in their natural environment, the soil. Although the methodology selected for this thesis provided substantial results, if the previous experiment (bacterial and fungal compatibility) is initially carried out, the EPN and EPF trials using hosts could be conducted using soil bioassays immediately as the initial interaction has already been predicted. This type of experimental setup is more reflective of how the pathogens will interact with the target species, as opposed to the insects being dipped directly into the conidial suspension, as occurred in this study. The movement of the larvae over soil previously applied with the fungal suspension is a better representation of field conditions.

Potential EPF and EPN combinations found to exhibit high levels of additivity or synergism in laboratory bioassays, should be considered for field trials. Because EPN species under early examination as potential control agents are usually not in a formulated suspension, greenhouse experiments are one method of overcoming certain detrimental environmental conditions that could negatively affect the unprotected EPN, while still obtaining field-like conditions. Ansari et al. (2004) commenced with greenhouse experiments on unformulated *M. anisopliae* CLO 53 and *H. megidis* and *S. glaseri* against third-instar *H. philanthus* once laboratory soil bioassays had led to additive and synergistic interactions. Choo et al. (2002) and Ansari et al. (2004) did make use of formulated MCA and thus were able to apply the EPN/EPF isolates in the field where the pest species occurred, to assess whether the interactions obtained from their laboratory studies were altered as a result of field conditions and whether the combination of two agents was more effective at inducing insect mortality than the agents acting alone.

Utilising these methodologies can streamline future interaction assessments for EPF and EPN isolates for control of FCM and other insect pests. The use of laboratory and field trials will provide vital information as to whether the combination of two agents is better than a single agent acting alone so that future IPM programmes can be improved to achieve greater control efficacies against a particular pest. If the results show that two agents bring about the same level of control as that achieved by a single agent, this information is equally important for IPM programmes for several reasons. Firstly, funding research on and development of a single agent is a lot cheaper and less time consuming than researching two, and this could allow the individual control agent to be studied in greater depth. Secondly, although synergism might be observed, a reduction in progeny production and MCA development might still occur when two agents are combined. Hence, the persistence of the two agents in the field might be reduced, ultimately resulting in necessary follow up applications. However, if a single agent is released, with similar control efficacies as the two combined agents, it might persist longer in the field because it is not competing with other agents and thus fewer follow up applications need to be carried out.

Lastly, the FCM IPM programme in South Africa relies on a multitude of control techniques and control agents and chemicals (Moore 2021). The application of these controls may overlap throughout the season, and although they might not be used to target the same life stage (or pest species) (Moore 2019, 2021), the agents may come into contact with one another. These indirect interactions could have no impact on the persistence of the agents. However, if shown that certain combinations or a particular control agent have sublethal or antagonistic effects on other control agents, the persistence of agents could be hindered. Hence, fully understanding how different control agents, interact with one another is an essential component in improving IPM programmes, regardless of whether these agents were intended for combined application or not.

### **Technology transfer**

- ESSA 2019 conference, 8-11 July, presented by Samantha Prinsloo
- SIP 2021 online conference, 28 June – 2 July, presented by Dr Candice Coombes on behalf of Samantha Prinsloo
- MSc thesis 2019 – 2021 Samantha Prinsloo, Rhodes University

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### 3.4.5 FINAL REPORT: Improving biocontrol of woolly whitefly in the Western and Eastern Cape regions.

Project 1194 (2018/19 – 2019/20) by M.J. Gilbert and C. Love (CRI)

#### Summary

Samples of woolly whitefly were collected from citrus trees near Robertson, Citrusdal and Stellenbosch. The leaf samples were retained in the lab and emerging parasitoids were collected for identification. *C. noacki* emerged from the samples from all three locations. The Stellenbosch sample, despite only coming from two woolly whitefly-infested trees, provided the most adult parasitoids. A sampling of woolly whitefly-infested leaves from a netted orchard (Middeltuyn farm) in the area between Citrusdal and Clanwilliam in November 2017 also revealed that *C. noacki* was present there. At a third location, between Robertson and Ashton, *C. noacki* was recovered from an organic lemon orchard. The parasitoid has therefore, by some unknown means, established in the Western Cape. *C. noacki* is not yet known from any other locations in this province. Insufficient parasitoid numbers prevented the transfer of adults to the Eastern Cape.

#### Opsomming

Monsters van wollerige witvlieg is van sitrusbome naby Robertson, Citrusdal en Stellenbosch versamel. Die blaarmonsters is na die laboratorium gebring en volwasse is vir identifikasie versamel. *C. noacki* het van die monsters van albei lokaliteite uitgekom. Die monster van Stellenbosch, alhoewel net van twee besmette bome, het die meeste parasitoïde gelewer tot op datum. Tussen Citrusdal en Clanwilliam by Middeltuyn plaas, in 'n boord onder nette in November 2017, is *C. noacki* ook gevind. By 'n derde lokasie, tussen Robertson en Ashton, is *C. noacki* vanuit 'n organiese suurlemoen boord gevind. Die parasitoïed het dus wel deur 'n onbekende manier, in die Wes Kaap self gevestig. Die teenwoordigheid van *C. noacki* is nog nie in ander areas in die Wes Kaap vasgestel nie.

#### Introduction

Woolly whitefly, *Aleurothrixus floccosus* (Maskell) (Hemiptera: Aleyrodidae) was first collected in Jamaica in 1896 (Martin & Mound 2007) but has only recently been recorded in South Africa (Giliomee & Millar 2009). Found initially on “backyard” citrus trees, it has become a pest on some commercial plantings (Grout et al. 2012). In addition, growers are reporting that the pest is becoming problematic in cooler areas where citrus trees are cultivated under netting (Stander & Cronjé 2016). Biological control of woolly whitefly has been successful in other parts of the world through the release of the Aphelinid parasitoid *Cales noacki* Howard (Mottern et al. 2011, Mottern & Heraty 2014). In recent years, this parasitoid was bred successfully at CRI-Nelspruit. Releases of *C. noacki* were carried out on citrus in the Nelspruit area and at Mooinooi in the North-West Province. The parasitoid established at both these sites (Tim Grout, personal communication). Unfortunately, the *C. noacki* colony died out at CRI-Nelspruit and no specimens of *C. noacki* were available for release in the Western Cape.

Citrus production, west of the Cedarberg, occurs in distinct geographical “pockets” e.g. around the towns of Piketberg, Porterville, Malmesbury, Riebeeek Kasteel etc which are in turn surrounded by large areas of cereal production where there is very little natural vegetation. Such “barren” areas (in terms of fruit production) would be unfavourable for the natural spread of a parasitoid bearing in mind the very dry summers (and increasingly dry winters). Elsewhere, there are cooler production areas such as the Hex River Valley and Stellenbosh where woolly whitefly is becoming a pest, particularly under netting. It is therefore important to try to improve the biological control of this emerging pest by confirming where *C. noacki* is present or absent in the Western Cape. Specimens of *C. noacki* can then be collected from the two locations where it is known to occur and distributed to other citrus-producing areas. In the Eastern Cape *C. noacki* is not known to occur as previous releases from CRI-Nelspruit did not establish. It is therefore of importance to also introduce *C. noacki* to this citrus-producing region using specimens from the Western Cape.

### Stated objectives

1. Identify woolly whitefly problem areas in the Western Cape.
2. Determine the distribution of *Cales noacki* in the Western Cape.
3. Increase the distribution area of *C. noacki* in the Western and Eastern Cape regions.
4. Determine if any other species of parasitoids are effective natural enemies of woolly whitefly in the Western Cape.

### Materials and methods

Reports of woolly whitefly infestation received from growers, technical personnel, chemical company representatives etc., were followed up by visiting the relevant orchards. Where woolly whitefly was found in citrus orchards, samples of leaves were collected into plastic Ziploc bags and placed in a cooler box. The samples were returned to the laboratory and examined under a dissecting microscope before placing them in emergence boxes. Parasitoids that emerged were sent to CRI, Nelspruit for identification and to ascertain whether *Cales noacki* was present in any of the samples.

### Results

*C. noacki* emerged from samples collected from three locations. The Stellenbosch sample, from trees at the Sustainability Institute (co-ordinates 33° 58' 59.05" S, 18° 46' 04.40" S) provided the greatest number of adult parasitoids. A sampling of woolly whitefly-infested leaves from a netted orchard at Middeltuyn farm, (co-ordinates 32° 20' 20.89" S, 18° 50' 38.39"E), in the area between Citrusdal and Clanwilliam also revealed that *C. noacki* was present there. The third location was between Worcester and Ashton at Tierhoek Cottages (co-ordinates 33° 42' 39.18" S, 19° 47' 21.89" E) where an organic lemon orchard is located.

Since then, no further samples of woolly whitefly could be found due to what seems to be a significant population decline. Whether this is due to the fact that the parasitoid has become established in the Western Cape, or to a lessening of the drought, or a combination of the two factors is not known. Nevertheless, any reported incidence of woolly whitefly outbreaks will continue to be investigated and sampling will be done in

order to hopefully confirm the presence of the parasitoid in other parts of the Western Cape. Insufficient parasitoid numbers prevented the transfer of adults to the Eastern Cape.

## Conclusion

Generally, the infestations of woolly whitefly that were found were not as serious as was expected. Before this project commenced the impression had been gained that serious infestations existed in commercial orchards in the Western Cape. This was during the time of a severe drought in the province. The drought ended in 2018. However, our field sampling revealed only sporadic presence of woolly whitefly and none that would be considered of a serious nature. Nevertheless it could be shown that *Cales noacki* had, by means as yet unknown, established in the Western Cape.

## Future research

Samples of woolly whitefly should continue to be collected on an *ad hoc* basis and monitored for parasitoid presence especially if an increase in pest incidence should occur. The parasitoid still needs to be released in the Eastern Cape as it is not yet known from that citrus production area.

## Technology transfer

None

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### 3.4.6 PROGRESS REPORT: New systemic insecticides for citrus

Project 1148 (RCE-2-25) (2016/7-2021/2) by T G Grout, P R Stephen (CRI), S M Faris and P Nderitu (*icipe*)

## Summary

In order to prepare for the arrival of *Diaphorina citri* in South Africa, we need to find more systemic insecticides that can be used frequently in nurseries and for non-bearing trees. Early research evaluated several treatments against the black citrus aphid *Aphis citricidus* and gave some promising results. Research is now aimed at evaluating several systemic products against *D. citri* from a culture on potted citrus plants in an insect-proof structure in SE Kenya in collaboration with *icipe*. However, numbers of *D. citri* on citrus in the culture declined, despite the addition of *Murraya koenigii* plants to the culture. It has therefore not yet been possible to conduct the first screening trial of several systemic insecticides against *D. citri* on potted citrus plants.

## Opsomming

Ten einde vir die aankoms van *Diaphorina citri* in Suid-Afrika voor te berei, moet meer sistemiese insekdoders gevind word wat gereeld in kwekerie en vir nie-draende bome gebruik kan word. Vroeë navorsing het verskeie behandelings teen die swart sitrus plantluis, *Aphis citricidus*, geëvalueer, en belowende resultate is verkry. Navorsing is nou daarop gemik om verskeie sistemiese produkte teen *D. citri* te evalueer, vanaf 'n kultuur op gepotte sitrusplante in 'n insek-bestande struktuur in SO Kenia, in samewerking met *icipe*. Getalle van *D. citri* op sitrus in die kultuur het egter afgeneem, ten spyte van die byvoeg van *Murraya koenigii* plante by die kultuur. Dit was dus nog nie moontlik om die eerste siftingsproef van verskeie sistemiese insekdoders teen *D. citri* op gepotte sitrusplante uit te voer nie.

### 3.4.7 **PROGRESS REPORT: Determine the primary cause for mealybug repercussions under netting** Project 1195 (2018/9 – 2021/2) by T G Grout, P R Stephen, L Serfontein and E Mauda (CRI)

#### **Summary**

In citrus orchards under 20% shade net in both Australia and South Africa, mealybug has become a primary pest. This research is being conducted to determine the main reasons for this. Shade net structures at the Citrus Research Centre in Nelspruit were built, potted Valencia trees placed under the shade net and in the open adjacent to the structures and citrus mealybug cultures initiated. Plans to compare the growth rate of mealybug in parasitoid-proof containers outside the structures and under 20% white shade net were not possible because the cultures heated up in the sun. Difficulty was also experienced in preventing contamination of mealybug cultures with the parasitoid *Coccidoxenoides perminutus* but this was finally resolved. However, placing 130 mealybug crawlers per plant did not result in infestation, neither did the careful transfer of adults to citrus plants, finally the attachment of a container to each plant with egg sacs was successful. After being infested for 65 days, plants under net had significantly more citrus mealybug nymphs per leaf than those in the open. After 112 days, no citrus mealybug could be found on plants in the open whereas plants under net had approximately four nymphs per leaf in the middle of the plant and a lower position. After 151 days, numbers per leaf under the net had declined but nymphs could still be found. Pest infestation of potted Valencia trees in the open adjacent to potted Valencia trees of the same age and source under 20% net at CRC, Nelspruit showed that under the net citrus red mite infestation was 75% higher, silver mite was 14% higher, red scale 33% higher and mealybug 6% higher. A photo-radiometer was used to measure PAR and UV-B radiation in adjacent commercial orchards in the open and under 20% netting at various locations in Mpumalanga and Limpopo during summer and autumn 2018/19. Results were variable on different occasions but in general the nets reduced PAR by approximately 20% and UV-B by 26%. Leaf samples at the same commercial sites were taken on two occasions in the open and under net to test for levels of phenolics. These results showed more difference between the northern and southern sides of trees than between net and no net. Tests of chemical residues on fruit under nets and in the open during February and March showed little difference between samples under net and in the open except for a trend for some strobilurin residues to be slightly higher under net. None of these differences would have been problematic at harvest time. Objective 5 on the effect of natural enemies under 20% net will be addressed in 2021. Insect proof cages were constructed at CRI to be used for testing the effects of 20% shade net in preventing the natural enemy *Anagyrus vladimiri* from finding the mealybug when released outside the net. Open and closed containers with 20% white shade net will be placed inside the insect proof cage where *A. vladimiri* will be released. The rate at which third instar citrus mealybugs on butternuts are parasitized will be recorded in both open and closed 20% shade net containers. Five hundred *A. vladimiri* were released inside insect cages with 20 butternut infested with mealybug in containers open without a net and 20 butternut containers under 20% white shade net. Mealybugs will be examined for parasitism weekly for thirty days in both control (open containers) and experimental containers (20% shade net), recording the number of parasitised mealybug in each replicate. Furthermore, objective 5 will be repeated using the natural enemy *Cryptolaemus montrouzieri* to access the ability of the beetle to find mealybugs from outside the 20% shade net and open containers with mealybug-infested butternuts.

#### **Opsomming**

Witluis het 'n belangrike plaag in sitrusboorde onder 20% skadunet in beide Australië en Suid-Afrika geword. Hierdie navorsing is uitgevoer ten einde die hoofredes hiervoor vas te stel. Skadunet strukture is by die Sitrus

Navorsing Sentrum (CRC) in Nelspruit gebou, Valencia bome is in potte geplant en onder die skadunet en in die oopte aangrensend die strukture geplaas, en sitrus witluis kulture is begin. Planne om die groeitempo van die witluis in parasiet-bestande houers buite die strukture, en onder 20% wit skadunet te vergelyk, was nie moontlik nie omdat die kulture in die son verhit het. Dit was ook moeilik om kontaminasie van witluis kulture met die parasiet *Coccidoxenoides perminutus* te voorkom, maar dit is uiteindelik opgelos. Die plasing van 130 witluis kruipers per plant het egter nie tot besmetting gelei nie, en ook nie die versigtige oordra van volwassenes na sitrusplante nie. Die vasheg van 'n houer met eiersakkies aan elke plant, was uiteindelik suksesvol. Ná besmetting vir 65 dae, het plante onder net betekenisvol meer sitrus witluis nimfe per blaar gehad, in vergelyking met plante in die oopte. Ná 112 dae, kon geen sitrus witluis op plante in die oopte gevind word nie, terwyl plante onder net ongeveer vier nimfe per blaar in die middel van die plant en by 'n laer posisie gehad het. Ná 151 dae het die aantal per blaar onder die net afgeneem maar nimfe kon nog gevind word. Plaagbesmetting van gepotte Valencia bome in die oop areas aangrensend die gepotte Valencia bome van dieselfde ouderdom en bron onder 20% net by CRC, Nelspruit, het getoon dat onder die net, sitrus rooimyt besmetting 75% hoër was, silwermyt was 14% hoër, rooi dopluis 33% hoër en witluis 6% hoër. 'n Foto-radiometer is gebruik om PAR en UV-B bestraling in aangrensende kommersiële boorde in die oopte en onder 20% net by verskeie liggings in Mpumalanga en Limpopo gedurende die somer en herfs van 2018/19 te meet. Resultate het by verskillende geleenthede gevarieer, maar oor die algemeen het die nette PAR met ongeveer 20% verminder en UV-B met 26%. Blaarmonsters is by dieselfde kommersiële areas by twee geleenthede in die oopte en onder net geneem om vir fenoliese vlakke te toets. Hierdie resultate het groter verskil tussen die noordelike en suidelike kante van bome getoon as tussen net en geen net nie. Toetse van chemiese residue op vrugte onder nette en in die oopte gedurende Februarie en Maart het min verskil tussen monsters onder net en in die oopte getoon, behalwe vir 'n neiging van strobilurie residue wat effens hoër onder net was. Geen van hierdie verskille sou problematies met oestyd gewees het nie. Doelwit 5 oor die effek van natuurlike vyande onder 20% net sal in 2021 aangespreek word. Insek-bestande hokke is by CRI gebou om te gebruik om die effekte van 20% skadunet te toets ten einde te voorkom dat die natuurlike vyand *Anagyrus vladimiri* die witluis vind wanneer buite die net vrygelaat word. Oop en toe houers met 20% wit skadunet gaan binne die insek-bestande hok geplaas word waar *A. vladimiri* vrygelaat gaan word. Die tempo waarteen derde instar sitrus witluis op botterskorsie geparasiteer word, gaan in beide oop en geslote 20% skadunet houers aangeteken word. Vyf honderd *A. vladimiri* is binne insekhokke vrygelaat met 20 botterskorsies wat met witluis besmet is in oop houers sonder 'n net, en 20 botterskorsies in houers onder 20% wit skadunet. Witluis sal weekliks vir parasitisme ondersoek word, vir dertig dae in beide kontrole (oop houers) en eksperimentele houers (20% skadunet), en die aantal geparasiteerde witluis in elke herhaling sal aangeteken word. Doelwit 5 sal verder herhaal word deur gebruik te maak van die natuurlike vyand *Cryptolaemus montrouzieri* ten einde die kweker se vermoë vas te stel om witluis van buite die 20% skadunet, en oop houers met witluis-besmette botterskorsies, te vind.

#### 3.4.8 PROGRESS REPORT: Controlling mites on budwood

Project 1203 (2018/9, 2021/2) by T G Grout, P R Stephen, K C Stoltz and E Mauda (CRI)

##### Summary

Citrus budwood often requires fumigation with methyl bromide on arrival in South Africa and this often kills many of the buds. In the search for a more benign means of control, preliminary work with Vapormate (ethyl formate) and carbon dioxide showed that the Vapormate was more efficacious but that it was also more phytotoxic. A heat treatment of bud sticks over a water bath at 45°C for 4 h was not very detrimental to navel orange buds with 93% taking but it was very detrimental to mandarin buds with only 18% alive three weeks after budding. This is therefore not an option for treating mites. Dips of bud sticks infested with citrus bud mite for different periods of time (30 seconds, 2 minutes and 8 minutes) in fenpyroximate at the registered rate of 150 ml/hl water gave promising results. The solution was constantly agitated when dips were made providing overall cover of the buds. Dips for 2 min and 8 min had no live bud mite in the leaf axil buds compared to high levels of infestation in the control. Further dip treatments in acaricides will be conducted before the better treatments are tested for bud viability.

##### Opsomming

Sitrus okuleerhout vereis dikwels beroking met metielbromied met aankoms in Suid-Afrika en dit maak dikwels baie van die ogies dood. In die soeke na 'n meer sagte wyse van beheer, het voorlopige werk met Vapormate (etielformaat) en koolstofdiksied getoon dat Vapormate meer doeltreffend was, maar dat dit ook meer fitotoksies was. 'n Hitte-behandeling van ogiestokkies oor 'n waterbad by 45°C vir 4 h was nie baie nadelig vir nawel lemoen ogies nie, met 93% wat gevat het, maar dit was baie nadelig vir mandaryn ogies met slegs 18% wat drie weke ná okulering lewendig was. Dit is dus nie 'n opsie vir die behandeling van myte nie. Doop van ogiestokkies wat met sitrus knopmyt besmet is, vir verskillende tydspanes (30 sekondes, 2 minute en 8 minute) in fenpiroksimaat teen die geregistreerde dosis van 150 ml/hl water, het belowende resultate gelewer. Die oplossing is konstant geroer wanneer die doopbehandeling uitgevoer is, wat algehele bedekking van die ogies verskaf het. Doopbehandelings vir 2 min en 8 min het geen lewendige knopmyt in die blaar aksiale knop gehad, in vergelyking met hoër vlakke van besmetting in die kontrole nie. Verdere doopbehandelings in mytdoders sal uitgevoer word voordat die beter behandelings vir ogie lewensvatbaarheid getoets word.

#### 3.4.9 **PROGRESS REPORT: Augmentation of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) for the control of California red scale (Hemiptera: Diaspididae) in citrus.**

Project 1259 (Apr 2020-Mar 2023) by Ernst de Beer, Martin Hill (RU), Tammy Marsberg and Sean Moore (CRI)

##### **Summary**

Pressure is mounting from markets and regulations to reduce chemical inputs in citrus production. The current chemical active ingredient residue scenario should be improved to maintain market access. By having an active ingredient of a red scale control remedy as the most common denominators, it would benefit the citrus industry to investigate alternative red scale control measures. *Aphytis lingnanensis* Compere was reared in South Africa for augmentation against red scale on citrus. However, it was concluded that there is little evidence that augmentation of *A. lingnanensis* had a major effect on the population dynamics of red scale. *A. melinus* is now for the first time commercially available for augmentation in South Africa. Consequently, it is important that the efficacy of augmentation of this species on red scale is determined locally. Six orchards were selected in the Eastern- and Western Cape. *A. melinus* was released in three orchards in each region and the remaining three orchards were studied as untreated controls. Release numbers were 240 000 parasitoids/ ha at one release/ week over a five-week period. Releases started in November 2019 in the biological orchards and in January 2020 in the conventional orchards. Four yellow sticky traps were hung in each orchard to monitor adult *Aphytis* spp. activity and replaced weekly. Fortnightly, levels of red scale infestation were determined by scouting ten fruit on each of ten trees in each orchard. Once a level of 5% red scale fruit infestation was reached, a sample of 20 infested fruit from each orchard was collected randomly every 4 weeks. Collected fruit was microscopically inspected to determine the levels of live, dead, and parasitized red scale. Parasitoid species responsible for parasitism were identified and share of control effect monitored and documented. *Aphytis* spp. were identified morphologically and confirmed by sequencing the CO1 gene samples. Preliminary results of this study during the first year of the trials suggests that the augmentation of *A. melinus* did not significantly control nor reduce red scale infestation and did not increase the overall level of parasitism above that of the untreated control. The overwhelming natural presence of *A. africanus* led to dominate the share of parasitism in both the control and treatment orchards.

##### **Opsomming**

Druk is besig om toe te neem vanaf markte en regulasies om chemiese insette te verminder tydens sitrus produksie. Die huidige chemiese aktiewe bestanddeel residu situasie moet verbeter word om mark toegang te behou. Deur 'n aktiewe bestanddeel van 'n rooidopluis beheer middel as die mees algemene deler te hê, sal die Suid-Afrikaanse sitrus bedryf daarby baat om alternatiewe dopluis beheer maatreëls te ondersoek. *Aphytis lingnanensis* Compere was geteel in Suid-Afrika vir die aanvulling in sitrus boorde teen rooidopluis. Maar, dit is egter bevind dat daar te min bewyse is dat hierdie aanvullings van *A. lingnanensis* 'n groot effek op rooidopluis populasies gehad het. *A. melinus* is nou vir die eerste keer kommersieel beskikbaar in Suid-Afrika. Gevolglik is dit belangrik dat die effektiwiteit van die aanvulling met hierdie spesie teen rooidopluis bepaal word. Ses boorde is gekies in die Oos- en Wes-Kaap. Vrylatings van *A. melinus* is gedoen in drie boorde in elke streek terwyl die oorblywende drie boorde in elke streek as kontrole gemonitor was. 240 000 *A.*

*melinus wespes/* ha was vrygelaat oor 'n vyf week periode. In die biologiese produksie boorde het vrylatings in November 2019 begin en in Januarie 2020 in die konvensionele produksie stelsels. Vier geel kleef kaartjies, wat weekliks vervang was, is in elke boord gehang om die aktiwiteit van volwasse *Aphytis* spp. wespes te monitor. Boord verkenning is elke twee weke gedoen om rooidopluis infestasiëvlakke te monitor. Sodra 'n infestasië vlak van meer as 5% waargeneem was, is 20 gëinfesteerde vrugte vanuit elke boord ewekansig geneem om die vlakke van lewendige, dooie en geparasiteerde rooidopluis mikroskopies te bepaal. Parasitoïede spesies wat verantwoordelik was vir die parasitisme van rooidopluis was geïdentifiseer en die aandeel in parasitisme was bepaal en aangeteken. *Aphytis* spp. was morfologies geïdentifiseer en moet nog bevestig word deur die opstelling van die CO1 geen volgorde. Voorlopige resultate van hierdie studie tydens die eerste jaar van proewe dui aan dat die aanvulling van *A. melinus* nie rooidopluis beheer nog verminder het in die boorde wat daarmee behandel is nie. Die algehele vlak van parasitisme het ook nie verhoog bokant die van die kontrole boorde nie. Die oorweldigende natuurlike teenwoordigheid *A. africanus* het die aandeel in parasitisme gedomineer in beide die behandeling en kontrole boorde.

#### 3.4.10 PROGRESS REPORT: IPM under Nets in the Western Cape

Project 1228 (2019/20 – 2021/22) by M Gilbert and Courtney Morris (CRI)

##### Summary

It is essential to determine what influence, if any, nets may have on different citrus pest populations as well as those of predators and parasitoids. Both netted and open orchards, of the same cultivar at the same site, were found in order to make a valid comparison. Two suitable sites in the Western Cape were identified. The first site with Clementines is just outside Porterville and is not involved in any sterile insect release (SIT) programmes. The second site, in the Breede River Valley, consists of Clementines and this site is included in both the FCM and fruit fly SIT programmes. The following traps were set out: FCM (yellow Delta traps with pheromone lures); fruit fly (Capilure in Sensus traps and Biolure in yellow bucket traps), thrips and other pests, predators and parasitoids (yellow sticky traps). Three traps of each type were placed under the netted and the open blocks and monitored weekly.

At Porterville, under fully enclosed netting with only narrow entrances, trapped fruit fly numbers were 80% and 90% fewer when monitoring with Biolure and Capilure respectively, compared with open orchards. At De Doorns, where netting does not fully enclose the orchard, results were less clear. Numbers of male (and a few female) fruit fly trapped with Capilure were higher under netting than in the open. The results of Biolure trapping (mostly catching females) showed a significant reduction of activity under the netting.

At De Doorns a 75% reduction in wild FCM trap catches was recorded under netting. There was also a 55% reduction in sterile FCM catches under netting compared to open orchards. Regarding citrus thrips, the trend of significantly greater numbers of thrips being trapped in open blocks compared to netted blocks continued at De Doorns. At Porterville, there was no significant difference. Trends were unclear regarding red scale males, whitefly and aphids as counts were too low for any conclusions to be drawn.

Green leafhopper numbers tended to be higher under fully enclosed nets whereas the opposite was observed where nets were not completely down to ground level. Leaf and fruit inspections are continuing but have not yet revealed any significant differences between orchards, except in the case of windscar and sunburn, which is significantly reduced under netting.

Clearly, not all netting is created equal. Trends as to whether netting has a significant effect on pest populations will have to take into account the degree to which the orchard is enclosed, in addition to how well the net is maintained with reference to repairing holes etc.

##### Opsomming

Dit is nodig om te bepaal watter invloed, indien enige, nette dalk op verskeie plaag populasies sowel roofdiere en parasitoïede mag hê. Albei oop boorde en onder nette, van die selfde kultivar op die selfde plaas is gevind, om 'n ware verlyking te kan doen. Twee geskikte proefpersele in die Wes Kaap was geïdentifiseer. Die eerste

proefpersel met sagtesitrus (Clementine), is naby Porterville en is nie by enige steriele insek loslaating program betrokke nie. Die tweede perseel, in die Breederivier vallei ook met sagtesitrus, is wel deel van albei VKM en vrugtevlug steriele insek loslaatings programme. Die volgende valletjies is uitgesit: VKM (geel Delta valletjies met feromoon lokmiddel); vrugtevlug (Capilure in Sensus valletjies en Biolure in geel emmer valletjies); blaaspootjies en ander plae, roofdiere en parasitoïede (geel kleefvalle). Drie van elke tipe valletjie is in die boorde met nette asook die oop boorde opgehang en weekliks gemonitor.

By Porterville, onder volledig toegeboude nette met nou ingange, is vrugtevlug vangste met 80% en 90% onderskeidelik verminder wanneer Biolure en Capilure is vir moniteering gebruik, in vergelyking met oop boorde. By De Doorns waar die nette nie volledig toegebou is nie, resultate is minder duidelik. Getalle mannetjie (en 'n paar wyfie) vrugtevlug, gevang met Capilure, is hoër onder nette as in die oop. Met Biolure (wat meestal wyfies vang) is 'n betekenisvolle vermindering in vlug aktiwiteit onder die nette aangeteken.

By De Doorns, 'n 75% vermindering in wilde VKM Vangste was onder nette aangeteken. Daar was ook 'n 55% vermindering in steriele VKM Vangste onder die nette in vergelyking met oop boorde. Wat sitrusblaaspootjie aanbetref, die neiging van betekenisvolle hoër vangste in oop blokke in vergelyking met toegeboude boorde het by De Doorns aangehou. By Porterville was daar nie 'n betekenisvolle verskil nie. Neigings wat rooidopluis, witvlug, en plantluis aanbetref was onduidelik omdat getalle te min was om gevolgtrekkings te trek.

Groenbladspringer vangste was geneig om onder volledige nette hoër te wees alhoewel die teenoorgestelde is opgemerk waar nette nie tot grondvlak was nie. Blaar en vrug inspeksies het geen betekenisvolle verskille tussen boorde gewys. Wat windskaad en sonbrand aanbetref is daar wel 'n betekenisvolle vermindering onder nette.

Duidelik is nie alle nette gelyk geskep nie. Neigings oor die vraag of nette 'n betekenisvolle invloed op plaag populasies het sal die mate waartoe die boorde toegemaak is in ag moet neem, asook hoe effektief die nette na gekyk word met verwysing na die herstel van gate.

#### 3.4.11 PROGRESS REPORT: IPM under nets in Mpumalanga Province

Project 1205 (April 2018 – December 2020) by Karlien Grobler, Martin Hill (RU) and Sean Moore (CRI)

##### Summary

The incidence and severity of citrus thrips throughout the 2020/2021 citrus growing season were found to be less under the 20% enclosed shade net in comparison to the open citrus orchard. The severity of thrips damage in comparison, confirms the scouting results. Mealybug populations were higher and more concentrated under the citrus netting than in the open orchard, confirming the populations observed in the previous growing season. *Coccidoxenoides perminutus* and *Nephus* sp. were released in equal quantities simultaneously under citrus netting and in the open orchard. FCM total trap catches seem to be higher in the open orchard this season in comparison to the netted orchard. Fruit fly species were once again caught in higher quantities in the open orchard. Low level of fruit infestations were observed during harvesting. Mummified mealybugs were collected in citrus sites and placed in emergence boxes. *Coccidoxenoides perminutus*, emerged. Red scale was collected and inspected for parasitism by *Aphytis* spp. Only one *Aphytis* adult was found in the netted orchard. Red scale (crawlers, white caps, male scales and adult females) were found in higher populations under the netted orchard when compared to the open orchard. Citrus fruit sampled for residue tests resulted in higher concentrations of methoxyfenozide and pyraclostrobin, with lower dithiocarbamates under the enclosed netting in comparison to the open orchard. Seychelles scale and pink wax scale were present at higher populations under litchi shade net in comparison to the control. Mango scale seems to be similar in population densities at both litchi sites. Litchi moth and false codling moth adults were trapped in the control orchard, with no catches in the netted orchard. Litchi moth larval infestation was detected in the control orchard. Fruit fly trap catches and larval infestation were found to be higher in the open orchard than under shade net. *Cryptolaemus montrouzieri* was released in equal quantities in the litchi netted orchard and open orchard for the control of Seychelles scale and pink wax scale. Both pest populations had reduced to acceptable IPM levels before harvest.

## Opsomming

Die intensiteit en populasie druk van sitrus blaaspootjie deur die seisoen was laer op vrugte onder 20% skadu net teenoor die oop boord. Die persentasie van skade ondervind tydens oes evaluasies bevestig die scout verslae. Witluis populasies was meer opgemerk en in hoër populasies teenoor die oop kontrole boord, wat ooreenstemming is met data van die vorige seisoen. *Coccidoxenoides perminutus* en *Nephus* sp. was vrygelaat op dieselfde dag in dieselfde hoeveelhede onder asook buite die net. FCM se totale mot vangste was meer buite die net teenoor die boord onder net. Meer vrugte vlieë was in die oop boord gelok en gevang teenoor die boord onder net. Minimale vrug infestasies was opgemerk tydens oes. Geparasiteerde witluis van beide boorde was geneem en in bokse geplaas vir parasiet ontwikkeling. *Coccidoxenoides perminutus* het uitgebroei. Rooi dopluis kruiers, 1ste tot finale instar was gevind op vrug monsters wat geneem en geïnspekteer was met n mikroskoop vir parasitisme deur *Aphytis* spp. Slegs een volwasse *Aphytis* sp. was gevind maar talle kruiers en 1ste tot finale instar dopluis was gevind op vrugte. 'n hoër intensiteit was opgemerk in die boord onder net, teenoor die oop boord. Drie verskeie sitrus vrug monsters van beide boorde was geneem vir residu analise. Die aktiewe dithiokarbamate was laer in konsentrasie in die boord onder net waar pyraclostrobin en methoxyfenozide in hoër konsentrasies onder die net teenoor vrugte van die kontrole boord. Seychelles dopluis en pienk was dopluis was in hoër populasies gevind onder die lietjie net teenoor die kontrole boord. Die net het nie n effek getoon op mango dopluis se voorkoms nie aangesien vlakke soortgelyk opgemerk was. Lietjie mot en FCM was gevang in valletjies buite die net, maar geen onder net. Lietjie mot larwes was gevind buite die net en geen onder die net nie. *Cryptolaemus montrouzieri* was vrygelaat in gelyke populasies op dieselfde dag onder die net asook in die kontrole boord vir die beheer van Seychelles dopluis en pienk was dopluis. Beide peste se populasies het verlaag tot aanvaarbare IPM vlakke voor oes.

### 3.4.12 PROGRESS REPORT: The influence of systemic phenology (citrus, mealybug and parasitoids) on the efficacy of *Anagyrus* augmentation for mealybug control

Project 1258 (April 2020 – March 2022) by S D Moore, W T Mommsen (CRI) and M P Hill (RU)

#### Summary

In previous trials where *Anagyrus vladimiri* was released into citrus orchards in the Eastern Cape for control of citrus mealybug, the results of relatively early releases during November showed they were effective. Similarly, the trials conducted in Burgersfort and Hoedspruit, where different timing of releases was compared, effectively showed reduction in mealybug infestation on citrus fruit before harvest in April of 2020. Studies on three different cultivars were done in three different regions. Mealybug infestation peaked at an average of 14.0% (Dec/Jan) in Burgersfort open orchards, 14.3% (Feb/Mar) in Burgersfort orchards under net and 10.0% (Dec/Jan) in Hoedspruit open orchards respectively. The respective mealybug infestations were reduced to averages of 1.5%, 5.3% and 0.8% in the few weeks prior to harvest. Unfortunately, the average mealybug infestation in control orchards was lower than the treated orchards and ranged between 5%-15%, 0%-2% and 0%-6% respectively. The orchards where *Anagyrus* releases were initiated the earliest (October 2019) showed the lowest incidence of mealybug-infested fruit over time and resulted in the lowest percentage mealybug-infested fruit before harvest at two of the three trial sites. Considering the positive result achieved in 2020, and in search of a statistically significant relationship between timing of releases of *Anagyrus* and control of mealybug, the study was repeated in the 2020/21 season. Compared to the previous season, mealybug infestation peaked at an average of 18% (Feb) in the Burgersfort open orchards, 55% (Feb) in Burgersfort orchards under net and 14% (Jan) in Hoedspruit open orchards. The average mealybug infestation before harvest was 15%, 4% and 0% at the three sites respectively. The respective mealybug infestation in control orchards peaked at an average of 13%, 35%, 13% which indicates a notable increase in mealybug infestation in the Burgersfort orchards under net and Hoedspruit open orchards compared to the previous season. Third instar mealybug samples were inspected for parasitism by placing mealybug in containers so that observations could be made of emerging parasitoids. From the Hoedspruit sites, predominantly two primary parasitoids of citrus mealybug emerged, *Anagyrus vladimiri* and *Leptomastix dactylopi*. From the Burgersfort region two primary parasitoids (*Anagyrus vladimiri* and *Coccidoxenoides perminutus*) and three hyperparasitoids (*Coccophagous rusti* Compere, *Chartocerus* sp. and *Pachyneuron* sp.) predominantly emerged. The level of hyperparasitism in 2021 was much higher than the previous season and it may have been the reason for the low levels of primary parasitism observed in the field. This is important for us to understand the potential impact

of hyperparasitoids on the efficacy of commercial augmentations of *Anagyrus vladimiri* in order for us to optimise control strategies reliant on effective biocontrol.

## Opsomming

In vorige experimente met vrylaatings van *Anagyrus vladimiri* in die Oos Kaap, was die effek van vroeë vrylaatings in November effektief om witluis te beheer. Dieselfde was gesien in Burgersfort en Hoedspruit met effektiewe afname van witluis infestasië op sitrus vrugte voor oestyd in April 2020. Die experiment het gekyk na die tydsberekening van *Anagyrus* vrylaatings om witluis te beheer op drie sitrus kultivars. Die gemiddelde witluis infestasië piek in Burgersfort oop boorde was 14.0% (Dec/Jan), 14.3% (Feb/Mar) in Burgersfort boorde onder net en 10.0% (Dec/Jan) in Hoedspruit oopboorde. Die gemiddelde witluis infestasië het gedaal voor oestyd tot 1.5%, 5.3% en 0.5% onderskeidelik. Ongelukkig was die gemiddelde witluis infestasië in kontrole boorde heelwat laer as die proef boorde, onderskeidelik in die drie gebiede het dit gewissel tussen 5-15%, 0-2% en 0-2%. In die boorde waar die eerste vrylaatings plaasgevind het in Oktober 2019 'n laer persentasie witluis opgebou in twee uit die drie experimente. Met die goeie witluis beheer in 2020 was dit besluit om die protokol te herhaal in die opvolgende seisoen, sodoende om 'n statistiese verhouding tussen die tyd van vrylaating van *Anagyrus* en witluis beheer te bepaal. In 2021 het die gemiddelde witluis infestasië in Burgersfort oopboorde gepieke op 18.0% (Feb), 55.0% (Feb) in Burgersfort boorde onder net en 14.0% (Jan) in Hoedspruit oopboorde. Die infestasië het gedaal voor oestyd tot 15.0%, 4.0% en 0% onderskeidelik. Die witluis infestasië was aansienlik meer in die Burgersfort netboorde en die Hoedspruit oopboorde in vergelyking met die vorige seisoen. Die witluis infestasië piek in die kontroleboorde was 13%, 35% en 13% in die drie gebiede. Inpeksie van witluis op vrugte was gedoen om te kyk na persentasie parasitisme van die volwasse witluis, wat die *Anagyrus vladimiri* sal aanval. Vrugte was in houers geplaas om te sien watter spesies parasiete sal uitbroei. In Hoedspruit het *Anagyrus vladimiri* en *Leptomastix dactylopi* hoofsaaklik uitgebroei. In Burgersfort het twee primêre parasiete van witluis uitgebroei (*Anagyrus vladimiri* en *Coccidoxenoides perminutus*) en 3 sekondêre parasiete (*Coccophagus rusti* Compere, *Chartrocerus* sp. en *Pachyneuron* sp.). Die persentasie van sekondêre parasitisme het heelwat toegeneem in 2021 en was moontlik die rede vir laer persentasies van primêre parasitisme wat waargeneem was in die boorde. Dit is belangrik om te kyk na die effek van sekondêre parasitisme op ons biologiese beheer program sodat ons die strategie van *Anagyrus vladimiri* vrylaatings vir witluis beheer verbeter kan word in die toekoms.

## 4 PORTFOLIO: DISEASE MANAGEMENT

### 4.1 PORTFOLIO SUMMARY

By Jan van Niekerk (Portfolio Manager: DM, CRI)

Sustainable production of high quality citrus fruit is dependent on successful management of various pre and postharvest pathogens. In order to focus on all of these, the Disease Management portfolio of CRI is divided into different research programmes. These are preharvest (Fruit and Foliar with CBS and Soilborne diseases), Graft Transmissible Diseases (GTD) and postharvest diseases programmes (PHD). In order to focus on the research aimed at preparing the South African citrus industry for the arrival of Asian Citrus Greening (HLB), the HLB Research Programme was furthermore established in 2021. Within each programme specific industry research needs are addressed while proactive research is done to prepare the industry for any future challenges pertaining to disease management.

Within the GTD programme the focus is aimed at preventing the damaging effects of these pathogens, such as citrus tristeza virus (CTV) and citrus viroids. Due to the importance of CTV cross-protection, field trials (Project 1173) are done to evaluate the best single-strain CTV source for their performance in different citrus types. These trials also serve to identify the suitability of strains to be used for CTV infectious clone construction in Project 1160. Within this project, several novel control strategies are developed for the management of the Liberibacter pathogen of HLB and also its vector, *Diaphorina citri*. In early results comparing the horticultural performance of field-cut propagation material versus budwood supplied by the Citrus Improvement Scheme (CIS) it is clear that the certified material from the CIS performs much better (Project 1074). However, despite the use of certified material, citrus viroids remain a recurring problem in the nursery due to their mechanical

transmission. This makes correct rootstock selection important. In Project 1155, current commercial and newly imported rootstocks are evaluated in a field trial to determine their sensitivity. Currently shoot-tip grafting is used to eliminate any graft transmissible pathogens from plant material. Successful elimination is furthermore at this stage determined by biological indexing and molecular screening. In an attempt to speed up this process, Project 1241 is aimed to investigate if high throughput sequencing (HTS) based diagnostics has potential to fast-track the currently used STG pipeline. Due to both '*Candidatus*' *Liberibacter asiaticus* (Las) and *Diaphorina citri* being present on the African continent and the need for early detection, a real-time diagnostics assay was developed that is able to detect and differentiate between Las and Laf (Project 1200).

The preharvest diseases programme focusses on soilborne diseases, fruit and foliar diseases as well as Citrus Black Spot (CBS). Strong emphasis is being placed on understanding the different pathogens better through epidemiological studies as well as finding alternative control strategies or improving the current strategies used. Projects 762 and 1030 investigate control of nematodes and soilborne pathogens. The data from 762 is showing that pre-plant soil fumigation is promoting tree growth. In Project 1030 it was seen that commercially available biocontrol agents are variably effective against the different soilborne pathogens. Effective biocontrol will therefore be achieved by mixing different agents. Project 1068 focusses on studying the complex Valley Bushveld Decline occurring in the Gamtoos and Sunday's River valleys. To date a pathogen complex involved has been identified along with some potential foliar treatments that can contribute to maintaining tree health. To identify a less susceptible rootstock, a rootstock trial was furthermore established in March 2021 in Addo. Project 1215 developed detection and quantification techniques that will enable better diagnostics for citrus soilborne pathogens and also to study them in more detail. Also in this project, potential bacterial and fungal biocontrol agents were isolated from citrus trees. These were evaluated for the control of different soilborne pathogens, *in vitro* and *in vivo*. However, as seen in Project 1030, the different biocontrol agents varied in efficacy against the different species of soilborne pathogens, indicating that a mixture of biocontrol agents will in all likelihood give the best control of these pathogens.

Due to CBS and *Alternaria* brown spot (ABS) having such a negative impact on the fruit exports, a large focus is given to the study of these two diseases. Projects 970 and 750 are respectively focused on improving CBS and ABS fruit protection programmes. This is done by evaluating new fungicides, adjuvants, oils and also contact fungicides that can replace currently used products. From these projects some promising actives have already been identified. Apart from investigating products for disease control, the application of these products is also getting attention in Project 1132. Here the use of the tree row volume concept has shown promise in reducing spray volumes without negatively affecting spray deposition.

Due to fruit and foliar disease management that cannot be reliant on chemical control only, studying disease or pathogen epidemiology is just as important. In Project 1187 the effect of protective netting on CBS and ABS disease development are being studied while Project 1186 investigate ontogenic resistance development towards CBS infection in different citrus types. The role of pruning debris as inoculum in CBS development forms the focus of Project 1223, while Project 1242 is aimed at understanding the differences in citrus types' sensitivity to CBS development. In previous projects, the CRI PhytRisk platform was established as a management tool for CBS management. In order to further refine the CBS prediction models used on this platform Project 1238 are being conducted in conjuncture with 1244 that are aimed at establishing the infection parameters of CBS ascospores. The last CBS focused project is 1235 that is aimed at better understanding CBS population structures, not only in South Africa but also in other citrus producing areas such as the USA.

In recent years, *Botrytis* has increased in prominence not only on lemons, but also on other citrus types. This led to the inception of Project 1236 where the focus is development of a *Botrytis* prediction model as well as finding effective control measures.

Management of postharvest disease is becoming more difficult due to market pressure on the fungicides used for the management of these diseases. Therefore Project 123 has over the years focused on evaluating any products that have potential as postharvest remedies. In the past season some promising sanitizers were identified. Due to the notification in 2019 about the potential loss of imazalil in future, two projects 1250 and 1251 were started. In 1250 the focus was to optimize the aqueous application of alternative postharvest fungicides that could potentially be used to replace imazalil. In Project 1251 on the other hand, these same

fungicides were tested in wax applications to determine if any of them have potential to replace imazalil in wax. From both these projects good results were obtained that will enable optimal use of these fungicides for the control of postharvest *Penicillium* decay. The last project in this programme, 1165, studied the occurrence of fungal decay on wooden pallet bases and the source of OPP residue contamination on fruit. In the outcome, products were identified that could replace OPP as treatment of the wood used in pallet manufacture as the OPP used to treat the wood was identified as the source of OPP residues on fruit.

## PORTEFEULJE-OPSOMMING

Volhoubare produksie van hoë kwaliteit sitrusvrugte is afhanklik van die suksesvolle bestuur van verskeie voor- en na-oes patogene. Ten einde op al bogenoemde te kan fokus, is die Siektebestuur portefeulje van CRI in verskillende navorsingsprogramme verdeel: Voor-oes Siektes (Vrug en Blaar met SSV en Grondgedraagde siektes), Ent-oordraagbare Siektes (GTD) en Na-oes Siektes programme (PHD). Ten einde die navorsing te fokus met die doel om die Suid-Afrikaanse sitrusbedryf voor te berei vir die aankoms van Asiatiese Sitrusvergroening (HLB), is die HLB Navorsingsprogram verder in 2021 gevestig. Spesifieke navorsingsbehoefte van die bedryf word binne elke program aangespreek, terwyl pro-aktiewe navorsing gedoen word om die bedryf vir enige toekomstige uitdagings met betrekking tot siektebestuur voor te berei.

Binne die GTD program is die fokus gerig op die voorkoming van skadelike effekte van hierdie patogene, soos sitrus tristeza virus (CTV) en sitrus viroïede. Weens die belang van CTV kruisbeskerming, word veldproewe (Projek 1173) gedoen om die beste enkel-ras CTV bron te evalueer vir hul effektiwiteit in verskillende sitrustipes. Hierdie projekte dien ook om die geskiktheid van rasse te bepaal vir hul gebruik vir CTV aansteeklike kloon konstruksie in Projek 1160. Binne hierdie projek word verskeie nuwe beheerstrategieë ontwikkel vir die bestuur van die Liberibacter patogeen van HLB en ook sy vektor, *Diaphorina citri*. Vroeë resultate, wat die hortologiese prestasie van veld-gesnyde voortplantingsmateriaal teenoor okuleerhout wat deur die Sitrus Verbeteringskema (SVS) voorsien is, vergelyk, dui duidelik daarop dat die gesertifiseerde materiaal vanaf die SVS baie beter vaar (Projek 1074). Ten spyte van die gebruik van gesertifiseerde materiaal, bly sitrus viroïede 'n probleem wat herhalend in die kwekery voorkom weens hul meganiese oordraging. Dit maak die korrekte onderstam seleksie belangrik. In projek 1155 word huidige kommersiële en nuut-ingevoerde onderstamme in 'n veldproef geëvalueer om hul sensitiwiteit vas te stel. Tans word loot-punt enting (STG) gebruik om ent-oordraagbare patogene vanuit plantmateriaal te elimineer. Suksesvolle eliminasië word verder op hierdie stadium bepaal deur biologiese indeksering en molekuleêre sifting. In 'n poging om die proses te verhaas, het Projek 1241 ten doel om vas te stel of hoë deurvloei volgorde-bepaling (*high throughput sequencing*, HTS) gebaseerde diagnostiek die potensiaal het om die huidig gebruikte STG pyplyn vinniger te maak. Weens die teenwoordigheid van beide '*Candidatus*' Liberibacter asiaticus (Las) en *Diaphorina citri* op die Afrika vasteland, en die behoefte aan vroeë opsporing, is 'n intydse diagnostiese toets ontwikkel, wat in staat is om Las en Laf waar te neem en tussen hulle te onderskei (Projek 1200).

Die voor-oes siekteprogram fokus op grondgedraagde siektes, vrugsiektes, asook Sitrus Swartvlek (SSV). Sterk klem word daarop gelê om die verskillende patogene beter deur epidemiologiese studies te verstaan, asook die vind van alternatiewe beheerstrategieë of die verbetering van huidige strategieë wat gebruik word. Projekte 762 en 1030 ondersoek beheer van aalwurms en grondgedraagde patogene. Die data vanaf 762 toon dat voor-plant grondberoking boomgroei bevorder. In projek 1030 is gesien dat kommersieel beskikbare bio-beheer agente varieer in hul effektiwiteit teen die verskillende grondgedraagde patogene. Effektiewe bio-beheer sal dus verkry word deur die verskillende agente te meng. Projek 1068 fokus op die bestudering van die komplekse *Valley Bushveld Decline* wat in die Gamtoos- en Sondagsriviervallei voorkom. Tot op datum is 'n patogeenkompleks geïdentifiseer wat betrokke is, tesame met 'n paar potensieël blaarbehandelings wat daartoe kan bydra om boomgesondheid in stand te hou. Ten einde 'n minder vatbare onderstam te identifiseer, is 'n onderstamproef in Maart 2021 in Addo gevestig. Projek 1215 het opsporings- en kwantifiseringstegnieke ontwikkel wat tot beter diagnostiek vir sitrus grondgedraagde patogene sal lei en dit moontlik maak om hul in meer besonderhede te bestudeer. Potensieël bakteriese en swam bio-beheer agente is ook in hierdie projek vanaf sitrusbome geïsoleer. Hulle is vir die beheer van verskillende grondgedraagde patogene, *in vitro* en *in vivo*, geëvalueer. Soos gesien in projek 1030, het die verskillende bio-beheer agente egter gevarieer in hul doeltreffendheid teen die verskillende spesies grondgedraagde patogene, wat aandui dat 'n mengsel van bio-beheer agente na alle waarskynlikheid die beste beheer van hierdie patogene gee.

Aangesien SSV en *Alternaria* Bruinvlek (ABV) so 'n negatiewe impak op vrug-uitvoere het, word groot fokus geplaas op die studie van hierdie twee siektes. Projekte 970 en 750 fokus onderskeidelik op die verbetering van SSV en ABV vrugbeskerminsprogramme. Dit word gedoen deur die evaluasie van nuwe fungisiedes, bymiddels, olies, en ook kontakfungisiedes wat produkte wat tans gebruik word, te kan vervang. 'n Paar belowende aktiewe is alreeds vanuit hierdie projekte geïdentifiseer. Behalwe vir die ondersoek van produkte vir siektebeheer, kry die toedien van hierdie produkte ook aandag in Projek 1132. Hier toon die gebruik van die boom-ry-volume-konsep belofte in die vermindering van spuitvolumes sonder om die spuitneerlegging negatief te beïnvloed.

Aangesien vrug- en blaarsiektebestuur nie net van chemiese beheer afhanklik kan wees nie, is die bestudering van siekte- of patoogeen-epidemiologie net so belangrik. In projek 1187 word die effek van beskermende net op SSV en ABV siekte-ontwikkeling bestudeer, terwyl projek 1186 ontogeniese weerstandsontwikkeling teenoor SSV infeksie in verskillende sitrustipes ondersoek. Die rol van snoeisels as inokulum in SSV ontwikkeling vorm die fokus van projek 1223, terwyl projek 1242 daarop gefokus is om die verskille in sitrustipes se sensitiviteit teenoor SSV ontwikkeling te verstaan. In vorige projekte is die CRI PhytRisk platform as 'n bestuursgereedskap vir SSV bestuur gevestig. Ten einde die SSV voorspellingsmodelle wat op hierdie platform gebruik word, verder te verfyn, word projek 1238 in samewerking met 1244 uitgevoer, wat daarop gemik is om die infeksie parameters van SSV askospore vas te stel. Die laaste SSV gefokusde projek is 1235 wat ten doel het om SSV populasie strukture beter te verstaan – nie net in Suid-Afrika nie, maar ook in ander sitrusproduserende areas soos in die V.S.A.

In meer onlangse jare, het die belang van *Botrytis* toegeneem, nie net op suurlemoene nie, maar ook op ander sitrustipes. Dit het gelei tot die ontstaan van Projek 1236 waar die fokus op die ontwikkeling van 'n *Botrytis* voorspellingsmodel is, asook die vind van effektiewe beheermaatreëls.

Bestuur van na-oes siektes word al moeiliker weens markdruk op die fungisiedes wat gebruik word vir die bestuur van hierdie siektes. Projek 123 het dus oor die jare gefokus op die evaluasie van enige nuwe produkte wat potensiaal as na-oes middels het. In die laaste seisoen is 'n paar belowende saniteerders geïdentifiseer. Weens die kennisgewing in 2019 oor die moontlike staking van imazalil in die toekoms, is twee projekte, 1250 en 1251, begin. In 1250 was die fokus op die optimalisering van die toediening in water van alternatiewe na-oes fungisiedes wat moontlik gebruik kan word om imazalil te vervang. In Projek 1251 aan die ander kant, is hierdie selfde fungisiedes in wakstoedienings getoets ten einde vas te stel of enige van hulle potensiaal het om imazalil in waks te vervang. Goeie resultate is vanuit hierdie projekte verkry wat die optimale gebruik van hierdie fungisiedes vir die beheer van na-oes *Penicillium* verval moontlik maak. Die laaste projek in hierdie program, 1165, het die voorkoms van verval van hout palletbasisse weens swamme bestudeer, en die bron van OPP residu kontaminasie op vrugte. In die uitkoms is produkte geïdentifiseer wat gebruik kan word om OPP, as behandeling van hout wat in pallet vervaardiging gebruik word, te vervang, aangesien die OPP wat gebruik is om die hout te behandel as die bron van OPP residue op vrugte geïdentifiseer is.

## 4.2 PROGRAMME: GRAFT TRANSMISSIBLE DISEASES

Programme coordinator: G. Cook (CRI)

### 4.2.1 Programme summary

Vegetative propagation by grafting carries the potential to disseminate pathogens that are present in budwood and include bacteria, viruses and viroids. The first line of defence is to ensure supply of pathogen-free propagation material, which is reliant on good diagnostic capabilities. In instances where pathogens are vectored by insects that occur endemically, further control strategies are required.

Cross-protection is a management strategy applied to mitigate the damaging effects of citrus tristeza virus (CTV). An ongoing research facet is to investigate the effect of CTV strains and to determine which are ultimately required for cross-protection. CTV is a complex of strains and variants and CTV strain diagnostics has progressed significantly, enabling identification and characterisation of single-strain sources. Field trials evaluating performance of single-strain CTV sources in various citrus types are ongoing with no significant

differences observed with tree growth or yield at the early stage of these trials. These trials are done with the additional aim of evaluating them as potential cross-protection sources (4.2.5).

Despite the use of certified budwood, viroids remain problematic in the industry due to their mechanical transmissibility. Newly planted orchards are especially vulnerable if susceptible rootstocks are used. A field trial is underway to evaluate which commercial and new rootstock selections are sensitive to viroids (4.2.4). Results of a field trial comparing the horticultural performance of field-cut propagation material to budwood supplied by the Citrus Improvement Scheme (CIS) confirms that better growth and tree health is achieved using CIS propagation material (4.2.3).

Shoot-tip grafting is the technique used to render all new citrus accessions entering the CIS pathogen free. Confirmation of the successful elimination of pathogens is done using both molecular and biological screening. Project 1241 (4.2.7) is aimed to investigate the use of high throughput sequencing (HTS) to potentially fast-track diagnostic processes in the STG pipeline and enable quicker release of cultivars to the industry. Additionally, a new virus was detected with HTS and further research was conducted to investigate the impact of the virus and develop diagnostic assays.

The confirmed presence of both 'Candidatus' *Liberibacter asiaticus* (Las) and *Diaphorina citri* on the African continent necessitates preparation for an incursion event. Detection capabilities are vital for early detection and control interventions. A real-time diagnostic assay was developed to detect and differentiate citrus Liberibacters in a single assay. The assay is included in the standard operating procedures for HLB detection. Additionally, a cheap, reliable DNA extraction was developed as a replacement for commercial kit extractions, allowing for the processing of more samples. These achievements are presented in the final report of Project 1200 (4.2.2).

## Opsomming

Vegetatiewe vermeerderingsmetodes soos enting dra die potensiaal om patogene, soos bakterieë, virusse en viroïedes, teenwoordig in die enthout, te versprei. Die eerste vereiste is dus om gesonde voortplantingsmateriaal te verskaf wat afhanklik is van goeie diagnostiese vermoëns. In gevalle waar patogene versprei word deur insekte wat endemies voorkom, word verdere beheerstrategieë vereis.

Kruisbeskerming is een van die bestuurstrategieë wat toegepas word om die skadelike effekte van sitrus tristeza-virus (CTV) te verminder. Navorsing is daarop gerig om die effek van CTV-rasse te ondersoek en om te bepaal watter benodig word vir kruisbeskerming. CTV is 'n kompleks van rasse en variante. Diagnostiek om CTV-rasse te identifiseer het aansienlik verbeter wat die identifikasie en karakterisering van enkelrasbronne moontlik maak en veldproewe van verkillende sitrustiepes word gedoen om die prestasie van verskillende enkel CTV rasse te toets. Tot dusver is daar nog nie verskille in boomgroei of oes waargeneem in hierdie vroeë stadium van hierdie proewe nie (4.2.5).

Ten spyte van die gebruik van gesertifiseerde enthout, is viroïede steeds problematies in die industrie vanweë hul meganiese oordraagbaarheid. Nuut aangeplante boorde is veral kwesbaar as vatbare onderstamme gebruik word. Om beter te verstaan watter kommersiële en potensieel belangrike onderstamme sensitief is, is 'n veldproef daargestel (4.2.4).

Huidige resultate van 'n veldproef waarin die tuinbouprestasies van veldgesnyde voortplantingsmateriaal vergelyk word met voortplantingsmateriaal verskaf deur die Sitrusverbeteringsskema (SVS), toon dat beter groei en boomgesondheid verkry word van CIS-voortplantingsmateriaal (4.2.3).

Groeipuntenting (GPE) word gebruik om sitrus materiaal te vrywaar van ent-oordraagbare patogene voor vrystelling van nuwe kultivars. Bevestiging van die suksesvolle eliminering van patogene word gedoen met behulp van beide molekulêre en biologiese tegnieke. Projek 1241 (4.2.7) is daarop gemik om die gebruik van diagnostiek, gebaseer op hoë-deurset volgordebepaling gebaseerde opsporing (HTS), te ondersoek om die diagnostiese prosesse van die GPE-pyplyn te bespoedig en vinniger vrystelling van kultivars aan die bedryf te

bewerkstellig. Verder is 'n nuwe virus met HTS opgespoor en ondersoek gedoen om die impak van die virus te bepaal asook 'n diagnostiese toets te ontwikkel.

Die bevestigde teenwoordigheid van beide 'Candidatus' *Liberibacter asiaticus* (Las) en *Diaphorina citri* op die Afrika-kontinent noodsaak voorbereiding vir die moontlike inbeweeg van die pes en patoogeen. Diagnostiese vermoëns is noodsaaklik vir vroeë opsporing en beheer. 'n Diagnostiese toets is ontwikkel om sitrus-Liberibacters op te spoor en te onderskei in 'n enkele toets. Die toets is ingesluit in die standaardprosedures vir HLB-opsporing. Daarbenewens is 'n goedkoop, betroubare DNA-ekstraksie ontwikkel as plaasvervanger vir ekstraksies voorheen gedoen met kommersiële reagense, wat die verwerking van aansienlik meer monsters moontlik maak. Hierdie prestasies word in die finale verslag van Project 1200 (4.2.2) aangebied.

#### 4.2.2 FINAL REPORT: Validation of primer regions used for the differentiation of Asian HLB, African greening and its subspecies.

Project 1200 (1 April 2018 – 31 March 2021) by Ronel Roberts (ARC-PHP) and Glynnis Cook (CRI)

##### Summary

Three *Liberibacter* species and two psyllid vectors are associated with citrus on the African continent. In East and Southern Africa, citrus is affected by the African Greening disease, associated with the bacterial pathogen '*Candidatus Liberibacter africanus*' (Laf). However, '*Ca. L. asiaticus*' (Las), the bacterium associated with Huanglongbing (HLB), was identified in northern Ethiopia in 2010 and recently in southern Kenya. '*Ca. L. africanus* subsp. *clausenae*' (LafCl), a bacterium first described from an indigenous Rutaceae species in South Africa, was misdiagnosed as Las in citrus in Uganda and Tanzania due to a non-specific diagnostic assay. *Trioza erytraeae*, the vector of Laf, is present in East and Southern Africa. Additionally, in 2016, *Diaphorina citri*, the natural vector of Las, was reported in Tanzania and later also in Kenya and Zanzibar. The presence of both Las and *D. citri* in East Africa and the probable dispersal on the continent is of concern for southern African citrus production. Early detection of Las is critical for rapid response to an incursion. However, the presence of three *Liberibacter* species occurring in citrus in Africa requires a diagnostic assay capable of differentiating the species, preferably in a single assay. The ribonucleotide reductase  $\beta$ -subunit (*nrdB*) gene region was used as a target region to develop a diagnostic assay to detect and differentiate the citrus *Liberibacter* species. The *nrdB* genome region of Laf samples from different geographical regions was sequenced and no sequence variation was observed indicating that this is a conserved region and a reliable target site for diagnostic development. Additionally, the nucleotide sequences of the *nrdB* gene region were determined for three Laf subspecies. The LafC, LafV and LafCl sequences share 89%, 88% and 89% sequence identity with Laf, 75%, 75% and 76% with Las and 79%, 78% and 80% with '*Candidatus Liberibacter americanus*' (Lam), respectively. A *Liberibacter* detection assay, targeting the *nrdB* gene, was developed which differentiates Laf and Laf sub-species from Las and Lam. Furthermore, a non-commercial DNA extraction method was validated to replace the commercial extraction kits for DNA extractions from psyllids for survey purposes.

##### Opsomming

Drie *Liberibacter* spesies en twee bladvlooi vektore word geassosieër met sitrus op die Afrika kontinent. In Oos en Suider Afrika word sitrus geaffekteer deur Vergroeningsiekte, geassosieër met die bakteriese patoogeen, '*Candidatus Liberibacter africanus*' (Laf). '*Ca. L. asiaticus*' (Las), die bakterieë geassosieer met Huanglongbing (HLB), was geïdentifiseer in die noorde van Ethiopia in 2010 en onlangs in die suide van Kenia. '*Ca. L. africanus* subsp. *clausenae*' (LafCl), 'n bakterie wat die eerste keer van 'n inheemse Rutaceae-spesie in Suid-Afrika beskryf is, is verkeerd gediagnoseer as Las in sitrus in Uganda en Tanzanië weens 'n nie-spesifieke diagnostiese toets. *Trioza erytraeae*, die vektor van Laf, is in Oos en Suider Afrika aanwesig. In 2016 is *Diaphorina citri*, die natuurlike vektor van Las gerapporteer in Tanzania en later ook in Kenya en Zanzibar. Die aanwesigheid van beide Las en *D. citri* in Oos Afrika, en die moontlike verspreiding op die kontinent, is 'n bedreiging vir sitrus produksie in suider Afrika. Vroeë opsporing van Las is van kritieke belang vir vinnige reaksie om verspreiding van die patoogeen te verhoed. Die aanwesigheid van drie *Liberibacter*-spesies wat in sitrus in Afrika voorkom, vereis egter 'n diagnostiese toets wat die spesies kan onderskei, verkieslik in 'n enkele toets. Die 'ribonucleotide reductase  $\beta$ -subunit' (*nrdB*) geen word tans ondersoek as 'n teikengebied vir die

ontwikkeling van so 'n diagnostiese toets. Die nukleotied volgorde van hierdie genoom gebied was bepaal vir Laf isolate afkomstig van verskillende geografiese streke en geen variasie is waargeneem nie wat daarop dui dat dit 'n betroubare teikengebied vir diagnostiek ontwikkeling is. Daarbenewens is die nukleotiedvolgorde van die *nrdB*-geengebied vir drie Laf-subspesies bepaal. Die nukleotiedvolgordes van LafC, LafV en LafCI deel 89%, 88% en 89% nukleotied ooreenkoms met Laf, 75%, 75% en 76% met Las en 79%, 78% en 80% met 'Candidatus Liberibacter americanus' (Lam), onderskeidelik. 'n Liberibacter-opsporingstoets, gerig op die *nrdB*-geen, is ontwikkel wat die Laf- en Laf-subspesie van Las en Lam onderskei. Verder is 'n nie-kommersiële DNA-ekstraksie-metode verfyn om die kommersiële ekstraksiepakette vir DNA-ekstraksies uit psylliede vir opname-doeleindes te vervang.

## Introduction

'Candidatus Liberibacter asiaticus' (Las), the bacteria that causes Asian citrus greening disease or Huanglongbing and its insect vector, *Diaphorina citri* (Psyllidae), have caused devastation in the citrus industries in Florida, USA and Brazil, apart from their region of origin in the Far East. Both are now present on the African continent, with Las identified from Ethiopia (Saporani *et al.*, 2010) and Kenya (Ajene *et al.*, 2020a), and *D. citri* detected in Tanzania (Shimwela *et al.*, 2016), Kenya (Rwomushana *et al.*, 2017) and Ethiopia (Ajene *et al.*, 2020b). Kaylebi *et al.* (2015) additionally reported the presence of Las in Uganda, however, CRI along with ARC-PHP and ICIPE demonstrated that this report was due to the non-specific amplification of 'Ca. L. africanus subsp. clausenae' (LafCI), initially identified from indigenous *Clausena anisata* (Rutaceae), with the real-time PCR assay described by Li *et al.* (2006) (Roberts *et al.*, 2017). This mis-identification demonstrated the need for a reliable Las-specific diagnostic test, especially in East Africa where LafCI is frequently found infecting citrus.

In addition to LafCI, four subspecies of Laf have been described from indigenous rutaceous species and include 'Ca. L. africanus subsp. capensis' (LafC) (Garnier *et al.*, 2000), 'Ca. L. africanus subsp. vepridis' (LafV), 'Ca. L. africanus subsp. zanthoxyli' (LafZ) (Roberts *et al.*, 2015) and 'Ca. L. africanus subsp. teclae' (LafT) (Roberts and Pietersen, 2016). The indigenous rutaceous species from which LafV and LafZ were characterized are known hosts of *Trioza erythrae* (Moran, 1968), but it is not known if these Laf subspecies can be transmitted to citrus, as was shown for LafCI.

Zheng *et al.* (2016) identified the reductase  $\beta$ -subunit gene (*nrdB*) as an alternative target region for Liberibacter detection due to a higher copy number of this gene in the Las genome compared to the 16SrDNA target region. Sequence data for this gene region is only available for Las and Laf, but not the Laf sub-species. Characterization of this region of the various Laf subspecies was required for diagnostic development.

An additional objective of the project was to compare DNA extraction methods from triozyds for DNA quality, ease of extraction and cost.

## Stated objective

Obtain complete *nrdB* sequences for Laf subspecies and validate the specificity of the RNR-based HRM assay.

## Materials and methods

### *Confirmation of Laf nrdB nucleotide (nt) sequence*

Overlapping primers were designed to span the *nrdB* gene of Laf based on the complete genome of Laf (Genbank accession CP004021) (Table 4.2.2.1). PCR amplification was performed using 0.5  $\mu$ l DNA of each extract in the following reaction mix; 5  $\mu$ l of 5 $\times$  Green GoTaq<sup>®</sup> Flexi buffer and 0.13  $\mu$ l GoTaq<sup>®</sup> G2 Flexi DNA polymerase (5U/ $\mu$ l) (Promega, Madison, WI, USA), 2  $\mu$ l 25mM MgCl<sub>2</sub>, 200nM of each primer and 200nM dNTP mix in a final reaction volume of 25  $\mu$ l. Cycling conditions included an initial denaturation at 94°C for 5min followed by 35 cycles of 94°C for 20s, 55°C for 20s and 72°C for 40s with a final extension step at 72°C for 10min. PCR amplification products were gel extracted and bi-directionally sequenced. Consensus sequences

were generated using BioEdit software and subjected to BLAST analyses for sequence verification. Sequences were aligned using the Mafft online tool.

**Table 4.2.2.1.** PCR primers designed for amplification of overlapping fragments of the Laf *nrdB* gene region. Primer sequences and expected product sizes are shown.

Primer Name	Forward Primer sequence	Reverse Primer sequence	Size (bp)
LafRNR_new1	TACCAAGGAATCCTCGTGGC	TGCTCTTTGGAAGTCGGCTG	665
LafRNR_new4	CGGTTCAAAAACGGGATCCAA	ACATTATGCGCGACGAATCAG	269
LafRNR_new5	GAGATGGACTGATTCGTCCG	TGCAGGTTTAGGACCCATTCA	358
LafRNR_new9	TCAAGACCGAGACTTCCACA	TGCAGGTTTAGGACCCATTCA	411

*Nucleotide sequencing of the nrdB gene of Laf samples from various geographical regions*

Eight previously identified Laf positive samples, from different geographical locations, were used to investigate possible sequence diversity of the Laf *nrdB* gene. Samples obtained in South Africa were from Pretoria in the Gauteng Province, Port Elizabeth in the Eastern Cape Province, Letsitele in the Limpopo Province, Nelspruit in the Mpumalanga Province as well as from KwaZulu-Natal Province. Samples from Tanzania, Uganda and Kenya were also included in the analysis. Primers LafRNR\_new4F and LafRNR\_new9R were used to amplify a 900 bp fragment and the amplicons bi-directionally sequenced as described above.

*Determination of the nrdB nucleotide sequences of Laf-subspecies:*

High-throughput sequencing reads previously obtained from a LafC positive sample were mapped to the Laf *nrdB* sequence using the CLC Genomics Workbench. The consensus sequence was extracted and used as a template for primer design (Table 4.2.2.2). Samples containing each of the Laf-subspecies (i.e. LafC, LafCl, LafT, LafV and LafZ) were amplified using these primers. Laf, Las and Lam were included as controls. LafCl samples from both citrus and *Clausena anisata* were included. PCR amplification products were bi-directionally sequenced as above. Sequences were aligned with equivalent sequence fragments of Laf, Las and Lam and phylogenetic analysis was done using MegaX software.

**Table 4.2.2.2.** PCR primers designed for amplification of overlapping fragments of the *nrdB* gene region for Laf-subspecies. Primer sequences and expected product sizes are shown.

Primer Name	Forward Primer sequence	Reverse Primer sequence	Size (bp)
LafCRn_new1	TCGTGGCATAGTATCTTCCCC	CTCGGTCTTGATGAAGGAGAGT	436
LafCRn_new4	GTATATTGTAAAGCCCAGCTTGC	TCGGTCTTGATGAAGGAGAGT	165
LafCRn_new5	CTCTCCTTCATCAAGACCGAGACT	TGCAGGCTTAGGGGATATTCA	421

*Diagnostic assay to differentiate citrus Liberibacter species*

Various primers were designed targeting the *nrdB* gene region and tested for simultaneous detection and differentiation of citrus Liberibacter species. Primers CLaHRM\_F 5'-CGCATDATRTATTGATATTGTTTC-3' and CLaHRM\_R 5'-GTKTTTGAAGGAATGTGGTTTAA-3' were designed to amplify a 103bp product in a SYBR Green real-time PCR assay followed by a melt analysis for species differentiation. Reaction components comprised SensiFast SYBR Green Master Mix (Bioline, USA) and 250nM of each primer. Cycling parameters included an initial denaturation of 3 min at 95°C and 40 cycles of 95°C for 10s, 57°C for 15s and 72°C for 15s. Acquisition on the green channel was recorded at the end of the extension step followed by a melt curve analysis to differentiate the Liberibacter species and to identify primer-dimers and non-specific amplifications.

*Comparative analysis of various DNA extraction methods from trioizids*

Orchards in Swellendam were identified with high infestations of *Trioza erytreae* the African citrus trioza (ACT). Traps were placed in these orchards for a month. In excess of 150 ACT were removed from the traps and placed in ethanol. Five DNA extraction methods were compared by extracting DNA from 20, single ACT per method. The methods included two commercially supplied kits and three non-commercial methods. The non-commercial methods included an extraction buffer containing hexadecyl-trimethyl-ammonium bromide (CTAB), a Tris buffer with sodium dodecyl sulphate (SDS) and the same Tris and SDS buffer with an additional proteinase K (protK) digestion step (method is provided as an addendum). The commercial kits included the Qiagen DNeasy Blood and Tissue extraction kit (currently prescribed in the HLB testing schedule of the SA HLB action plan) and a prepGEM Universal kit (microGEM) which were used as prescribed by the manufactures.

The extraction efficiencies were determined using a probe-based real-time PCR targeting the cytochrome oxidase I gene of ACT. The Ct values obtained using these single trioza DNA extractions were compared statistically.

## Results and discussion

### *Confirmation of nrdB sequence of Laf*

A partial fragment of the *nrdB* gene was assembled for Laf by sequencing overlapping amplicons using various primers. A consensus sequence of 936bp for Laf was obtained. A BLAST analysis showed 99% sequence identity with the published Laf genome (Genbank accession: CP004021) and 75%, 81% and 78% nucleotide identity with Las, Lam and *Liberibacter crescens* respectively.

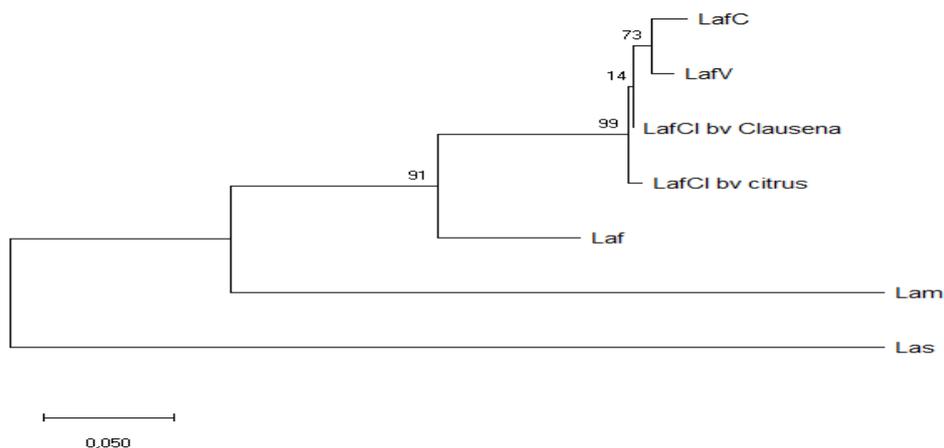
### *Nucleotide sequencing of the nrdB gene of Laf samples from different geographical regions*

Partial nucleotide sequences of the Laf *nrdB* gene were obtained from 8 Laf samples from different geographical regions in Africa. Sequence alignments of the respective nucleotide sequences from these samples showed 100% nucleotide sequence identity between the samples indicating that this is a conserved region and a reliable target site for diagnostic development.

### *The nrdB nucleotide sequences of Laf-subspecies*

PCR amplification of the *nrdB* region was successful for samples containing LafC, LafCI (from both citrus and *C. anisata*) and LafV. Nucleotide sequences of 830bp were assembled for these Laf-subspecies. Sequences were not obtained for LafT and LafZ.

The partial sequences for the *nrdB* gene of LafC, LafV and LafCI shared 89%, 88% and 89% sequence similarity with Laf, 75%, 75% and 76% sequence similarity with Las and 79%, 78% and 80% sequence similarity with Lam, respectively. These differences are illustrated in Figure 4.2.2.1. Additionally, differences in the nucleotide sequences were observed for LafCI from citrus and those obtained from *C. anisata*. These shared 99% sequence identity with 6 bp pair changes identified between samples from the two hosts.



**Figure 4.2.2.1.** Maximum-likelihood phylogeny of *Liberibacter* species based on a partial sequence of the *nrdB* gene.

*Diagnostic assay to differentiate citrus Liberibacter species*

Primer set, CLaHRM, amplified a 103 bp product from citrus *Liberibacter* species including Laf, Las and Lam as well as Laf sub-species LafC, LafCI, LafV and LafZ. The real-time melt analysis of the amplification products differentiated Laf, Las and Lam. Melt curve peak for Laf was between 75-76°C, for Las 79-80°C and Lam 77-78°C. The melt curves of amplification products of the Laf subspecies were similar to that of Laf, thus not enabling differentiation of Laf from Laf-subspecies. This assay is none the less an improvement to the standard 16SrDNA assay (Bao *et al.*, 2020) for Las which non-specifically amplifies LafCI, resulting in a false positive result. This assay also enables simultaneous detection of the three citrus *Liberibacter* species.

*Comparative analysis of various DNA extraction methods from triozids*

Efficiencies of the extraction methods differed statistically (Table 4.2.2.3 and 4.2.2.4). The Tris buffer+SDS+protK was the best extraction based on this assessment. Lower Ct values were obtained, indicating higher DNA yields. Additionally, greater consistency in Ct values were obtained as indicated by low standard deviation between samples. This protocol is provided as an addendum.

No amplification was achieved for three samples, two of which were extracted with the prepGEM kit and one using the CTAB method. Both these methods showed high standard deviations in Ct values, indicating lower consistencies with the extractions.

**Table 4.2.2.3.** Real-time PCR Cycle threshold (Ct) statistical values for the various extraction methods.

Extraction method	Min	Max	1st Quartile	Median	3rd Quartile	Mean	Std dev.
CTAB	20.0	31.6	21.0	21.3	22.4	22.3	2.8
Qiagen DNeasy kit	19.1	26.8	19.5	19.8	20.4	20.4	1.7
Tris buffer+SDS	19.4	25.7	20.7	21.2	22.0	21.7	1.5
Tris buffer+SDS+ProtK	15.8	21.1	18.0	18.6	18.9	18.5	1.2
prepGEM	26.8	39.2	27.9	29.5	30.9	30.0	2.9

**Table 4.2.2.4.** Average Cycle Threshold values (Ct) obtained using DNA from various extraction methods.

Extraction Method	Number of positive reactions	Mean Ct	Groups
prepGEM	18	30.0	A
CTAB	19	22.3	B
Tris buffer+SDS	20	21.7	B
Qiagen DNeasy kit	20	20.4	C
Tris buffer+SDS+ProtK	20	18.5	D

Treatments with the same letters for Fisher least square difference do not statistically differ at  $P = 0.05\%$ .

A further 58 specimens from the same traps were tested with the Tris buffer+SDS+protK method. The Ct values obtained were in the same range as the initial 20 samples confirming the consistency of the extraction method.

## Conclusion

Nucleotide sequences generated for Laf and the Laf subspecies indicated that the *nrdB* gene region has greater inter species divergence compared to the 16S rDNA gene, commonly used for Liberibacter diagnostics, but the intra-species diversity was low which enabled the use of this genome region to develop a diagnostic assay to differentiate citrus Liberibacter species. The assay is currently included in the standard operating procedures for HLB detection and has been tested in a workshop with participants of the sub-committee on HLB diagnostics (a sub-committee of the HLB Steering Committee).

A cheap, reliable DNA extraction was developed as a replacement for the commercial kit extraction currently prescribed in standard operating procedures. This will enable the processing of more samples.

The successful results of this research will provide more effective tools for the identification of HLB in trapped vectors and citrus plants.

## Future research

The single assay diagnostic cannot differentiate Laf from the Laf sub-species, however, this assay is an improvement on existing citrus Liberibacter diagnostics, further assay development is required once additional sequence data is available for the various Liberibacter species.

The routine use of this diagnostic for citrus Liberibacter detection in psyllids and triozids indicated the presence of other, non-citrus Liberibacters. This highlights that a greater understanding is also required regarding Liberibacter diversity present in the insect vectors.

## Technology transfer

R. Roberts and G. Cook: The complexity of HLB on the African continent. Presented at the 10th biennial Citrus Research Symposium hosted by CRI. From 19-22 August 2018 at Champagne Sports Resort, Drakensberg, KZN.

Roberts R, Cook G, Steyn C, *et al.* Detection of 'Candidatus Liberibacter species' from citrus in east Africa. Presented at the 51st Conference of the South African Society of Plant Pathology, 20-24 January 2019, Club Mykonos Langebaan, Western Cape.

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## Addendum: ACP ACT DNA Extraction Protocol

1. Prepare Extraction Mix in the following ratio for the required number of samples:  
[500µl Tris ACP/ACT Buffer + 20µl 20% SDS + 2.5µl Proteinase K (20mg/ml)] per sample.
2. Place 1-10 psyllids in a 1.5ml microfuge tube and crush with micro-pestle.
3. Add 500µl Extraction Mix.
4. Incubate at 55°C for 1 hour.
5. Incubate at 70°C for 10 min in a water bath to inactivate proteinase K.

6. Add 200µl 5M potassium acetate, shake vigorously, and incubate on ice for 20 min.
7. Centrifuge at 4°C for 20 min (12000 rpm).
8. Recover 600µl of the aqueous phase to a new 1.5ml tube.
9. Add 600µl isopropanol, invert to mix and incubate at room temp for 30 min.
10. Centrifuge at 15°C for 20 min (12000 rpm).
11. Discard supernatant and wash pellet with 200µl of 75% ethanol.
12. Centrifuge at 4°C for 15 min (12000 rpm).
13. Discard supernatant and dry DNA pellet.
14. Re-suspend DNA pellet in 15µl distilled water for single insect. See resuspension table for more than 1 insect:

Insects/sample	Resuspension volume (µl)	Insects/sample	Resuspension volume (µl)
1	15	6	75
2	25	7	90
3	40	8	100
4	50	9	115
5	65	10	125

#### Reagent components:

##### Tris ACP/ACT Buffer pH 8.0 (Per 100 ml):

100 mM Tris-base	1.21 g
50 mM EDTA	1.861 g
500 mM NaCl	2.922 g

##### 5 M Potassium Acetate pH 5.5 (Per 100 ml):

Potassium acetate	35.4 g
Acetic Acid	23.0 ml

#### 4.2.3 PROGRESS REPORT: Comparison of shoot tip grafted citrus with field-cut (old clone) material

Project 1074 (2013 - 2023) by G. Cook, J.H.J. Breytenbach, R. de Bruyn and C. Steyn (CRI)

#### Summary

The assertion that some cultivars are more profitable when trees are made from field-cut material compared to that supplied by the Citrus Improvement Scheme (CIS) is investigated in a trial using two navel and one Valencia cultivar. Graft transmissible pathogens, including viroids and viruses, are removed by shoot tip grafting from accessions submitted to the CIS. Thereafter a citrus tristeza virus (CTV) source is introduced to each accession within the cross-protection programme. Field trees can however acquire a range of graft transmissible pathogens over time, either by means of insect vector transmission or mechanically during routine orchard practices. The objective of this study is to compare tree health, production and fruit characteristics of CIS supplied material with that of field-cut and viroid infected material. Budwood was collected from original field sources of the cultivars, which contained various populations of CTV strains and citrus viroids. These were budded to 'Swingle' citrumelo, 'Carrizo' citrange and 'C35' citrange rootstocks. The same was done for budwood obtained from the CFB and for CFB budwood to which a non-cachexia causing hop stunt viroid (CVd-IIa) source was additionally inoculated. A field trial was established at Burgersfort in 2016. After four and a half years, significant differences in tree growth were observed between treatments. Reduced canopy volumes were associated with field-cut material of all three cultivars. Trees made from CFB supplied budwood were consistently the largest. The 2020 season was the second harvest from the trial. Average yields of trees made with CIS budwood were higher than yields obtained from trees made with field-cut material. A week prior to harvest cold weather conditions with probable freeze damage impacted fruit internal quality resulting in lower acids and juice content of the two navel cultivars. No inference can therefore

be made from the internal fruit quality this season. Results show that tree size was significantly reduced using field-cut budwood for tree production which was also reflected in a yield reduction.

## Opsomming

Sektore binne die sitrusbedryf beweer dat sommige kultivars meer winsgewend is wanneer bome gemaak word van veld-gesnyde materiaal, eerder as dié wat deur die Sitrus Verbeteringskema (SVS) verskaf word. Hierdie aanname word ondersoek in 'n proef wat twee nawel en een Valencia-kultivar insluit. Oordraagbare patogene, insluitend viroïede en virusse, word verwyder van kommersieële kultivars deur middel van groeipuntenting en word daarna geïnkuleer met 'n citrus tristeza virus (CTV) bron vir kruisbeskerming binne die SVS. Veldbome kan egter oor 'n tydperk 'n verskeidenheid ent-oordraagbare patogene optel deur middel van insekvektoroordraging of meganies tydens roetine-boordpraktyke. Die doel van hierdie studie is om boomgesondheid, produksie en vrugteienskappe van veld-gesnyde materiaal, wat verskeie CTV-rasse en sitrusviroïede bevat, te vergelyk met dié van SVS materiaal, asook met SVS materiaal waarop CVd-IIa addisioneel geïnkuleer is. Okuleerhout is op 'Swingle' citrumelo, 'Carrizo' citrange en 'C35' citrange onderstamme ge-inkuleer en 'n veldproef is in 2016 op Burgersfort geplant. Na vier en 'n half jaar is beduidende verskille in boomgroei waargeneem. Verminderde boom volumes is geassosieer met bome gemaak met veld-gesnyde materiaal van al drie kultivars. Bome van SVS materiaal was deurgans groter. Die 2020 seisoen was die tweede oes van die proef. Die gemiddelde opbrengste van bome gemaak met SVS enthout was hoër as die opbrengste verkry van bome was met veld-gesnyde materiaal gemaak was. Een week voor oes het koue weertoestande, met gepaardgaande vriesskade, die interne kwaliteit van die vrugte beïnvloed. Dit het veroorsaak dat die twee nawelkultivars laer sure en sapinhoud gewys het en geen gevolgtrekking kan dus uit die interne vrugkwaliteit hierdie seisoen gemaak word nie. Resultate toon dat boomgrootte aansienlik verminder was met bome gemaak van veldgesnyde enthout en dit het ook in 'n opbrengsvermindering weerspieël.

### 4.2.4 PROGRESS REPORT: Field testing of commercial or potentially important rootstock selections for viroid sensitivity

Project 1155 (2016/7 – 2025/6) by G. Cook, J.H.J. Breytenbach, C. Steyn, R. de Bruyn and J. Joubert (CRI)

## Summary

The choice of rootstock is an important consideration for the establishment of a citrus orchard. Apart from climate and soil suitability, rootstock selection should include considerations for resistance or tolerance to diseases and pests. Viroids are graft-transmissible agents which can induce a range of symptoms dependent on the sensitivity of the rootstock and scion. They are also mechanically transmitted by cutting tools and can unintentionally be introduced to, and spread in nurseries and orchards. Apart from the diseases, Exocortis and Cachexia, viroids can also induce symptoms such as bark cracking and stunting. There is limited experience regarding the effect of viroids on hybrid rootstocks introduced in the past two decades, including new selections from the USA. A field trial is underway to test the sensitivity of these newer commercial or potentially commercial rootstocks to citrus dwarfing viroid and the non-cachexia variant of hop stunt viroid. The trial was planted in October 2019 in the Nelspruit district. The trial trees were inoculated and the transmission success to each trial plant was tested by RT-PCR prior to planting. Despite successful graft take, viroid detection was erratic. The plants were re-inoculated and transmission verification was repeated. Although more transmissions were confirmed, the results indicated erratic distribution in the plants. A relative quantitative assay was developed and viroid distribution and titre were investigated in scions on four rootstock selections. Preliminary analysis suggests that the rootstocks may influence viroid translocation within the scion. Growth parameters including canopy volumes, scion and rootstock circumferences were measured. Differences in growth were observed between rootstocks in accordance with the expected vigour of the respective rootstocks, but the influence of viroids on these growth parameters was not detected at this early stage.

## Opsomming

Die keuse van onderstam is belangrik in die vestiging van 'n sitrus boord. Benewens klimaats- en grondgeskiktheid moet hierdie oorweging weerstand of verdraagsaamheid teenoor siektes en plaë insluit. Viroïede is entoordraagbare entiteite wat verskeie simptome kan veroorsaak op sensitiewe bo- en onderstamme. Weens maklike meganiese oordraging deur snygereedskap en besmette enthout, word viroïede soms, per ongeluk, in kwekerye en boorde versprei. Afgesien van die siektetoestande, 'Exocortis' en 'Cachexia', kan viroïede ook simptome soos baskraak en verdwering veroorsaak. Daar is beperkte ervaring met betrekking tot die effek van viroïede op onderstamme wat die afgelope twee dekades bekendgestel is, insluitende nuwe onderstamme afkomstig uit die VSA. 'n Veldproef is voorberei om die sensitiwiteit van hierdie nuwer, kommersiële of potensieel kommersiële, onderstamme teen 'citrus dwarfing' viroïed en die nie-patogeniese variant van 'hop stunt' viroïed, te toets. Die proef is in Oktober 2019 in die Nelspruit omgewing geplant. Die proefbome was geïnkuleer met die viroïed-behandelings en die oordragsukses na elke proefplant was deur PKR getoets voor uitplant. Viroïed oordrag was oenskynlik nie optimaal nie en die plante was weer geïnkuleer. Alhoewel oordragsukses hoër was, blyk dit of die verspreiding van viroïede in die plant net onegalig is. 'n Kwantitatiewe PKR toets was ontwikkel om die verspreiding en relatiewe hoeveelheid van die viroïede in die bostamme wat gevestig was op vier onderstam seleksies, te ondersoek. Voorlopige resultate dui daarop dat die verspreiding van die viroïede moontlik deur die onderstam beïnvloed word. Die groei van die bome i.t.v. boomvolumes en stam omtrek was gemeet en verskille in lyn met verwagte groeikragtigheid vir elke onderstam is waargeneem, maar die invloed van die viroïede was nog nie op hierdie vroeë stadium sigbaar nie.

### 4.2.5 PROGRESS REPORT: Field evaluation of three single-strain CTV isolates on navel and soft citrus cultivars

Project 1173 (2017/8-2022/3) by G. Cook, J.H.J. Breytenbach, C. Steyn and R. de Bruyn (CRI)

#### Summary

Single-strain citrus tristeza virus (CTV) isolates were characterized and evaluated in various industry cultivars in a glasshouse trial (Project 1056). No detrimental symptoms were associated with these isolates. Selected cultivars and treatments of the trial were planted at various sites to evaluate field performance and to monitor the CTV translocation to new growth of the trees. This is done with the aim of testing the suitability of these isolates for use in CTV clone construction (Project 1160), in addition to evaluating them as potential cross-protection sources. Previous grapefruit field trials indicated that single-strain CTV sources were associated with better horticultural performance compared to the multi-strain sources (Project 742). Grapefruit and Valencia trees were planted in the Northern Cape, Navels in Mpumalanga and a Clementine and a mandarin hybrid were planted in Limpopo Province. The Northern Cape trials were terminated as numerous trial trees were lost due to a lack of water shortly after planting. Tree canopy volumes were determined for the third year for the navels and soft citrus and no significant differences were observed between treatments of the 3 Navel cultivars or the 2 soft citrus cultivars. Yield differences between treatments were not observed for the second harvest from the two soft citrus cultivars, but yields were still low. The first harvest of the navel trees is also not yet informative. The trees are still young and it is too soon to draw any conclusions regarding the potential impact of the different strains.

#### Opsomming

Enkel-ras citrus tristeza virus (CTV) isolate is gekarakteriseer en geëvalueer in verskeie bedryfskultivars in 'n glashuis proef (Projek 1056). Geen nadelige simptome was geassosieer met hierdie isolate nie. Geselekteerde kultivars en handelings van hierdie proef is in verskeie proefpersele geplant om veldprestasie en CTV-translokasie in die plante te evalueer. Die doel hiermee is om die verskeie CTV isolate te evalueer as kandidate vir gebruik in CTV-kloonkonstruksie in Projek 1160, asook om hulle te evalueer as moontlike kruisbeskermingsbronne. Vorige pomelo proewe het aangedui dat enkelras CTV bronne beter presteer as CTV bronne bestaande uit ras mengsels (Projek 742). Dit is dus van waarde om die enkel-ras CTV bronne as potensieële kruisbeskermingsbronne te evalueer. Die pomelo en Valencia-bome is in die Noord-Kaap geplant en Navels in Mpumalanga. 'n Clementine- en 'n Mandaryn is in Limpopo geplant. Die Noord-Kaap proef is

beëindig aangesien talle proefbome gevrek het weens 'n tekort aan water kort na plant. Boomvolumes was vir die 3de jaar bepaal vir die navels en sagte sitrus. Daar was geen beduidende verskille tussen behandelings vir die 3 Navel kultivars of die 2 sagte sitrus kultivars. Opbrengsverskille tussen behandelings is nie waargeneem vir die tweede oes van die sagte sitrus kultivars nie en die opbrengste was nog laag. Die eerste oes van die navel bome was ook nie insiggewend nie. Die bome is nog jonk en dit is te vroeg om enige gevolgtrekkings te maak rakende die impak van die verskillende CTV rasse.

#### 4.2.6 **PROGRESS REPORT: Application of CTV infectious clones to combat HLB**

Project 1160 (2016/17 – 2021/2) by R Bester (CRI), D Aldrich (SU), G Cook, JHJ Breytenbach (CRI), JT Burger (SU), WO Dawson (University of Florida, USA), HJ Maree (CRI)

##### **Summary**

The confirmed presence of both 'Candidatus' *Liberibacter asiaticus* (CLAs), and *Diaphorina citri* in East Africa, requires a proactive approach from the South African citrus industry to prepare for the eventual incursion of HLB. The aim of this project is to establish a suite of citrus tristeza virus (CTV) infectious clones with a range of silencing targets (payloads) that would form part of a management strategy to contain HLB and limit its impact. CTV infectious clones of genotype T36 were imported from collaborator Prof W.O. Dawson (University of Florida) at the start of the project and protocols optimized to successfully infect citrus using the T36 clone. The T36 clone was converted into the local RB (asymptomatic) genotype. However, no systemic infection in *Nicotiana benthamiana* could be observed. Plasmid DNA was sent for low coverage high-throughput sequencing and three mutations in critical open reading frames were identified that can influence virus infectivity and spread. These mutations will be repaired by replacing fragments of the RB genotype. A dual reporter infectious clone based on the T36 clone that expresses GFP and also contains a PDS silencing cassette was also constructed and evaluated in citrus. The clone proved to be an effective expression and silencing vector. This clone will be used as a test platform to screen different 'payloads' while the RB clone is still under construction. Four RNAi gene targets in *Diaphorina citri* have been identified from literature and homologues for *Trioza erytreae* have been identified using the HTS data generated from *Trioza erytreae* RNA. CTV vectors are being constructed to contain payloads directed towards both *Trioza erytreae* and *Diaphorina citri* for each gene target. CTV vectors targeting all 4 genes per insect will also be constructed.

Additionally, this project also included the identification of CTV-induced stem pitting determinants. All CTV infectious clone mutants for the stem pitting trials of this study have been successfully assembled and proved to be replication competent in *N. benthamiana*. These clones have also been sent for low-coverage high-throughput sequencing to validate plasmid sequences. Recombinant virions were then purified from systemically infected *N. benthamiana* plants by ultracentrifugation. All available Duncan and Mexican lime test plants for the stem pitting trials have been bark-patch infected with the relevant CTV clones and wild-type CTV sources. These plants will be pruned back as soon as they have been confirmed to be infected with the correct CTV virus/clone. Stem pitting characterisations will be carried out 4 to 6 months after prune-back.

##### **Opsomming**

Die bevestigde teenwoordigheid van beide 'Candidatus' *Liberibacter asiaticus* (CLAs), en *Diaphorina citri* in Oos-Afrika, vereis 'n proaktiewe benadering van die Suid-Afrikaanse sitrusbedryf om voor te berei op die uiteindelijke indringing van HLB. Die doel van hierdie projek is om 'n paneel van CTV infektiewe klone te vestig met 'n verskeidenheid 'silencing' teikens (payloads) wat sal deel uitmaak van 'n beheerstrategie om die impak van HLB te beperk. Infektiewe klone van die T36 genotipe is ingevoer vanaf medewerker, Prof W.O. Dawson (Florida University) aan die begin van die projek en protokolle is geoptimaliseer om sitrus suksesvol met die T36-kloon te besmet. Die T36-kloon is omgeskakel in die plaaslike RB (asimptomaties) genotipe. Geen sistemiese infeksie in *N. benthamiana* kon egter waargeneem word nie. Plasmied-DNA is gestuur vir lae-dekking hoë-deurset-volgordebepaling en drie mutasies in kritiese leesrame is geïdentifiseer wat die infektiwiteit en verspreiding van virusse kan beïnvloed. Hierdie mutasies sal herstel word deur fragmente van die RB-genotipe te vervang. Daar is ook 'n dubbel-rapporteerder infektiewe kloon gebou, gebaseer op die T36-kloon (Dawson), wat beide GFP uitdruk asook 'n PDS 'silencing cassette' bevat. Hierdie kloon is reeds geëvalueer in sitrus en het bewys dat die kloon 'n doeltreffende uitdrukking en 'silencing' vektor is. Hierdie

kloon sal gebruik word as 'n platform om verskillende 'payloads' te toets terwyl die RB-kloon gefinaliseer word. Vier RNAi-teiken gene in *Diaphorina citri* is uit literatuur geïdentifiseer en homoloë vir *Trioza erytrae* is geïdentifiseer met behulp van die HTS-data wat gegenereer is uit *Trioza erytrae* RNA. CTV-vektore met 'payloads' wat na beide *Trioza erytrae* en *Diaphorina citri* gerig is, word tans gemaak. CTV-vektore wat op al 4 die gene per insek gerig is, sal ook gemaak word.

Verder sluit hierdie projekdoelwitte die bepaling van CTV-geïnduseerde stamgleuf determinante in. Al die CTV-kloonmutante vir die stamgleuf proewe is suksesvol saamgestel en bewys dat dit in *Nicotiana benthamiana* repliseer. Hierdie klone is ook gestuur vir lae-dekking hoë-deurset-volgordebepaling. Rekombinante virions is dan gesuiwer van *N. benthamiana* plante deur ultrasentrifugasie. Die Duncan and Mexican lime proefplante vir die stamgleufproewe is besmet met die relevante CTV-klone en wilde-tipe CTV-bronne. Hierdie plante sal teruggesnoei word sodra daar bevestig is dat hulle met die korrekte CTV-virus/kloon besmet is. Stamgleufkarakterisering sal 4 tot 6 maande na die terugsnoui uitgevoer word.

#### 4.2.7 **PROGRESS REPORT: Application of high-throughput sequencing (HTS) for routine virus and viroid detection in high value accessions.**

Project 1241 (2019/20 – 2021/22) by R Bester, G Cook, JHJ Breytenbach, C Steyn, R. De Bruyn, P Fourie, HJ Maree (CRI)

##### **Summary**

The ultimate aim of this project is to validate high throughput sequencing (HTS) based detection of known viruses and viroids of citrus for routine detection and to open up the possibility to fast-track multiplication of clean material in the CIS without additional risk. Plant material infected with a range of viruses (positive and negative stranded viruses) and viroids was established. Total RNA was extracted from three representative samples of each plant (4 plants) using two different methods (CTAB vs Zymo Research RNA kit) and sent for HTS (Macrogen, S. Korea). One representative sample of each plant was also sent to the Central analytical facility (CAF) in Stellenbosch for HTS on an Ion Torrent platform. The data were evaluated for biological and technical variation focussing on RNA extraction method, platform used and bioinformatic analysis. The study evaluated the influence of different HTS protocols on the sensitivity, specificity and repeatability of HTS as a detection tool. Both extraction methods and sequencing platforms resulted in significant differences between the datasets. Using a de novo assembly approach, complemented with read mapping, the Illumina data allowed a greater proportion of the expected pathogen scaffolds to be inferred, and an accurate virome profile was constructed. The complete virome profile was also constructed using the Ion Torrent data but analyses showed that more sequencing depth is required to be comparative to the Illumina protocol and to produce consistent results. The CTAB extraction protocol lowered the proportion of viroid sequences recovered with HTS, and the Zymo Research kit resulted in more variation in the read counts obtained per pathogen sequence.

The project also included a direct comparison between an HTS-based detection assay and the conventional methods as applied in the citrus improvement scheme (CIS). Seven accessions were selected for this comparison. Total RNA was extracted from these samples before and after shoot tip grafting and sent for HTS (Macrogen). Data will arrive by 30 April 2021. One of these samples was selected as source material and was used to deliberately infect healthy seedlings. The infection status of these seedlings was monitored for seven months at five time points using both HTS and RT-PCR to test the sensitivity of both approaches to detect viruses and viroids.

Additionally, one of the objectives of this project is to investigate diseases with unknown aetiology (such as the Psorosis-like diseases) using HTS. Psorosis-like symptomatic plants were identified and subjected to HTS. Citrus virus A (CiVA) was identified in these samples, however the association of the fruit skin symptom and the presence of CiVA will need to be investigated further. A new detection assay has also been developed for citrus coguviruses. This assay was evaluated for sensitivity to detect citrus virus A (CiVA). The complete genome of a South African variant of CiVA was also assembled using the HTS data and validated using Sanger sequencing.

## Opsomming

Die hoofdoel van die projek is om the gebruik van hoë-deurset volgordebepaling gebaseerde opsporing (HTS) van virusse en viroïede in sitrus as 'n roetine toets te valideer. In die proses mag dit die geleentheid bied om die vermeerdering van plantmateriaal in die SVS te bespoedig sonder addisionele risiko. Plant materiaal geïnfecteer met 'n verskeidenheid virusse (positiewe en negatiewe string virusse) en viroïede is gemaak. Totale RNA is uit drie verteenwoordigende monsters van elke plant (4 plante) met behulp van twee verskillende metodes (CTAB vs Zymo Research RNA kit) uitgehaal en vir HTS (Macrogen, S. Korea) gestuur. Een verteenwoordigende monster van elke plant is ook na die Sentrale analitiese fasiliteit (CAF) in Stellenbosch vir HTS gestuur om data met die Ion Torrent-platform te genereer. Die data is geëvalueer vir biologiese en tegniese variasie met die fokus op RNA-ekstraksie-metode, volgordebepalingsplatform en bioinformatiese analise. Die studie het die invloed van verskillende HTS-protokolle op die sensitiviteit, spesifisiteit en herhaalbaarheid van HTS as 'n opsporingsinstrument geëvalueer. Beide ekstraksie metodes en platform keuse het gelei tot beduidende verskille tussen die datastelle. Met behulp van 'n de novo-samestellingsbenadering, aangevul met volgorde kartering, kon die Illumina-data 'n groter deel van die verwagte patoogeen op spoor, en 'n akkurate viroomprofiel is saamgestel. Die volledige viroomprofiel is ook saamgestel met behulp van die Ion Torrent-data, maar ontleding het getoon dat meer data diepte nodig is om vergelykend met die Illumina-protokol te wees en konsekwente resultate te lewer. Die CTAB-ekstraksieprotokol het die hoeveelheid viroïed-geassosieerde volgordes wat met HTS herwin is gelewer, en die Zymo Research-kit het gelei tot meer variasie in die hoeveelheid volgordes wat per patoogeen verkry is.

Die projek bevat ook 'n direkte vergelyking tussen 'n HTS-gebaseerde opsporingstoets en die konvensionele metodes soos toegepas in die sitrusverbeteringskema (CIS). Sewe monsters is vir hierdie vergelyking gekies. Totale R

NA is voor en na die inplanting van lootpunte uit hierdie monsters onttrek en vir HTS (Macrogen) gestuur. Data sal teen 30 April 2021 ontvang word. Een van hierdie monsters is ook as bronmateriaal gekies en gebruik om gesonde saailinge doelbewus aan te steek. Die infeksiestatus van hierdie saailinge is op vyf tydpunte vir sewe maande gemonitor met behulp van beide HTS en RT-PCR om die sensitiviteit van beide benaderings om virusse en viroïede op te spoor, te toets.

Addisioneel is een van die doelstellings van hierdie projek om siektes met onbekende etiologie (soos die Psorose-agtige siektes) met behulp van HTS te ondersoek. Psorose-agtige simptomatiese plante is geïdentifiseer en aan HTS onderwerp. Sitrusvirus A (CiVA) is in hierdie monsters geïdentifiseer, maar die assosiasie van die vrugteskielintoom en die teenwoordigheid van CiVA sal verder ondersoek moet word. 'n Nuwe opsporingstoets is ook ontwikkel vir sitrus coguvirusse. Hierdie toets is geëvalueer vir sensitiviteit om sitrusvirus A (CiVA) op te spoor. Die volledige genoom van 'n Suid-Afrikaanse variant van CiVA is ook saamgestel met behulp van die HTS-data. Hierdie konsepvolgorde was gevalideer met behulp van Sanger-volgorde bepaling.

### 4.3 PROGRAMME: PREHARVEST DISEASES

Programme coordinator: Jan van Niekerk (CRI)

#### 4.3.1 Programme summary

Within the preharvest disease programme the research focus is soilborne diseases of citrus and fruit and foliar diseases, including citrus black spot, of citrus. The research focusses on finding alternative, softer management options, studies into the epidemiology of the different pathogens and optimizing the control of these pathogens through better application technology or better chemical control programmes.

Projects 762 (4.3.13) and 1030 (4.3.3) are specifically aimed at finding alternative means of control for *Phytophthora* and citrus nematode. Data have been recorded in Project 762 since 2011. It is becoming clear that the different pre-plant soil fumigation treatments, specifically the 1,3 dichloropropene and metham sodium treatments, have caused the trees in these treatments to be taller with thicker trunks compared to the other treatments and the untreated control. It is therefore becoming clear that these treatments are beneficial as

preplant treatments to manage soilborne pathogens. Within Project 1030, commercially available *Trichoderma*-based biocontrol products were evaluated *in vitro* and *in planta* for their ability to inhibit citrus replant pathogens. Results clearly showed that the products, and strains within products, vary significantly in controlling the different replant pathogens. A final management plan will therefore have to rely on a mixture of different products or strains.

The decline and death of citrus trees have been reported from the Gamtoos and Sunday's River valleys for a number of years. Surveys of diseased trees were done as part of Project 1068 (4.3.11) in the Gamtoos and Sunday's River Valley production areas. A pathogen complex was identified with all pathogens being known as stress pathogens. Investigations indicated that high soil pH and EC probably contributed to predisposing trees to infection by the different *Neocosmospora* spp. identified. This could also explain why orchards planted on Rough Lemon do not have any problems. To identify other rootstock options, a trial containing 10 different rootstocks was planted in March 2021 and will be monitored to identify better rootstock options. It was furthermore seen that a treatment programme based on specific plant extract products can maintain tree health at the same level as trees grown in fumigated soils.

Project 1215 (4.3.2) concluded in April 2021. In this project species-specific qPCR primers were developed successfully for the detection and quantification of citrus replant pathogens. These were subsequently used to study the *in planta* interaction of the different replant pathogens. It was seen that *Neocosmospora solani* promoted root system colonization by oomycete soilborne pathogens. This could be a significant discovery for the development of better management strategies for soilborne pathogens. Also in this project two bacterial and two *Trichoderma harzianum* isolates were identified as potential biocontrol agents (BCA's) of soilborne pathogens. *In vitro* these BCA's were found to provide good inhibition of oomycete pathogens but not as good inhibition of *Neocosmospora* spp. *In planta* it was seen that these BCA's did colonize citrus seedling roots but were not successful in reducing root infections by the different soilborne pathogens. This indicated that further work is probably needed to optimize the application of these BCA's.

Citrus black spot (CBS) and *Alternaria* brown spot (ABS) are two major fruit and foliar diseases that hamper the export of citrus fruit to fresh markets by South African producers. Research is therefore focused on the epidemiology and management of these pathogens. Project 750 (4.3.4) focusses on the chemical management of ABS while Project 970 (4.3.5) focusses on CBS chemical management. In the Western Cape trial of 750 it was seen that a mixture of azoxystrobin-difenoconazole in a control programme showed promise and needed further evaluation. Unfortunately these results were not mirrored in the Eastern Cape trial. At this trial the standard programme containing azoxystrobin and copper gave the results. In Project 970 it was seen that fenbuconazole has great potential for inclusion in a CBS control programme while some adjuvants were seen to be viable replacement options for mineral oil in strobilurin applications.

Apart from evaluating different fungicides and spray programmes for CBS and ABS control, research is also done to improve spray application and spray calibration methods. In Project 1132 (4.3.16), trials over years showed that higher spray volumes were better in controlling insect pests such as mealybug and red scale. This was seen as being due to these volumes leading to better deposition uniformity and quality compared to other volumes. In order to further improve these two parameters, trials were done to evaluate tree row volume (TRV) as the basis for spray calibration. Spray deposition results showed that reducing volumes based on TRV gave similar deposition values on fruit and leaves compared to higher volume applications. It was furthermore seen that air volume during application had no significant effect on spray deposition. Unfortunately, due to low pest pressure in trial orchards, no meaningful conclusions could be made regarding the biological efficacy of TRV based spray volumes.

In the area of CBS epidemiology, several projects are being conducted. In Project 1187 (4.3.7) it was observed that in netted orchards, due to more conducive environmental conditions, higher levels of CBS and ABS as well as *Botrytis* could be expected, compared to open orchards. It was furthermore seen that fungicide residues persist longer under nets compared to open orchards.

Project 1186 (4.3.6) focusses on the period of CBS fruit susceptibility. Results indicated that both Valencia and mandarins' susceptibility does decline with increasing fruit age and that the fruit protection periods used in

control programmes are valid. This project links to Project 1242 (4.3.14) where it was found that the rind phytochemistry of five citrus types, all differing in CBS susceptibility, changed with fruit maturity. It was also seen that types with different levels of susceptibility, had different rind phytochemical profiles. These findings could help explain the interaction between CBS and different fruit types and their level of susceptibility. Project 1223 (4.3.8) is aiming to determine how chopped pruning debris plays a major role as a CBS inoculum source. It was seen that the ascospore production from shredded pruning debris is lower compared to un-chopped material. Shredding of pruning debris will therefore be important in CBS management. Project 1244 (4.3.10) ties in with 1223 and is focusing on the infection parameters of CBS pycnidio and ascospores. This is with the aim of improving CBS prediction models. In a breakthrough, CBS ascospores were produced in culture and this allows further studies on the infection parameters of this important infection source. Results from Project 1244 will eventually feed into Project 1238 (4.3.9) that is aimed at improving the prediction model in CRI-PhytRisk. Apart from improving the CBS prediction model, CRI-PhytRisk was also expanded to include ABS and Botrytis prediction models. However, these two models still need some refinement for South African conditions. To improve our understanding of the CBS pathogen's population structure, Project 1235 (4.3.12) is looking at this aspect. Using high throughput sequencing (HTS) the clonal nature of the *Phyllosticta citricarpa* population in the USA was confirmed. This tool will now also be used to characterise the South African population in the same manner.

The last project in the programme is 1236 (4.3.15) that is investigating the epidemiology of Botrytis on lemons. Work from this study indicated that lemon blossoms and broad leaf weeds are the primary sources of inoculum in lemon orchards. It was furthermore seen that some fungicides have potential to control this disease and should be pursued. Validation of the Botrytis prediction model incorporated in CRI PhytRisk is still ongoing and will be a valuable management tool.

## Programopsomming

Binne die voor-oes siekteprogram, is die fokus van navorsing op grondgedraagde siektes van sitrus, en vrug- en blaarsiektes, insluitende Sitrus Swartvlek, van sitrus. Die navorsing fokus op die vind van alternatiewe, sagter bestuur-opsies, studies in die epidemiologie van die verskillende patogene, en die optimalisering van beheer van hierdie patogene deur beter toedieningstechnologie of beter chemiese beheerprogramme.

Projekte 762 (4.3.13) en 1030 (4.3.3) het spesifiek ten doel om alternatiewe maniere van beheer vir *Phytophthora* en sitrus-aalwurm te vind. Data is sedert 2011 in projek 762 aangeteken. Dit het duidelik geword dat die verskillende voor-plant grondberokingsbehandelings, spesifiek die 1,3-dichloropropreen en metamnatrium behandelings, veroorsaak het dat die bome in hierdie behandelings langer is en dikker stamme het, in vergelyking met die ander behandelings en die onbehandelde kontrole. Dit is duidelik dat hierdie behandelings as voor-plant behandelings voordelig is om grondgedraagde patogene te bestuur. Binne projek 1030 is kommersieel beskikbare *Trichoderma*-gebaseerde bio-beheer produkte *in vitro* en *in planta*, vir hul vermoë om sitrus herplant patogene te inhibeer, geëvalueer. Resultate het duidelik getoon dat die produkte, en isolate binne produkte, betekenisvol verskil in beheer van die verskillende herplant patogene. 'n Finale bestuursplan sal dus op 'n mengsel van verskillende produkte of isolate moet staatmaak.

'n Agteruitgang en afsterwing van sitrusbome is vir 'n aantal jare vanaf die Gamtoos- en Sondagsriviervallei aangemeld. Opnames van siek bome is as deel van Projek 1068 (4.3.11) in die Gamtoos- en Sondagsriviervallei produksie-areas gedoen. 'n Patogeenkompleks is geïdentifiseer met al die patogene bekend as stres patogene. Ondersoeke het aangetoon dat hoë grond pH en EC moontlik tot die predisponering van bome vir infeksie deur die verskillende *Neocosmospora* spp., wat geïdentifiseer is, bydra. Dit kan ook verduidelik waarom boorde wat op Rough Lemon aangeplant is, geen probleme toon nie. Ten einde ander onderstam opsies te identifiseer, is 'n proef, bevattende 10 verskillende onderstamme, in Maart 2021 aangeplant, en sal gemonitor word ten einde beter onderstam opsies te identifiseer. Daar is verder waargeneem dat 'n behandelingsprogram, gebaseer op spesifieke plant-ekstrak produkte, boomgesondheid tot dieselfde vlak kan handhaaf, as bome wat in beroekte gronde groei.

Projek 1215 (4.3.2) het in April 2021 tot 'n einde gekom. In hierdie projek is spesie-spesifieke qPKR inleiers suksesvol ontwikkel vir die opsporing en kwantifisering van sitrus herplant patogene. Dit is daarna gebruik om

die *in planta* interaksie van die verskillende herplant patogene te bestudeer. Daar is gesien dat *Neocosmospora solani* wortelsisteem kolonisasie deur oömiseet grondgedraagde patogene bevorder. Dit kan 'n betekenisvolle ontdekking wees vir die ontwikkeling van beter bestuurstrategieë vir grondgedraagde patogene. In hierdie projek is ook twee bakteriese en twee *Trichoderma harzianum* isolate as potensiële bio-beheer agente (BBA's) van grondgedraagde patogene geïdentifiseer. Hierdie BBA's het *in vitro* goeie inhibisie van oömiseet patogene verskaf, maar nie sulke goeie inhibisie van *Neocosmospora* spp. nie. *In planta* is gesien dat hierdie BBA's sitrusaailingwortels koloniseer, maar was nie suksesvol in die vermindering van wortel-infeksies deur die verskillende grondgedraagde patogene nie. Dit het aangedui dat verdere werk moontlik benodig word om die toediening van hierdie BBA's te optimaliseer.

Sitrus Swartvlek (SSV) en *Alternaria* Bruinvlek (ABV) is twee belangrike vrug- en blaarsiektes wat die uitvoer van sitrusvrugte, deur Suid-Afrikaanse produsente, na varsmarkte belemmer. Navorsing is dus gefokus op die epidemiologie en bestuur van hierdie patogene. Projek 750 (4.3.4) fokus op die chemiese bestuur van ABV, terwyl Projek 970 (4.3.5) op SSV chemiese bestuur fokus. In die Wes-Kaap proef van 750 is gesien dat 'n mengsel van asoksistrobien-difenokonasool in 'n beheerprogram belofte toon en verdere evaluasie benodig. Hierdie resultate kon egter nie in die Oos-Kaap proef herhaal word nie. In hierdie proef het die standaard program, bevattende asoksistrobien en koper, die resultate gegee. In projek 970 is gesien dat fenbukonasool groot potensiaal toon vir insluiting in 'n SSV beheerprogram, terwyl sommige bymiddels lewensvatbare vervangingsopsies vir minerale olie in strobilurien toedienings is.

Afgesien van die evaluasie van verskillende fungisiedes en spuitprogramme vir SSV en ABV beheer, word navorsing ook gedoen om spuittoediening en spuit kalibrasiemetodes te verbeter. In Projek 1132 (4.3.16) het proewe oor jare getoon dat hoër spuitvolumes beter gevaar het in die beheer van insekplae soos witluis en rooi dopluis. Dit kan die gevolg wees van hierdie volumes wat tot beter neerleggingsuniformiteit en -kwaliteit lei, in vergelyking met ander volumes. Ten einde hierdie twee parameters verder te verbeter, is proewe gedoen om boom-ry-volume (BRV) as basis vir spuitkalibrasie te evalueer. Spuitneerleggingsresultate het getoon dat verminderde volumes, gebaseer op BRV, soortgelyke neerleggingswaardes op vrugte en blare lewer in vergelyking met hoër volume toedienings. Daar is verder gesien dat lugvolume gedurende toediening, geen betekenisvolle effek op spuitneerlegging gehad het nie. Weens lae plaagdruk in die proefboorde, kon ongelukkig geen betekenisvolle gevolgtrekking rakende die biologiese doeltreffendheid van BRV-gebaseerde spuitvolumes gemaak word nie.

Verskeie projekte word in die area van SSV epidemiologie uitgevoer. In Projek 1187 (4.3.7) is waargeneem dat verwag kan word dat boorde onder net, weens meer bevorderlike omgewingstoestande, hoër vlakke van SSV en ABV, asook *Botrytis*, sal hê, in vergelyking met oop boorde. Daar is verder gesien dat fungisiedresidue langer onder net agter bly, in vergelyking met oop boorde.

Projek 1186 (4.3.6) fokus op die periode van SSV vrugvatbaarheid. Resultate het getoon dat beide Valencia en mandaryn se vatbaarheid afneem met toenemende vrug-ouderdom, en dat die vrug beskermingsperiodes soos gebruik in beheerprogramme, geldig is. Hierdie projek skakel met Projek 1242 (4.3.14) waar gevind is dat skil fitochemie van vyf sitrustipes, almal verskillend in SSV vatbaarheid, met vrugvolwassenheid verander. Daar is ook gesien dat tipes met verskillende vlakke van vatbaarheid, verskillende skil fitochemiese profiele het. Hierdie bevindinge kan help om die interaksie tussen SSV en verskillende vrugtipies en hul vlak van vatbaarheid te verduidelik. Projek 1223 (4.3.8) het ten doel om vas te stel hoe versnipperde snoeisels 'n belangrike rol as SSV inokulumbron speel. Daar is gesien dat die askospore produksie vanaf versnipperde snoeisels laer is in vergelyking met nie-versnipperde materiaal. Versnippering van snoeisels sal dus belangrik wees in SSV bestuur. Projek 1244 (4.3.10) skakel met 1223 en fokus op die infeksie parameters van SSV piknidio- en askospore. Dit is met die doel om SSV voorspellingsmodelle te verbeter. In 'n deurbraak is SSV askospore in kultuur geproduseer, en dit laat verdere studies op die infeksie parameters van hierdie belangrike infeksiebron toe. Resultate van Projek 1244 sal uiteindelik in Projek 1238 (4.3.9) gebruik word, wat ten doel het om die voorspellingsmodel in CRI-PhytRisk te verbeter. Behalwe vir die verbetering van die SSV voorspellingsmodel, is CRI-PhytRisk ook uitgebrei om ABV en *Botrytis* voorspellingsmodelle in te sluit. Hierdie twee modelle benodig egter nog verfyning vir Suid-Afrikaanse toestande. Projek 1235 (4.3.12) kyk na die aspek om ons kennis oor die SSV patoogen se populasiestruktuur uit te brei. Deur gebruik te maak van hoë deurvloei volgorde-bepaling (HTS), is die klonale aard van die *Phyllosticta citricarpa* populasie in die V.S.A.

bevestig. Hierdie gereedskap sal nou ook gebruik word om die Suid-Afrikaanse populasie op dieselfde manier te karakteriseer.

Die laaste projek in die program, 1236 (4.3.15), ondersoek die epidemiologie van Botrytis op suurlemoene. Die studie dui daarop dat suurlemoenbloeisels en breëblaar onkruid die hoofbronne van inokulum in suurlemoenboorde is. Daar is ook gesien dat sommige fungisiedes potensiaal het om hierdie siekte te beheer en moet ondersoek word. Validasie van die Botrytis voorspellingsmodel wat in CRI PhytRisk geïnkorporeer is, gaan nog voort en sal 'n waardevolle bestuursgereedskap wees.

#### 4.3.2 FINAL REPORT: Potential biocontrol agents and host/pathogen interaction of citrus replant pathogens

Project 1215 (RCE-2-07A) by Jan van Niekerk (CRI), Prof Lizel Mostert, Dr Elodie Stempien, Gray-Lee Carelse and Sonè Reens (USPP)

#### Summary

The first objective of this study was to study the *in planta* interaction between different citrus replant pathogens identified before. The second objective was to find potential biocontrol agents from the roots of citrus trees showing no disease symptoms in an orchard that has severe decline due to root disease problems. To address the first objective, species specific primers along with qPCR protocols, were developed to detect and quantify the different replant pathogens. This was done successfully and the primer sets developed were shown to be species specific and sensitive. Single and combined inoculation of citrus seedlings with the different replant pathogens showed that especially *Neocosmospora solani* has a synergism with the oomycete pathogens in the replant complex. In these combinations between the oomycetes and *N. solani*, the oomycetes were always re-isolated from the seedling roots compared to when they were inoculated alone. In the evaluation of these interactions, the species specific qPCR reactions developed were found to be effective in detecting and quantifying the replant pathogens in the roots. In the second objective, a *Bacillus subtilis*, *Pseudomonas fluorescens* and two *Trichoderma harzianum* isolates were selected as potential biocontrol agents following extensive *in vitro* screening, using various techniques, against the replant pathogen complex. Results indicated that, although the selected BCA isolates varied in efficacy against different replant pathogens, inhibition of the oomycete pathogens by the selected BCA isolates was higher compared to the inhibition achieved against the *Neocosmospora* spp. It was furthermore seen that the mechanisms of inhibition by the selected BCA isolates were most likely through the production of non-volatile metabolites and competition. Despite the inhibition observed *in vitro*, the *in planta* evaluation of the selected BCA isolates were not as successful. Despite the BCA's being found to colonize the citrus seedling roots, they were unable to reduce the level of the inoculated replant pathogens in the roots. It illustrated that although effective *in vitro*, further studies are needed to optimise the use of the BCA's for the *in planta* protection of citrus seedlings against replant pathogen infection.

#### Opsomming

Die eerste doelwit van hierdie studie was om die *in planta* interaksie van die verskillende sitrus herplant patogene wat tevore identifiseer is, te bestudeer. Die tweede doelwit was om potensiële biobeheeragente te isoleer van die wortels van simptomevrye sitrusbome in boorde wat ernstige terugsterwing toon. Ten einde die eerste doelwit aan te spreek, is spesie spesifieke primers, tesame met kwantitatiewe PCR (kPKR) protokolle, ontwikkel om die verskillende herplant patogene te identifiseer en te kwantifiseer. Hierdie is suksesvol gedoen en die inleierstelle is bevind om spesifiek en sensitief te wees. Enkel en gekombineerde inokulasies van sitrussaailinge het getoon dat veral *Neocosmospora solani* 'n sinergisme het met die oomycete patogene in die komplekse. Wanneer die oomycete patogene saam met *N. solani* geïnkuleer is, is bevind dat die oomycete altyd teen 'n hoër persentasie herisoleer is as wanneer die alleen inokuleer is. Met die evaluasie van hierdie interaksies is verder bevind dat die kPKR reaksies wat ontwikkel is effektief is in die opsporing en kwantifisering van herplant patogene in die sitruswortels. In die tweede doelwit is 'n *Bacillus subtilis*, *Pseudomonas fluorescens* en twee *Trichoderma harzianum* isolate gekies as potensiële biobeheeragente. Seleksie is gedoen deur die gebruik van verskeie *in vitro* tegnieke en teen die verskillende herplantpatogene. Resultate het aangetoon dat, hoewel die geselekteerde biobeheeragente wissel in hul effektiwiteit teen verskillende herplantpatogeenisolate, inhibisie van die oomycete patogene beter was as die inhibisie behaal teen die

verskillende *Neocosmospora* spp. dit is verder waargeneem dat die inhibisiemeganisme van die geselekteerde biobeheeragente waarskynlik deur middel van nie-vlugtige metaboliet produksie of kompetisie is. Ondanks die waargenome *in vitro* inhibisie, was die *in planta* evaluasie van die beheeragente nie so suksesvol nie. Dit is gesien dat hoewel die biobeheeragente die saailingwortels koliniseer, hulle onsuksesvol was om te verhoed dat die wortels deur die herplantpatogene koloniseer word. Dit het illustreer dat, hoewel die biobeheeragente *in vitro* effektief was, verdere studies nodig is om hulle *in planta* gebruik te optimiseer ten einde sitrus saailinge teen herplant patoëen infeksie te beskerm.

## Introduction

Replant disease is a phenomenon that is observed in cases where young, healthy nursery trees are planted on old orchard sites. The phenomenon is characterised by the newly planted trees being stunted with small leaves and showing low vigour (Derrick and Timmer, 2000). The retarded and stunted growth of newly planted trees has been ascribed to accumulation of phytotoxins in the soil, development of nutrient imbalances, deterioration of soil physical characteristics and the establishment of soil pathogens that damage the root systems of the young trees (Cronje *et al.* 2002). The growth of citrus production in South Africa has necessitated that growers establish new orchards on sites where citrus has been cultivated previously for many years (Burger and Small, 1983). Replanting on old sites has therefore led to replant disease also being observed in South African citrus orchards like in many other parts of the world (Le Roux *et al.* 1998).

Previously the causal agents associated with replant disease in South African citrus orchards have been regarded as the citrus nematode, *Tylenchulus semipenetrans* and the soilborne pathogens, *Phytophthora nicotianae* and *P. citrophthora* (Le Roux *et al.* 1998). However, a recent study by Swart (2018) found that apart from abovementioned pathogens and nematode, *Pythium* spp. and *Fusarium* spp. might also potentially be involved in citrus replant disease. These organisms were shown to build up in an orchard soil during the life time of the orchard. Once the orchard is removed, inoculum of the pathogens remains in the soil and reinvest the newly planted trees (Matheron and Porchas, 2009). As these pathogens were found to occur together in the same soils, it could be expected that they would interact to cause typical citrus replant symptoms. Dandurand and Menge (1992) reported that the severity of citrus root rot caused by *Phytophthora nicotianae* and *P. citrophthora* were increased when co-inoculation with *Fusarium solani* was done. However, several *Fusarium* spp. were identified by Swart (2018) along with several *Pythium* spp. The interaction of this whole complex are therefore at this stage unclear and needs to be studied, both in controlled co-inoculation trials, as well as in naturally infested orchard soils.

Methyl bromide (MeBr) was previously regarded as a one-stop solution to treating replant soils due to its wide range of activity. Le Roux *et al.* (1998) found that if soil fumigation with methyl bromide was done prior to replanting, the net income is significantly higher compared to replant sites where no pre-plant fumigation was done. The treated site also had significantly higher volumes of exportable fruit compared to the untreated site. However, the use of methyl bromide to treat citrus replant soils is no longer an option. This is due to the phasing out of methyl bromide in South Africa. Over the years many fumigant chemicals have been evaluated as possible replacement for MBr. Many were shown to be effective against only fungal soilborne pathogens, while others were effective only against nematodes. However, abovementioned soil fumigants have also been shown to not provide the long term control effect of soilborne diseases and nematodes. Concerns about resistance development by over-use of other fungicides such as metalaxyl (mefenoxam), have furthermore led to a reluctance to use these chemicals in management of soilborne pathogens in a replant situation (Zhang *et al.* 2016; Liu *et al.* 2018). Due to these concerns, the development and implementation of alternative control strategies such as biological control have gained traction in recent years (Pane *et al.* 2012; Liu *et al.* 2018). To this end, a variety of biological control agents (BCA's), including various genera of bacteria and fungi have been tested and even registered as biopesticides for the control of soilborne plant diseases (Pane *et al.* 2012). What makes the use of different biocontrol agents so attractive is that they have different modes of action in their interaction with the pathogen. This includes direct parasitism and competition while plant growth promoting characteristics are an added benefit (Aleandri *et al.* 2015; Jimtha *et al.*, 2016; Liu *et al.* 2017). These characteristics therefore make them suited to develop as part of an integrated management system for soilborne pathogens (Abd-Elgawad *et al.* 2010), even if repeated applications are needed for optimal efficacy (Mazzola and Freilich, 2017).

Rhizospheres and root endophyte populations are regarded as a rich source of microflora that could have potential as biocontrol agents (Lu *et al.* 2017). The ability of these microorganisms to colonize the rhizosphere and host roots and their beneficial effect on plant growth have especially led to these microorganisms attracting more attention (Zhang *et al.* 2016). These BCA's must have the ability to survive or tolerate challenging soil conditions such as high alkalinity, making it very important to search for these microorganisms in the rhizosphere of the crop where they are to be applied as well as under the soil conditions that these crops are grown in (Zouari *et al.* 2016). Apart from surviving challenging soil conditions, the potential BCA's should also have the ability to successfully colonize the rhizosphere and root system of the host plant where protection is needed (Mafia *et al.* 2009). Rhizosphere competent bacteria and fungi such as *Bacillus* and *Pseudomonas* spp. along with *Trichoderma* spp. have been studied extensively as biocontrol agents of soilborne oomycete and fungal pathogens of a variety of crops (Aleandri 2015; Zhang *et al.* 2016).

Finding a suitable carrier for the application of a BCA is often a challenge (Wei *et al.* 2015). However, citrus trees are produced in nurseries before being transplanted into orchards. This provides an opportunity to establish a population of the BCA on and within the roots of the young citrus tree during its time in the nursery (Fang and Tsao, 1995; Nemeč *et al.* 1996). Nemeč *et al.* (1996) found that when *Bacillus subtilis* and *Trichoderma harzianum* inoculum were incorporated into the medium used for seedling production, these two organisms were successful in colonizing the seedling roots. It was furthermore found that, if these seedlings were planted into orchard soils infested by oomycete pathogens, the root rot severity observed for BCA inoculated seedlings were significantly lower compared to the untreated controls. Plant growth in BCA inoculated seedlings were also significantly better or similar to the untreated seedlings. Results obtained by Abd-Elgawad *et al.* (2010) furthermore found that applications of bacterial BCA's to mature citrus trees in orchards, could reduce the levels of *Fusaria* in the soil from 38% to in the region of 4.0%.

Shinde and Sadgir (2016) found that *in vitro* specific strains of *B. subtilis* and *Pseudomonas fluorescens* were effective in inhibiting growth of *Phytophthora nicotianae*, *Pythium* spp., and *Fusarium* spp. They also found that if these strains were incorporated into *P. nicotianae* infested growing medium before seedling transplant, the root rot caused by the *P. nicotianae* were reduced substantially. However, from their results it was evident that strains and genera varied in their effectivity against different pathogens and that testing of different strains are very important. In an earlier study Weideman and Wehner (1993) screened and tested a very limited number of *T. harzianum* and non-pathogenic *Fusarium oxysporum* isolates for their ability to reduce root rot of citrus seedlings caused by *P. nicotianae*. Their results indicated that the tested isolates had a limited effect in reducing root rot severity and that in the two different soils used in the trials, the performance of the isolates were different. These results underline that a range of isolates of different BCA's should be tested and evaluated under different soil conditions. In another citrus field trial evaluating BCA's, it was found that when *Pseudomonas putida* was applied weekly through micro-sprinklers to the soil of newly planted citrus trees or to soil in a 50-year-old orchard, the bacteria did establish over a three year period in the tree rhizosphere and that it reduced the rhizosphere populations of *P. nicotianae* to statistically similar levels as that seen for trees where yearly applications of metalaxyl was done during spring (Steddom *et al.* 2002). From these studies it is evident that the use of BCA's in integrated management of root pathogens of citrus has potential to provide a long term solution to managing citrus replant pathogens. However, these studies focussed only the effect of the different BCA's on *Phytophthora* spp. associated with citrus and did not consider other oomycete pathogens such as *Pythium* spp. or fungal pathogens such as *Fusarium* spp. or instances where these pathogens occur together.

It was also evident from abovementioned studies on citrus and similar studies on other crops that to find an effective BCA that will establish in the citrus rhizosphere, the citrus rhizosphere and roots of trees growing under different soil conditions, should be the source of the specific BCA. To isolate potential BCA's from rhizosphere soil and plant roots, most studies used simple techniques that involved shaking of samples in water before plating out onto selective media targeting bacteria and fungi (Aranda *et al.* 2011; Acebo-Guerrero *et al.* 2015; Subhashint, 2015). For the isolation of *Pseudomonas* spp. King's B and nutrient agar (NA) were used (Acebo-Guerrero *et al.* 2015; Subhashint, 2015) while for the isolation of *Bacillus* spp. NA and a semi-selective *Bacillus* medium was used (Turner and Backman, 1991; Pane *et al.* 2012). *Trichoderma* spp. were isolated through plating out onto selective and semi-selective *Trichoderma* media (Elad *et al.* 1981; Promwee

*et al.* 2017). *In vitro* evaluation of the different isolated BCA's against different soilborne pathogens included normal dual plate cultures, testing of culture filtrates for their ability to inhibit pathogen growth as well as determining if the specific BCA can colonize the host plant root system (Alves Silva *et al.* 2003; Bae *et al.* 2016; Inglis and Kawchuk, 2002; Kotze *et al.* 2011; Lui *et al.* 2017; 2018; Lu *et al.* 2017; Mafia *et al.* 2009; Mutawila *et al.* 2016; Raaijmakers *et al.* 1994). *In vivo* studies included the incorporation of the BCA in growing medium, planting of seedlings in the amended medium and then challenge by the pathogen. After a period of incubation, the seedlings were evaluated using specific growth parameters, while root infestation by the pathogen along with re-isolation of the BCA was often also done (Weideman and Wehner, 1993; Fang and Tsao, 1995; Nemeč *et al.* 1996; Aleandri *et al.* 2015; Shinde and Sadgir, 2016). This *in vivo* testing under controlled conditions was often followed by field experiments, testing the identified BCA under field conditions (Steddom *et al.* 2002; Promwee *et al.* 2017).

As mentioned above, Swart (2018) found that *Fusarium*, *Phytophthora* and *Pythium* spp. often occur together in old citrus soils and that they can infect roots. While the interaction of certain of these pathogens have been studied for citrus, the role of *Pythium* spp. in such an interaction is still unknown. To address this, dual or multiple co-inoculation of citrus seedlings with the most virulent species from these genera, will be done under controlled conditions. The level of root infestation by the different pathogens as well as their interactions will then be done through qPCR and or T-RFLP using new or existing (Ippolito *et al.* 2004; Tewoldemedhin *et al.* 2011) primers. The findings of this controlled study will subsequently be validated by taking root samples from old, existing orchards and subjecting them to the same analyses.

For this purpose, two orchards showing replant problems, will be identified in the Western and Eastern Cape production areas. Within these two orchards, escape trees and diseased trees will be identified. From the escape trees, root and soil samples will be collected for the isolation of the rhizosphere and endophyte bacterial and fungal communities. Isolations will be done using classical techniques described above. Collected bacterial and fungal isolates will then be subjected to *in vitro* screening to determine their ability to inhibit the growth of replant pathogens identified by Swart (2018). The most successful isolates will then be used in further *in vivo* studies to determine if the identified BCA's are effective in colonizing citrus seedling roots and if it can protect these seedlings against root pathogen infection. The roots collected from both escape and diseased trees will then be used to study the interaction of the various mentioned replant pathogens *in planta*.

This study therefore aim to provide better insight into the interaction of the various citrus replant pathogens that could lead to better, more sustainable management strategies. The results from this study could also lead to a BCA or mixture of BCA's that can be used in an integrated management systems to provide long-term protection against soilborne pathogens not only in citrus nurseries but also orchards.

### **Stated objectives**

1. Study the interaction of the citrus replant pathogen complex under controlled and field conditions.
2. Identify and evaluate potential biocontrol agents that can be used in the integrated management of citrus replant pathogens.

### **Materials and methods**

*Objective 1. Study the interaction of the citrus replant pathogen complex in planta under controlled and field conditions.*

1. Develop new species-specific qPCR primers for the detection of citrus replant pathogens.

Primers were designed for detection of *Phytophthora* spp. and *Neocosmospora* spp. by using the Geneious Prime software 2019.1.3 (<https://www.geneious.com>).

The region of the ras-related protein gene *Ypt1* was used to developed species specific primers for the identification of *Phytophthora citrophthora* and *Phytophthora nicotianae*. Reference sequences of *Ypt1* genes from the two *Phytophthora* spp. were obtained from the nucleotide database, the National Centre for

Biotechnology Information (NCBI) GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide/>) and aligned using MAFFT v. 7388 (Kato and Standley, 2013; Kato *et al.* 2002 in order to design multiple pair of primers.

Gene sequences for *N. ferruginea* and *N. citricola* isolates were obtained from a previous study (Guarnaccia *et al.*, 2021). The ITS gene was used to develop primers for the identification of *Neocosmospora citricola* and the *RPB2* gene was used for the identification of *Neocosmospora ferruginea*. For *Neocosmospora solani*, the beta tubulin gene ( $\beta$ -tubulin) was chosen to develop species-specific primers. In order to have sequences for *N. solani*, the primers Bt2A (5' GGTAACCAAATCGGTGCTGCTTTC 3') and Bt1B (5' GACGAGATCGTTCATGTTGAACTC 3') (Boyogueno, 2010) were used to amplify and sequence the  $\beta$ -tubulin gene of selected isolates from Guarnaccia *et al.* 2021. The PCR-amplified products were sent to the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University for DNA sequencing. Following sequencing, consensus sequences were obtained for the different isolates. From these sequences primers were subsequently developed.

Primer design was based on primer length, primer melting temperature ( $T_m$ ), GC content, annealing temperature, product size and chance of hairpin/dimer formation. The specificity of these primers was initially assessed *in silico* by using the Basic Local Alignment Search Tool (BLAST) with the NCBI database prior to testing by PCR amplification. All primers were synthesized by Inqaba Biotechnical Industries (South Africa) and stored at -20°C.

For *P. irregulare*, existing specific qPCR primers designed by Spies *et al.* (2011), based on the internal transcribed spacer region, were used.

## 2. Validate existing and new species-specific qPCR primers for replant pathogen detection in soil and plant material.

### *Fungal isolates and culturing conditions*

All the *Phytophthora* spp., *Neocosmospora* spp. and *Pythium irregulare* isolates used in this study are listed in Table 4.3.2.1. *Phytophthora* and *P. irregulare* isolates were obtained from a previous study (van der Merwe, 2017; van der Merwe, 2019). The *Neocosmospora* isolates, which were identified using ITS (internal transcribed spacer region), TEF (translation elongation factor 1- $\alpha$ ) and *RPB2* (RNA polymerase II gene) sequence data, were collected during 2018 from two citrus production regions in South Africa (Guarnaccia *et al.*, 2021). Other fungal and oomycete species (close relatives) were made available from the STE-U culture collection at the Department of Plant Pathology (Stellenbosch University, South Africa). Isolates were cultured for 7-14 days at 25°C on potato dextrose agar (PDA, 39 g/L), with the exception of *Phytophthora infestans*, which was grown on wheat medium (120 g of crushed pearled wheat seeds, blended, boiled and filtered, plus 15 g of sucrose and agar, per litre of water) and were maintained by regular sub-culturing on their respective media. All isolates were stored either in sterile distilled water with citrus leaf pieces at room temperature (*Phytophthora* spp. and *P. irregulare* isolates) or on water agar (WA, 12 g/L) slants and in sterile, distilled water at 4°C (*Neocosmospora* spp.).

### *gDNA extraction from replant pathogen cultures and citrus roots*

For the isolation of gDNA from the different replant pathogen isolates, each isolate was grown on PDA at 25°C for 7-14 days (with the exception of *P. infestans*, which was grown on wheat medium), and the mycelium was harvested and used for gDNA extraction (Table 4.3.2.1). The mycelia harvested were lyophilized, and ~100 mg mycelia were powdered in 2 mL Eppendorf™ tubes (Eppendorf, Germany) containing glass beads (3 mm) by shaking for 5 min at 30 Hz in a Retsch® MM400 mixer mill (Fisher Scientific, United States). The extraction was performed using the NucleoSpin® Plant II kit (Macherey-Nagel GmbH and Ko, Germany) according to the manufacturer's instructions with minor modifications. Cell lysis Buffer PL1 was used instead of Buffer PL2, and following the addition of Buffer PC, samples were not mixed by pipetting, but rather vortexed. Close relatives of *P. irregulare*, *Phytophthora* spp. and *Neocosmospora* spp. were also extracted to validate the existing and newly designed primers.

Root samples from pathogen free citrus roots were collected and were surface sterilized in 70% ethanol for 30 s, rinsed in distilled water for 60 s, and allowed to air-dry on sterile paper towels. The root samples were placed into 15 mL falcon tubes and freeze dried. The falcon tubes were stored at -80°C until lyophilization.

Root DNA extractions were conducted by first lyophilizing the root samples for 48 h at a chamber temperature of -52°C to -55°C. This was followed by grinding of the roots, in the Falcon tubes, into a powder using a flame sterilized tweezer. Root samples were further powdered in 30 mL tubes each containing 5 g glass beads (2 mm diameter) by shaking for 5 min at 30 Hz in a Retsch® MM400 mixer mill (Fisher Scientific, United States). A subsample of 20 mg of roots were transferred into 1.5 mL Eppendorf™ tubes (Eppendorf, Germany) containing 8 g of 3 mm diameter glass beads. The Eppendorf™ tubes (Eppendorf, Germany) were shaken for 5 min at 30 Hz using a Retsch® MM400 mixer mill (Fisher Scientific, United States) after which the DNA was extracted. Genomic DNA was extracted from the powdered roots using the Nucleospin® Plant II kit (Macherey-Nagel GmbH and Ko, Germany) according to the manufacturer's instructions with minor modifications as described above. The quantity and purity of extracted DNA were measured using a NanoDrop™ spectrophotometer (Thermo Fischer Scientific, USA). DNA was stored at 4°C until further analysis.

**Table 4.3.2.1.** Fungal isolates used during validation of qPCR assays for the detection of *Phytophthora citrophthora*, *Phytophthora nicotianae*, *Neocosmospora citricola*, *Neocosmospora ferruginea*, *Neocosmospora solani* and *Pythium irregulare*.

Species name	Number of isolates	Hosts
<i>Pythium irregulare</i> s.s	6	Citrus (6)*
<i>Phytophthora nicotianae</i>	10	Citrus (10)
<i>Phytophthora citrophthora</i>	12	Citrus (12)
<i>Neocosmospora ferruginea</i>	6	Citrus (6)
<i>Neocosmospora citricola</i>	8	Citrus (8)
<i>Neocosmospora solani</i>	14	Citrus (14)
<i>Phytophthora</i> close relatives		
<i>P. cactorum</i>	1	Strawberry (1)
<i>P. infestans</i>	1	Potato (1)
<i>P. capsica</i>	1	Tomato (1)
<i>P. citricola</i>	1	Agathosma (1)
<i>P. menzei</i>	1	Avocado (1)
<i>Neocosmospora</i> close relatives		
<i>F. solani</i>	4	Grapevine (2), Apple (2)
<i>F. oxysporum</i>	7	Citrus (5), Apple (2)
<i>F. equiseti</i>	1	Apple (1)
<i>F. verticillioides</i>	2	Maize (2)
<i>F. graminearum</i>	2	Wheat (2)

\*Number in parenthesis indicates the number of isolates from each host.

#### Primer specificity in PCR

Conventional PCR assays were performed in order to validate the existing and new species-specific qPCR primers for *Phytophthora nicotianae*, *P. citrophthora*, *P. irregulare* and *Neocosmospora* spp. PCR-parameters for the newly designed primer sets were evaluated and optimized with two isolates of each species, in order to find the optimal annealing temperature.

For the two *Phytophthora* spp., each PCR amplification (25 µL) contained 2 µL of gDNA, 12.5 µL of Taq (GoTaq™ green master mix from Promega Corporation, USA or KAPA Taq ReadyMix PCR Kit from KAPA BIOSYSTEMS, USA), 0.5 µL of each primer set (10 µM), and 9.5 µL of nuclease-free water (QIAGEN, Germany). Amplification reactions were carried out in an Applied Biosystems Veriti Dx Thermal Cycler (ThermoFischer Scientific, USA) with the following cycling conditions: initial denaturation step at 95°C for 2

min followed by 30 cycles of denaturation at 94°C for 30 s, 30 s at annealing temperature (50-65°C was used to optimize primer performance) and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min.

For *Neocosmospora* spp. each PCR amplification (12.5 µL) contained 2 µL of gDNA, 6.25 µL of Taq (GoTaq™ green master mix from Promega Corporation, USA or KAPA Taq ReadyMix PCR Kit from KAPA BIOSYSTEMS, USA), 0.25 µL of each primer set (10 µM), and 3.75 µL of nuclease-free water (QIAGEN, Germany). For *N. FSSC 28* and *N. species 1* the PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing temperature (50-62°C was used to optimize primer performance) for 30 s, elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. For *N. solani* the PCR conditions consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing temperature (52-64°C was used to optimize primer performance) for 30 s, elongation at 72°C for 1 min and a final elongation at 72°C for 1 min. The primer sets that did not amplify were removed and the annealing temperature was chosen to be at 59-60°C.

PCR assays were then performed to validate the specificity of the chosen sets of primers for *Phytophthora* spp. and *Neocosmospora* spp. The specificity of the newly designed primers was tested against DNA extracted from the isolates listed in Table 4.3.2.2. Isolates for each species were selected as well as negative control isolates and water.

For *Phytophthora* spp. in a total reaction volume of 25 µL, the PCR reaction contained 2 µL of DNA, 12.5 µL Taq (GoTaq™ green master mix from Promega Corporation, USA or KAPA Taq ReadyMix PCR Kit from KAPA BIOSYSTEMS, USA), 0.5 µL at 10 µM of each set of primers and 9.5 µL of nuclease-free water (QIAGEN, Germany). The PCR cycle consisted of an initial denaturation step at 95°C for 2 min followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C with a final extension step at 72°C for 5 min.

For *Neocosmospora* spp. each PCR amplification (12.5 µL) contained 2 µL of gDNA, 6.25 µL of Taq (GoTaq™ green master mix from Promega Corporation, USA or KAPA Taq ReadyMix PCR Kit from KAPA BIOSYSTEMS, USA), 0.25 µL of each primer set (10 µM), and 3.75 µL of nuclease-free water (QIAGEN, Germany). For *N. FSSC 28* and *N. species 1* the PCR conditions consisted of an initial denaturation step at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, 30 s at 59°C annealing temperature and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. For *N. solani*, the PCR conditions consisted of an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, 30 s at 60°C annealing temperature and extension at 72°C for 1 min, with a final extension step at 72°C for 1 min.

All PCR products were analyzed by agarose gel electrophoresis (1% w/v) in TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5). Gels were stained with SYBR™ Safe DNA Gel Stain (ThermoFischer Scientific, USA) and visualized under ultraviolet (UV) light with Gel Doc™ XR+ Imager and Image Lab software 5.2.1 (Biorad, Hercules, USA).

**Table 4.3.2.2.** Fungal and oomycete isolates used during validation of qPCR assays for the detection of *Phytophthora citrophthora*, *Phytophthora nicotianae*, *Neocosmospora citricola*, *Neocosmospora ferruginea*, *Neocosmospora solani* and *Pythium irregulare* s.s.

Species name	Number of isolates	Isolate code	Hosts
<i>Pythium irregulare</i> s.s.	6	149B, 59B, 174B, 80B, 81B, 51.1	Citrus (6) <sup>a</sup>
<i>Phytophthora nicotianae</i>	10	33.1, 37.1, 14.2, 15.1, 4.2, 19.1, 12.1, 16.2, 35.1, 22.1	Citrus (10)
<i>Phytophthora citrophthora</i>	12	C3A, C35A, C10A, K12A, C25B, C8B, K9A, C26A, C2B, C41B, C5B, C6A	Citrus (12)
<i>Neocosmospora ferruginea</i>	6	VG109, VG205, VG133, VG195, VG289, VG394	Citrus (6)
<i>Neocosmospora citricola</i>	8	VG17, VG139, VG30, VG302, VG183, VG203, VG343, VG140	Citrus (8)

<i>Neocosmospora solani</i>	14	VG99, VG63, VG78, VG46, VG169, VG68, VG38, VG53, VG93, VG147, VG175, VG193, VG36, VG115	Citrus (14)
<i>Phytophthora</i> close relatives			
<i>P. cactorum</i>	1	DC 316/14	Strawberry (1)
<i>P. infestans</i>	1	PI3B	Potato (1)
<i>P. capsici</i>	1	STE-U 6697	Tomato (1)
<i>P. citricola</i>	1	STE-U 6270	Agathosma (1)
<i>P. menzei</i>	1	P1165	Avocado (1)
<i>Neocosmospora</i> close relatives			
<i>Fusarium solani</i>	4	STE-U 6187, STE-U 6188, STE-U 7213, STE-U 7214	Grapevine (2), Apple (2)
<i>Fusarium oxysporum</i>	7	STE-U 8512, STE-U 8511, STE-U 8514, STE-U 8510, STE-U 7210, STE-U 7211, STE-U 8489	Citrus (5), Apple (2)
<i>Fusarium equiseti</i>	1	STE-U 7209	Apple (1)
<i>Fusarium verticillioides</i>	2	452, 458	Maize (2)
<i>Fusarium graminearum</i>	2	W-2-922, W-2-952	Wheat (2)

<sup>a</sup> Number in parenthesis indicates the number of isolates from each host.

### Primer specificity in qPCR

#### Singleplex

Primer sets for each species that were suitable and specific in conventional PCR, were tested for use in qPCR. For *Phytophthora* spp., *P. irregulare* and *Neocosmospora* spp. conditions were optimized for each primer set using a gradient of annealing temperatures of 58-65°C for *Phytophthora* spp., 61-67°C for *N. ferruginea* and *N. citricola*, 62-68°C for *N. solani*, and 60-64°C for *P. irregulare*. Primers that amplified only the species for which the primers were designed and that resulted in the highest fluorescence of target DNA were selected for subsequent qPCRs (Table 4.3.2.3).

Once qPCR cycling conditions were optimized for the detection of *Phytophthora* spp., *P. irregulare* and *Neocosmospora* spp., the specificity of assays was assessed. Specificity was tested against the specific species and the same collection of close relatives used above, as well as other citrus replant pathogens. For *Phytophthora* spp. and *Neocosmospora* spp. each qPCR reaction contained 2 µL of gDNA, 10 µL SensiFAST SYBR No-ROX Mix (Bioline, USA), 1 µL at 10 µM of each set of primers, and 6 µL of nuclease-free water (QIAGEN, Germany). For *P. irregulare* each qPCR reaction contained 2 µL of gDNA, 10 µL SensiFAST SYBR No-ROX Mix (Bioline, USA), 0.6 µL of primer *PirF1* at 10 µM, 1.8 µL of primer *PirR3* at 10 µM, and 5.6 µL of nuclease-free water (QIAGEN, Germany). The qPCR conditions were as follows: an initial denaturation at 95°C for 3 min; followed by 40 cycles of 95°C for 5 s, annealing temperature for 10 s, and 72°C for 20 s, concluding with a melt curve. The exception was the *P. irregulare* assay, which used a 10 s denaturation time, and a 15 s annealing time. All qPCR assays were performed in a CFX96™ Real-Time System (Bio-Rad Laboratories, USA).

To test the repeatability of each qPCR assay, DNA from one sample was used in three technical repeats. Nuclease-free water was used as a negative control to replace template DNA.

#### Multiplex

A multiplex real-time PCR assay was developed for the simultaneous detection of *P. nicotianae* and *P. citrophthora*. Similar to the singleplex assays, the qPCR reaction conditions (i.e. annealing temperature and primer concentrations) were adjusted experimentally. Reaction conditions were optimised for selected primer sets using annealing temperatures of 58-65°C. Conditions that resulted in the highest fluorescence of target DNA were selected for subsequent qPCRs. Primers were adjusted to their optimal concentration to ensure balanced amplification of each targeted amplicon and to compensate for varying amplification efficiencies. Once qPCR conditions were optimized for the detection of *Phytophthora* spp., the specificity of assays were

assessed. Specificity was tested alone and in mixtures with the specific species and a collection of close relatives as well as other citrus replant pathogens.

All qPCR reactions were performed in a CFX96™ Real-Time System (Bio-Rad Laboratories, USA). The qPCR reaction contained 10 µL SensiFAST SYBR No-ROX Mix (Bioline, USA), 1.5 µL of each *P. citrophthora* primer at 10 µM, 0.25 µL of each *P. nicotianae* primer at 10 µM, 2 µL of gDNA, and 4.5 µL nuclease-free water (QIAGEN, Germany) up to a final volume of 20 µL. The final primer concentrations were 125 nM for each *P. nicotianae* primer and 750 nM for each *P. citrophthora* primer. The real-time PCR condition was programmed as follows: initial denaturation of 95°C for 3 min, then 40 cycles of 95°C for 5 s, 65°C for 10 s, and 72°C for 20 s, followed by a ramp from 60 to 95°C for melting curve stage. For this stage, the reaction temperature is increased from 60 to 95°C at a rate of 0.5°C every 5 s with a continuous fluorescence monitoring. Each set of qPCR reactions included three technical replications of each sample and a negative (nuclease-free water) control.

#### *Optimization and sensitivity of qPCR*

##### Tests for linearity (Singleplex)

Standard curves were constructed for each of the investigated citrus replant pathogens including *P. irregulare*, *P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola* (Table 4.3.2.2). Isolates of the different species were obtained as previously described. One isolate each of *P. irregulare* (51.1), *P. citrophthora* (C8A), *P. nicotianae* (22.1), *N. solani* (VG36), *N. ferruginea* (VG289), and *N. citricola* (VG17) was grown on PDA. DNA was extracted using the NucleoSpin® Plant II kit (Macherey-Nagel GmbH and Ko, Germany) according to the manufacturer's instructions as described above. The quantity and purity of extracted DNA were measured using a NanoDrop™ spectrophotometer (Thermo Fischer Scientific, USA). A standard curve for each pathogen was constructed from four fold (*P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola*) and fivefold (*P. irregulare*) serial dilutions of gDNA, which yielded 5 to 9 gDNA concentration points depending on the pathogen. For this purpose, gDNA were serially diluted in nuclease-free water as well as in a fixed background of root DNA extracted from pathogen free citrus roots. The qPCR assays were conducted for each of the concentrations using three technical replicates per concentration.

All qPCR assays developed in this study were performed using the SensiFAST SYBR No-ROX Mix (Bioline, USA). The qPCR reactions were performed in a total volume of 20 µL, with primers at the concentrations described in Table 4.3.2.3. Reaction conditions consisted of an initial denaturation step of 95°C for 3 min followed by 40 cycles of denaturing at 95°C for 5 s, the relevant annealing temperature and times (Table 4.3.2.3), and extension at 72°C for 20 s. The exception was the *P. irregulare* assay, which used a 10 s denaturing time. Amplifications were performed in a CFX96™ Real-Time System (Bio-Rad Laboratories, USA). Each dilution point was assayed in triplicate. All assays included a non-template control (i.e. nuclease-free water).

Standard curves were constructed by plotting the cycle threshold (Ct) values against a logarithm of the initial DNA concentration. Standard curves were acceptable if the amplification efficiency was 90-110% and the correlation coefficient (R<sup>2</sup>) was above 0.980 (Taylor *et al.*, 2010).

**Table 4.3.2.3.** Primers selected for specific amplification of *Phytophthora citrophthora*, *Phytophthora nicotianae*, *Neocosmospora citricola*, *Neocosmospora ferruginea*, *Neocosmospora solani* and *Pythium irregulare* by means of quantitative real-time PCR (qPCR), with their respective concentrations and amplification conditions.

Target species	Target region	Primers (nM) <sup>a</sup>	Primer sequence (5' to 3')	T <sub>m</sub> <sup>b</sup> (°C)	Product size (bp)	Annealing	
						Temperature	Time
<i>P. citrophthora</i>	Ypt1	PC_YPT1_FW2 (500)	GAAGAAGAGATCCAGTGAGGTTTC	62.5	113	65	10
		PC_YPT1_RV2 (500)	GGTGGATGACGAGTCTTAAACA	63.2			
<i>P. nicotianae</i>	Ypt1	PN_YPT1_FW17 (500)	GTGTGTGTCTGTAGTGGGACACG	67.1	147	65	10
		PN_YPT1_RV17 (500)	GGATCTTCTCTCGATAAGTCGGAC	65.4			
<i>N. citricola</i>	ITS	NSP1_FW1_ITS (500)	GAGGACCCCTATCTCTGTTA	61.4	130	67	10
		NSP1_RV1_ITS (500)	ATGTGCGTTCAAAGATTCCG	61.8			
<i>N. ferruginea</i>	RPB2	NFSSC28_FW1_RPB2 (500)	CTTTACTACCCGCAAAAACCC	63.6	162	67	10
		NFSSC28_RV1_RPB2 (500)	CTTCTCTTGGTCAGAGTAAGATCG	62.6			
<i>N. solani</i>	β-tubulin	BTSOL_FW5 (500)	GTCGACCAGGTCCTCGAT	63.4	170	69	10
		BTSOL_RV5 (500)	CTCGACGACGGTGTCTGAG	65.4			
<i>P. irregulare</i>	ITS	PirF1 (300)	AGTGTGTGTGGCACGTTGTC	65.5	±120	65	15* <sup>c</sup>
		PirR3 (900)	GATCAACCCGGAGTATACAAAAC	62.6			

<sup>a</sup> Primer concentrations used in qPCRs.

<sup>b</sup> Primer melting temperature (T<sub>m</sub>) was calculated using the Multiple Primer Analyzer (<https://www.thermofisher.com/za/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html>).

<sup>c</sup> Values followed by \* were modified from the published assay.

### Limit of quantification and limit of detection (Singleplex and multiplex)

The limit of quantification (LOQ) is defined as the highest dilution of template that is still linear. The limit of detection (LOD) is defined as the concentration at which the assay is no longer linear. That is, a dilution that gives a signal but is no longer co-linear with the other dilutions (Bustin *et al.*, 2010). To determine the LOD/LOQ of the different target species, 10-fold serial dilutions of the pathogen gDNA were prepared in nuclease-free water as well as in a fixed background of pathogen free citrus root DNA. With the singleplex qPCR, both the LOQ and the LOD of the new assays were evaluated. While for the multiplex assay only the limit of detection of *P. citrophthora* and *P. nicotianae* was calculated.

Each 20 µL reaction mixture consisted of 10 µL SensiFAST SYBR No-ROX Mix (Bioline, USA), the relevant primers and primer concentrations (stock at 10 µM) (Table 4.3.2.3), 2 µL of gDNA, and nuclease-free water. For the multiplex assay, the qPCR reaction contained 10 µL SensiFAST SYBR No-ROX Mix (Bioline, USA), 1.5 µL of each *P. citrophthora* primer at 10 µM, 0.25 µL of each *P. nicotianae* primer at 10 µM, 2 µL of gDNA, and 4.5 µL nuclease-free water up to a final volume of 20 µL. The final primer concentrations were 125 nM for each *P. nicotianae* primer and 750 nM for each *P. citrophthora* primer. qPCR assays were performed in a CFX96™ Real-Time System (Bio-Rad Laboratories, USA) and consisted of an initial denaturation step of 95°C for 3 min followed by 40 cycles of denaturing at 95°C for 5 s, the relevant annealing temperature and times (Table 4.3.2.3), and extension at 72°C for 20 s. The exception was the *P. irregulare* assay, which used a 10 s denaturing time. Each dilution point was tested in triplicate along with negative controls (i.e. nuclease-free water).

Standard deviations between the Ct values of the technical replicates were recorded at each LOQ and LOD. Standard deviations below 0.35 were considered acceptable (Hellemans and Vandesompele, 2011).

### 3. Prepare citrus seedlings for dual and multiple pathogen inoculation studies.

Inoculation studies were conducted on healthy 1-year-old Carrizo citrange rootstock seedlings grown under controlled conditions (temperatures of 25°C) in the glasshouse. The rootstock seeds, two to three per bag were planted in March 2019 into 1 L self-draining plastic bags (100 mm x 214 mm), filled with sterile potting medium. Seedlings were watered as needed. The potting medium (Reliance Compost) was made up of compost and pine bark, which was steam sterilized for 180 min at 80°C prior to planting.

### 4. Study replant pathogen interaction through dual and multiple pathogen inoculations of citrus seedlings

#### *Inoculum preparation*

Sand bran inoculum was prepared using the method described by Lamprecht *et al.*, (1986), with minor adjustments. The sand-bran inoculum was prepared by mixing 400 g washed river sand with 40 g wheat bran and 60 mL distilled water in 1000 mL Schott bottles (Duran®). The mixture was autoclaved for 15 min at 121°C for two consecutive days prior to use and left to cool. Prior to preparing the sand bran inoculum, each isolate was cultured on potato dextrose agar (PDA, 39 g/L) for 7-14 days at 25°C. Ten (6 mm diameter) PDA plugs, containing *Phytophthora*, *Neocosmospora* spp. and *P. irregulare* mycelium were used to inoculate the sand-bran mixture (one isolate per bottle). The control bottles were inoculated with sterile PDA agar plugs only. Additionally, combinations of these species were included. The inoculum was incubated for 5 weeks at 25°C without direct light exposure and shaken every second day to ensure even growth of the mycelium throughout the inoculum mixture.

#### *Inoculation of seedlings*

The seedlings were exposed to inoculations with the different replant pathogens alone and in combination to study colonization of seedling roots by the different replant pathogens mentioned above.

Prior to inoculation, the shoot length (from soil surface to tip of shoot) of each seedling was recorded. For inoculation, 50 g/L of sand bran inoculum was added to each planting bag. Inoculation was done by making

two holes (opposite sides of the root system) with an aluminium spoon and placing the sand bran inoculum in equal parts into these holes. After inoculation, the seedlings were watered with normal tap water until the trial was terminated. Seedlings were sprayed once a week (ensuring good coverage of the foliage), either with Oleum (720 a.i. g/L; Agro-Serve), Crystal 550 SC (550 a.i. g/L; Villa Crop Protection) or Spirofen 240 SC (240 a.i. g/L; Villa Crop Protection) to manage sucking insect pests. Oleum, Crystal 550 SC and Spirofen 240 SC solutions were prepared using tap water. For each inoculation treatment 16 seedlings were inoculated, two biological repetitions with eight technical replicates (eight seedlings) each.

## Trial evaluation

### *Seedling growth parameters*

After ~3 months of growth, the trial was destructively evaluated by measuring the shoot length (cm), seedling fresh weight (g), root fresh weight (g) and volume (cm<sup>3</sup>). Accordingly, seedlings were removed from the plastic planting bags and soil was gently rinsed from the roots with running tap water before determining abovementioned measurements. Their root systems were separated from the aerial parts with a sterilised pruning shear (spray-sterilised with 70% ethanol). Using 70% ethanol and running tap water, the scale and volumetric cylinders were cleaned thoroughly between each treatment. Root systems were then taken to the laboratory in brown paper bags (124 mm x 260 mm) and prepared for pathogen and gDNA isolations.

### *Preparation of citrus roots*

In order to fulfil one of Koch's postulates, re-isolations were made from the roots of inoculated seedlings. Roots were surface sterilized in 70% ethanol (Moralejo *et al.*, 2009; Spies *et al.*, 2011; Sandoval-Denis *et al.*, 2018) for 30 s, rinsed in distilled water for 60 s, and allowed to air-dry on sterile paper towels. At this point, each root sample was divided into two groups for (i) isolation of *Neocosmospora*, *Pythium* and *Phytophthora* spp. and (ii) gDNA extraction.

### *Re-isolation of fungi (Neocosmospora spp.) and oomycetes (Pythium and Phytophthora spp.) from roots*

For replant pathogen re-isolation, segments of root tissue were plated onto three different growth mediums (in 90 mm Petri dishes). Five small root pieces, each approximately 5 mm in length were removed from the root systems using a flame sterilized scalpel and plated onto different mediums according to the species inoculated (three Petri dishes per root system). PARP (Jeffers and Martin, 1986) was used to isolate for *Pythium*, PARPH (Jeffers and Martin, 1986) to isolate *Phytophthora* spp. and PDA (Biolab, Merck, 39 g/L), amended with streptomycin (0.04 g/L) for *Neocosmospora* spp. isolation. All plates were incubated at room temperature for at least 6-7 days, with the exception of PARP and PARPH plates, which were incubated in the dark (in brown paper bags at 28°C). When fungal growth emerging from the roots was observed, the fungi and oomycetes were identified based on morphological characteristics. Per treatment the percentage pathogen colonised root pieces was noted per pathogen and recorded.

## Detection and quantification of pathogens in roots

### *DNA extraction from roots*

Portions of seedling root systems selected for qPCR pathogen detection and quantification, were immediately wrapped in labelled aluminium foil squares, flash frozen in liquid nitrogen, and stored at -80°C before further analyses. For each treatment, two seedlings from the same block were pooled and mixed together into one sample and subdivided for gDNA extraction.

Root gDNA extractions were conducted by first lyophilizing the root samples for 48 hr at a chamber temperature of -52°C to -55°C, followed by grinding the roots into a powder using a sterile tweezer. Root samples were further powdered in 30 mL Falcon tubes, each containing 5 g of glass beads (2 mm diameter), by shaking for 5 min at 30 Hz in a Retsch® MM400 mixer mill (Fisher Scientific, United States). After crushing, subsamples of 20 mg of freeze dried root were transferred into 1.5 mL Eppendorf™ tubes (Eppendorf, Germany) containing

0.5 g of 2 mm diameter glass beads. The Eppendorf™ tubes (Eppendorf, Germany) were shaken for 5 min at 30 Hz using a Retsch® MM400 mixer mill (Fisher Scientific, United States) after which the gDNA was extracted. Genomic DNA was extracted from the powdered roots using the Nucleospin® Plant II kit (Macherey-Nagel GmbH and Ko, Germany) according to the manufacturer's instructions with minor modifications. Cell lysis Buffer PL1 was used instead of Buffer PL2, and following the addition of Buffer PC, samples were not mixed by pipetting, but rather vortexed. DNA was stored at 4°C until further analysis. The quantity and purity of extracted DNA were measured using a NanoDrop™ spectrophotometer (Thermo Fischer Scientific, USA).

#### *Detection and qPCR quantification of citrus replant pathogens from roots*

Singleplex or multiplex qPCR reactions were carried out for each root sample depending on the different treatments. *Phytophthora citrophthora*, *P. nicotianae*, *N. citricola*, *N. ferruginea*, *N. solani* and *P. irregulare* were quantified from root DNA extracts using the newly developed singleplex qPCR assays described above. Reactions for qPCR quantification were conducted by using the same reaction and amplification conditions that were used for constructing the standard curves. Each 20 µL qPCR reaction contained 2 µL of undiluted gDNA, and each DNA sample was analysed in triplicate. A negative control (i.e. nuclease-free water) and a standard curve control sample were included in each qPCR assay. Amplifications were conducted in a CFX96™ Real-Time System (Bio-Rad Laboratories, USA).

The root DNA extracts were applied in multiplex qPCR for the detection of *P. nicotianae* and *P. citrophthora*. The reaction and amplification conditions were the same as those used to determine the limit of detection (LOD) of the assay above. The qPCR reactions consisted of a total volume of 20 µL, each containing 2 µL of undiluted gDNA, and each sample was assayed in triplicate. For each qPCR assay, a negative control (i.e. nuclease-free water) and a standard curve control sample were included. All qPCR reactions were performed in a CFX96™ Real-Time System (Bio-Rad Laboratories, USA).

Melt curve analysis was performed at the end of the amplification cycle in order to detect the two *Phytophthora* species. For this stage, the reaction temperature was increased from 60°C to 95°C at a rate of 0.5°C every 5 s with a continuous fluorescence monitoring. Positive reactions for *P. citrophthora* were recognized by a melt peak with a temperature of 82°C whereas positive reactions for *P. nicotianae* were characterized with a melt peak at 85.50°C. *Neocosmospora citricola*, *N. ferruginea* and *N. solani* were recognized by a melt peak with a temperature of 80.50°C, 84.50°C and 87°C, respectively. Positive reactions for *P. irregulare* were recognized by a melt peak with a temperature of 80°C.

#### Statistical analysis

For the glasshouse trial, the experimental design was a randomised block with the 11 treatments replicated in four blocks. An experimental unit consisted of 4 seedlings in total. The increase in length per seedling were calculated and analysed. For each pathogen, the percentage of re-isolation was calculated per experimental unit. The average qPCR values per treatment and block were subjected to analysis of variance (ANOVA). Log transformation was applied to normalise the data distribution. Principal component analysis (PCA) were performed using the means of the 11 treatment combinations.

*Objective 2. Identify and evaluate potential biocontrol agents that can be used in the integrated management of citrus replant pathogens.*

#### 1. Isolation of potential biocontrol agents (BCA's) from sampled roots and rhizosphere soil

##### *Sampling of roots and rhizosphere soil*

Forty soil and root samples were taken from old orchards in the Citrusdal (Western Cape) and Kirkwood (Eastern Cape) citrus production areas that were grown on Carrizo citrange and Rough lemon rootstocks. In each of these orchards, 10 visually healthy trees and 10 trees showing decline symptoms were selected. At each tree, soil and root samples were collected from two points: from each side of the trunk under the tree

canopy, at a depth of 10–20 cm. The two samples per tree were mixed into one composite sample per tree (Aranda *et al.*, 2011) and were transported to the laboratory for further analyses.

#### *Isolation of bacteria and fungi from rhizosphere soil and roots*

The isolation of potential BCAs was focused on *Bacillus* and *Pseudomonas* spp. as well as *Trichoderma* spp. The collected roots were shaken gently for the removal of excess soil, leaving only the closely adhering soil on the root surface. Two grams of roots from each sample were placed into 15 mL Falcon tubes, along with 10 mL sterile distilled water and vortexed for 1 minute to put the rhizosphere soil into suspension (Aranda *et al.*, 2011).

The roots were removed from the Falcon tubes and the resulting suspensions were serially diluted to  $10^{-3}$  and  $10^{-4}$  for the isolation of fungi and bacteria respectively (Shinde and Sadgir, 2016). For the isolation of *Bacillus* spp., 100  $\mu$ L of suspension were spread onto Nutrient Agar (31 g.L $^{-1}$ ; NA, Merck) and incubated at 28°C for 48 hrs (Turner and Backman, 1991). From these initial isolations, single colonies with different morphology were selected and streaked repeatedly onto NA to obtain pure single colony isolates. *Pseudomonas* spp. isolations were done by plating out 100  $\mu$ L of suspension onto King's B media (King *et al.* 1954) and NA media. Plates were incubated at 28°C for 48 h (Acebo-Guerrero *et al.*, 2015; Shinde and Sadgir, 2016). Single colony isolates were again obtained by re-streaked single colonies from the initial plates onto KB and NA (Acebo-Guerrero *et al.*, 2015; Shinde and Sadgir, 2016). *Trichoderma* spp. were isolated by plating 100  $\mu$ L of the suspension onto potato dextrose agar (PDA, 39 g.L $^{-1}$ , Merck) and potato dextrose agar with streptomycin sulphate (40 mg.L $^{-1}$ ; PDAs) to inhibit bacterial growth. From these initial plates, *Trichoderma* spp. were hyphal tip transferred to clean PDAs plates and stored at 28°C for 3 days. For the initial isolations from the soil suspensions, five plates per growing medium were used.

For the isolation of potential BCAs from citrus roots, root samples were removed from the Falcon tubes and were surface sterilized in 70% ethanol for 30 seconds, rinsed in distilled water and air-dried in a laminar flow. Four root pieces of 5 mm each were plated out on five plates for each of abovementioned media. Bacteria that oozed from the roots, plated on NA and KB media, were selected and streaked repeatedly on the same media to obtain single isolate cultures. When fungal growth, originating from the roots was observed, pure cultures were obtained by transferring a hyphal tip to fresh PDAs (Sandoval- Denis *et al.*, 2018).

#### 2. Preliminary identification of selected BCA's.

Preliminary identification to genus level of bacteria isolated from the roots and rhizosphere soils was based on morphological observations, such as colony colour on NA and KB media (King *et al.*, 1954). Further identification included physiological and biochemical testing based on Bergeys' Manual of Systematic Bacteriology (King *et al.*, 1954; Suslow *et al.*, 1982; Bergey, 2004; Zhang *et al.*, 2016). The biochemical tests included the assessment of anaerobic growth, pigmentation (KB medium), and Potassium hydroxide (KOH) solubility test. These tests were done for the identification of *Bacillus* and *Pseudomonas* spp. For the preliminary identification of *Trichoderma* spp., morphological traits (colony colour, hyphal growth) were recorded within 4-5 days from cultures that were grown on PDAs at 28°C (Aleandri *et al.*, 2015). Based on this preliminary identification, isolates from the different genera were selected for *in vitro* screening.

#### 3. *In vitro* screening of potential BCA for inhibition of the growth of citrus replant pathogens.

For the selection of potential BCAs from the bacterial and *Trichoderma* isolates that were collected from sampled roots and rhizosphere soil, different preliminary *in vitro* screening tests were conducted. These tests evaluated the mycelial growth inhibition of single isolates of the different citrus replant pathogens (Table 4.3.2.4) by the selected BCAs according to different parameters.

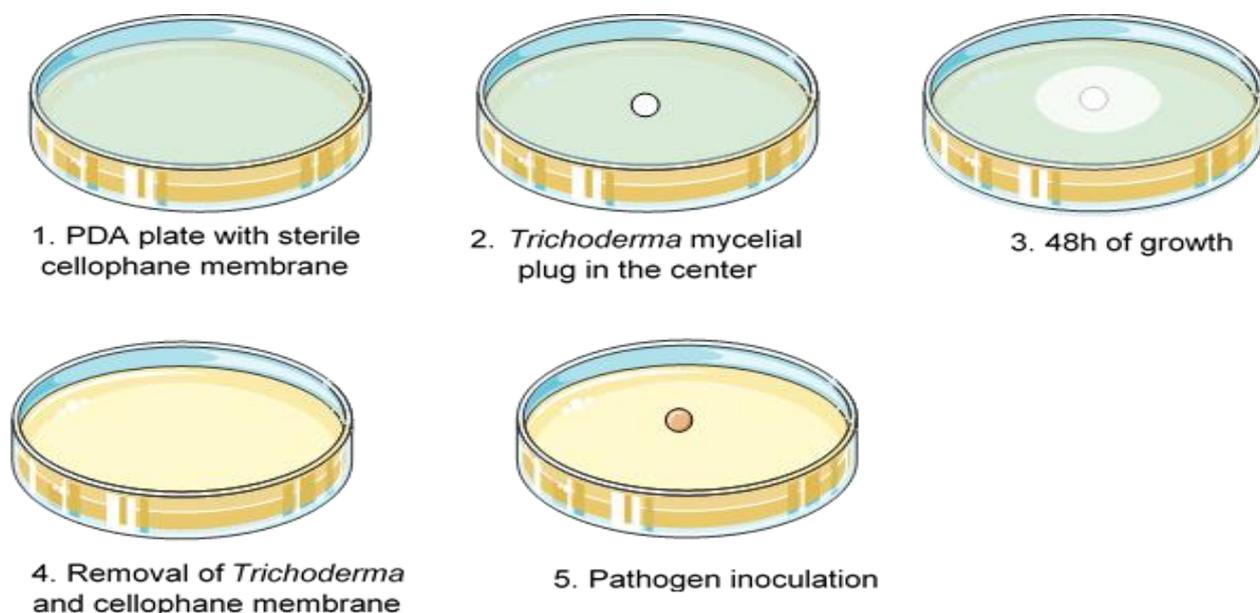
#### *Preliminary screening for the selection of Trichoderma isolates as biocontrol agents*

To determine the best candidates with biocontrol potential of all the preliminary selected *Trichoderma* isolates towards the citrus replant pathogens (Table 4.3.2.4), a non-volatile *in vitro* test according to the Petri-dish

based assay was realized (Dennis and Webster, 1971a). *Trichoderma* isolates and pathogens were grown on PDA in the dark at 28°C for 7 days. Mycelial plugs (6 mm diameter) from the 7-day old *Trichoderma* colonies were placed face down on PDA plates in which the PDA was covered with autoclaved 50 µm thick cellophane membranes (Sigma, Germany; 90 mm in diameter). The *Trichoderma* inoculated PDA plates were then incubated in the dark at 28°C. After 48 hrs, the cellophane membranes were removed, ensuring that the PDA plates were completely free of *Trichoderma* spp. propagules. The Petri dishes were subsequently inoculated with a mycelial plug of the respective citrus replant pathogens, one isolate from each species (Table 4.3.2.4), and incubated in the dark at 28°C as follows: 2 days for *P. irregulare*, 5 days for *P. citrophthora*, *Neocosmospora* spp, and 10 days for *P. nicotianae* (Figure 4.3.2.1). For the control plates, *Trichoderma* were not inoculated on the cellophane membranes. Four technical repetitions were done for each *Trichoderma* isolate × pathogen isolate combination. At the end of the incubation period, the colony diameters of the pathogens on each PDA plate were measured and calculated according to  $[(A-B)/A \times 100]$  where A is the colony diameter of the control plates and B is the diameter of pathogen colony (Lui *et al.* 2017).

**Table 4.3.2.4.** List of citrus replant pathogen isolates used for preliminary *in vitro* screening for the selection of potential biocontrol agents.

Species name	Isolates
<i>Neocosmospora solani</i>	VG 38
<i>Neocosmospora ferruginea</i>	VG 109
<i>Neocosmospora citricola</i>	VG 197
<i>Phytophthora citrophthora</i>	C3A
<i>Phytophthora nicotianae</i>	15.1
<i>Pythium irregulare</i>	81 B

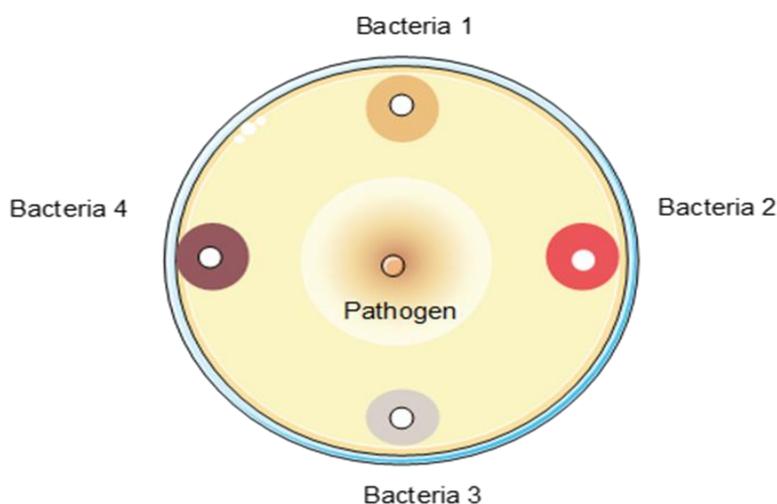


**Figure 4.3.2.1.** Illustration of the non-volatile test of *Trichoderma* against one isolate from each of six citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

#### *Preliminary screening for the selection of bacterial isolates as biocontrol agents*

To select the isolates with the best BCA potential, bacterial isolates obtained from the roots and rhizosphere soil were tested *in vitro* for their ability to inhibit the mycelial growth of replant pathogens. This was done by performing a dual culture assay as described by Alvarez-Pérez *et al.* (2017). One isolate of each pathogen

(Table 4.3.2.4) was grown on PDA in the dark at 28°C for 7 days, whereas the bacterial isolates were cultivated on NA for 72 h at 28°C. For each pathogen isolate, a mycelial plug (6 mm) was plated onto the centre of a new PDA plate before 4 colonies, representing 4 different bacterial isolates, were inoculated at 4 points around the pathogen plug, 1 cm from the edge of the plate (Acebo-Guerrero *et al.* 2015; Alvarez-Pérez *et al.* 2017; Lui *et al.*, 2017, Figure 4.3.2.2). Four replicates were done for each bacterial isolate x pathogen isolate combination and the plates were incubated at 28°C. After incubation period of 2 days for *P. irregulare*, 5 days for *P. citrophthora*, *Neocosmospora* spp, and 10 days for *P. nicotianae*, the colony diameter of the pathogen colonies were measured. The inhibitory efficiency of the bacterial isolates was calculated according to  $[(A-B)/A \times 100]$  where A is the colony diameter of colonies on the control plates and B is the colony diameter of pathogens on the bacterial inoculated plates (Lui *et al.* 2017).



**Figure 4.3.2.2.** Illustration of the competition test of different bacteria isolates against one isolate from each of the citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

#### Molecular species identification of selected BCA

Following the preliminary screening described above, the selected bacterial and *Trichoderma* spp. isolates were identified to species level using DNA sequence data.

#### DNA extraction of selected fungi

DNA was extracted from selected *Trichoderma* isolates grown on PDA plates for 7 days at 28°C. A CTAB based extraction protocol was followed as described by Damm *et al.* (2008) with some adjustments. Mycelia was recovered and placed in 2 mL Eppendorf tubes containing 0.5 g of 2mm glass beads and 600 µL of CTAB mix (2% w/v CTAB; 1M Tris, pH 7.5; 5M NaCl; 0.5M EDTA, pH 8.0) and were mixed by inverting the tubes. The tubes were shaken for 5 min at 30 Hz using a Retsch Mixer Mill (Retsch MM 400, Germany) and incubated for 15 min at 65°C. Four hundred µL of chloroform: isoamylalcohol (24:1) was then added and samples were centrifuged at 14 000 rpm for 5 min. The supernatant was transferred into new Eppendorf tubes containing 250 µL of ammonium acetate (7.5 M) and 600 µL of isopropanol. The tubes were inverted and incubated at room temperature for 15 min. The samples were again centrifuged for 15 min at 14 000 rpm and the supernatant was discarded. The resulting pellet was suspended in 70% EtOH after which the tubes were centrifuged for 5 min at 14 000 rpm. The supernatant was discarded by pipetting and the DNA pellet were dried in the oven at 65°C for 10 min. Finally, the DNA pellet was dissolved in 100 µL of sterile nuclease free water (QIAGEN, Germany).

#### DNA extraction of selected bacteria

Genomic DNA was extracted from bacteria grown on NA plates for 48 h at 28°C. One colony of each bacteria was suspended into a PCR tube containing 100 µL of sterile nuclease free water (QIAGEN, Germany). The samples were subjected to two cycles of 5 min at 96°C followed by 5 min at 4°C. The suspension was tenfold diluted and was used as a DNA template for PCR reactions.

#### Polymerase chain reaction

Polymerase chain reactions (PCRs) for the identification of *Trichoderma* isolates were performed using primer sets ITS4/ITS6 (White *et al.* 1990; Cooke and Duncan, 1997; Cooke *et al.*, 2000) to amplify the internal transcribed spacer (ITS) region and the EF1/EF2 (O'Donnell *et al.*, 1998) to amplify the partial Elongation Factor 1 $\alpha$  (EF-1 $\alpha$ ) gene. For ITS4/ITS6, PCR reactions were set up in 12.5 µL volumes which consisted of 2 µL of genomic DNA, 12.5 µL of KAPA Taq ReadyMix Taq (KAPA BIOSYSTEMS, USA), 0.375 µL of each primer (10 µM) and 3.5 µL of nuclease free water (QIAGEN, Germany). The PCR cycle consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 S at 72°C, and a final extension step at 72°C for 5 min. For the EF1/EF2, a total reaction volume of 12.5 µL, the PCR reaction contained 2 µL of genomic DNA, 6.25 µL of KAPA Taq ReadyMix Taq (KAPA BIOSYSTEMS, USA), 0.4 µL of each primer (10 µM), and 3.45 µL of nuclease free water (QIAGEN, Germany). PCR cycle consisted of an initial denaturation step at 95°C for 3 min followed by 35 cycles of annealing temperature at 52°C for 1 min and a final extension of 1 min at 72°C.

Species identification of selected bacterial isolates from preliminary screening was done by sequencing the 16S rDNA gene. The 16s rDNA region was amplified using the 16S DNA primers A1 and B6 (Manceau & Horvais, 1997). In a total reaction volume of 25 µL, the PCR reaction contained 2 µL of genomic DNA, 12.5 µL of KAPA Taq ReadyMix Taq (KAPA BIOSYSTEMS, USA), 0.5 µL of each primer (10 µM), and 9.5 µL of sterile nuclease free water (QIAGEN, Germany). The PCR reaction conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 minute at 72°C, and a final extension step at 72°C for 1 min.

#### Electrophoreses and Sequencing.

The PCR products were visualised by gel electrophoresis using a 1% agarose (1% w/v) in TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5) stained with SYBR™ Safe DNA Gel Stain (ThermoFischer Scientific, USA) alongside with a 1000-bp DNA ladder (GeneRuler™, Thermo Fisher Scientific, USA). The Gel Doc™ XR+ Imager and Image Lab software 5.2.1 (Biorad, USA) was used to visualize the gel under ultraviolet (UV) light. The PCR products were sent to the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University for purification and sequencing in both directions using the respective forward and reverse PCR primers. Consensus sequences were created with the respective forward and reverse sequences in Geneious R10.1.3. (Biomatters Ltd., New Zealand). To identify the genus of the isolates, the consensus sequences were run in The Basic Local Alignment Search Tool (BLAST) of NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

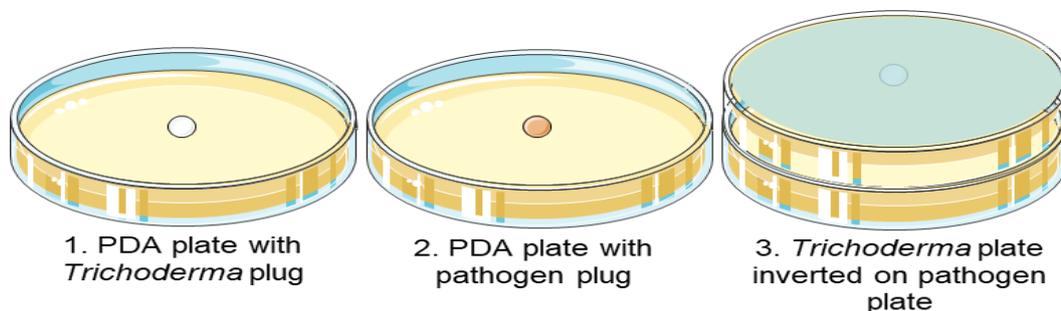
#### *Second in vitro screening of selected Trichoderma isolates for their antagonistic activity*

The preliminary screening of the fungi allowed us to select *Trichoderma* isolates that showed the greatest potential as biocontrol agents based on the effect of their non-volatile inhibitors on the different replant pathogens (Table 4.3.2.4). Subsequently, these selected *Trichoderma* isolates were then further tested to additional *in vitro* screening for their ability to reduce the mycelial growth of one isolate from each of six citrus replant pathogens according to the volatile and dual culture assays in order to study different mod of actions.

#### Production of volatile organic compounds

The selected *Trichoderma* isolates were subjected to an *in vitro* screening to evaluate the potential production of volatile compounds able to inhibit the mycelial growth of the citrus replant pathogens. *Trichoderma* isolates and the replant pathogen isolates (Table 4.3.2.4) were grown on PDA in the dark at 28°C for seven days. Mycelial plugs (6 mm), taken from the actively growing cultures of *Trichoderma* or replant pathogens, were

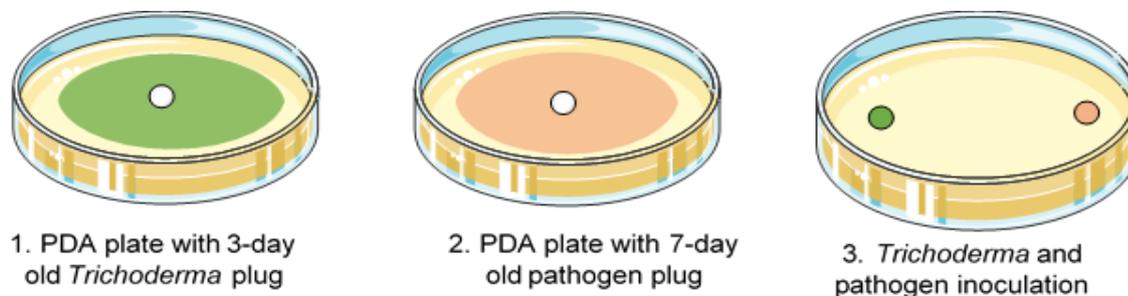
placed face down on PDA plates (Figure 4.3.2.3). The PDA plates with the pathogen plugs were then inverted over the plates containing the *Trichoderma* isolates and sealed with parafilm. For the control, clean PDA plates were used. Four replications were done for each pathogen isolate x *Trichoderma* isolate combination and plates were incubated as describe before. Mycelial inhibition was calculated in the same manner as previously mentioned.



**Figure 4.3.2.3.** Illustration of the volatile test of *Trichoderma* against one isolate from each of the citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

#### Competition test

As a final test, the selected *Trichoderma* isolates were subjected to *in vitro* testing via a competition test. The dual culture method of Dennis and Webster (1971b) was followed to observe the effect of the *Trichoderma* isolates on the growth of the six replant pathogens (Table 4.3.2.1). Mycelial plug (6 mm in diameter) was taken from the actively growing, seven days isolates of *Trichoderma* and pathogens and placed 5 cm apart on the PDA plates (Figure 4.3.2.4). The control plates consisted of pathogen plugs only. As before, four repetitions were done for each combination, and the dual culture plates were incubated and measured as stated earlier. In addition, microscopic photos were taken of any *Trichoderma* x pathogen interactions observed.



**Figure 4.3.2.4.** Illustration of the competition test of *Trichoderma* against one isolate from each of the citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

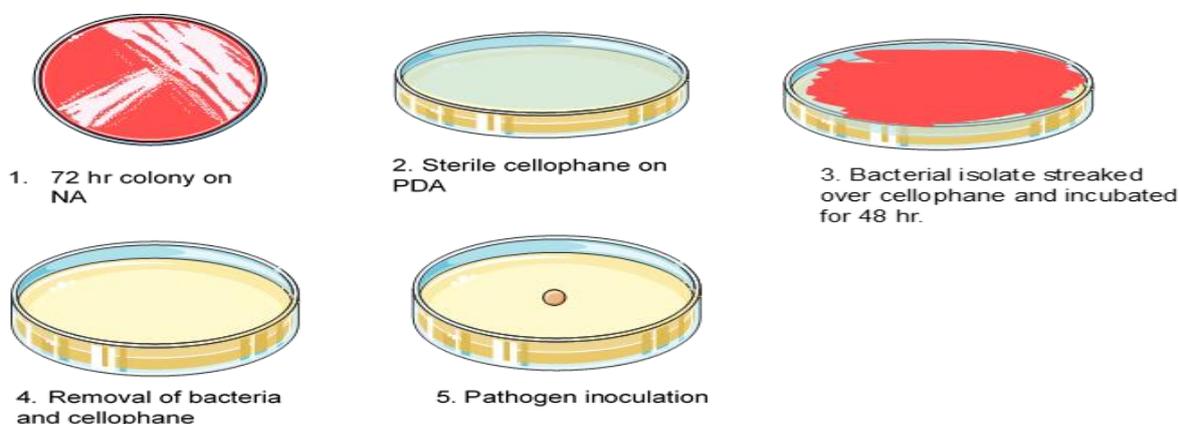
#### Second in vitro screening of bacterial isolates for their antagonistic activity

The preliminary competition test screening of the bacteria described above allowed us to select bacterial isolates that showed the greatest inhibition and therefore the best potential as biocontrol agents for replant pathogens. These selected bacterial isolates were subjected to a second preliminary screening using a non-volatile and a volatile test against one isolate of each of the replant pathogens.

#### Diffusible antifungal compound production

The potential of the bacterial strains was further tested by an *in vitro* screening aimed at determining if they can produce diffusible secondary metabolites that could inhibit citrus replant pathogens. Cellophane membranes (Sigma, Germany; 90 mm in diameter) were autoclaved and were placed aseptically on the surface of PDA plates. One colony of a 72 h old culture of the bacterial isolate was streaked out over the entire

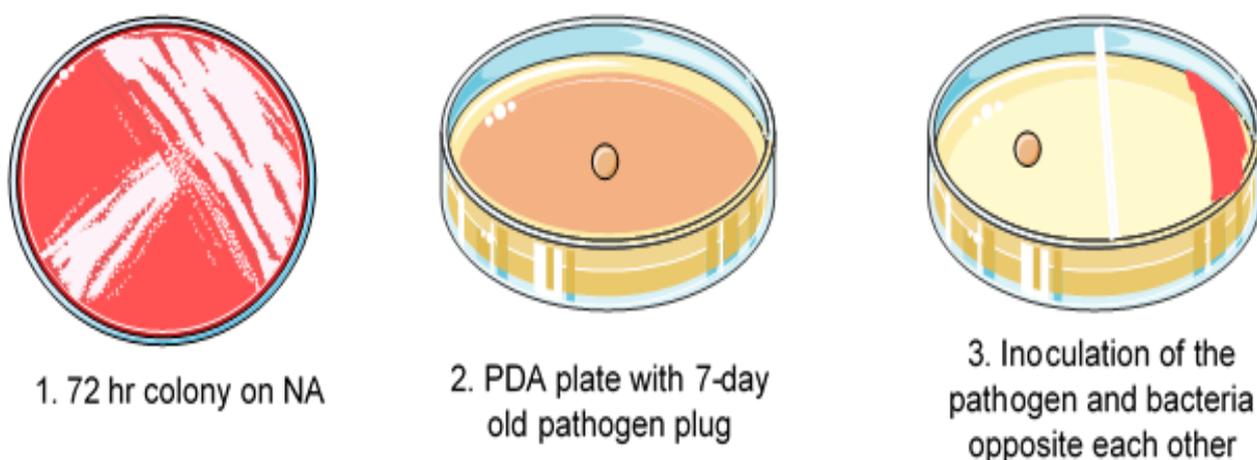
surface of the cellophane. For the control plates, no bacteria were streaked onto the cellophane. The plates were incubated in the dark at 28°C for 48 h after which the cellophane membrane with the adhering bacterial culture was removed (Nourozian *et al.*, 2006). Mycelial plugs (6 mm) from 7-day-old citrus replant pathogen cultures (Table 4.3.2.4) were placed in the centre of the PDA plates and incubated in the dark at 28°C for 2 days for *P. irregulare*, 5 days for *P. citrophthora*, *Neocosmospora* spp. and 10 days for *P. nicotianae* (Figure 4.3.2.5) after which colony diameters were measured and percentage inhibition calculated as above. Four replications were done for each pathogen x bacteria combination.



**Figure 4.3.2.5.** Illustration of the non-volatile test of selected bacterial isolates against six citrus replant pathogens with one isolate each: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

#### Production of volatile organic compounds

For this test, PDA was poured in Petri dishes containing a separation in the middle (half plates). Mycelial plugs (6 mm) were cut from 7-day-old citrus replant pathogen cultures (Table 4.3.2.4) and placed in the centre of the one side of the plate. A 72 h old culture of the bacterial isolate were streaked out in the opposite side (Figure 4.3.2.6). Petri dishes with only the pathogen served as the control. Four replicates were done for each combination, after which the inhibition was determined as stated above.



**Figure 4.3.2.6.** Illustration of the volatile test of selected bacterial isolates against one isolate from each of the citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

*Third In vitro screening of the selected bacterial and Trichoderma harzianum isolates for their antagonistic activity against five isolates of each replant pathogen*

The different preliminary screenings, as described above, enabled us to select the best candidate and the best screening methods that show the greatest inhibition against the replant pathogens and are therefore

considered to have the best potential as an antagonistic screening method. If the candidate or the screening method showed an efficiency of 50% in mycelial inhibition, these selected *Trichoderma* and bacterial isolates were evaluated in the appropriate screening method a second time against the replant pathogens with 5 isolates each, using the following methods.

#### Diffusible anti-fungal compound production

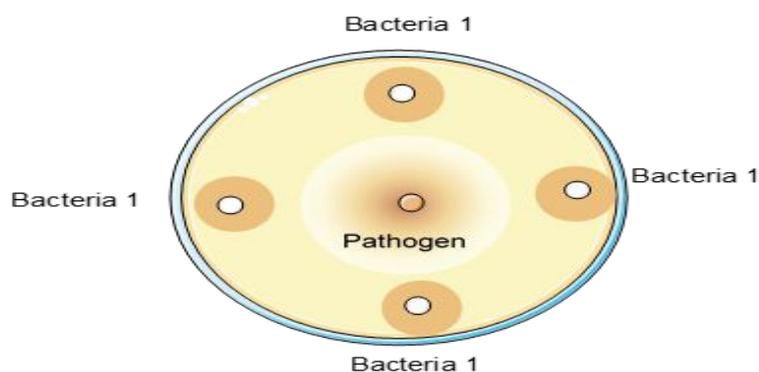
The selected *Trichoderma* isolates were subjected to a new non-volatile test, following the experimental design methodology as stated above. However, in this test, 5 isolates of each citrus replant pathogen (Table 4.3.2.5) were used. For each *Trichoderma* x pathogen isolate, four replicate plates were used as described above. These plates were incubated in the dark at 28°C as follows: 2 days for *P. irregulare*, 5 days for *P. citrophthora*, *Neocosmospora* spp. and 10 days for *P. nicotianae*. After the different incubation periods, the mycelial growth was calculated as before.

**Table 4.3.2.5.** List of citrus replant pathogen, five isolates per pathogen, used for *in vitro* screening for the selection of potential biocontrol agents.

Species name	Isolate number	Species name	Isolate number
<i>Neocosmospora solani</i>	VG 38	<i>Phytophthora citrophthora</i>	C5
<i>Neocosmospora solani</i>	VG 93	<i>Phytophthora citrophthora</i>	C25B
<i>Neocosmospora solani</i>	VG 147	<i>Phytophthora citrophthora</i>	C3A
<i>Neocosmospora solani</i>	VG 175	<i>Phytophthora citrophthora</i>	K9A
<i>Neocosmospora solani</i>	VG 193	<i>Phytophthora citrophthora</i>	K12A
<i>Neocosmospora ferruginea</i>	VG 109	<i>Phytophthora nicotianae</i>	4.2
<i>Neocosmospora ferruginea</i>	VG 133	<i>Phytophthora nicotianae</i>	7.2
<i>Neocosmospora ferruginea</i>	VG 195	<i>Phytophthora nicotianae</i>	15.1
<i>Neocosmospora ferruginea</i>	VG 205	<i>Phytophthora nicotianae</i>	34.2
<i>Neocosmospora ferruginea</i>	VG 394	<i>Phytophthora nicotianae</i>	37.1
<i>Neocosmospora citricola</i>	VG 139	<i>Pythium irregulare</i>	14.1
<i>Neocosmospora citricola</i>	VG 140	<i>Pythium irregulare</i>	24
<i>Neocosmospora citricola</i>	VG 183	<i>Pythium irregulare</i>	43.1
<i>Neocosmospora citricola</i>	VG 197	<i>Pythium irregulare</i>	51.1
<i>Neocosmospora citricola</i>	VG 203	<i>Pythium irregulare</i>	81B

#### Competition test

The selected bacteria were then subjected to a new competition screening, following the same methodology as outline above. In this test, only one bacterium, representing the same bacterial isolate, was inoculated four times around the pathogen and 5 isolates per pathogen were used (Table 4.3.2.5 and Figure 4.3.2.7). Plates were incubated at 28°C at the different incubation periods (2 days for *P. irregulare*, 5 days for *P. citrophthora*, *Neocosmospora* spp. and 10 days for *P. nicotianae*) and the pathogen growth inhibition were calculated as previously indicated.



**Figure 4.3.2.7.** Illustration of the competition test of one bacterial isolate against five isolates from each of the citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

#### Diffusible antifungal compound production

The selected bacterial isolates were then subjected to a new non-volatile test, following the methodology as describe above. However, in this test, 5 isolates of each citrus replant pathogen (Table 4.3.2.5) were included. For each bacterium x pathogen isolate four replicate plates were used and incubated as described above. The mycelial growth was calculated as indicated earlier.

#### Statistical analysis

The raw growth inhibition data of the experiments were subjected to analyses of variance (ANOVA) using General Linear Models (GLM) Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA) to assess the effect of the biocontrol agents on the pathogens. Shapiro-Wilk test was conducted to test for deviation from normality (Shapiro and Wilk, 1965). Fisher's least significant difference was performed at the 5% level to compare factor means (Ott and Longnecker, 2001), where a probability level of 5% was considered significant for all significance tests.

#### In planta evaluation of selected BCA's for their ability to reduce replant pathogen root infection

##### *Preparation of plant material*

Troyer citrange seeds were obtained from the Citrus Foundation Block, Uitenhage, Eastern Cape. These seeds were surface sterilized under the laminar flow by placing them into 2.5% sodium hypochloride + 2g/L captan fungicide mixture for 10 min. The seeds were then washed 5 min in autoclaved water. Washing was repeated twice. Subsequently, the seeds were soaked in sterile water for 1 h. The water-soaked seeds were then placed onto autoclaved paper towels under the laminar flow to dry. The rest of the seeds were then peeled carefully with sterilised tweezers. The peeled seeds were carefully placed on wet sterile paper towels in sterilised plastic containers. The plastic containers were covered with foil and the peeled seeds were left for at least 2 weeks for germination. The peeled seeds were checked every second day, and moistened if necessary, to make sure they stay hydrated.

The pot experiment was carried out using pasteurized perlite. One week prior to planting, the perlite was pasteurized for 180 min at 80°C. After cooling down, it was placed inside 1 L planting black bags (214 mm x 100 mm), which were sealed off at the bottom with a plastic cover to prevent the loss of drainage water. The germinating Troyer citrange seeds were planted 2 cm deep in the bags with the pasteurized perlite, two seeds per bag. One hundred mL of water was given every 2 weeks and nutrient solution (Nutrifeed, 5 g/1 L; Stark Ayres), were applied in the between weeks to ensure that there were enough nutrients for the plants. Insect control with Oleum (20mL/1L; EFEKTO), Crystal 550 SC (0.55mL/1L; Villa Crop Protection) and Spirofen 240 SC (0.15 mL/1 L, Villa Crop Protection) were also done as needed. These insecticides were mixed with water and were applied for the control of aphids, Red spider mite and mealy bug, respectively. These germinating seeds were grown in the glasshouse for nine months at a constant temperature of 25°C before being used for the *in planta* evaluation of BCAs.

##### *Treatments and experimental design*

The experiment tested the effect of the biological control agent (BCA) on replant pathogens in the glasshouse using 9-month-old seedlings. Selected bacterial isolates, one *B. subtilis* and one *P. fluorescens* isolate, along with the two *Trichoderma* isolates, P4 and P16, were evaluated as BCA treatments for the glasshouse experiment. The replant pathogens used to evaluate the BCA efficacy included *N. solani*, *P. irregulare* and *P. nicotianae*. The experiment was set up as a completely randomized block design that consisted of 10 treatments (Table 4.3.2.6) with three replant pathogens, which were replicated 10 times, giving a total of 300 plants. The randomized block design included five blocks with 2 plants per pathogen x treatment combination.

**Table 4.3.2.6.** List of different treatments applied on Troyer citrange seedlings in the glasshouse to evaluate the ability of the selected BCA isolates to prevent seedling root infections by different replant pathogens.

<p>T1 - (Control plant with BCA) Seedling in perlite drenched with <i>B. subtilis</i>.  T2 - (Control plant with BCA) Seedling in perlite drenched with <i>P. fluorescens</i>.  T3 - (Control plant with BCA) Seedling in perlite drenched with <i>Trichoderma</i> P4.  T4 - (Control plant with BCA) Seedling in perlite drenched with <i>Trichoderma</i> P16.  T5 - (Control plant with pathogen) Seedling in perlite were inoculate with sand-bran pathogen inoculum at a dosage of 50 g.L<sup>-1</sup>  <i>Pythium irregulare</i> 43.1 (Pi); <i>Phytophthora nicotianae</i> 15.1 (PN); <i>Neocosmospora solani</i> 175 (NS) inoculum  T6 - (Control plant) Seedlings received 100 ml water/ 100 nutrient broth.  T7 - (Plant with BCA + Pathogen) Seedling in perlite drenched with <i>B. subtilis</i> + pathogen inoculum at a dosage of 50g.L<sup>-1</sup>  T8 – (Plant with BCA + Pathogen) Seedling in perlite drenched with <i>P. fluorescens</i> + pathogen inoculum at a dosage of 50g.L<sup>-1</sup>  T9 - Plant with BCA + Pathogen) Seedling in perlite drenched with <i>Trichoderma</i> P4 + pathogen inoculum at a dosage of 50g.L<sup>-1</sup>  T10 - - Plant with BCA + Pathogen) Seedling in perlite will be drenched with <i>Trichoderma</i> P16 + pathogen inoculum at a dosage of 50g.L<sup>-1</sup></p>
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#### *Preparation of the pathogen inoculum*

Sand-bran medium inoculum was prepared by following the method of Lamprecht *et al.* (1986) to infest the soil with one isolate of either *Neocosmospora solani* or *Pythium irregulare* or *Phytophthora nicotianae*. The sand-bran medium consisted of a mixture 400 g of sterile and washed river sand, 40 g of wheat bran and 60 mL distilled water in 1000 mL Schott bottles (Duran Wheaton Kimble) The sand-bran mixtures were autoclaved for 20 min at 120°C on the first day and shaken before it was autoclaved a second time on the following day (Lamprecht *et al.* 1986). The autoclaved sand-bran mixtures were inoculated with 30 mycelial plugs (6 mm in diameter) of 10-day-old cultures grown on PDA (De Villiers *et al.*, 2006). The control bottles were inoculated with discs of PDA only (De Villiers *et al.*, 2006). The inoculated sand-bran bottles were incubated for 14 days at 28°C without being exposed directly to light (De Villiers *et al.*, 2006; Lamprecht *et al.*, 2011). The sand-bran mixtures were shaken every second day to ensure thorough colonization.

#### *Preparation of the BCAs treatments*

##### Spore suspension of *Trichoderma* isolates, P4 and P16

To obtain the spore suspension of *Trichoderma* isolates P4 and P16, the fungus was grown on Potato Dextrose Agar (39 g/L, PDA, Merck) for 7 days at 25°C until abundant green spores were present. Spores were harvested from the surface of plates by adding small amounts of sterile distilled water to the culture plates and scraping gently with a sterile hockey stick. The harvested spore suspension was then filtered through a single layer of sterile Miracloth (Merck KGaA, Darmstadt, Germany) to prepare a stock solution. The spore concentration was counted by using a Haemocytometer and adjusted to a final concentration of 1 x 10<sup>6</sup> spores/mL (El-Mohamedy, 2009, Aleandri *et al.*, 2015; Vargas-Inciarte *et al.*, 2019) with sterile distilled water. A total volume of 6 L spore suspension per *Trichoderma* isolate was required (10 plants x 3 pathogens x 2 treatments x 100 mL) and was prepared 1 hr prior to inoculation.

##### Liquid culture of *Bacillus subtilis* and *Pseudomonas fluorescens*

##### Determine growth curve

Liquid cultures of *Bacillus subtilis* and *Pseudomonas fluorescens* were prepared to determine the stationary phase of the bacteria. The bacteria were streaked on Nutrient Agar (31 g.L<sup>-1</sup>, NA, Merck) and grown for 72 hrs. One single colony of *B. subtilis* from the 72 hr-streaked plates was inoculated into Falcon tubes with 10 mL of sterile Nutrient Broth (16 g.L<sup>-1</sup>, NB). The tubes were incubated overnight in a shaking incubator (Labcon) at 150 rpm at 37°C (Koni and Hanim, 2017). The Optical Density (OD<sub>600</sub>) of the overnight culture was determine

using a spectrophotometer (CECIL, CE 1021 1000 series) and diluted to an OD<sub>600</sub> of 0.05 in a 50 mL final volume of NB in 150 mL Erlenmeyer flasks with a cotton and foil cap. OD<sub>600</sub> was recorded at time zero. *B. subtilis* was then incubated again as mentioned above and the OD<sub>600</sub> was taken every 1 hr until the stationary phase has been reached.

The same methodology was used to determine the stationary phase of *P. fluorescens* except for the incubation conditions that were adjusted to 180 rpm at 28°C. The experiment was repeated three times at the same time. To measure the growth rate of the bacteria, the OD<sub>600</sub> readings were taken hourly until a stable reading was obtained, and a bacterial growth curve was generated to indicate the stationary phase. Serial dilutions were made up to 10<sup>6</sup> every 2 hrs using 1 mL from the bacterial suspension used for the OD<sub>600</sub> readings. One hundred µL of each dilution factor was transferred to the NA plate and spread using a sterile glass hockey stick until the agar surface was dry. Three NA plates were used and were sealed with clingwrap and incubated at 28°C for 24 hrs, after which the colonies were counted between a range of 25-300 colonies and average together and multiplied by the appropriate dilution factor to obtain an average CFUs/mL per bacteria concentration.

#### Production of the bacteria suspension for the glasshouse trial

By determining the growth curve and the number CFU/mL for bacteria, *B. subtilis* and *P. fluorescens*, we were able to prepare the bacterial inoculum for drench treatment of the Troyer citrange seedlings. To prepare the bacterial inoculum, 10 single colonies of the 72 h old *B. subtilis* grown on NA was suspended in 100 mL NB in a 250 mL Erlenmeyer flask. This was shaken overnight at 150 rpm at 37°C. The OD<sub>600</sub> of the starter culture were taken again and was time diluted to 0.05 in 500 mL NB in a 1000 mL Erlenmeyer flasks with cotton and foil cap. The OD<sub>600</sub> was taken again every hour and was used to construct a growth curve to compare with the previously generated growth curve, to see if the conditions under which the bacteria grew remained the same when a larger volume was used. For the inoculum preparation of *P. fluorescens*, the same steps were followed as outlined above, with only changes in the incubation conditions that were adjusted to 180 rpm at 28°C.

*Bacillus subtilis* and *Pseudomonas fluorescens* were grown for the time based on the growth curve previously generated to see how long the bacterium takes to transition from the lag phase to the stationary phase. At the stationary phase, the desired bacterial concentration was confirmed by dilution plating after a 24 h incubation period. A total of 6 L per bacterial suspension for direct application (10 plants x 3 pathogens x 2 treatments x 100 mL) for the glasshouse experiment was prepared 24 h prior to the inoculation.

#### Pathogen inoculation and BCA application

The trial was done on 9-month-old Troyer citrange seedlings. Prior to BCA application, seedling height was measured to allow assessment of the growth increase of the seedlings at the end of the experiment. Application of the freshly prepared BCA suspensions was done by drench application. Treatment groups 1 to 4 and 7 to 10 were treated with the BCA by drenching 100 mL of the pre-prepared suspensions as described above. The BCAs was applied at the start of the trial and then again 7, 14 and 21 days after the first application (Steddom *et al.* 2002; Aleandri *et al.* 2015; Shinde and Sadgir, 2016). Twenty-four hrs after the last application of the BCAs, treatment groups 5,7,8,9 and 10 were inoculated with the replant pathogens. This was done by making four holes of 4 cm deep each in the perlite near the root system using a sterile wooden planting stick. The pathogen inoculum was applied at a dosage of 50 g/L, approximately 12.5 g into each hole (De Villiers *et al.*, 2006; Haq *et al.*, 2014). Treatment 5 in each block was an untreated control (without the pathogen or BCA) and only 100 mL water or 100 mL NB was applied according to the BCAs in the same schedule as the BCA applications.

#### Trial analysis

##### Plant measurements

The experiment was terminated at 13 weeks after pathogen inoculation. At this stage the growth of the citrus plants was assessed using the following parameters: seedling height (cm), seedling mass (g), root fresh mass (g) and root volume (cm<sup>3</sup>). For the seedling height measurements, the seedlings were measured from soil

surface to the tip of the shoot. The seedlings were removed from each planting bag and shaken to remove the perlite and seedling weight were recorded in grams (g). For the root mass, the root systems were removed with a sterilised (70% ethanol) pruning shear and weighed. Root volume (cm<sup>3</sup>) of these roots were also obtained. These roots were then placed in labelled plastic bags and were transported to the Department of Plant Pathology where re-isolation took place.

#### Isolation of replant pathogens and BCAs from roots

Re-isolation of the fungi and BCAs were done from the roots of the seedlings. Roots of each treatment were surface sterilised with 70% ethanol for 30 sec. Four root segments (5 mm) were placed on the surface of three different mediums in 90 mm Petri dishes, potato dextrose agar (PDA, 39 g.L<sup>-1</sup>, Merck) amended with streptomycin sulphate (40 mg.L<sup>-1</sup>; PDAs), King's B media (20.0 g.L<sup>-1</sup>; Proteose peptone, 15 mL Glycerol, 2.5 g.L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O, 6 g.L<sup>-1</sup> MgSO<sub>4</sub>.7H<sub>2</sub>O and 15 g.L<sup>-1</sup> Agar; KB, King *et al.*, 1954) and PARP (17 g.L<sup>-1</sup> Corn meal agar (CMA) + antibiotic solution (0.01 g Pimaricin, 0.125 g Ampicillin, 0.01 Rifampicin, 0.1 g PCNB), Sigma-Aldrich). The KB media were used for the isolation of the bacterial BCAs and the PDAs media for *Trichoderma* isolates. The PARP media was used as isolation media for the oomycetes. Three plates per medium were used as replicates for each root system. The PDAs and KB inoculated plates were incubated at 25°C and inspected daily until fungal and bacterial growth were observed. The PARP plates were placed in brown paper bags, to avoid denaturing of the antibiotics and were placed at 28°C until the growth of oomycetes was observed.

#### Identification of re-isolations

Morphological identification of the re-isolations was performed to confirm their presence in the roots. For the identification of the *P. fluorescens*, the KB plates were observed under ultraviolet light, to see the fluorescing pigment. *Bacillus subtilis* was identified based on their colony morphology (colour and shape). *Trichoderma* isolations were identified on their colony colour and hyphal growth. *Neocosmospora solani* reisolations were characterised based on conidial morphology PDAs (Guarnaccia *et al.* 2021). Identification of the oomycetes, was based on colony characteristics on PARP.

#### Statistical analysis

The growth measurements of seedlings and re-isolation percentages of pathogens and biocontrol agents were subjected to Analysis of variance (ANOVA). These were assessed according to the experimental layout to test for the pathogen and BCAs interactions using GLM (General Linear Models) Procedure of SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA). The experimental results for the seedling growth parameters and re-isolations, respectively, were combined in one analysis of variance after validation of experiment homogeneity of variance using Levene's test (Levene, 1960). Shapiro-Wilk test was performed to test data for normality (Shapiro, 1965), while Fisher's least significant difference at the 5% level was calculated to compare treatment means for significant effects (Ott, 1998), where a probability level of 5% was considered significant for all significance tests.

## Results and discussion

*Objective 1. Study the interaction of the citrus replant pathogen complex in planta under controlled and field conditions.*

#### Develop new species-specific qPCR primers for the detection of citrus replant pathogens.

Multiple sequences of several *Phytophthora* and *Neocosmospora* species were aligned to identify a characteristic region for *P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola*. Genomic and reference sequences from different *Phytophthora* isolates were obtained from NCBI and aligned using MAFFT v. 7388. Consequently, five new sets of qPCR primers were designed for each *Phytophthora* spp., targeting the *Ypt1* region. For *P. citrophthora*, the primers were designed based on the GenBank sequence KJ755172.1, *Phytophthora citrophthora* strain JKI 6/08-1 ras-related protein Ypt1 (*ypt1*) gene, partial cds (Schwenkbier *et*

al., 2014). For *P. nicotianae*, the primers were designed based on the GenBank sequence DQ162981.1, *Phytophthora nicotianae* strain IMI268688 ras-like protein (ypt1) gene, partial cds (Schena and Cooke, 2006). Sequences from numerous *Neocosmospora* isolates were aligned and various primers were designed. Consequently, six specific primers were designed for *N. citricola* from the ITS gene (internal transcribed spacers region). For *N. ferruginea* four specific primers were designed targeting the RNA polymerase II (*RPB2*) gene. For *N. solani* nine primers were designed based on the  $\beta$ -tubulin gene.

All primers were designed to have lengths of 18-25 bp, 40-60% GC content, have a 1-2°C difference in annealing temperature between forward and reverse primers, produce product sizes of 70-200 bp, and to produce no primer dimers. The primer T<sub>m</sub> values, and primer-dimer formation were calculated using the Multiple Primer Analyzer tool available online from Thermo Fisher Scientific <https://www.thermofisher.com/za/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html>). Overall, 29 pairs of primers were designed from conserved regions and their combinations were tested by conventional and real-time PCR reactions with different primer concentrations and cycling conditions to evaluate the suitability of the designed primers for qPCR. The sequences of each forward and reverse primer pair designed *in silico* are shown in Table 4.3.2.7.

**Table 4.3.2.7.** The sequences of the primer pairs designed *in silico* for the detection of citrus replant and citrus decline syndrome pathogens (*Phytophthora citrophthora*, *Phytophthora nicotianae*, *Neocosmospora citricola*, *Neocosmospora ferruginea*, *Neocosmospora solani* and *Pythium irregulare*) in citrus roots.

Primer name	Primer sequence (5' to 3')	Length	Reference	
PC_YPT1_FW2	GAAGAAGAGATCCAGTGAGGTTC	23	This study	
PC_YPT1_RV2	GGTGGATGACGAGTCTTAAACA	22		
PC_YPT1_FW4	GAAGAAGAGATCCAGTGAGGTTC	23		
PC_YPT1_RV4	GTCCAGAGTCTCAGGTGGATG	21		
PC_YPT1_FW5	GAAGAAGAGATCCAGTGAGGTTC	23		
PC_YPT1_RV5	GACGAGTCTTAAACATTGGTTGCT	24		
PC_YPT1_FW10	AAGCAACCAATGTTAAGACTCG	23		
PC_YPT1_RV10	GGAGTTGGCACAGCCATTAA	20		
PC_YPT1_FW13	TAATTAATGGCTGTGCCAACTCC	23		
PC_YPT1_RV13	CTGGTAAAATAATTCTGCCACGAAC	25		
PN_YPT1_FW7	AGCTCCAGATTGTACGTCTTTC	22		This study
PN_YPT1_RV7	ACACGTGATTTGGTACATCG	20		
PN_YPT1_FW9	CGTCTTTCAATAGACTTGTATCACT	25		
PN_YPT1_RV9	CACAATAATGCCGTGTGC	18		
PN_YPT1_FW14	CGATGTACCAAATCACGTGTG	21		
PN_YPT1_RV14	ACCACAATAATGCCGTGTG	19		
PN_YPT1_FW17	GTGTGTGTCTGTAGTGGGACACG	23		
PN_YPT1_RV17	GGATCTTCTCTCGATAAGTCGGAC	24		
PN_YPT1_FW19	GTGCACACGGCATTATTGTG	20		
PN_YPT1_RV19	TGGAATATCACTCAGCTCTTTCC	24		
NSP1_FW1_ITS	GAGGACCCCTATCTCTGTTA	21	This study	
NSP1_RV1_ITS	ATGTGCGTTCAAAGATTCCG	19		
NSP1_FW2_ITS	GAGGACCCCTATCTCTGTTA	21		
NSP1_RV2_ITS	GGGTTGTAATGACGCTCG	18		
NSP1_FW3_ITS	GGATCATTACCGAGTGTA	18		
NSP1_RV3_ITS	TAGTAACAGAGATAGGGGG	19		
NSP1_FW4_ITS	GATCATTACCGAGTGTA AAAACTCT	25		
NSP1_RV4_ITS	GCAATTCACATTACTTATCGC	21		
NSP1_FW5_ITS	GGATCATTACCGAGTGTA AAA	20		
NSP1_RV5_ITS	CATATAGTAACAGAGATAGGGGG	23		
NSP1_FW6_ITS	TTACCGAGTGTA AAAACTCT	20		

<i>NSP1_RV6 ITS</i>	TAGTAACAGAGATAGGGGG	19	
<i>NFSSC28_FW1_RPB2</i>	CTTTACTACCCGCAAAAACCC	21	This study
<i>NFSSC28_RV1_RPB2</i>	CTTCTCTTGGTCAGAGTAAGATCG	24	
<i>NFSSC28_FW2_RPB2</i>	ATCGTCGCTATCGCTTGC	18	
<i>NFSSC28_RV2_RPB2</i>	CTTCTCTTGGTCAGAGTAAGATCG	24	
<i>NFSSC28_FW3_RPB2</i>	GGACGACCCCAACAGGAG	18	
<i>NFSSC28_RV3_RPB2</i>	TGTGAATCAAAGGCACATACCTG	23	
<i>NFSSC28_FW4_RPB2</i>	GATTTGCATGACCCCTGAG	19	
<i>NFSSC28_RV4_RPB2</i>	TGTGAATCAAAGGCACATACCT	22	
<i>BTSOL_FW1</i>	CGAGGTATGTTGCCCGAAGC	20	This study
<i>BTSOL_RV1</i>	CTCGGCACCCTCAGTGTAATG	21	
<i>BTSOL_FW2</i>	TCAACGAGGTATGTTGCCCGAAGC	24	
<i>BTSOL_RV2</i>	CCTCGCGCGGACGACA	17	
<i>BTSOL_FW3</i>	CGTCGACCAGGTCCTCGAT	19	
<i>BTSOL_RV3</i>	GGAGAGGGAACGACGGAGAAT	21	
<i>BTSOL_FW4</i>	CGTCGACCAGGTCCTCGAT	19	
<i>BTSOL_RV4</i>	GGACTGAGAGGGTGGCGTTA	20	
<i>BTSOL_FW5</i>	GTCGACCAGGTCCTCGAT	18	
<i>BTSOL_RV5</i>	CTCGACGACGGTGTCTGAG	19	
<i>BTSOL_FW6</i>	CCCTTCCCCGTCTGCAC	18	
<i>BTSOL_RV6</i>	GCCACGGCTGTGAAATGTTAGG	22	
<i>BTSOL_FW7</i>	AGACACCGTCGTCGAGCCTTAT	22	
<i>BTSOL_RV7</i>	GACGAGGTAGTTGAGATCGCCGTAC	25	
<i>BTSOL_FW8</i>	GACCGAATGATGGCCACA	18	
<i>BTSOL_RV8</i>	GAGGTAGTTGAGATCGCCGTAC	22	
<i>PirF1</i>	AGTGTGTGTGGCACGTTGTC	20	Spies <i>et al.</i> ,
<i>PirR3</i>	GATCAACCCGGAGTATACAAAC	23	2011

Validate existing and new species-specific qPCR primers for replant pathogen detection in soil and plant material.

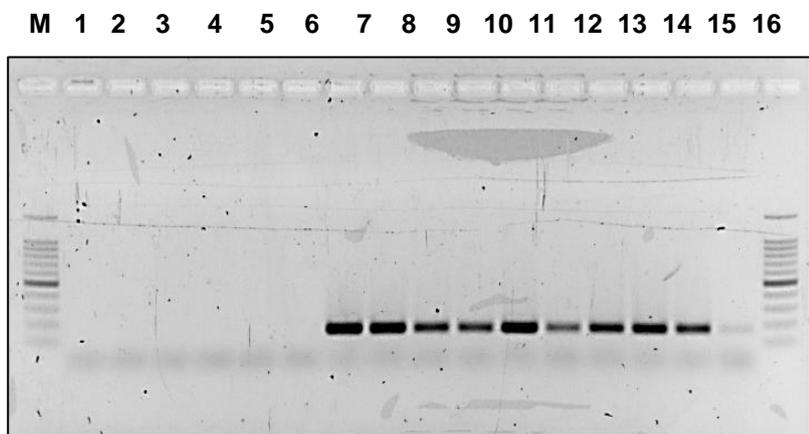
#### Primer specificity in PCR

Conventional PCR assays were realised first in order to select the most suitable primer sets for qPCR. For *Phytophthora* spp. and *P. irregulare*, six different annealing temperatures were chosen for the optimisation: 50°C, 54°C, 57°C, 60°C, 62°C and 65°C. For *N. citricola* and *N. ferruginea*, five different annealing temperatures were chosen: 50°C, 54°C, 57°C, 60°C and 62°C. For *N. solani*, six different annealing temperatures were chosen: 52°C, 54°C, 57°C, 60°C, 62°C and 64°C. For each optimisation assay, two isolates of either *Phytophthora* spp., *P. irregulare* or *Neocosmospora* spp. and a negative water control were used.

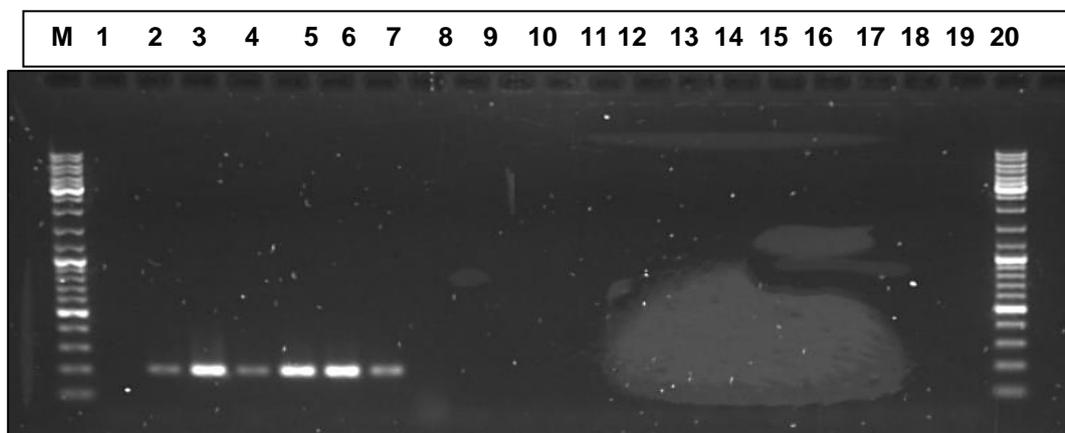
According to the PCR results, the most suitable set of primers for *P. citrophthora* were primer pairs 2, 4 and 5. The most suitable primer sets for *P. nicotianae* were 14, 17 and 19. Ideally, qPCR primers should anneal at 59°C or 60°C (Thornton and Basu, 2011) and therefore 60°C was selected as annealing temperature for both *P. citrophthora* and *P. nicotianae* primer pairs, as it was found to be the most suitable temperature to realise qPCR assays. The same optimal annealing temperature chosen for *Phytophthora* species primers (60°C) were also suitable for the primer set published previously for *P. irregulare* (Spies *et al.*, 2011). According to the PCR results, the most suitable set of primers for *N. citricola* were primer pairs 1, 3, 4 and 6. For *N. ferruginea* the most suitable primer sets were 1, 3 and 4. Regarding the annealing temperature, the optimal annealing temperatures were 59°C and 60°C for *N. citricola* and *N. ferruginea* respectively. According to the PCR results, the most suitable set of primers for *N. solani* were 1, 5, 6 and 8, and the optimal annealing temperature were 60°C in conventional PCR. Primer sets that did not amplify were removed and not studied further

The specificity of the newly designed primers was tested on a range of isolates and close relatives (Figures 4.3.2.8 and 4.3.2.9). Subsequently, one primer pair were selected for each species based on the *in silico* and *in vitro* test results. Both the *in silico* and *in vitro* tests confirmed that the selected primer sequences were

specific to the target species only. Primer pair *PC\_YPT1\_FW2* and *PC\_YPT1\_RV2* amplified a unique band of 113 bp from *P. citrophthora* isolates, and for *P. nicotianae* isolates, primer pair *PN\_YPT1\_FW17* and *PN\_YPT1\_RV17* amplified a 147 bp band. Primers *NSP1\_FW1\_ITS* and *NSP1\_RV1\_ITS* gave a 130 bp product from *N. citricola* isolates, whereas primers *NFSSC28\_FW1\_RPB2* and *NFSSC28\_RV1\_RPB2* gave a 162 bp product from *N. ferruginea* isolates. The primer pair *BTSOL\_FW5* and *BTSOL\_RV5* amplified a 170 bp PCR product from *N. solani* isolates (Table 4.3.2.3).



**Figure 4.3.2.8.** Specificity of primer pair *PN\_YPT1\_FW17* and *PN\_YPT1\_RV17* for detection of *Phytophthora nicotianae*. The products were electrophoresed on 1% (w/v) agarose gels for 50 min at 100 V. Lane M represent a 100 bp DNA ladder. Lane 1 contained the negative control (nuclease-free water). Lanes 2 to 6 are closely related species. Lanes 7 to 16 represent *Phytophthora nicotianae* isolates (one DNA fragment, 147 bp).



**Figure 4.3.2.9.** Specificity of primer pair *NFSSC28\_FW1\_RPB2* and *NFSSC28\_RV1\_RPB2* for detection of *Neocosmospora ferruginea*. The products were electrophoresed on 1% (w/v) agarose gels for 50 min at 100 V. Lane M represent a 1000 bp DNA ladder. Lane 1 contained the negative control (nuclease-free water). Lanes 2 to 7 represent *Neocosmospora ferruginea* isolates (one DNA fragment, 162 bp). Lanes 8 to 20 are closely related species.

Primer specificity in qPCR

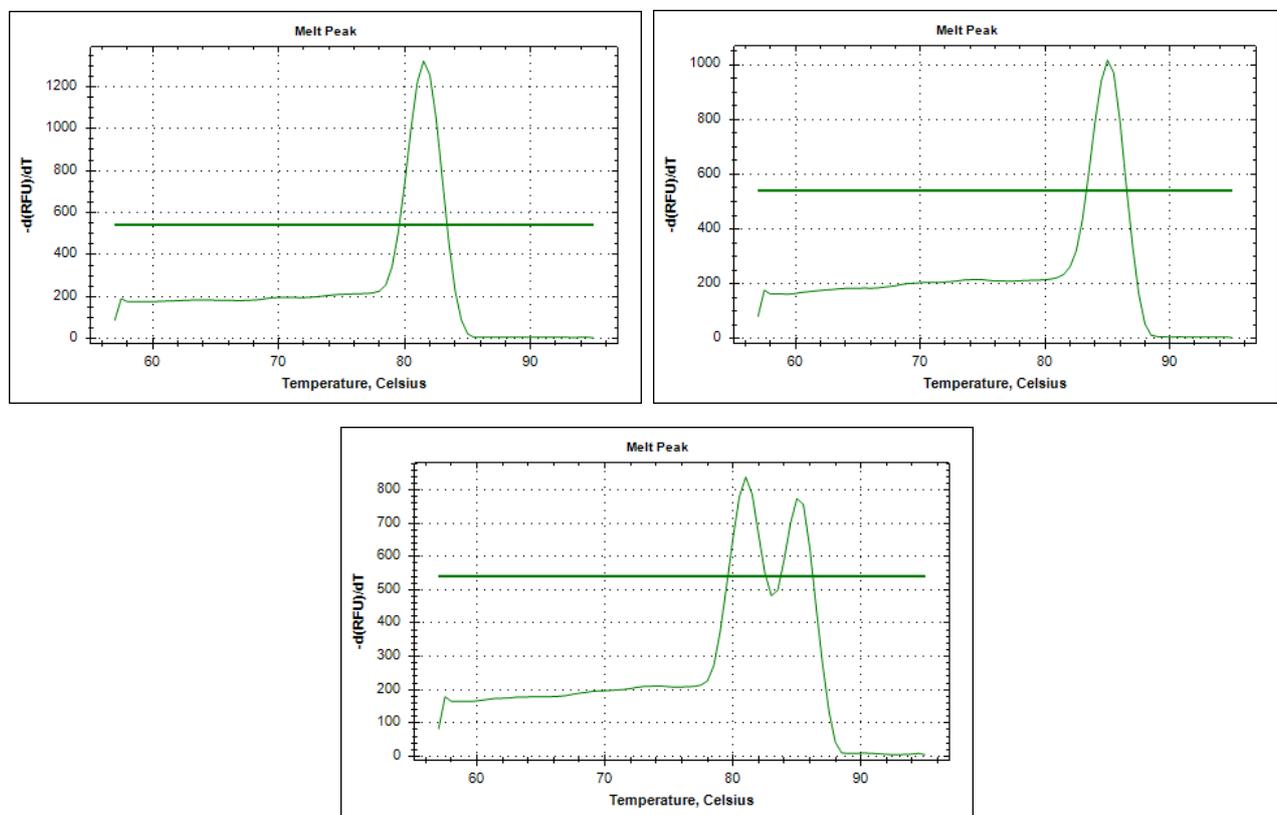
*Singleplex*

For the singleplex, the *Phytophthora* spp. and *P. irregulare* primers performed best at a 65°C annealing temperature, with SYBR SensiFAST chemistry and were specific for the detection of all the *Phytophthora* and *P. irregulare* isolates. *Neocosmospora citricola* and *N. ferruginea* primers performed best at a 67°C annealing

temperature and were also specific in detecting the *N. citricola* and *N. ferruginea* isolates. The optimal annealing temperature for the *N. solani* primers was 69°C and were specific in detecting all the *N. solani* isolates. All primer sets worked best at a 0.5 µM primer concentrations with the exception of *P. irregulare*, which worked best with 0.3 µM (forward) and 0.9 µM (reverse) primer concentrations. The melting points of the target amplicons were 82°C and 85.50°C for *P. citrophthora* and *P. nicotianae* respectively. For *N. citricola* and *N. ferruginea* the melting points were 80.50°C and 84.50°C. The melting points of the target amplicons were 80°C and 87°C for *P. irregulare* and *N. solani* respectively.

### Multiplex

For multiplex qPCR, two sets of primers were combined to simultaneously detect *P. citrophthora* and *P. nicotianae*. Annealing temperatures of 58-65°C were tested, and 65°C was considered as optimal annealing temperature. The primer concentrations had to be optimized to achieve balanced amplification of all targeted DNA. The final primer concentrations were 750 nM for each *P. citrophthora* primer and 125 nM for each *P. nicotianae* primer. The melting points of the target amplicons were 82°C and 85.50°C for *P. citrophthora* and *P. nicotianae* respectively (Figure 4.3.2.10). The multiplex for *P. citrophthora* and *P. nicotianae* were all specific.



**Figure 4.3.2.10.** Melting curves of PCR products used for the detection of *Phytophthora citrophthora* and *Phytophthora nicotianae*. (A) Melt curve analysis of *Phytophthora citrophthora* when tested by singleplex real-time PCR with melting points of 82°C. (B) Melt curve analysis of *Phytophthora nicotianae* amplified by singleplex real-time PCR with melting points of 85.50°C. (C) Melting-curve analysis corresponding to the two amplicons generated by multiplex real-time PCR showing the peaks of *Phytophthora citrophthora* and *Phytophthora nicotianae* simultaneously.

### Optimization and sensitivity of qPCR

#### Tests for linearity (Singleplex)

Standard curves were established, for each targeted gene, to quantify the amount of fungal genomic DNA. The standard curves of all the qPCR assays had acceptable  $R^2$  values and efficiencies. Standard curves showed a linear correlation between input DNA and Ct values with  $R^2$  of 1.000 for *P. irregulare*, 0.990 for *P. citrophthora*, 0.999 for *P. nicotianae*, 0.998 for *N. solani*, 0.996 for *N. ferruginea* and 0.985 for *N. citricola* (water background). The amplification efficiency for each target DNA in water was 96.3% (*P. irregulare*), 94.9% (*P. citrophthora*), 91.0% (*P. nicotianae*), 93.0% (*N. solani*), 99.6% (*N. ferruginea*) and 90.4% (*N. citricola*), respectively. The corresponding slopes were within a slope range of between -3.577 and -3.332, which indicated good qPCR efficiency (Carneiro *et al.*, 2017).

DNA amplification efficiencies for each target DNA in a root background was 93.3% (*P. irregulare*), 91.0% (*P. citrophthora*), 93.3% (*P. nicotianae*), 98.4% (*N. solani*), 85.5% (*N. ferruginea*) and 82.0% (*N. citricola*), respectively. The results of the target DNA in a root background, however, will not be used since DNA extracted from different sample types can be severely affected by various components of the sample matrix. For example, DNA extracted from infected roots will provide a very different matrix than DNA extracted from non-infected roots.

#### *Limit of quantification and limit of detection (Singleplex and multiplex)*

LODs and LOQs for each primer pair were measured in singleplex PCRs to describe the lowest concentration of the sample that can be reliably detected and quantified. Sensitivity of the primer pairs was tested using serial dilutions of total DNA extracted from *P. irregulare*, *P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola*. In singleplex PCR, the LOD for the *P. citrophthora* and *N. ferruginea* primer pairs was  $10^{-3}$  ng/ $\mu$ L, while the LOD for *P. nicotianae* was  $10^{-4}$  ng/ $\mu$ L. The limit of detection for the *P. irregulare*, *N. solani* and *N. citricola* primer pairs were  $10^{-5}$  ng/ $\mu$ L (water background). The LOD in roots for the respective primer pairs was  $10^{-4}$  ng/ $\mu$ L for *P. citrophthora*, *P. nicotianae*, *N. solani* and *N. ferruginea*,  $10^{-5}$  ng/ $\mu$ L for *N. citricola*, and  $10^{-7}$  ng/ $\mu$ L for *P. irregulare*. The LOQ of the singleplex PCR assays was  $10^{-1}$  ng/ $\mu$ L for both the *P. citrophthora* and *N. citricola* primer pairs, while the LOQ for *N. ferruginea* was  $10^{-2}$  ng/ $\mu$ L. The LOQ for the *P. nicotianae* primer pair was  $10^{-3}$  ng/ $\mu$ L, and  $10^{-4}$  ng/ $\mu$ L for *N. solani* and *P. irregulare* (water background).

Sensitivities for *P. citrophthora* and *P. nicotianae* DNA were tested in multiplex real-time PCR. Serial dilutions of *P. citrophthora* and *P. nicotianae* template DNA were utilised to identify the LOD of the multiplex reaction. In multiplex PCR, the limit of detection for the respective primer pairs was  $10^{-4}$  ng/ $\mu$ L for *P. citrophthora* and  $10^{-2}$  ng/ $\mu$ L for *P. nicotianae* (water and root background). These results show that all seven qPCR assays were sensitive and specific enough to detect the target species in citrus roots.

### Study replant pathogen interaction through dual and multiple pathogen inoculations of citrus seedlings

#### Plant measurements

Analyses of variance (ANOVA) of seedling measurements indicated a significant treatment (inoculation) effect for mean seedling height ( $P = 0.0031$ ), mean seedling mass ( $P = 0.0001$ ), mean root mass ( $P < 0.0001$ ) and mean root volume ( $P = 0.0007$ ). The control seedlings (45.2 g) had a mean seedling height that was statistically similar to that of the *Pythium irregulare* (44.9 cm), *Phytophthora nicotianae* (46.5 cm), *Neocosmospora solani* (51.5 cm), *N. citricola* (47.6 cm), *N. solani* + *P. citrophthora* (51.1 cm) and *N. solani* + *P. nicotianae* (45.3 cm) inoculated seedlings (Table 4.3.2.8). The mean seedling height of *P. citrophthora* seedlings (43.5 cm) were also statistically the same as that of the control seedlings and the *P. irregulare*, *P. nicotianae*, *N. ferruginea* (43.5 cm), *N. citricola* and *N. solani* + *P. nicotianae* inoculated seedlings. The mean seedling height of the control seedlings and *P. irregulare*, *P. citrophthora*, *P. nicotianae*, *N. ferruginea*, *P. nicotianae* + *P. irregulare* (40.2 cm) and *N. solani* + *P. nicotianae* inoculated seedlings (Table 4.3.2.8). The statistically lowest mean seedling height was seen for the *N. solani* + *P. nicotianae* + *P. irregulare* inoculated seedlings (34.1 cm).

In terms of mean seedling mass, variation in mean mass was again seen between the different inoculation treatments (Table 4.3.2.8). Treatments that had means similar to the control seedlings (23.5 g) were *P. irregulare* (23.0 g), *N. solani* + *P. nicotianae* (23.9 g) and *N. solani* + *P. citrophthora* (26.3 g) inoculated seedlings. *Phytophthora nicotianae* (19.6 g), *N. citricola* (20.1 g) and *N. solani* (20.6 g) seedlings had means that were similar to aforementioned group. The *P. citrophthora* (15.5 g) inoculated seedlings had a mean that

was statistically similar to that of *N. ferruginea* (18.9 g), *P. nicotianae* + *P. irregulare* (17.7 g) and *N. solani* + *P. nicotianae* + *P. irregulare* inoculated seedlings (17.0 g; Table 4.3.2.8).

Mean root mass results showed that the control (16.9 g), *N. solani* + *P. citrophthora* (18.3 g) and *N. solani* + *P. nicotianae* (16.7 g) inoculated seedlings had the highest mean root mass (Table 4.3.2.8). Means falling between 12 g and 15 g were *P. irregulare* (14.9 g), *N. solani* (13.9 g), *N. ferruginea* (12.9 g) and *N. citricola* (14.1 g). Inoculation treatments with means below 12 g were *P. nicotianae* (11.2 g), *P. nicotianae* + *P. irregulare* (11.5 g), *N. solani* + *P. nicotianae* + *P. irregulare* (11.1 g), with *P. citrophthora* (9.5 g) inoculated seedlings having the lowest mean root mass (Table 4.3.2.8). The mean root volumes of most treatments were below 10.0 cm<sup>3</sup>, ranging from 8.1 cm<sup>3</sup> to 9.8 cm<sup>3</sup> (Table 4.3.2.8). These were also statistically quite similar. Treatments that had mean root volumes above 10.0 cm<sup>3</sup>, were *P. nicotianae* + *P. irregulare* (10.2 cm<sup>3</sup>), *N. citricola* (9.8 cm<sup>3</sup>), *N. ferruginea* (9.8 cm<sup>3</sup>), *N. solani* (9.7 cm<sup>3</sup>), *N. solani* + *P. nicotianae* + *P. irregulare* (9.7 cm<sup>3</sup>), *P. nicotianae* (8.4 cm<sup>3</sup>) and *P. citrophthora* (8.1 cm<sup>3</sup>) inoculated seedlings (Table 4.3.2.8).

#### Pathogen re-isolation percentages

Analyses of variance of the pathogen re-isolation data indicated a significant treatment effect for *Neocosmospora* spp. ( $P < 0.0001$ ), *P. citrophthora* ( $P < 0.0001$ ), *P. nicotianae* ( $P < 0.0001$ ) and *P. irregulare* ( $P < 0.0001$ ) re-isolations from inoculated seedlings. From seedlings that were inoculated with *Neocosmospora* spp., no other pathogen was isolated. The control seedlings also did not yield any isolates of *Neocosmospora* (Table 4.3.2.9). In seedlings inoculated with *N. solani*, the mean re-isolation percentage was 62.1%. *Neocosmospora ferruginea* inoculated seedlings yielded 66.7% during re-isolation, while the seedlings inoculated with *N. citricola* yielded 66.7%. When seedlings were co-inoculated with *N. solani* and *P. citrophthora*, the re-isolation percentage of *N. solani* was 66.7%. This mean was statistically the same as when *N. solani* was inoculated on its own. Similarly, when *N. solani* was co-inoculated with *P. nicotianae*, the re-isolation percentage was 62.1%, exactly the same as when *N. solani* was inoculated on its own. Also, in the combination of *N. solani*, *P. nicotianae* and *P. irregulare*, the re-isolation percentage was again statistically the same as in the *N. solani* alone inoculation (Table 4.3.2.9).

The seedlings inoculated with *P. citrophthora* alone yielded a mean re-isolation percentage of 24.2%. In comparison, when co-inoculated with *N. solani*, the re-isolation percentage of *P. citrophthora* was significantly higher at 45.5%. From the control seedlings no *P. citrophthora* isolates were obtained. A similar trend was observed in *P. nicotianae* inoculated seedlings. When inoculated on its own, the seedlings yielded a mean re-isolation percentage of 20.8%. However, this increased significantly to 45.0% when co-inoculated with either *P. irregulare* or *N. solani*. Also, in the three pathogen co-inoculation, the seedlings yielded 37.1% *P. nicotianae* isolates. This mean was also significantly higher than observed in the *P. nicotianae* alone inoculation as well as the control seedlings (Table 4.3.2.9). This was also seen in seedlings where *P. irregulare* was inoculated alone or in combinations. When inoculated on its own, the seedlings yielded a mean re-isolation percentage of 41.3%. The mean increased significantly to 61.7% when *P. irregulare* was co-inoculated with *P. nicotianae* and was at 55.0% when inoculated with *N. solani* and *P. nicotianae* (Table 4.3.2.9).

#### qPCR detection and quantification from seedling roots

The ANOVA of the qPCR DNA concentration values obtained from the inoculated roots indicated a highly significant ( $P < 0.0001$ ) treatment effect for *Neocosmospora* spp. and *P. citrophthora* DNA concentrations. A significant treatment effect was also seen for *P. nicotianae* ( $P = 0.0061$ ) and *P. irregulare* ( $P = 0.0005$ ) DNA concentration values.

In all the control seedlings, low levels of pathogen DNA of all pathogens was detected (Table 4.3.2.10). In the *Neocosmospora* spp. inoculated seedlings, a mean of -2.3 was observed in the *N. solani* inoculated seedlings and in the *N. ferruginea* inoculated seedlings the mean concentration was -1.9, which was statistically similar to that observed for *N. solani*. In the *N. citricola* inoculated seedlings, the concentration of this pathogen was -3.9 that was statistically similar to the *N. solani* DNA concentration but significantly lower than the *N. ferruginea* DNA concentration (Table 4.3.2.10). When *N. solani* was co-inoculated with *P. citrophthora*, the DNA concentration was -2.1, statistically similar to when *N. solani* was inoculated alone. On the other hand, the

DNA concentration of *N. solani* was -3.4 when co-inoculated with *P. nicotianae*, which was statistically lower than when it was inoculated alone (Table 4.3.2.10).

In the *P. citrophthora* inoculated seedlings the DNA concentration was -3.5 that was statistically higher compared to the concentration observed in the seedlings where *N. solani* and *P. citrophthora* (-4.0) was co-inoculated. In the seedlings where *P. nicotianae* was inoculated alone or in combination with other pathogens, the mean DNA concentration of this pathogen was statistically similar and ranged between -1.2 and -1.9 (Table 4.3.2.10). When seedlings were inoculated with *P. irregulare* alone the mean DNA concentration of -3.7 was significantly lower compared to the -2.9 concentration observed in seedlings where this pathogen was co-inoculated with *P. nicotianae*. In the seedlings where the three pathogens, *N. solani*, *P. nicotianae* and *P. irregulare* were co-inoculated, the *P. irregulare* concentration was at -4.2 significantly lower than in the combination with *P. nicotianae*. On the other hand, the mean of -4.2 was statistically the same as when *P. irregulare* was inoculated alone (Table 4.3.2.10).

**Table 4.3.2.8.** Mean seedling height (cm), seedling mass (g), root mass (g) and root volume (cm<sup>3</sup>) of seedlings inoculated with the replant pathogens (*P. irregulare*, *P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola*.) alone and in different combinations.

Treatment	N	Seedling height (cm) <sup>1</sup>	Seedling mass (g) <sup>1</sup>	Root mass (g) <sup>1</sup>	Root volume (cm <sup>3</sup> ) <sup>1</sup>
<b>Control</b>	4	45.2 abc	23.5 abc	16.9 ab	12.2 bc
<b><i>P. irregulare</i></b>	4	44.9 abc	23.0 abc	14.9 bc	12.3 abc
<b><i>P. citrophthora</i></b>	4	43.5 bc	15.5 e	9.5 f	8.1 d
<b><i>P. nicotianae</i></b>	4	46.5 abc	19.6 cd	11.2 def	8.4 d
<b><i>N. solani</i></b>	4	51.5 a	20.6 bcd	13.9 bcde	9.7 cd
<b><i>N. ferruginea</i></b>	4	43.5 bc	18.9 de	12.9 cde	9.8 cd
<b><i>N. citricola</i></b>	4	47.6 ab	20.1 bcd	14.1 bcd	9.8 cd
<b><i>P. nicotianae</i> + <i>P. irregulare</i></b>	4	40.2 cd	17.7 de	11.5 def	10.2 cd
<b><i>N. solani</i> + <i>P. citrophthora</i></b>	4	51.1 a	26.3 a	18.3 a	15.3 a
<b><i>N. solani</i> + <i>P. nicotianae</i></b>	4	45.3 abc	23.9 ab	16.7 ab	13.3 ab
<b><i>N. solani</i> + <i>P. nicotianae</i> + <i>P. irregulare</i></b>	4	34.1 d	17.0 de	11.1 ef	9.7 cd
<b>LSD</b>		7.36	4.11	3.03	3.01

<sup>1</sup>Means with the same letter are not significantly different at a 95% confidence level.

**Table 4.3.2.9.** Mean re-isolation percentage from seedling roots inoculated with *P. irregulare*, *P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola*, alone and in different combinations.

Treatment	N	% <i>N. solani</i> <sup>1</sup>	% <i>P. citrophthora</i> <sup>1</sup>	% <i>P. nicotianae</i> <sup>1</sup>	% <i>P. irregulare</i> <sup>1</sup>
<b>Control</b>	4	0.0 b	0.0 c	0.0 d	0.0 c
<b><i>P. irregulare</i></b>	4	0.0 b	0.0 c	0.0 d	41.3 b
<b><i>P. citrophthora</i></b>	4	0.0 b	24.2 b	0.0 d	0.0 c
<b><i>P. nicotianae</i></b>	4	0.0 b	0.0 c	20.8 c	0.0 c
<b><i>N. solani</i></b>	4	62.1 a	0.0 c	0.0 d	0.0 c
<b><i>N. ferruginea</i></b>	4	66.7 a	0.0 c	0.0 d	0.0 c
<b><i>N. citricola</i></b>	4	66.7 a	0.0 c	0.0 d	0.0 c
<b><i>P. nicotianae</i> + <i>P. irregulare</i></b>	4	0.0 b	0.0 c	45.0 a	61.7 a
<b><i>N. solani</i> + <i>P. citrophthora</i></b>	4	66.7 a	45.4 a	0.0 d	0.0 c
<b><i>N. solani</i> + <i>P. nicotianae</i></b>	4	62.1 a	0.0 c	45.0 a	0.0 c
<b><i>N. solani</i> + <i>P. nicotianae</i> + <i>P. irregulare</i></b>	4	67.5 a	0.0 c	37.1 b	55.0 a
<b>LSD</b>		8.72	9.32	6.90	7.72

<sup>1</sup>Means with the same letter are not significantly different at a 95% confidence level.

**Table 4.3.2.10.** Mean log values of pathogen DNA concentration obtained by qPCR analyses from seedling roots inoculated with *P. irregulare*, *P. citrophthora*, *P. nicotianae*, *N. solani*, *N. ferruginea* and *N. citricola* alone and in different combinations.

Treatment	N	<i>LN. solani</i> <sup>1</sup>	<i>LP. citrophthora</i> <sup>1</sup>	<i>LP. nicotianae</i> <sup>1</sup>	<i>LP. irregulare</i> <sup>1</sup>
<b>Control</b>	4	-4.6 d	-4.0 b	-2.9 b	-5.6 c
<i>P. irregulare</i>	4	-	-	-	-3.7 ab
<i>P. citrophthora</i>	4	-	-3.5 a	-	-
<i>P. nicotianae</i>	4	-	-	-1.7 a	-
<i>N. solani</i>	4	-2.3 ab	-	-	-
<i>N. ferruginea</i>	4	-1.9 a	-	-	-
<i>N. citricola</i>	4	-3.1 bc	-	-	-
<i>P. nicotianae</i> + <i>P. irregulare</i>	4	-	-	-1.2 a	-2.9 a
<i>N. solani</i> + <i>P. citrophthora</i>	4	-2.1 a	-4.000 b	-	-
<i>N. solani</i> + <i>P. nicotianae</i>	4	-3.4 c	-	-1.9 a	-
<i>N. solani</i> + <i>P. nicotianae</i> + <i>P. irregulare</i>	4	-1.6 a	-	-1.9 a	-4.2 b
LSD		0.86	0.12	0.73	0.89

<sup>1</sup>Means with the same letter are not significantly different at a 95% confidence level.

*Objective 2. Identify and evaluate potential biocontrol agents that can be used in the integrated management of citrus replant pathogens.*

1. Isolation of potential BCA's from sampled rhizosphere soil.

A total of 123 bacterial isolates were obtained from sampled roots and rhizosphere soil collected in orchards in the Citrusdal and Kirkwood production areas.

2. Preliminary identification of selected BCA's.

Fourteen isolates were preliminary identified to be *Bacillus* spp. and 22 isolates as *Pseudomonas* spp. based on morphological observations and biochemical characteristics. Samples that could not be classified into the *Pseudomonas* spp. category, but were negative for anaerobic growth, were labelled as "Other" samples (Results not shown). For the fungal species, eight isolates were isolated from the roots and were morphologically identified as *Trichoderma* spp. based on their macroscopic characteristics. The isolates were stored in water at 4°C for further analyses. In accordance with the preliminary identification, these isolates were then subjected to *in vitro* screening.

3. *In vitro* screening of potential BCA for antifungal activity against citrus replant pathogens

*Preliminary screening for the selection of Trichoderma isolates as biocontrol agents*

Preliminary *in vitro* screening in the form of a non-volatile, diffusible antifungal compound production test were conducted in order to identify potential *Trichoderma* isolates that shows pathogen mycelial growth inhibition of at least 50%. Eight *Trichoderma* isolates were screened against one isolate of the replant pathogens (Table 4.3.2.11). Of these eight *Trichoderma* isolates, only 2 isolates exhibited growth inhibition of the whole replant complex of at least 50% from the control (Data not shown). Based on this observation, these two *Trichoderma* isolates, P4 and P16, were then subjected to a volatile and a competition test against one isolate of each replant pathogens to determine the different modes of action and interactions *Trichoderma* may have against the pathogens.

**Table 4.3.2.11.** List of citrus replant pathogen isolates used for preliminary *in vitro* screening for the selection of potential biocontrol agents.

Species name	Isolates
<i>Neocosmospora solani</i>	VG 38
<i>Neocosmospora ferruginea</i>	VG 109
<i>Neocosmospora citricola</i>	VG 197
<i>Phytophthora citrophthora</i>	C3A
<i>Phytophthora nicotianae</i>	15.1
<i>Pythium irregulare</i>	81 B

*Preliminary screening for the selection of bacterial isolates as biocontrol agents*

A total of 48 bacterial isolates were preliminary identified as *Bacillus*, *Pseudomonas* and Other species and were screened for mycoparasitism action towards the replant pathogens as a preliminary screening. For each pathogen, we selected the bacteria that inhibited the pathogen growth by 50% or more from the control. Of these, *Bacillus* N19 and *Pseudomonas* N83 spp. exhibited the maximum growth inhibition of the whole replant pathogen complex (Data not shown). Based on this observation, *Bacillus* N19 and *Pseudomonas* N83 were selected and furthermore subjected to a non-volatile and a volatile test against the six citrus replant pathogens with one isolate each.

### Molecular species identification of selected BCA

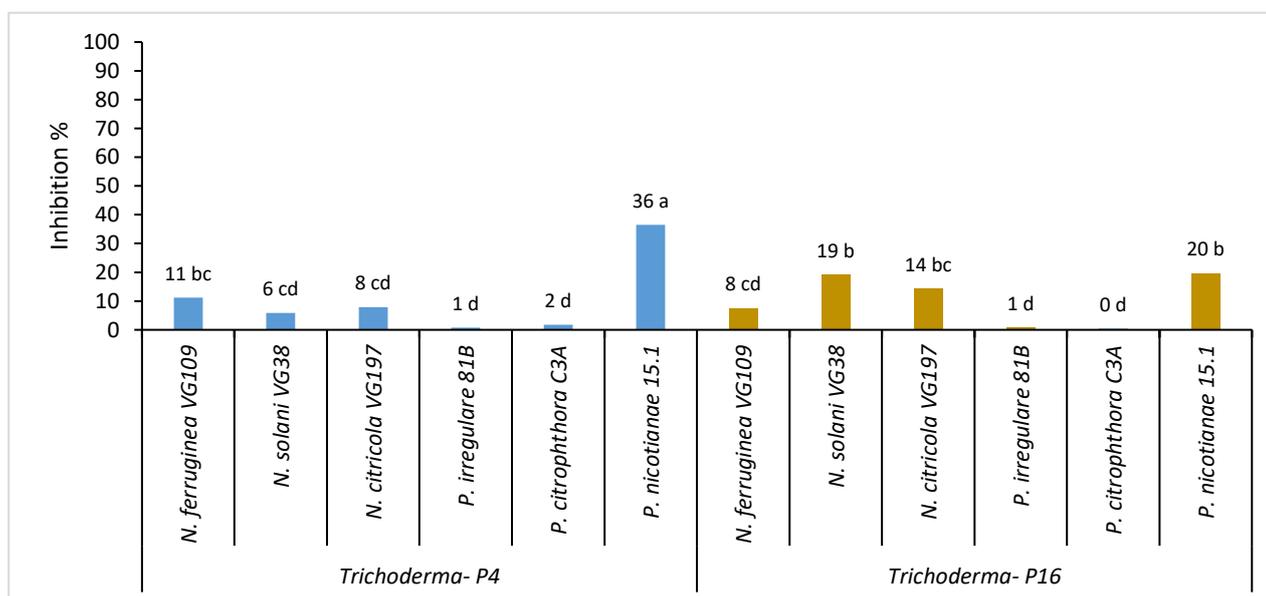
The BLAST results of the different sequences confirmed the genus identified with the physiological, biochemical and morphological tests. The selected bacterial isolates were identified by molecular analysis as *B. subtilis* and *P. fluorescence*. The candidate fungal isolates both belonged to the genus *Trichoderma harzianum*.

### Second in vitro screening of selected *Trichoderma harzianum* isolates for their antagonistic activity

#### Production of volatile organic compounds

The antifungal activity of the two selected *Trichoderma harzianum* isolates, P4 and P16 were further evaluated with a volatile test to study the inhibitory effect of any volatile anti-fungal compounds that the isolates might produce. Analyses of variance of the mean percentage inhibition data revealed a statistically significant *Trichoderma* isolate x pathogen interaction ( $P < 0.0019$ ). The mycelial growth of *Phytophthora nicotianae* was significantly more inhibited by *Trichoderma* isolate P4 (36%), in comparison to the inhibition achieved for the other pathogens (Figure 4.3.2.11). For the other pathogens the percentage inhibition was between 1 and 11 %, which was statistically similar. Isolate P16 inhibited *P. nicotianae* by 20% that was significantly less than the inhibition by P4. However, in comparison to the other pathogens, this inhibition was statistically similar to the inhibition percentage observed for *N. solani* (19%) and *N. citricola* (14%) but significantly better than the inhibition observed for the other pathogens (Figure 4.3.2.11). Interestingly, P16 caused significantly higher inhibition of *N. solani* (19%), in contrast to P4 (6%, Figure 4.3.2.11).

Nonetheless, these results underline that the *Trichoderma harzianum* isolates, P4 and P16 did not show a growth inhibition of 50 % or more in the volatile test against the whole replant pathogen complex. Consequently, the volatile assay was not repeated with five additional isolates per pathogen.



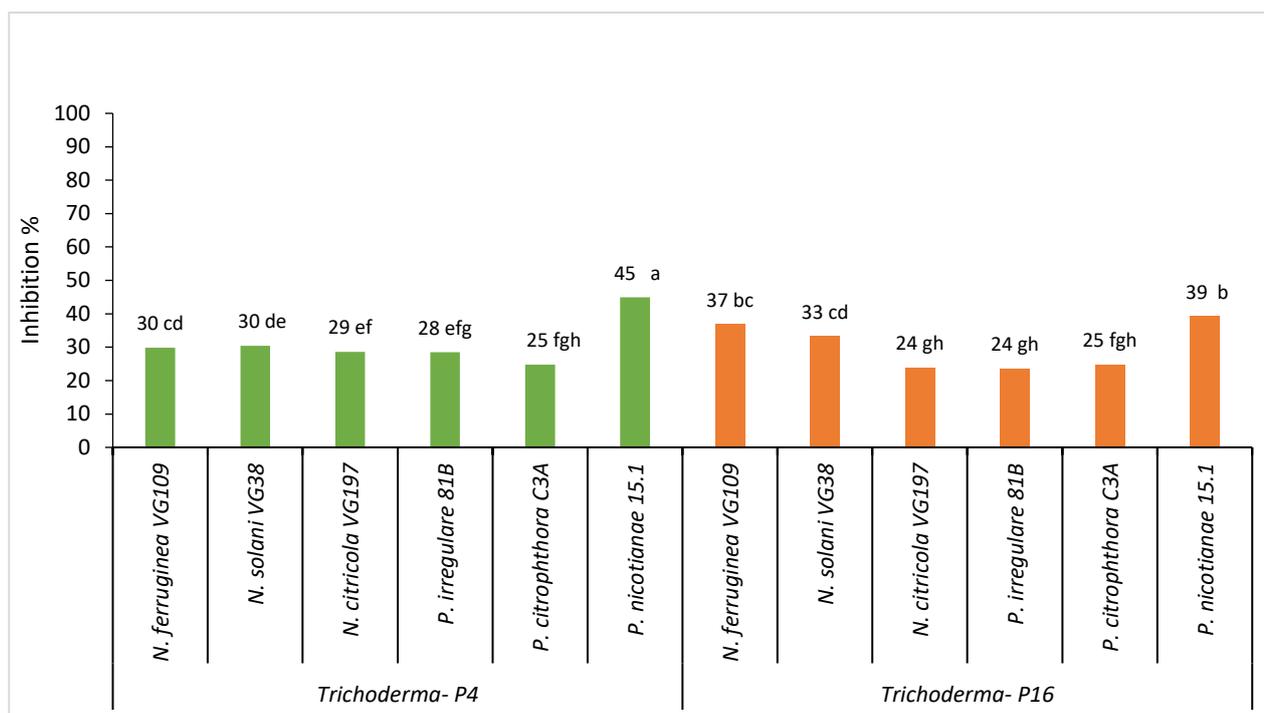
**Figure 4.3.2.11.** Mean percentage mycelial growth inhibition of six citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae* by two *Trichoderma* isolates, P4 and P16, resulting from a volatile compound test.

#### Competition test

*Trichoderma harzianum* isolates, P4 and 16 were evaluated *in vitro* for their competitive action against replant pathogens by using a dual culture assay. The ANOVA of the mean percentage mycelial growth inhibition showed a significant *Trichoderma* isolate x pathogen interaction ( $P < 0.0034$ ). The mycelial growth of *P. nicotianae* was found to be inhibited by P4 by 45% (Figure 4.3.2.12). This was significantly more than the

inhibition seen for the other pathogens. The inhibition for the other pathogens was between 25% and 30%, which was statistically similar (Figure 4.3.2.12). P16 inhibited *P. nicotianae* by 39%, statistically similar to the inhibition of *N. ferruginea* (37%; Figure 4.3.2.12). The latter inhibition percentage was statistically similar to the inhibition of 33% achieved by P16 for *N. solani*. The mean inhibition of these three pathogens were all significantly more than the inhibition for the remaining three pathogens that was statistically the same and ranged between 24% and 25% (Figure 4.3.2.9).

However, the competition effect was not effective as it could not inhibit the growth of mycelia up to 50% compared to the control and were therefore not subjected to another dual culture assay with five isolates of each citrus replant pathogens.



**Figure 4.3.2.12.** Mean percentage mycelial growth inhibition achieved by two *Trichoderma harzianum* isolates, P4 and P16, in a competition test against one isolate from each of six citrus replant pathogens: *Neocosmospora ferruginea*, *Neocosmospora solani*, *Neocosmospora citricola*, *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae*.

#### Second in vitro screening of bacterial isolates for their antagonistic activity

##### Production of volatile organic compounds

The ability of the two selected bacterial isolates, *B. subtilis* N19 and *P. fluorescence* N83, to produce volatile compounds with an antagonist effect against the replant pathogens were evaluated with a volatile test. The results did not show mycelia growth inhibition of the replant pathogens by the volatile compounds produced by the isolates of *B. subtilis* and *P. fluorescence*, above 50% (Data not shown).

##### Diffusible antifungal compounds production

The two bacterial isolates, one each of *B. subtilis* and *P. fluorescence*, were subjected to further *in vitro* screening to evaluate the potential production of non-volatile compounds able to inhibit citrus replant pathogens with a non-volatile test. The results showed that *B. subtilis* and *P. fluorescence* were able to inhibit the mycelia growth of the complex replant pathogens to at least 50% from the control (Data not shown). These isolates were consequently subjected to a new non-volatile test with five isolates of each citrus replant pathogen.

Third in vitro screening of the selected bacterial and *Trichoderma harzianum* isolates for their antagonistic activity against five isolates of each replant pathogens

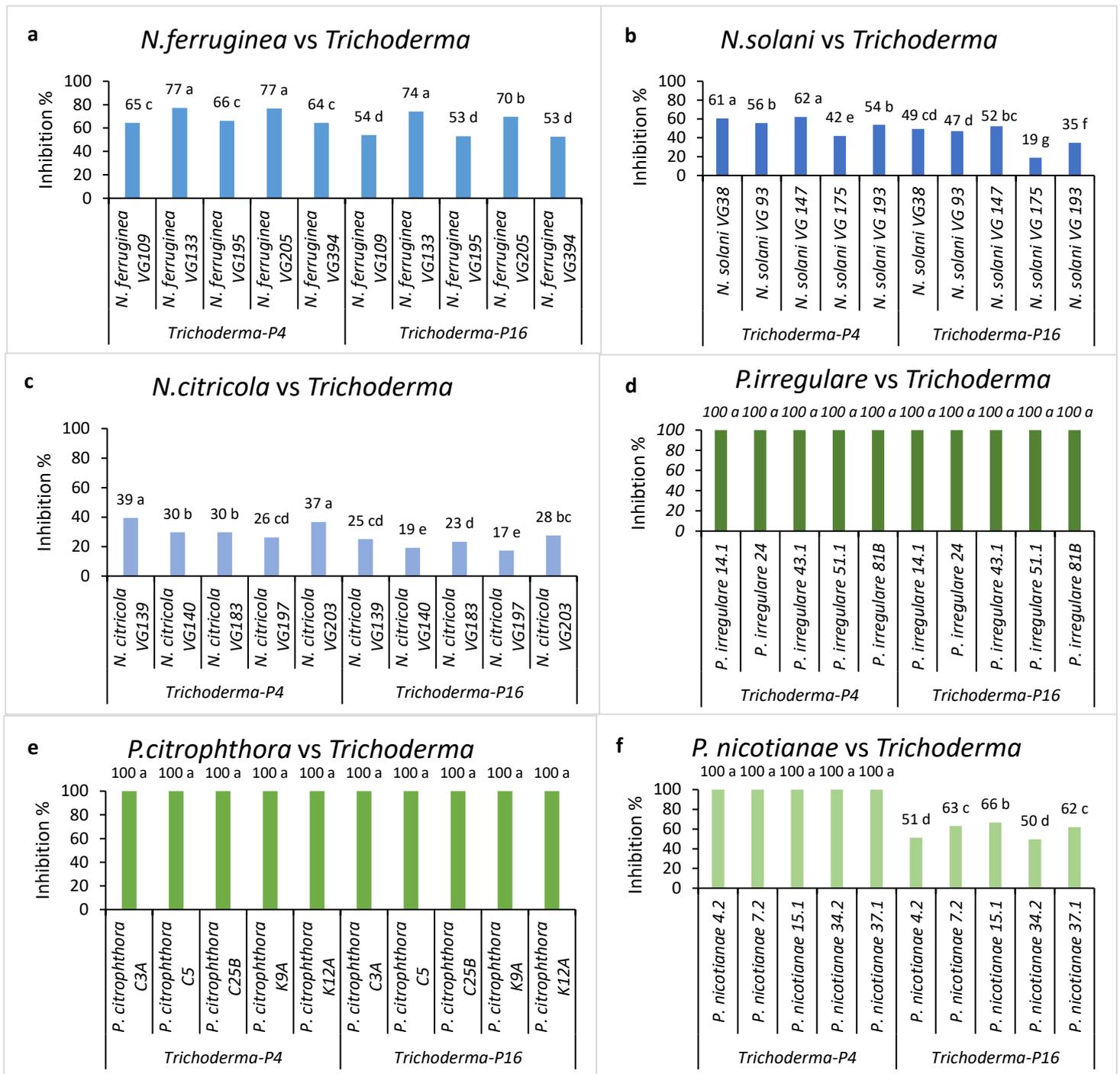
Following the preliminary screenings for the volatile and competition test described above, the selected *Trichoderma harzianum* isolates P4 and P16 were found not to be able to inhibit the growth of citrus replant pathogens to at least 50% through these two mechanisms. The same trend was observed for the volatile test when *B. subtilis* isolate N19 and *P. fluorescence* isolate N83 were evaluated against the replant pathogens. Consequently, these assays were not repeated with five additional isolates per pathogen.

Diffusible anti-fungal compound production

*Trichoderma harzianum* isolates, P4 and P16 significantly reduced the mycelia growth of the whole replant pathogen complex by at least 50% when compared with the non-treated control. This was the best screening method for the *Trichoderma harzianum* isolates and were therefore repeated with five isolates of the replant pathogens and is shown in Figure 4.3.2.13.

Analysis of variance (ANOVA) of growth inhibition percentage data revealed a significant *Trichoderma harzianum* isolate × pathogen isolate interaction for *N. ferruginea* ( $P = 0.0010$ ), *N. solani* ( $P < 0.0001$ ), and *N. citricola* ( $P = 0.0172$ ), *P. nicotianae* ( $P < 0.0001$ ), *P. citrophthora* ( $P < 0.0001$ ) and *P. irregulare* ( $P < 0.0001$ ). Isolate P4 exhibited a reduction in the mycelial growth of 54%-77% for *N. ferruginea* (Figure 4.3.2.13a). P16 resulted in a statistically similar inhibition for *N. ferruginea* with percentage inhibition ranging from 53% to 74%. In the case of *N. solani*, P4 inhibited mycelial growth of the different pathogen isolates by between 42% and 62% that varied significantly between isolates (Figure 4.3.2.13b). Inhibition for *N. solani* isolates by P16 again varied significantly between isolates (19%-52%) as seen in Figure 4.3.2.13b. This statistically significant variation in inhibition was also observed for *N. citricola* where the inhibition by P4 was between 26% and 39%, while P16 inhibited the growth of the pathogen isolates by between 17% and 28% (Figure 4.3.2.13c).

Maximum mean percentage inhibition of mycelial growth was achieved in the case of *P. irregulare* and *P. citrophthora*, with 100% inhibition observed for P4 and P16 against all pathogen isolates (Figure 4.3.2.13 d, e). All *P. nicotianae* isolates were furthermore inhibited 100% by P4, in comparison to P16 where the inhibition percentages (50%-66%) were significantly lower compared to the inhibition of P4 and also varied significantly between pathogen isolates (Figure 4.3.2.13f).



**Figure 4.3.2.13. a-f [(a) *N. ferruginea*, (b) *N. solani*, (c) *N. citricola*, (d) *P. irregulare*, (e) *P. citrophthora* and (f) *P. nicotianae*]- mycelial inhibition activity of *Trichoderma* isolates, P4 and P16 against 5 isolates of each of six replant pathogens evaluated by a non-volatile test. Results represents the mean of four biological replicates. Means with the same letter are not significantly different at a 95% confidence level.**

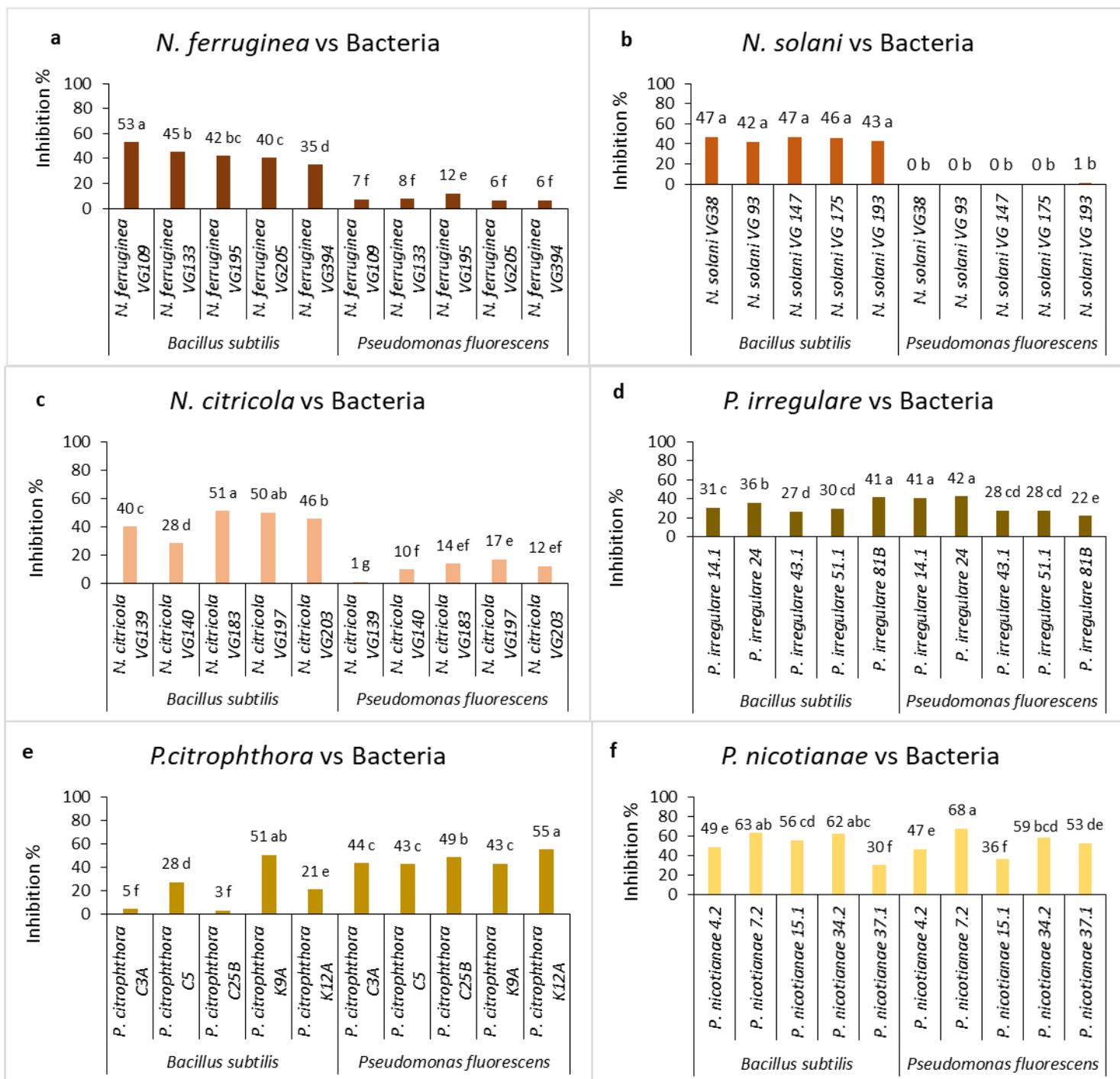
#### Competition test

The results of the first dual culture screening showed that the bacterial isolates, *B. subtilis* N19 and *P. fluorescence* N83 inhibited the growth of the replant pathogen complex by competition by 50% or more from the control and this technique was therefore used for further screening.

The competition assay was repeated with the two selected bacterial isolates, *B. subtilis* N19 and *P. fluorescence* N83 and five additional isolates per pathogen were used (Table 4.3.2.5). Results obtained by the

two selected bacterial isolates from the repetitive competition test are shown in Figure 4.3.2.14. Analysis of variance (ANOVA) of mean percentage mycelial growth inhibition revealed a significant bacterial species × pathogen isolate interaction for *N. ferruginea* ( $P < 0.0001$ ), *N. solani* ( $P = 0.0055$ ), *N. citricola* ( $P < 0.0001$ ), *P. irregulare* ( $P < 0.0001$ ), *P. citrophthora* ( $P < 0.0001$ ) and *P. nicotianae* ( $P < 0.0001$ ).

*Bacillus subtilis* N19 gave statistically the highest mycelial growth inhibition of *N. ferruginea* (35%-53%), with significant variation seen in the inhibition of the different isolates. In comparison, *P. fluorescence* N83 inhibited the *N. ferruginea* isolates by 6%-12%, which was statistically lower than the inhibition by *B. subtilis* N19 (Figure 4.3.2.14a). Similarly, *N. solani* isolates were inhibited by *B. subtilis* N19 by statistically similar percentages of 43%-47%, whereas no growth inhibition of any isolates of *N. solani* were observed for *P. fluorescence* N83 (0%, Figure 4.3.2.14b). *Neocosmospora citricola* was inhibited by *B. subtilis* N19 at between 28% and 51%. Again, the percentage inhibition for the different pathogen isolates varied significantly. In comparison the *P. fluorescence* N83 isolate inhibited the different *N. citricola* isolates by between 1% and 17%, that was statistically similar but significantly poorer compared to *B. subtilis* N19 (Figure 4.3.2.14c). *Pythium irregulare* mycelial growth was inhibited by *B. subtilis* by 27%-41%, again with the inhibition between isolates varying significantly. Though not glaringly different, the inhibition by *P. fluorescence* N83 ranged between 22% and 42% that was again statistically different between isolates (Figure 4.3.2.14d). *Pseudomonas fluorescence* N83 had a higher mycelial growth inhibition for *P. citrophthora* (43%-53%), in contrast to *B. subtilis* N19 (3%-51%). However, for both bacterial isolates, the significant variation in inhibition between isolates were observed. Maximum growth inhibition was recorded by *P. fluorescence* N83 when tested against *P. nicotianae* (36%-68%), in a similar range to that observed for *B. subtilis* N19 (30%-62%; Figure 4.3.2.14f).



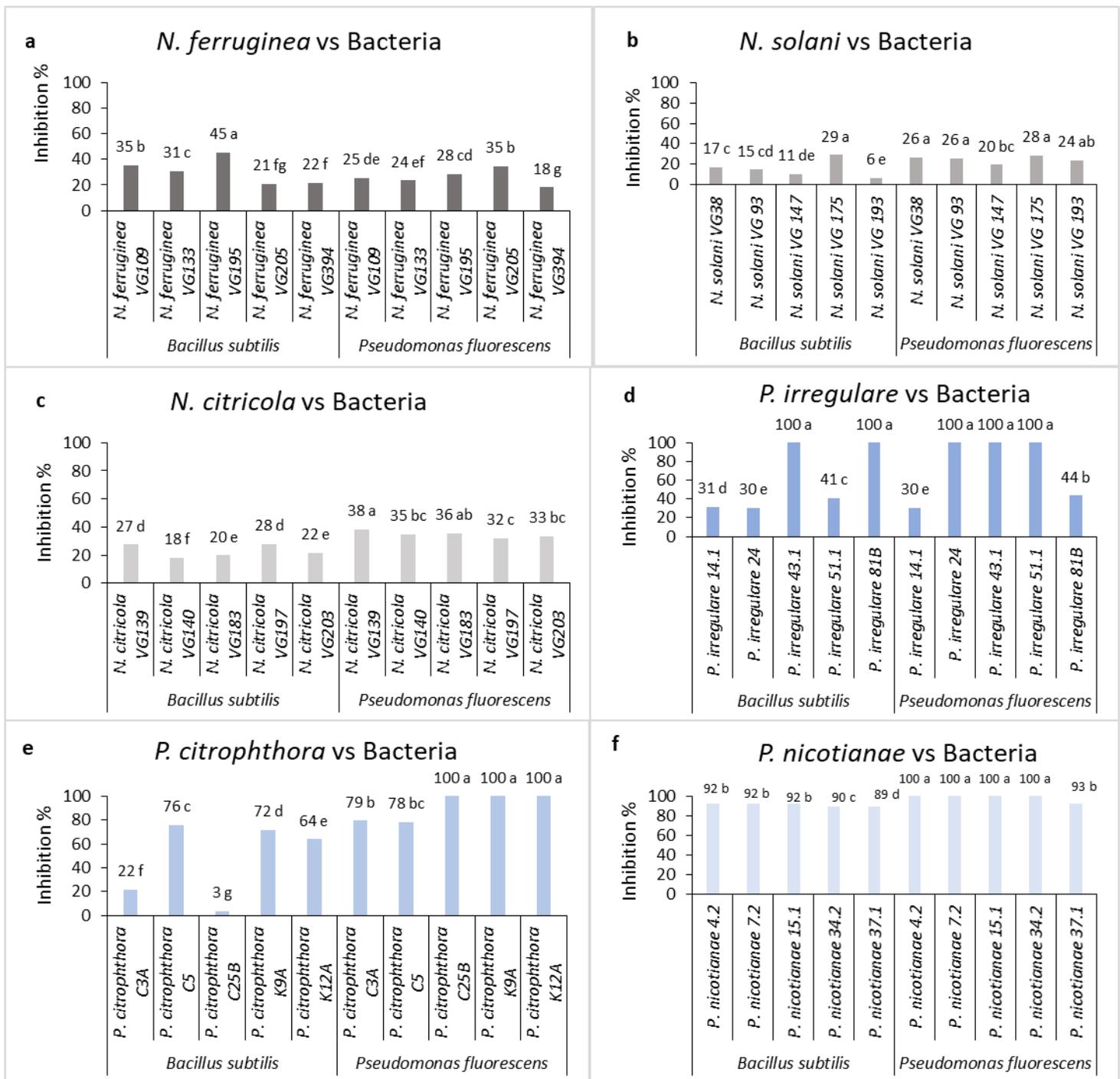
**Figure 4.3.2.14.** Mean percentage mycelial growth inhibition accomplished by two bacterial species, *B. subtilis* and *P. fluorescens*, in a competition test against five isolates from each of six citrus replant pathogens. (a) *N. ferruginea*, (b) *N. solani*, (c) *N. citricola*, (d) *P. irregulare*, (e) *P. citrophthora* and (f) *P. nicotianae*. Each data set represents the mean of four biological replicates. Means sharing similar letter are not significantly different at a 95% confidence level.

#### Diffusible antifungal compounds production

*Bacillus subtilis* N19 and *Pseudomonas fluorescens* N83 were subjected to a second non-volatile test based on the first screening, where they inhibited the replant pathogens mycelial growth to at least 50% of the control. The non-volatile test was repeated with the two selected bacterial isolates, *B. subtilis* N19 and *P. fluorescens* N83 and this time five isolates per replant pathogen were used (Table 4.3.2.5). Analyses of variance of inhibition data showed a significant bacterial species × pathogen isolate interaction for *N. ferruginea* ( $P < 0.0001$ ), *N. solani* ( $P = 0.0004$ ), *N. citricola* ( $P < 0.0001$ ) and  $P < 0.0001$  for all of *P. irregulare*, *P. citrophthora* and *P. nicotianae*.

The effect of the non-volatile compounds on the growth of the isolates from the six replant pathogens are presented in Figure 4.3.2.12-15. For *B. subtilis* N19, the inhibition varied significantly from 21% to 45% between isolates, whereas *P. fluorescence* N83 inhibited mycelial growth by 18% to 35% when tested against *N. ferruginea* (Figure 4.3.2.15a). In the case of *N. solani*, average mycelial inhibition by *B. subtilis* N19 was between 11% and 29% for the different isolates, again a significant variation and for *P. fluorescence* N83 the inhibition ranged from 20% to 28%, also a significant variation (Figure 4.3.2.15b).

Similarly for *N. citricola*, the inhibition by *B. subtilis* N19 was statistically different between 18% and 28% for the different isolates. However, for *P. fluorescence* N83 the mean inhibition was slightly higher, again varying significantly but this time between 32% and 38% (Figure 4.3.2.15c). For two of the *P. irregulare* isolates inhibition by *B. subtilis* N19 was 100%, significantly more than for the other isolates where inhibition was 30% to 41%. This was also seen for *P. fluorescence* N83 where three of the isolates were inhibited 100%, while the inhibition of the other two were statistically lower at 30% and 44%, respectively (Figure 4.3.2.15d). *Phytophthora citrophthora* isolates' inhibition by *B. subtilis* N19 again varied significantly. For two isolates inhibition was below 30%, while for the other three isolates it was above 64%. The *P. fluorescence* N83 isolate also inhibited three isolates by 100%, significantly more than the other two isolates where inhibition was 78% and 79%, respectively (Figure 4.3.2.15e). Inhibition of *P. nicotianae* by the bacterial isolates was excellent. *Bacillus subtilis* N19 inhibited the different isolates at statistically varying percentages of between 89% and 92% while the inhibition by *P. fluorescence* N83 for all isolates were 100%, statistically better than *B. subtilis* (Figure 4.3.2.15f).



**Figure 4.3.2.15.** Mean percentage mycelial growth inhibition by bacterial isolates, *B. subtilis* and *P. fluorescens* against five isolates of each of six citrus replant pathogens with a non-volatile test (a) *N. ferruginea*, (b) *N. solani*, (c) *N. citricola*, (d) *P. irregulare*, (e) *P. citrophthora* and (f) *P. nicotianae*. Each value is an average of four biological replicates. Means sharing similar letter are not significantly different at a 95% confidence level.

4. In planta evaluation of selected BCA's for their ability to reduce replant pathogen root infection

*Plant measurements*

Analyses of variance (ANOVA) of plant measurement data from seedlings inoculated with the different replant pathogens and treated with the selected biocontrol agents indicated the following significant effects. From the *N. solani* inoculated seedlings, the seedling height change data showed a significant ( $P = 0.0302$ ) pathogen control effect. In terms of seedling height percentage change a significant ( $P = 0.0157$ ) effect was observed.

The seedlings that were inoculated with *N. solani* had a mean change in seedling height of 4.4 cm that was significantly lower compared to the seedling height change observed for the control seedlings at 5.8 cm.

In terms of the mean percentage height change, the control seedlings from the *N. solani* group had a mean increase in height of 18.7% during the trial. In comparison, the *B. subtilis* N19 treated seedlings had a mean increase of 31.1%, significantly higher than the control seedlings. The *P. fluorescens* N83 treated seedlings had a mean increase of 27.9%, statistically the same as the control seedlings but significantly better than the seedlings treated with the two *T. harzianum* isolates. The two *T. harzianum* isolates had mean percentages increase of 15.9% (isolate P4) and 14.9% (Isolate P16; Table 4.3.2.12).

The plant measurements in the *P. nicotianae* group of plants through ANOVA showed from the seedling height change data a significant ( $P = 0.0016$ ) biocontrol agent effect. In this group of seedlings the control seedlings had a mean height increase of 7.2 cm. In comparison the *B. subtilis* N19 treated seedlings had a mean height increase of 4.6 cm that was statistically similar to the mean height change of the other biocontrol agents, which ranged between 3.4 cm (*P. fluorescens* N83) and 5.6 cm (*T. harzianum* P16; Table 4.3.2.12).

ANOVA of the *P. irregulare* seedlings plant measurement data a significant biocontrol agent effect was seen for mean seedling height change ( $P = 0.0132$ ), seedling height percentage change ( $P = 0.0219$ ) and seedling mass ( $P = 0.0063$ ). The control seedlings in this group had a mean height increase of 7.4 cm that was significantly more than observed for the four biocontrol treatments. The mean height increase for the biocontrol agents was statistically the same and ranged between 4.1 cm and 5.1 cm. In terms of mean percentage height change, the control seedlings, *P. fluorescens* and *B. subtilis* treated seedlings had statistically similar means at 29.4%, 30.3% and 26.6%, respectively (Table 4.3.2.12). Statistically similar, but markedly lower to the *B. subtilis* means were the means of the two *T. harzianum* isolates. In this case it was 14.5% for isolate P4 and 14.6% for P16.

#### *Re-isolation percentages*

The re-isolation data per pathogen group of seedlings showed for the *N. solani* group of seedlings a significant biocontrol agent effect of  $P < 0.0001$  for the *B. subtilis*, *P. fluorescens* and *T. harzianum* re-isolation data. In this group a significant  $P < 0.0001$  pathogen effect was furthermore observed for the *N. solani* re-isolation data. From the seedlings treated with *B. subtilis* N19, this bacterium was isolated with a mean of 89.2% while it was not isolated from any of the seedlings treated with the other biocontrol agents or the control seedlings (Table 4.3.2.13). *Pseudomonas fluorescens* N83 was re-isolated at a percentage of 75.8%, while *T. harzianum* isolate P4 was re-isolated at 73.3% and P16 at 76.3% (Table 4.3.2.13). *Neocosmospora solani* was re-isolated at a mean percentage of 53.0% from inoculated seedlings but never from the control seedlings (Table 4.3.2.14).

The re-isolation data from the *P. nicotianae* group of seedlings again showed a significant ( $P < 0.0001$ ) biocontrol agent effect for the biocontrol re-isolation data and a significant ( $P < 0.0001$ ) pathogen effect for the *P. nicotianae* re-isolation data. Here *B. subtilis* was re-isolated at a mean percentage of 87.9%, *P. fluorescens* at 75.0% and *T. harzianum* P4 at 79.6% and P16 at 74.6%. From the inoculated seedlings, *P. nicotianae* was re-isolated at a mean of 59.33% (Tables 4.3.2.13 and 4.3.2.14).

In the *P. irregulare* seedling group the re-isolation data also showed a significant ( $P < 0.0001$ ) biocontrol effect for the four biocontrol agent re-isolation data and a significant ( $P < 0.0001$ ) pathogen effect for *P. irregulare* re-isolations. As before, *B. subtilis* was re-isolated at a high percentage of 87.9%, while *P. fluorescens* was re-isolated at 78.3%. The two *T. harzianum* isolates were re-isolated at 83.8% (P4) and 80.4% (P16), respectively (Table 4.3.2.13). Here the pathogen, *P. irregulare*, was re-isolated at a percentage of 70.16% from the inoculated seedlings (Table 4.3.2.14).

**Table 4.3.2.12.** Mean seedling height, seedling height percentage (%) change and seedling mass (g) of Troyer citrange seedlings treated with biocontrol agents, *B. subtilis* N19, *P. fluorescens* N83, *Trichoderma harxianum* strains P4 and P16, 3 weeks before inoculation with *Neocosmospora solani*, *Phytophthora nicotianae* and *Pythium irregulare* and grown for 13 weeks in perlite under glasshouse conditions.

Treatment	<i>N. solani</i>			<i>P. nicotianae</i>			<i>P. irregulare</i>		
	Mean seedling height change (cm)	Mean seedling height % change	Mean seedling mass (g)	Mean seedling height change (cm)	Mean seedling height % change	Mean seedling mass (g)	Mean seedling height change (cm)	Mean seedling height % change	Mean seedling mass (g)
Control	-	18.7 bc*	-	7.2 a	-	-	7.4 a	29.4 a	20.2 ab
<i>B. subtilis</i> N19	-	31.1 a	-	4.6 bc	-	-	5.1 b	26.6 ab	12.2 bc
<i>P. fluorescens</i> N83	-	27.9 ab	-	3.4 c	-	-	4.9 b	30.3 a	9.0 c
<i>P4-Trichoderma</i>	-	15.9 c	-	5.3 b	-	-	4.9 b	14.5 b	24.0 a
<i>P16-Trichoderma</i>	-	14.9 c	-	5.6 ab	-	-	4.1 b	14.6 b	20.1 ab
LSD	-	11.13	-	1.68	-	-	1.84	12.58	8.66

\*Means sharing similar letters are not significantly different at a 95% confidence level.

**Table 4.3.2.13.** Mean percentage of re-isolations of biocontrol agents from Troyer citrange seedlings treated with biocontrol agents *B. subtilis* N19, *P. fluorescens* N83 and *Trichoderma harzianum* strains P4 and P16, 3 weeks before inoculation of *N. solani*, *P. nicotianae* and *P. irregulare* and grown for 13 weeks in perlite in the greenhouse.

Treatment	<i>N. solani</i>			<i>P. nicotianae</i>			<i>P. irregulare</i>		
	Mean % <i>B. subtilis</i>	Mean % <i>P. fluorescens</i>	Mean % <i>Trichoderma</i>	Mean % <i>B. subtilis</i>	Mean % <i>P. fluorescens</i>	Mean % <i>Trichoderma</i>	Mean % <i>B. subtilis</i>	Mean % <i>P. fluorescens</i>	Mean % <i>Trichoderma</i>
Control	0.0 b*	0.00 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
<i>B. subtilis</i>	89.2 a	0.00 b	0.0 b	87.9 a	0.0 b	0.0 b	87.9 a	0.0 b	0.0 b
<i>P. fluorescens</i>	0.0 b	75.8 a	0.0 b	0.0 b	75.0 a	0.0 b	0.0 b	78.3 a	0.0 b
<i>T. harzianum</i> -P4	0.0 b	0.00 b	73.3 a	0.0 b	0.0 b	79.6 a	0.0 b	0.0 b	83.8 a
<i>T. harzianum</i> -P16	0.0 b	0.00 b	76.3 a	0.0 b	0.0 b	74.6 a	0.0 b	0.0 b	80.4 a
LSD	2.22	0.00 b	7.32	3.36	2.94	6.97	2.91	3.32	0.0 b

\*Means sharing similar letters are not significantly different at a 95% confidence level.

**Table 4.3.2.14.** Mean percentage of re-isolations of replant pathogens from Troyer citrange seedlings treated with biocontrol agents, 3 weeks before inoculation of *Neocosmospora solani*, *Phytophthora nicotianae* and *Pythium irregulare* and grown for 13 weeks in perlite in the greenhouse.

Treatment	Mean % <i>N. solani</i>	Mean % <i>P. nicotianae</i>	Mean % <i>P. irregulare</i>
Control	0.0 b*	0.0 b	0.0 b
Pathogen	53.0 a	59.3 a	70.2 a
LSD	7.10	5.76	4.29

\*Means sharing similar letters are not significantly different at a 95% confidence level.

## Discussion

Le Roux *et al.* (1998) and Swart (2018) reported that the primary pathogens associated with citrus replant disease in South Africa were *Phytophthora citrophthora*, *Phytophthora nicotianae*, *Neocosmospora solani* and *Pythium* species. This complex disease phenomenon is an emerging threat to the citrus industry in many parts of the world. Accurate and timely identification of citrus replant pathogens present in a specific situation is therefore necessary to prevent huge losses and limit the spread of disease through the application of suitable management strategies. The correct identification largely relies on the availability of sensitive and specific detection techniques.

Previous studies have used several primer sets for the detection of *P. citrophthora* and *P. nicotianae* in citrus roots and soils; however, the primers designed by Ippolito *et al.* (2002) were not suitable for real-time PCR. At present, there are no species-specific qPCR primers available for the newly described *Neocosmospora* spp. as identified by Guarnaccia *et al.* (2021). Although several real-time PCR-based methods are available, some of them showed sensitivity and specificity issues (Ippolito *et al.*, 2004). Therefore, new species-specific qPCR primers based on *Ypt1* (*P. citrophthora* and *P. nicotianae*), ITS (*N. citricola*),  $\beta$ -tubulin (*N. solani*) and *RPB2* (*N. ferruginea*) DNA sequences were developed and validated for the detection of citrus replant pathogens in roots. For *Pythium irregulare*, a previously published (Spies *et al.*, 2011) primer pair was used to develop a species-specific qPCR assay for detecting this species in the field. The specific primer pair used was originally designed to detect this species in South African grapevine nurseries where it is regarded as an important pathogen, impacting on nursery plant quality. However, in the current study, the real-time PCR parameters used previously were optimized and validated to improve sensitivity and ensure high specificity of the assay for use in the detection of this species in citrus roots.

Primer specificity is crucial for PCR-based diagnosis (Bi *et al.*, 2019). It usually is dependent on the length of the primer and the annealing temperature of the reaction (Dieffenbach *et al.*, 1993). Primers with low specificity tend to produce non-specific PCR products, which can result in lower yields of the desired product (Dieffenbach *et al.*, 1993). The primers proposed in the present study were tested for specificity and sensitivity. The results showed that all the primer sets were species-specific. The primers developed for *Phytophthora* spp. amplified only a single PCR band from *P. citrophthora* and *P. nicotianae* isolates, respectively. Primers for *Neocosmospora* spp. similarly amplified only a single PCR band from *N. solani*, *N. ferruginea* and *N. citricola* isolates, respectively. Moreover, the isolates were collected from a range of geographical locations and different host plants showing that they can amplify DNA across populations.

In order to quantify the sensitivity of the newly developed species specific primer, the limit of detection (LOD) in terms of DNA concentration was used. The LODs obtained in the different sample matrices showed correlation between them. In singleplex PCR, the LOD of the *N. citricola* assay were similar to those achieved by De la Lastra *et al.* (2018) ( $10^{-5}$  ng/ $\mu$ L) in strawberry plants. The LOD found by Li and Hartman (2003) in soybean roots were much lower than all three *Neocosmospora* assays, which demonstrates the high sensitivity of the optimised *Neocosmospora* qPCR assays. However, the LOD of the *N. citricola* assay were slightly lower than those achieved by Costa *et al.* (2017) ( $10^{-6}$  ng/ $\mu$ L) in black pepper roots.

For the development of a suitable multiplex PCR assay, annealing temperatures and primer concentrations have to be optimized (Markoulatos *et al.*, 2002). In this study, a SYBR Green-based method was used for the optimization of the protocol as it is generally less expensive than using TaqMan probes (Bustin *et al.*, 2010). Furthermore, by using SYBR Green, amplification products can be differentiated by their different  $T_m$  by analysing the melting curve (Wittwer *et al.*, 2001). The presence of a single melting peak at the corresponding  $T_m$  indicates the specificity of the reaction. This also reduces the likelihood of detecting false positives (Matthews *et al.*, 2020). The multiplex PCR assay developed for *P. citrophthora* and *P. nicotianae* in this study yielded two peaks with different  $T_m$ -values which confirmed the specificity of the primers. Although the multiplex assay was developed to only provide qualitative results (i.e. quick detection of the presence or absence of the target species), it provides a faster and cheaper alternative to the conventional methods.

Sensitivity obtained for the previously published multiplex PCR assay was 10 pg  $\mu\text{L}^{-1}$  for *P. nicotianae* and 100 pg  $\mu\text{L}^{-1}$  for *P. citrophthora* (Ippolito *et al.*, 2004). In this study, the detection level was 10 pg for *P. nicotianae* and 0.1 pg for *P. citrophthora*. The difference in reported detection limits could be due to primer design, variation between PCR instruments, different DNA extraction methods, or PCR reagents.

The *in planta* evaluation of the newly designed primers aimed at the detection of the replant pathogens in inoculated citrus seedlings, yielded good results. It furthermore also provided some insight into the interaction of the different pathogens in the seedlings.

In terms of plant growth parameters, none of the pathogen inoculations led to a significant reduction compared to the untreated control. This could possibly be attributed to the trial not continuing long enough for the inoculation to have a more pronounced effect on plant growth. The only exception to this was where inoculations of *N. solani*, *P. nicotianae* and *P. irregulare* were done together. This inoculation led to a significant reduction in seedling height, seedling mass and root mass compared to the untreated control. However, this reduction was not in all cases significantly more than the reduction caused by the other pathogens. Dandurand and Menge (1992) reported that root rot of citrus was more severe when *P. nicotianae* and *P. citrophthora* were co-inoculated with *N. solani*. However, in the present study this was not observed but only when *P. irregulare* was added to the inoculation.

Re-isolation data from the seedlings inoculated with the different pathogens alone or in combinations indicated all the *Neocosmospora* spp. was re-isolated at statistically same percentages. This included *N. solani* that was isolated at between 62% and 68%, whether it was inoculated alone or in combination with the different oomycete pathogens. Interestingly, all the oomycete pathogens were re-isolated at significantly higher percentages when inoculated in combination with *N. solani*, compared to when they were inoculated alone. It would therefore appear that the *N. solani* promoted the colonization of the citrus roots by oomycetes when co-inoculated with the oomycetes. This observation was not for all pathogens supported by the qPCR pathogen quantification from the seedling roots. In the case of the *Neocosmospora* spp. it showed the same trend. These results showed that the DNA concentrations of the *Neocosmospora* spp., as determined by qPCR, were again statistically similar in most cases, irrespective if they were inoculated alone or in combination with oomycetes. However, in contrast with the re-isolation results, the DNA levels of the three oomycete determined by qPCR, were statistically the same, regardless if they were inoculated alone or in combination with *N. solani*.

Several bacterial and fungal genera having multiple plant beneficial qualities were found to be associated with citrus roots and rhizosphere soil (Trivedi *et al.*, 2011, Ramezani *et al.*, 2015). *Trichoderma*, *Bacillus* and *Pseudomonas* spp. were found to be important biocontrol agents with potential antifungal activity against several pathogens (Chet and Baker, 1980; Bell *et al.*, 1982; Papavizas, 1985, Elad, 2000, El-katatny *et al.*, 2001, Howel, 2003, Gade and Lad, 2018). However, these studies did not focus on the effect of the different BCAs on the complex of replant pathogens.

In 2012, Ommati and Zaker screened eight native *Trichoderma* isolates against *Neocosmospora solani* using a non-volatile test and found an inhibition of 27.25% to 61.65% compared to the control. This inhibition range was very similar to our study (19%-62%), which may indicate that *Trichoderma* isolates could slow the growth of the *Neocosmospora* spp. The *Trichoderma* isolates were also found to have a great efficacy against the oomycetes, *P. irregulare*, *P. citrophthora* and *P. nicotianae* when evaluated in the non-volatile test and a maximum mycelial inhibition of 100% was obtained. Studies by Muthukumar *et al.* (2011) and Nath *et al.* (2014), reported maximum growth inhibition of 88% and 78% for *Pythium* and *Phytophthora* spp. respectively, where it was shown that *Trichoderma* spp. produced non-volatile compounds able to inhibit the pathogens. The efficacy of the *Trichoderma* isolates against the oomycetes pathogens in our study was higher compared to the efficacy found by these studies (Muthukumar *et al.*, 2011 and Nath *et al.*, 2014) and may be due to the difference in the strain or specie of the *Trichoderma* or pathogen used. From these results, it is clear that the *Trichoderma* isolates had good antagonistic efficacy against the replant pathogens and that they may be important producers of diffusible antifungal compounds that can inhibit the growth of the complex of pathogens.

Our study found that *B. subtilis* N19 and *P. fluorescens* N83 in the volatile test were the least effective at inhibiting the replant pathogens. This was in contrast to what was found in a study by Guevara-Avenidaño *et al.* (2019) where they found a higher mycelial growth inhibition against the pathogens. The antimicrobial metabolites produced by *B. subtilis* from the non-volatile test were effective against *P. irregulare* (30%-100%), *P. citrophthora* (3%-76%) and *P. nicotianae* (89%-92%). This range was similar to the inhibition observed by *P. irregulare* (30%-100%), when treated with *P. fluorescens*. For *P. citrophthora* (78%-100%) and *P. nicotianae* (93%-100%), a greater inhibition was achieved. A lower inhibition of mycelial growth was observed against the *Neocosmospora* spp. when they were evaluated against *B. subtilis* (11%-45%) and *P. fluorescens* (18%-38%). The results of the non-volatile metabolite activity in the reduction of the mycelial growth of *F. graminearum* with *B. subtilis* and *P. fluorescens* strains ranged from 51.5%-97% and 97% respectively (Nourozian *et al.*, 2006), which were relatively higher than the inhibition against the *Neocosmospora* spp. seen in the current study.

This might be due to the difference in the pathogens used as well as the differences in the BCA strains. Furthermore, the method of application of the bacteria on the cellophane differed, where Nourozian *et al.* (2006) suspended a 48-hour-old culture of bacterial isolate in sterile distilled water and smeared it with a sterile hockey stick over the entire surface of the cellophane, while the current study streaked the bacteria over the surface of the cellophane. Since the plating method of these bacteria differs on the cellophane, it may be a factor that could have influenced the results obtained.

Overall, the results of the non-volatile assays were encouraging, since they demonstrated that *Trichoderma*, *Bacillus* and *Pseudomonas* spp. have different inhibitory ability towards replant pathogens and their interaction may also be varied. However, pathogens might show different tolerance levels to the BCAs, as observed in this study. Harman (2000) and Vinale *et al.* (2006) found that although antibiotics may be the major factor for the biocontrol activity for a given strain, it may not be the case for others. Consequently, the selection of a biocontrol agent as well understanding their mode of action is important in designing effective biocontrol strategies. Antagonistic interactions showed excellent activity between the BCAs and the six replant pathogens *in vitro*. However, these biocontrol agents may perform outstanding under *in vitro* conditions but may not perform well *in vivo* as environmental conditions and competition with other microorganisms are much more restrictive. Based on abovementioned results, *B. subtilis*, *P. fluorescence*, *Trichoderma* isolates, P4 and P16 seemed to be good candidates and were therefore evaluated *in vivo* in a glasshouse trial.

It was expected that the treatment of citrus seedlings with the different BCA's, will have a growth promoting effect. However, this was not seen in the current study. In most cases the growth parameters of the treated seedlings were the same or even less compared to the untreated control seedlings. This could again be due to the trial period not being long enough, allowing the growth promoting effect of the BCA's to take hold. This was furthermore surprising as the re-isolation data indicated that the bacterial as well as *T. harzianum* isolates were re-isolated at high mean percentages from the roots of the treated seedlings. Colonization of the roots by the BCA's was therefore quite good. Unfortunately, despite the good root colonization by the BCA's, they were not effective in significantly reducing the pathogen colonization of the roots. This is furthermore evident from the fact that the three pathogens, *N. solani*, *P. nicotianae* and *P. irregulare* were re-isolated at mean percentages above 50%. The lack of effect by the BCA's could be the result of them not colonizing the roots to such an extent that they could effectively compete once they were challenged by the pathogens. Further optimization is therefore still needed.

Despite the BCA isolates identified in this study not having the anticipated effect on root infections by replant pathogens, the current study still produced some good results. This will form a solid basis for further studies into the optimisation of using BCA's for the management of citrus replant pathogens.

## Conclusion

This study showed that BCA's can successfully be used for the management of especially oomycete citrus pathogens. However, it was seen that biocontrol of *Neocosmospora* spp. will be more challenging. The sensitive, species specific primers developed in the study for the different replant pathogens will be valuable tools in the future study of this pathogen complex. It will furthermore play an important role in the species identification of oomycete pathogens found in citrus nurseries during routine nursery testing, reducing the turnaround time associated with the identification of the different species.

### Future research

In a follow-up study it is proposed that the following objectives are addressed.

1. GFP transformation of the BCA's found in this study.
2. Study seedling colonization by BCA's and pathogens using the transformed BCA's.
3. Optimise the application of BCA's for pathogen control using the transformed BCA strains.

### Technology transfer

Results from this project will lead to at least two peer reviewed papers. The results will furthermore be presented to industry through various platforms. Results will also be presented at scientific congresses and symposia.

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#### 4.3.3 **PROGRESS REPORT: Evaluation of alternative products for control of citrus nematode and *Phytophthora* spp. in citrus**

Project 1030 (2008 – 2021/2022) by Jan van Niekerk, Siyethemba Masikane (CRI) and Elodie Stempien (USPP)

##### **Summary**

Results obtained from the various evaluations done in 2020/2021 indicated that in a citrus replant situation, preplant fumigation with a mixture of chloropicrin and 1.3 dichloropropene is beneficial to tree growth while also suppressing citrus nematode and *Phytophthora nicotianae* levels in the soil. *In vitro* and *in planta* evaluations of *Trichoderma* and bacteria-based biocontrol agents for the control of different citrus replant pathogens, furthermore indicated that the effect on the pathogens is varied between different species and strains of the biocontrol agents. Effective control will therefore in all likelihood be achieved by using the different products in combination.

##### **Opsomming**

Resultate verkry uit die verskillende evaluasies wat in 2020/2021 gedoen is het aangedui dat in 'n sitrus herplant situasie, beroking voor plant met 'n mengsel van chloorpikrien en 1.3 dichloropropene, voordelig is vir boom groei. Dit onderdruk ook die vlakke van citrus nematode en *P. nicotianae* in die grond. *In vitro* en *in planta* evaluasies van biologiese produkte wat *Trichoderma* of bakterieë bevat het aangedui dat die verskillende spesies en rasse van die agente grootliks verskil in hulle effek op die verskillende herplant patogene. Effektiewe beheer sal dus in alle waarskynlikheid behaal word deur die verskillende produkte in kombinasie te gebruik.

##### **Introduction**

Nematodes are a diverse group of invertebrates, abundant as parasites or free living forms in soil, freshwater and marine environments. Soils are a particularly rich environment for nematodes, with about 26% of described genera inhabiting soil as bacterivores, fungivores, omnivores, predators or plant parasites (McSorley, 2005).

The citrus nematode, *Tylenchulus semipenetrans* Cobb, infects citrus worldwide (Van Gundy and Meagher, 1977; Heald and O'Bannon, 1987) and is the most abundant and frequent plant-parasitic nematode in citrus groves. Yield losses are estimated at about 10% worldwide. The citrus nematode is associated with poor growth of young citrus trees planted in infested groves and with poor performance of mature citrus trees. The host range of *T. semipenetrans* includes all *Citrus* species and most hybrids of citrus with other members of the rutaceous family such as trifoliolate orange (*Poncirus trifoliolate* L.Raf). Non-rutaceous plants such as grape (*Vitis vinifera*, L), olive (*Olea europea*, L) and persimmon (*Diospyrus spp.*) are also hosts (Verdejo-Lucas, 2002).

Damage thresholds, nematode population densities that suppress tree growth and yield, are influenced by several factors including aggressiveness of the nematode population, soil type, rootstock, other diseases and grove management practices (Garabedian *et al.* 1984). Threshold values in South Africa have been set at 10 000 juveniles/250 cc soil and a 1000 females/10 g roots in samples.

*T. semipenetrans* migrates very slowly on its own power and therefore does not readily spread from tree to tree in existing orchards. Infestation of new orchards occurs mainly through infested planting material and contaminated irrigation water (Tarjan, 1971; Baines, 1974). It is recorded that the sheath nematode, *Hemicycliophora* spp. occurs in combination with the citrus nematode in certain citrus producing countries in the world (Van Gundy, 1959) but the effect of the nematode on yields is not known. The sheath nematode was also detected in certain citrus producing regions in South Africa (L. Huisman, personal communication, CRI Diagnostic Centre, Nelspruit, 2007).

In the past, the nematicide 1,2 dibromo-3-chloropropane (DBCP) was widely used in the irrigation water against this nematode with great success. The nematode was effectively controlled while yields were also substantially

increased (O'Bannon *et al.*, 1963; Philis, 1969). The activity on eggs is the most important difference between the soil fumigants used to control nematodes earlier this century, and today's non-fumigant chemicals. Following the withdrawal of DBCP, non-fumigant post-plant nematicides (carbamate or organophosphate acetylcholinesterase inhibitors) were introduced. These chemicals, however, could not eliminate, or greatly reduce, nematode populations even if applied every year. Fenamiphos is translocated systemically in the vascular system of plants, whilst the other nematicides are non-systemic and reduce nematode populations through their initial contact action only. This explains the quick recovery of nematode populations once the nematicide has been degraded in soil and emphasizes the adverse effect of enhanced degradation, as eggs hatching after the nematicide has been degraded can continue the nematode's life-cycle. In 2002, the following nematicides were registered on citrus in South Africa: aldicarb, cadusafos, fenamiphos, terbufos, ethoprophos, fosthiazate and furalfural (Nel *et al.*, 2002). When multiple nematicide applications were introduced on a commercial scale to citrus orchards in South Africa, situations occurred where growers were not successful in disrupting the nematode's life cycle despite adhering strictly to prescribed procedures. In an investigation to determine the efficacy of cadusafos in soil where aldicarb and fenamiphos failed as a result of accelerated degradation, it was found that in the absence of sufficient irrigation water none of the nematicides were distributed thoroughly through the soil profile and they consequently failed to eliminate the citrus nematode (Le Roux *et al.*, 1998).

Due to safety, environmental concerns and market pressure, only a few registered chemical nematicides remain worldwide for utilization by farmers, and the use of these products is highly restricted. Developing alternatives to chemical nematicides is essential and a great concern to researchers worldwide. Recent attempts to develop alternative methods to manage plant-parasitic nematodes include the use of entomopathogenic nematodes and various biologically derived nematicides and other organic compounds. The aim of this experiment is to: evaluate less toxic compounds for the control of the citrus nematode as well as for the control of *Phytophthora* spp. in citrus orchards.

## **Objective**

The development and evaluation of new products or existing products for the control of soilborne pests and diseases in citrus orchards and nurseries.

## **Materials and methods**

### *Evaluation of pre-plant soil fumigation of a replant soil on *Phytophthora* spp. and citrus nematode levels in soil and growth of young citrus trees*

In September 2017 an old Midnight Valencia on Carrizo citrange rootstock orchard was removed in the Kirkwood area, Eastern Cape. The aim was to replant immediately with Tango on Carrizo citrange trees. Prior to tree removal soil and root samples were taken at 40 sites in the old orchard. These were analyzed at the CRI Diagnostic Centre (DC) in Nelspruit. The analyses indicated that on the number of citrus nematode juveniles in the soil were on average 2068 per 250 cc soil. In the root samples on average 1532 female nematodes were present. Both *Phytophthora citrophthora* and *P. nicotianae* were also shown to be present in the orchard soil.

Soil preparation was done and rows pegged out. Certain rows were fumigated with a 60:40 chloropicrin: 1.3 dichloropropene mixture. The fumigation dosage was 60 g/m<sup>2</sup>. For evaluation purposes, in 2019, 10 trees were marked in the fumigated rows and 10 in the non-fumigated rows. In 2019, at these trees soil samples were taken for nematode and *Phytophthora* analyses at the CRI Diagnostic Centre (DC) in Nelspruit. Tree height and stem diameter were also measured. This evaluation was repeated in March 2021. The recorded data were subjected to statistical analyses.

### *In vitro evaluation of commercially available bacterial and *Trichoderma* spp. based biological control agents*

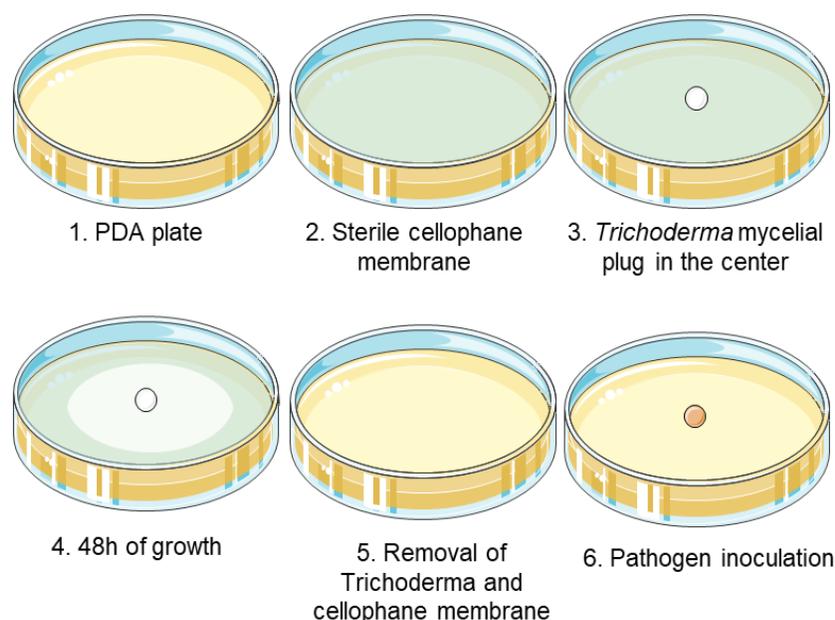
The screening of the *Trichoderma* and bacterial based products for their ability to inhibit citrus replant pathogens was done using a non-volatile test. Details of the products are listed in Table 4.3.3.1.

*Trichoderma* products and three isolates of each of the replant pathogens were grown on PDA in the dark at 28°C for three days. Mycelial plugs (6 mm) colonized by the *Trichoderma* products were placed face down on PDA plates covered with autoclaved 50 µm thick cellophane membranes (Sigma, Germany) (84 mm in diameter). The cellophane PDA plates were then incubated in the dark at 28°C. After 48 hours, the cellophane membranes with *Trichoderma* growth were removed ensuring that the PDA plates were completely free of *Trichoderma*. Mycelial plugs (6 mm diameter) of the citrus replant pathogens were inoculated in the centre of the PDA plates and incubated in the dark at 28°C for 2, 6, and 10 days for *Pythium irregulare*, *Neocosmospora* spp. and *Phytophthora citrophthora* and *Phytophthora nicotianae* respectively (Figure 4.3.3.1). For the control plates, *Trichoderma* were not inoculated on the cellophane membranes and three technical repetitions were done for each pathogen isolate. At the end of the incubation period, the mycelial diameters of the pathogens from each PDA plate were measured three times and the percentage inhibition was calculated according to  $[(A-B)/A \times 100]$  where A is the mycelial diameter of the control plates and B is the mean of three mycelial diameters of pathogens.

The bacterial products were, prior to plating onto the cellophane, streaked out onto NA and incubated at 28°C in the dark for 48 hrs. The resulting cultures were then used to streak the bacterial products out onto the cellophane membranes on the PDA plates. These plates were then also incubated for 48 hours prior to the removal of the membranes and plating of the replant pathogens. The rest of the evaluation was done as above.

**Table 4.3.3.1.** Details of commercial *Trichoderma* spp. or bacterial based biological control agents evaluated in a non-volatile test against replant pathogens.

Product	Biological control agent species	Company
USPP-T1	<i>Trichoderma atroviride</i>	Department of Plant Pathology, University of Stellenbosch
TrichoPlus	<i>Trichoderma asperelloides</i>	BASF
TriCure-SP	<i>Trichoderma harzianum</i>	MBFI
Eco-T	<i>Trichoderma asperellum</i>	Plant Health Products
Eco-77	<i>Trichoderma atroviride</i>	Plant Health Products
Rizofos Fruit & Veg	<i>Pseudomonas fluorescens</i>	MBFI
B-Rus	<i>Bacillus subtilis</i>	Stimuplant



**Figure 4.3.3.1.** Illustration of the non-volatile test of commercial *Trichoderma* and bacterial biological control products against citrus replant pathogens.

*Glasshouse evaluation of commercially available Trichoderma formulations for the control of Phytophthora root rot*

Swingle citrumelo seedlings were grown in the glasshouse in a mixture of 50:50 composted pine bark and steam sterilized topsoil. Seedlings were grown until they were on average 40 cm tall. Watering was done as needed and insect management and sprays were also done based on weekly scouting.

The *Trichoderma* based products were selected based on them having a registration to control *Phytophthora* spp. on tree and other crops. Details of the products are presented in Table 4.3.3.2.

**Table 4.3.3.2.** Details of commercial *Trichoderma* spp. biological control agents evaluated in a glasshouse trial for the control of *Phytophthora* root rot of Swingle citrumelo seedlings.

Product	Biological control agent species	Company
USPP-T1	<i>Trichoderma atroviride</i>	Department of Plant Pathology, University of Stellenbosch
TrichoPlus	<i>Trichoderma asperelloides</i>	BASF
TriCure-SP	<i>Trichoderma harzianum</i>	MBFI
Eco-T	<i>Trichoderma asperellum</i>	Plant Health Products
Eco-77	<i>Trichoderma atroviride</i>	Plant Health Products
Rootguard	<i>Trichoderma harzianum</i>	Biocult

These products were all applied as a monthly drench application at 25g of product per 100 L water. A total of 4 applications were done prior to inoculation with a *Phytophthora nicotianae* mycelium/zoospore suspension.

Inoculations were done weekly for a period of two months. This was to ensure proper colonization of the growing medium by the pathogen. Included were two control treatments; an untreated, un-inoculated treatment and an inoculated only treatment. Eight weeks after the last inoculation, the trial were evaluated destructively. Plants were measured for total wet plant mass and total wet root mass. The level of *P. nicotianae* infection in the roots of treated seedlings was determined by isolation onto PARPH media. Prior to isolation, root systems were washed under running tap water before surface sterilization by dipping for 30 s in 70% ethanol. After sterilization the root systems were air-dried in a laminar flow. All data obtained was subjected to statistical evaluation. Each treatment was replicated on 8 seedlings, split into 2 blocks with 4 replicate plants per block.

*Evaluation of a Paecilomyces lilacinus based biological control product for the control of the citrus nematode, Tylenchulus semipenetrans*

The ultimate aim is to evaluate this product at two trial sites. One will be located outside Nelspruit, Mpumalanga and one is located in Citrusdal, Western Cape. The trial in the Western Cape commenced in 2020/2021, while the Nelspruit trial will commence in October 2021. Trial site selection is based on pre-determined female nematode counts present in the orchards. Sites should have a count in excess of 5 000 per 10 g of roots. At the Citrusdal site, which is a 43-year old Navel orange orchard with Rough lemon rootstock, the average female nematode count of the trial trees was 6 795 per 10g roots. At each trial site, each treatment program is replicated on 8 trees that are split into 2 tree plots in each of 4 blocks. The different treatment programs for the two trial sites are given in Tables 4.3.3.3 and 4.3.3.4. These programs are based on the recommended application intervals of the product and a commercial cadusafos or phenamiphos product. Applications were done as a drench around the tree trunk, using a 10 L watering can. After each application, the products were washed into the soil profile with at least 35mm irrigation. At the Citrusdal trial, soil and root samples were collected in November 2020 and February and April 2021 and analysed for the presence of juvenile citrus nematodes in the soil and female nematodes in the roots. Results from the first two samplings are presented here. At the Nelspruit site, root and soil samples will be collected in December 2021 and again in February, March and May 2021.

*Evaluation of A22011B for the control of the citrus nematode, Tylenchulus semipenetrans*

The product A22011B was provided by Syngenta with the purpose of CRI testing it on citrus for the control of citrus nematode. A trial was therefore initiated in October 2020 in the same orchard where the abovementioned PL trial was established. In this case the requirement was for the trial trees to have an infestation level of at least 4000 nematode females per 10 g roots. In this case, the trial trees had an average count of 5 878 nematode females per 10 g roots. The trial treatments, as provided by Syngenta, are presented in Table 4.3.3.5 below. Applications were done as a drench around the tree trunk, using a 10 L watering can. After each application, the products were washed into the soil profile with at least 35 mm irrigation. Soil and root samples for nematode analyses were collected 30 days after application 1 (30 DAA1; November 2020), 60 DAA1 (December 2020), 30 DAA2 (January 2021) and 60 DAA2 (February 2021).

**Table 4.3.3.3.** Treatment programs for the evaluation of a commercial *Paecilomyces lilacinus* (PL) product in the Nelspruit area of South Africa.

Treatment programme	Treatment	Oct 2021	Nov 2021	Dec 2021	Jan 2022	Feb 2022	Mar 2022
1	Untreated	None	None	None	None	None	None
2	PL @ 125 g/ha	PL	PL	PL	PL	PL	PL
3	Cadusafos	Cadusafos		Cadusafos		Cadusafos	
4	PL + cadusafos 1	Cadusafos	PL	PL	PL	PL	PL
5	PL + cadusafos 2	Cadusafos	PL	Cadusafos	PL	Cadusafos	PL

**Table 4.3.3.4.** Treatment programs for the evaluation of a commercial *Paecilomyces lilacinus* (PL) product in the Citrusdal area of South Africa.

Treatment programme	Treatment	Sept 2020	Oct 2020	Nov 2020	Dec 2020	Jan 2020	Feb 2020
1	Untreated	None	None	None	None	None	None
2	PL @ 125 g/ha	PL	PL	PL	PL	PL	PL
3	Phenamiphos	Phenamiphos		Phenamiphos		Phenamiphos	
4	PL + Phenamiphos 1	Phenamiphos	PL	PL	PL	PL	PL
5	PL + Phenamiphos 2	Phenamiphos	PL	Phenamiphos	PL	Phenamiphos	PL

**Table 4.3.3.5.** Treatments for the evaluation of the product A22011B for the control of the citrus nematode in the Citrusdal area.

	Treatment	Product / ha Per m <sup>2</sup>	Product (ml) per tree	Timing
1	Untreated control	-	-	-
2	A22011B	444.4 ml	0.67 ml per tree	Drench in October
3	A22011B	555.5 ml	0.83 ml per tree	Drench in October
4	A22011B	1111.0 ml	1.67 ml per tree	Drench in October
5	A22011B	444.4 ml	0.67 ml per tree and 60 DAA1 0.67 ml per tree	Drench in October and 60 days after application 1 (December 2020; 60 DAA1)
6	A22011B	555.5 ml	0.83 ml per tree and 60 DAA1 0.83 ml per tree	Drench in October and 60 DAA1
7	Fluopyram	0.4125 ml/m <sup>2</sup>		Drench in October
8	Phenamiphos	10 ml / m <sup>2</sup> basin area		Drench in October

## Results and discussion

Objective / Milestone	Achievement
Apr –Jun 2020 <ol style="list-style-type: none"> <li>Annual report</li> <li>Continue with glasshouse evaluation of <i>Trichoderma</i> based products.</li> <li>Nematode trial planning</li> </ol>	<ol style="list-style-type: none"> <li>Annual report was written and submitted.</li> <li>Trial was continued as per the trial protocol.</li> <li>Nematode trials were planned and products obtained.</li> </ol>
Jul – Sept 2020 <ol style="list-style-type: none"> <li><i>In vitro</i> evaluation of bacterial and <i>Trichoderma</i> based biocontrol agents</li> <li>Continue with glasshouse evaluation of <i>Trichoderma</i> based products</li> <li>Commence with PL nematode trial in Citrusdal, Western Cape.</li> </ol>	<ol style="list-style-type: none"> <li><i>In vitro</i> evaluation was completed and results captured.</li> <li>Trial was continued as per the trial protocol.</li> <li>PL trial was started successfully.</li> </ol>
Oct – Dec 2020 <ol style="list-style-type: none"> <li>A22011B nematode trial was started in Citrusdal</li> <li>Evaluation of <i>Trichoderma</i> product glasshouse trial</li> </ol>	<ol style="list-style-type: none"> <li>A22011B trial was started successfully</li> <li>Trial was evaluated destructively and data was captured.</li> </ol>
Jan – Mar 2021 <ol style="list-style-type: none"> <li>Evaluation of fumigation trial in Kirkwood</li> <li>Data analysis</li> </ol>	<ol style="list-style-type: none"> <li>Trial was evaluated by measuring tree height and trunk diameter and doing nematode analysis from soil samples</li> <li>Data analyses were done.</li> </ol>

### *Evaluation of pre-plant soil fumigation of a replant soil on Phytophthora spp. and citrus nematode levels in soil and growth of young citrus trees*

In 2019 the mean stem diameter of fumigated trees was significantly more at 26.4 mm versus the mean for the unfumigated trees of 22.6 mm. In 2021 this trend continued with the mean stem diameter of fumigated trees being significantly thicker at 53.9 mm compared to 49.3 mm (Table 4.3.3.6). Mean tree height of fumigated trees was in 2019 120.7 cm, significantly more than the 110.7 cm of the control trees. In 2021, the mean tree height for the fumigated trees was 234.3 cm in comparison to the 226.7 cm mean for the unfumigated trees (Table 4.3.3.6).

In terms of juvenile nematode count, in 2019 the fumigated trees had no nematodes (mean of 0.0) which was significantly lower than the mean of 745.0 per 250 cc soil recorded for the unfumigated trees. In 2021 this trend was continued with the mean juvenile count of the unfumigated trees increasing to 3040 per 250 cc soil, significantly more than the 170 recorded for the fumigated trees. The same effect of fumigation was observed for *Phytophthora nicotianae* infested leaf discs. In 2019 the mean percentage infested discs was 17.1% for the untreated trees and significantly lower at 0.0% in the fumigated soil. In 2021 the mean percentage in the unfumigated soil increased to 79.4%, significantly more than the mean percentage of 25.4% of the fumigated trees (Table 4.3.3.6).

In this trial a 60:40 chloropicrin: 1.3 dichloropropene mixture was used to fumigate soil prior to planting. The results clearly indicate that 4 years after fumigation, the level of nematodes and also *P. nicotianae* is lower in the treated soil than in the untreated soil. This corresponds to the reported efficacy of chloropicrin and 1.3 dichloropropene having efficacy against oomycete pathogens and nematodes respectively (Duniway, 2002; Ruzo, 2006).

### *In vitro evaluation of commercially available bacterial and Trichoderma spp. based biological control agents*

Inhibition of mycelium growth of *P. nicotianae* and *P. citrophthora* was 100% for 4 out of the five products tested. The one product that performed significantly poorer was the *T. harzianum* based product of MBFI that achieved only 50% to 60% inhibition of the respective *Phytophthora* spp (Figures 4.3.3.2 and 4.3.3.3). However, in the case of *Pythium irregulare*, 100% inhibition of mycelium growth was obtained by all the products tested. However, inhibition of *Neocosmospora solani* was poor for all the products tested. The mean inhibition was below 40%. The best performing product was USPP-T1 followed by Eco-77. The inhibition achieved by these two products were between 30% and 40%. Inhibition achieved by the other 3 products was below 20% (Figure 4.3.3.5).

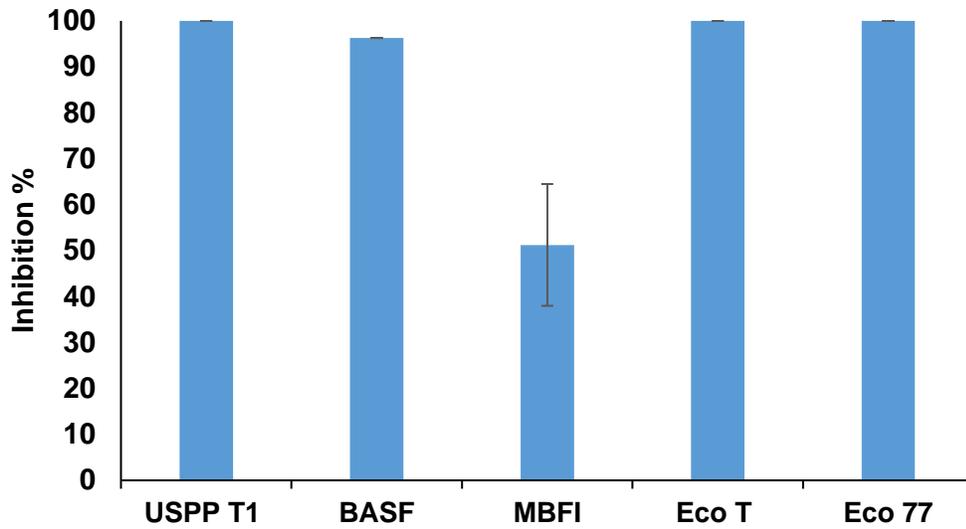
Mycelium growth inhibition achieved by the two tested bacterial biological control products varied greatly. The *Pseudomonas fluorescens* inhibited the mycelial growth by more than 60% while the *Bacillus subtilis* product did not achieve any inhibition (Figure 4.3.3.6). Inhibition of *P. citrophthora* was almost similar to *P. nicotianae*. The *P. fluorescens* product inhibited the mycelium growth to by almost 60%, with the *B. subtilis* product again resulted in no inhibition (Figure 4.3.3.7). *Pythium irregulare* mycelium growth was inhibited 100% by *P. fluorescens* while the *B. subtilis* product inhibited the mycelium by less than 30% (Figure 4.3.3.8). However, results obtained by the two bacterial products were good in the case of *N. solani*. The *B. subtilis* product inhibited this pathogen by more than 80% while the *P. fluorescens* product inhibited the growth significantly less at almost 70% (Figure 4.3.3.9).

Results from the *in vitro* screening clearly indicate that the effectivity of the products varies greatly between the products, the specific species they contain and the target pathogen. It is clear that, to control the complex of citrus soilborne pathogens, a single biocontrol agent will not be effective. It is more likely that the optimal effectivity will be obtained by using a combination of different biocontrol species or strains.

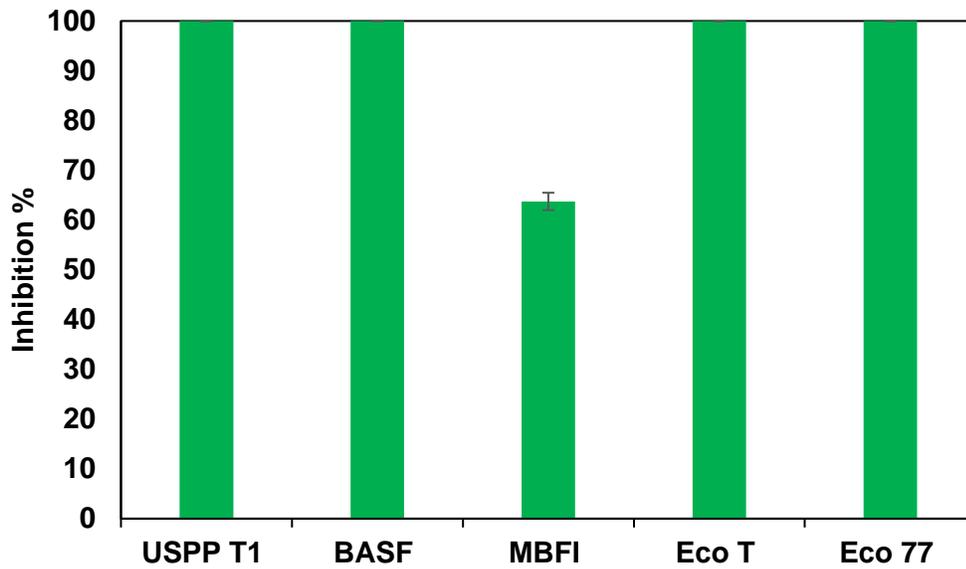
**Table 4.3.3.6.** Mean tree stem diameter (mm), tree height (cm), juvenile nematode counts and percentage *Phytophthora nicotianae* infested leaf discs recorded for fumigated and unfumigated trees in the Kirkwood area, Eastern Cape.

Treatment	Stem diameter (mm)		Tree height (cm)		Juvenile nematode counts		<i>Phytophthora nicotianae</i> (%)	
	2019	2021	2019	2021	2019	2021	2019	2021
Fumigated	26.4 a <sup>1</sup>	53.9 x	120.7 a	234.3 x	0.0 b	170.0 y	25.4 b	0.0 y
Unfumigated	22.6 b	49.3 y	110.7 b	226.7 y	745.0 a	3040.0 x	79.4 a	17.1 x
LSD	3.47	3.62	7.59	14.83	520.77	2673.0	43.88	21.43
<i>P</i> -value	0.076	0.040	0.035	0.387	0.023	0.079	0.021	0.186

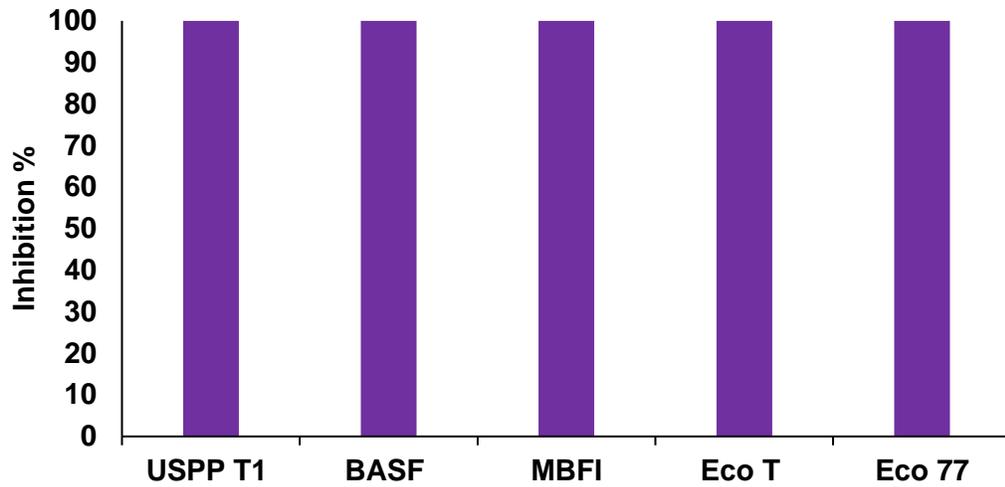
<sup>1</sup>Means followed by the same letter are not significantly different at a 90% confidence level.



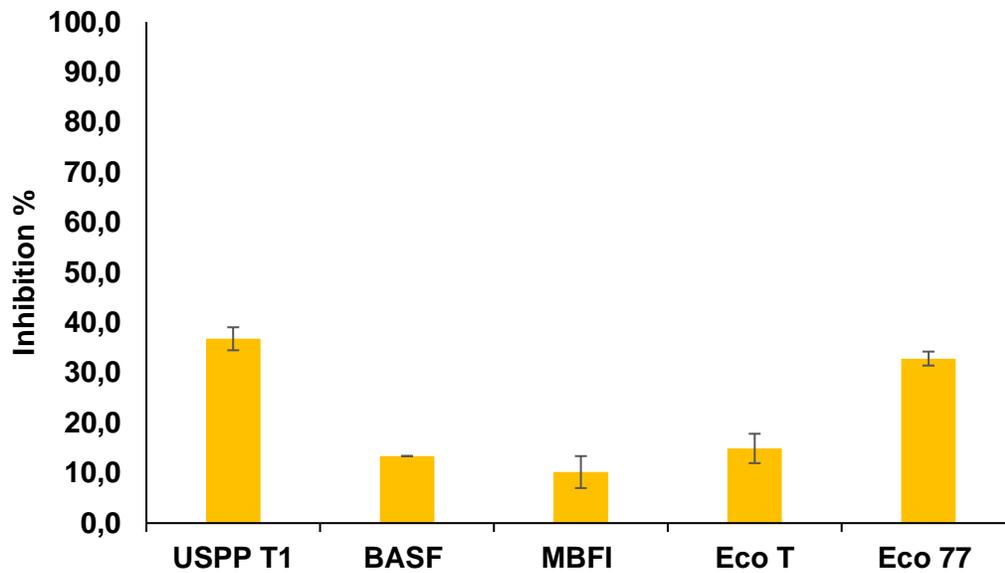
**Figure 4.3.3.2.** Percentage inhibition of *P. nicotianae* mycelium growth by non-volatile compounds secreted by five commercial *Trichoderma* spp. based biological control agents.



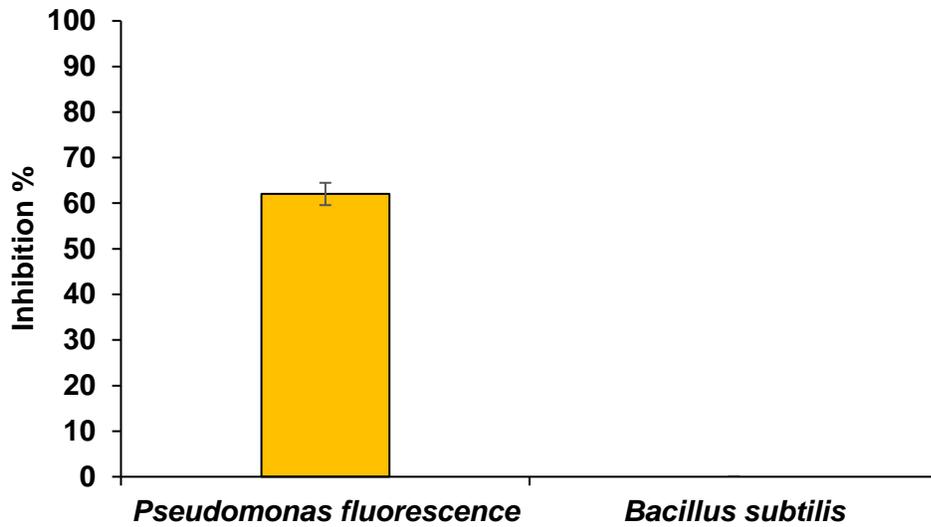
**Figure 4.3.3.3.** Percentage inhibition of *P. citrophthora* mycelium growth by non-volatile compounds secreted by five commercial *Trichoderma* spp. based biological control agents.



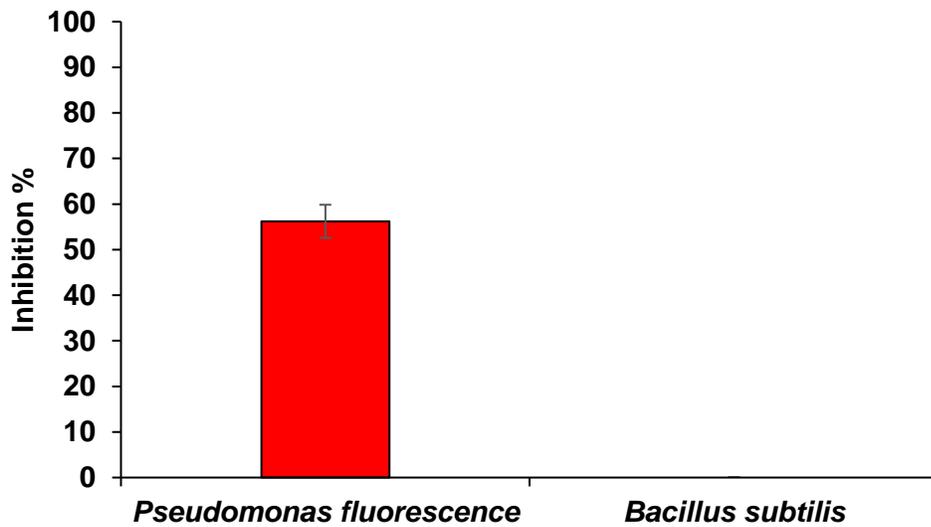
**Figure 4.3.3.4.** Percentage inhibition of *Pythium irregulare* mycelium growth by non-volatile compounds secreted by five commercial *Trichoderma* spp. based biological control agents.



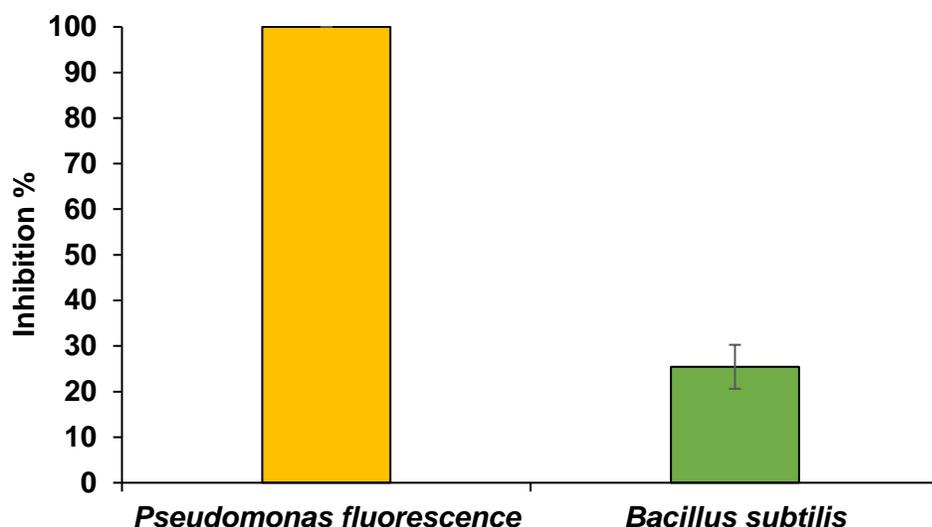
**Figure 4.3.3.5.** Percentage inhibition of *Neocosmospora solani* mycelium growth by non-volatile compounds secreted by five commercial *Trichoderma* spp. based biological control agents.



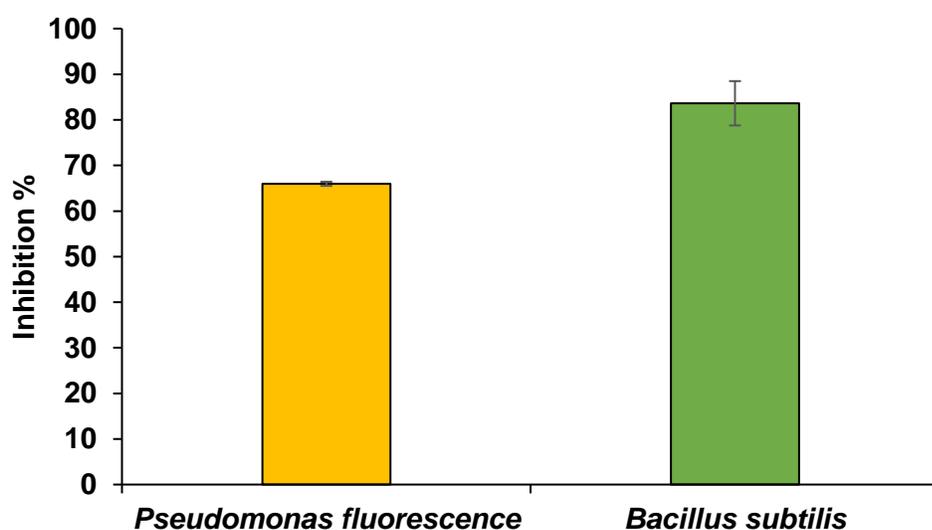
**Figure 4.3.3.6.** Percentage inhibition of *Phytophthora nicotianae* mycelium growth by non-volatile compounds secreted by two commercial bacterial biological control agents.



**Figure 4.3.3.7.** Percentage inhibition of *Phytophthora citrophthora* mycelium growth by non-volatile compounds secreted by two commercial bacterial biological control agents.



**Figure 4.3.3.8.** Percentage inhibition of *Pythium irregulare* mycelium growth by non-volatile compounds secreted by two commercial bacterial biological control agents.



**Figure 4.3.3.9.** Percentage inhibition of *Neocosmospora solani* mycelium growth by non-volatile compounds secreted by two commercial bacterial biological control agents.

*Glasshouse evaluation of commercially available Trichoderma formulations for the control of Phytophthora root rot*

Results from the trial evaluation indicated that the different *Trichoderma* based products did not have a significant effect on the mean root mass of treated seedlings. The means varies between 59.5g and 43.8g for the different treatments (Table 4.3.3.7). However, in terms of mean plant mass, a significant difference was observed between the different treatments. Interestingly, the highest means were recorded for the inoculated and negative control plants (48.8g and 43.4g respectively) and Eco-77 treated plants (43.2g; Table 4.3.3.7). The mean plant mass of the remaining treatments was below 40.0g. It would be expected that the mean of the inoculated control plants would be the lowest, while the means of the *Trichoderma* treated seedlings will be significantly higher than both controls. It is possible that the trial duration was not long enough to allow for the *Trichoderma* products to have

their full effect on plant growth parameters. Similarly, a longer duration could have led to the *P. nicotianae* inoculation taking a bigger effect, having a significant impact on seedling growth.

However, in terms of *P. nicotianae* infested root pieces, a marked effect of the different *Trichoderma* products was seen. The inoculated control plants had a mean of 87.8% infested root pieces. Compared to this level, the Eco-T treated plants had a mean of 40.9% followed by Tri-Cure SP (48.9%) and USPP-T1 (49.6%). The remaining treatments had means that were between 50.9% (Rootguard) and 67.5% (TrichoPlus) (Table 4.3.3.7). These results indicate that the different products, that are different in the *Trichoderma* species and strains they contain, vary greatly in their ability to reduce *P. nicotianae* infection of seedling roots.

**Table 4.3.3.7.** Mean root mass (g), plant mass (g) and percentage *Phytophthora nicotianae* infested root pieces of seedlings treated with different *Trichoderma* based biocontrol products and inoculated with *Phytophthora nicotianae*.

Treatments	Measurements		
	Mean root mass (g)	Mean plant mass (g)	Mean % <i>Pn</i> <sup>2</sup> infested root pieces
Eco-77	59.5 a <sup>1</sup>	43.2 ab	59.9 xy
Eco-T	49.6 ab	35.3 c	40.9 yz
Inoculated control	50.5 ab	48.8 a	87.8 x
Negative control	43.8 b	43.4 ab	0.0 z
Rootguard	51.3 ab	34.1 c	50.9 y
TrichoPlus	44.3 b	38.6 bc	67.5 xy
Tri-Cure SP	49.9 ab	39.6 bc	48.9 y
USPP-T1	47.8 ab	38.4 bc	49.6 y
LSD	13.95	6.94	46.89
<i>P</i> -value	0.703	0.015	0.054

<sup>1</sup>Means followed by the same letter are not significantly different at a 95% confidence level.

<sup>2</sup> *Pn* = *Phytophthora nicotianae*

*Evaluation of a Paecilomyces lilacinus based biological control product for the control of the citrus nematode, Tylenchulus semipenetrans*

The results obtained in November 2020 resulted from only two treatments from the different treatment programs applied to trees at the Citrusdal trial site (Table 4.3.3.4). Results from the February and April 2021 samplings will be presented in the 2021 progress report.

In terms of mean juvenile count, all treatment programmes reduced the counts by between 38.1% and 72.2%. In November 2020, the best treatment programme was programme 3 that led to a marked reduction in mean juvenile count. At that stage only one fenamiphos application had been applied. The second best treatment programme, also leading to a marked reduction in juvenile count, was program 5 that had received one fenamiphos and one PL application by November 2020. The results obtained by these two programmes by November 2020 were most likely due to the fenamiphos application done. Programmes 2 and 4 also reduced the mean counts in comparison to the untreated control but not significantly. They were furthermore statistically similar to programmes 3 and 5 in terms of mean juvenile count (Table 4.3.3.8).

In terms of mean female citrus nematode count, in November 2020, there were no significant differences between the different treatment programmes (Table 4.3.3.8). The only treatment that had a noticeable effect on the mean female count, was programme 4 that reduced the mean counts by 22.0% compared to the untreated control (Table 4.3.3.8).

**Table 4.3.3.8.** Mean citrus nematode juvenile and female counts obtained from soil and root samples collected from trees subjected to different treatment programmes containing a *Paecilomyces lilacinus* based product and fenamiphos at Citrusdal, Western Cape.

Treatment programme	Mean nematode juvenile count Nov 2020	Mean female nematode count Nov 2020
1. Untreated	4581.3 A <sup>2</sup>	4325.0 A
2. PL <sup>1</sup> @ 125 g/ha	2837.5 AB (-38.1) <sup>3</sup>	6262.5 A (44.8)
3. Fenamiphos	1275.0 B (-72.2)	4600.0 A (6.4)
4. PL + fenamiphos 1	1862.5 AB (-59.3)	3375.0 A (-22.0)
5. PL + fenamiphos 2	1631.3 B (-64.4)	4287.5 A (-0.90)
LSD	2777.0	4541.0
P-value	0.133	0.777

<sup>1</sup>PL = *Paecilomyces lilacinus*

<sup>2</sup>Means followed by the same letter are not significantly different at a 95% confidence level.

<sup>3</sup>Percentage reduction in count in comparison to the untreated control.

#### *Evaluation of A22011B for the control of the citrus nematode, Tylenchulus semipenetrans*

Results indicate that 30 days after application 1 (30 DAA1), no significant differences were evident in terms of mean juvenile or female counts (Table 4.3.3.9). Programmes 3 and 4 were the only two that reduced the mean female counts compared to the untreated control. The best was programme 4, A22011B applied at 1111 ml/ha, that reduced the count by 25.4%. Programme 3, (A22011B at 555.5 ml/ha) reduced the counts by 15.7%. At 30DAA1 all the treatment programmes reduced the mean juvenile counts compared to the untreated control. The best programme, with a 78.4% reduction, was programme 5. This was followed by programme 7 at a 76.4% reduction and then third best programme 6 with a reduction of 57.7% (Table 4.3.3.9).

At 60 DAA1, again none of the treatment programmes led to a significant reduction in mean juvenile and female citrus nematode counts (Table 4.3.3.9). Programme 8 was the best at this point with a 68.5% reduction in female count followed by programmes 4 (52.5% reduction) and 6 (51.1% reduction). At this stage a very low mean juvenile count was recorded in the untreated control. Consequently, only treatment programmes 2 (55.7%), 5 (33.6%) and 7 (49.6%) reduced the juveniles in comparison to the untreated control (Table 4.3.3.9).

At 60DAA1, a second application of A22011B was done in certain treatment programmes (Table 4.3.3.9). However, despite second applications in some programmes, at 30 days after application two (30 DAA2), the best two programmes in terms of mean female counts were programme 8 with a 65.4% reduction and programme 7 with a 49.4% reduction compared to the control. However, all treatments did reduce the mean female counts compared to the untreated control although these reductions were not significant. At 30 DAA2 all treatment programmes reduced the mean juvenile count by more than 50% with programme 7 reducing the mean count by 95%. Again, no significant effect was seen for any of the treatment programmes (Table 4.3.3.9).

The full picture regarding the efficacy of A22011B to control citrus nematode will only be seen once all the data is available from all the analyses. These will be presented in the progress report of 2021.

**Table 4.3.3.9.** Mean citrus nematode female and juvenile counts recorded from soil and root samples collected from trees at three different time points after one or more application of the experimental product A22011B, fluopyram or fenamiphos to trees at the Citrusdal trial site.

Treatment programme	Female 30 DAA1	Juvenile 30 DAA1	Female 60 DAA1	Juvenile 60 DAA1	Female 30 DAA2	Juvenile 30 DAA2
1. Untreated control	4557 ab <sup>1</sup>	3542 a	6442 a	935 a	5250 a	1492 a
2. A22011B; 444.4 ml/ha, spring drench application	5885 ab (29.2) <sup>2</sup>	2264 a (-36.1)	4628 a (-28.2)	414 a (-55.7)	4571 a (-12.9)	364 ab (-75.6)
3. A22011B; 555.5 ml/ha, spring drench application	3842 ab (-15.7)	3171 a (-10.5)	5342 a (-17.1)	1985 a (112.2)	4528 a (-13.7)	428 ab (-71.3)
4. A22011B; 1111 ml/ha, spring drench application	3400 ab (-25.4)	2714 a (-23.4)	3057 a (-52.5)	2150 a (129.8)	3371 a (-35.8)	407 ab (-72.7)
5. A22011B; 444.4 ml/ha, spring drench application + 60DAA1	7442 a (63.3)	764 a (-78.4)	5042 a (-21.7)	621 a (-33.6)	3333 a (-36.5)	571 ab (-61.7)
6. A22011B; 555.5 ml/ha, spring drench application + 60DAA1	5457 ab (19.7)	1500 a (-57.7)	3085 a (-52.1)	1535 a (64.1)	2942 a (-43.9)	507 ab(-66.0)
7. Fluopyram 0.4125 ml/m <sup>2</sup> ; spring drench application	3300 b (27.6)	835 a (-76.4)	4385 a (-31.9)	471 a (-49.6)	2657 a (-49.4)	71 b (-95.2)
8. Fenamiphos 10 ml/m <sup>2</sup> , spring application	5385 ab (18.2)	2657 a (-25.0)	2028 a (-68.5)	1928 a (106.1)	1814 a (-65.4)	707 ab (-52.6)
LSD	4134	3847	6016	2853	3940	1376
<i>P</i> -value	0.479	0.756	0.856	0.812	0.688	0.640

<sup>1</sup>Means followed by the same letter are not significantly different at a 95% confidence level.

<sup>2</sup>Percentage reduction in count in comparison to the untreated control.

## Conclusions to date

Results obtained to date indicate that in a replant situation, preplant fumigation with a mixture of chloropicrin and 1.3 dichloropropene has a marked effect on tree growth and the levels of nematodes and *Phytophthora nicotianae*. Trees planted in fumigated soil are bigger with thicker trunks compared to trees in unfumigated soil. In the fumigated soil, the level of juvenile citrus nematode and *Phytophthora nicotianae* are furthermore also significantly lower than in the unfumigated soil.

*In vitro* evaluation of *Trichoderma* and bacteria based biocontrol agents indicated that they varied greatly in their ability to inhibit the growth of different citrus replant pathogens. It is therefore likely that in order to effectively control these pathogens using biocontrol agents, you would have to use a mixture of these products in an integrated programme. This was further supported by the *in planta* evaluation of commercial *Trichoderma* products for the control of *P. nicotianae* infection of citrus rootstock seedlings. It was seen that the different products, containing different species or strains, varied in their ability to reduce root infections by *P. nicotianae*.

Due to the last results from the nematode trials not available yet, no final conclusions are possible yet.

## Technology transfer

Relevant results will be presented at the appropriate grower forums.

## Further objectives and work plan

Continue to search for alternative products and methods for the control of the citrus nematode and *Phytophthora* spp. in citrus orchards. Any reports of phytotoxic damage caused by existing applications to control *Phytophthora* on new cultivars will be investigated along with any new products to use in the citrus nursery industry for the control of soilborne pathogens.

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**4.3.4 PROGRESS REPORT: Evaluation of new spray programmes for the control of Alternaria brown spot in the summer rainfall regions of South Africa**  
Project 750 (Ongoing) by Providence Moyo and Jan van Niekerk (CRI)

**Summary**

Different fungicide spray programmes were evaluated for the control of *Alternaria alternata* which causes Alternaria brown spot (ABS) on citrus. The spray programmes were evaluated on 'Nova' mandarins in the Kirkwood and Buffeljagsrivier areas in the Eastern Cape (EC) and Western Cape (WC) provinces, respectively. Treatments tested included programmes consisting of tank mixtures of various products sprayed at certain spray intervals. None of the treatments or spray programmes was good enough to satisfactorily control the disease for fruit destined for the fresh fruit market, but a higher level of control was achieved in the WC orchard (between 75 and 82.5% ABS free fruit) compared to the EC orchard (between 9 and 52.8% ABS free fruit). The highest amount of ABS free fruit (82.5%) was achieved in the 'Nova' orchard in the Western Cape, with the spray programme involving two applications of azoxystrobin-difenoconazole plus mancozeb plus mineral oil followed by four applications of mancozeb. None of the tested programmes were effective in the EC orchard. The highest percentage of clean fruit achieved in the EC was 52.8% and was produced by a treatment consisting of two copper oxychloride sprays followed by two tank mixtures of mancozeb plus azoxystrobin plus mineral oil and finally two sprays of mancozeb. The lack of efficacy of the treatments in the EC could be due to the high level of ABS inoculum historically present or the complications associated with the change of personnel responsible for applying the treatments.

**Opsomming**

Verskillende fungisied spuitprogramme is vir die beheer van *Alternaria alternata*, wat Alternaria bruinvlek (ABS) op sitrus veroorsaak, geëvalueer. Die spuitprogramme is op 'Nova' mandaryne in die Kirkwood en Buffeljagsrivier areas in onderskeidelik die Oos-Kaap- (OK) en Wes-Kaap-provinsies (WK) geëvalueer. Behandelings wat getoets is, het programme ingesluit wat uit tenkingsels van verskeie produkte bestaan het, wat teen seker spuit-intervalle gespuit is. Geen van die behandelings of spuitprogramme was goed genoeg om die siekte vir vrugte wat vir die varsvrugtemark bestem is, bevredigend te beheer nie, maar 'n hoër vlak van beheer is in die WK boord verkry (tussen 75 en 82.5% ABS-vry vrugte) in vergelyking met die OK boord (tussen 9 en 52.8% ABS-vry vrugte). Die hoogste hoeveelheid ABS-vry vrugte (82.5%) is in die Nova boord in die Wes-Kaap bereik, met die spuitprogram wat twee toedienings van asoksistrobien-difenokonasool plus mankoseb plus minerale olie, gevolg deur vier toedienings van mankoseb, ingesluit het. Geen van die getoetste programme was effektief in die OK boord nie. Die hoogste persentasie van skoon vrugte wat in die

OK bereik is, was 52.8% en is verkry deur 'n behandeling bestaande uit twee koper-oksichloried spuite, gevolg deur twee tenkmengsels van mankoseb plus asoksistrobien plus minerale olie, en laastens twee spuite mankoseb. Die tekort aan doeltreffendheid van die behandelings in die OK kan weens die hoë vlak van ABS inokulum, histories teenwoordig, wees, of die komplikasies wat gepaard gegaan het met die verandering in personeel wat verantwoordelik was vir die toedien van die behandelings.

## Introduction

*Alternaria* brown spot (ABS) is a serious disease of tangerines (*Citrus reticulata*) and their hybrids in all citrus producing regions of South Africa (Dalikilic *et al.*, 2005; Peever *et al.*, 2005). Susceptibility to ABS is a dominant trait that is transferred from 'Dancy' mandarin to its progeny. Dancy mandarin hybrids and some cultivars of unknown origin, such as 'Murcott', 'Emperor' and 'Ponkan', are affected by the disease (Dalikilic *et al.*, 2005). The presence of ABS in South Africa is still a serious problem on all cultivars derived from crosses with Dancy tangerine such as the 'Nova', 'Minneola' and 'Mor'.

The ABS disease is caused by *Alternaria alternata*. This fungus attacks young leaves, twigs and fruit, causing small black necrotic spots after a 24 to 36 h incubation period. The ABS pathogen sporulates abundantly on lesions on mature leaves remaining in the canopy (Timmer *et al.*, 1998, 2003; Reis *et al.*, 2006). The pathogen produces a host-specific toxin that causes lesions to expand, often resulting in leaf and fruit drop as well as twig dieback (Pegg 1966; Peever *et al.*, 2004, 2005). On more mature fruit, lesions may vary from small necrotic spots to large sunken pockmarks. Leaves are susceptible until they are fully expanded and hardened whereas fruits are susceptible from petal fall until harvest.

Cultural measures, such as wider tree spacing and pruning to allow air movement and drying-off of trees, the elimination of overhead irrigation and avoidance of excess nitrogen fertilizer, can assist in reducing disease severity in some orchards (Dalikilic *et al.*, 2005). However, fungicide applications are essential for disease control and production of blemish-free fruit (Schutte *et al.*, 1992). In South Africa, it is important to protect fruit and flushes of cultivars such as tangerines and their hybrids with fungicides from September to April/May, often requiring 8+ spray applications. The number of sprays and the products being used are not economically sustainable and may result in unacceptable residues on fruit.

## Objectives

To evaluate different spray programmes on susceptible 'Nova' mandarin orchards.

## Materials and methods

Two 'Nova' mandarin orchards, one located in Kirkwood in the Eastern Cape and one located in Buffeljagsrivier in the Western Cape, were used as trial sites for the 2019-2020 season. Spray programmes included alternating mancozeb and dipotassium phosphate and the inclusion of azoxystrobin-difenoconazole as part of a spray programme (Table 4.3.4.1). Similar treatments were applied in both orchards except that the treatment that incorporated a silica drench (treatment 6 in Table 4.3.4.1) was not applied in the Eastern Cape and the treatment that constituted the outright alternation of dipotassium phosphate and mancozeb (treatment 5 in Table 4.3.4.1) was not applied in the Western Cape orchard. Each treatment consisted of five single data trees as replicates. Guard trees were located between plots within rows. Unsprayed trees served as controls. The treatments in the Eastern Cape orchard were applied by the grower using his own machinery, but treatments in the Western Cape were applied with a Stihl® SR 420 motorized backpack mist blower.

At fruit maturity in May 2020, 100 and 40 fruit per data tree in the Eastern Cape and Western Cape, respectively, were evaluated according to an infection scale where: 0 = fruit with no brown spot lesions, 1 = fruit with one to five lesions and 2 = fruit with six or more lesions. ANOVA was carried out on the data using XLSTAT, Version 2014.5.03 (Addinsoft, New York, USA) to determine the efficacy of each treatment on ABS incidence. Tukey's least significant difference (LSD) test ( $P = 0.05$ ) was used to compare means.

## Results and discussion

The person who always applied the treatments in the EC orchard relocated and someone had to take over the application of the treatments. It is possible that complications associated with the change of personnel negatively impacted the project.

The 2019-2020 season in the Eastern Cape (EC) orchard was marred with high *Alternaria* brown spot inoculum pressure and all treatments did not perform well in controlling the disease in this orchard. The ABS inoculum pressure in the Western Cape (WC) orchard was lower compared to the orchard in the EC, as higher percentages (between 75 and 82%) of ABS free fruit were achieved with the treatments applied in comparison to a range of between 9 and 52% ABS free fruit achieved in the EC (Table 4.3.4.1). All the treatments tested in the WC orchard produced significantly similar levels of ABS control, but the spray programme with two applications of azoxystrobin-difenoconazole plus mancozeb plus mineral oil followed by four applications of mancozeb produced the highest amount of ABS free fruit (82.5%) (Table 4.3.4.1). A program consisting of a silica drench applied with mancozeb or copper in the WC produced 76.1% clean fruit, an amount of ABS control significantly similar to other treatments (Table 4.3.4.1). The highest percentage of clean fruit (52.8%) in the EC was achieved by treatment 1 which incorporated copper oxychloride, mancozeb and tank mixtures of azoxystrobin, mancozeb and mineral oil (Table 4.3.4.1). Strangely, the standard spray programme used by the grower (treatment 7) in the EC yielded the least amount of clean fruit (1.2%) compared to other fungicide treatments, including the untreated fruit which yielded 5% clean fruit (Table 4.3.4.1). The amount of clean ABS free fruit achieved with the treatments applied in both orchards is not high enough for consideration for fruit destined for fresh fruit markets.

A multi-disciplinary strategy is important for effective control of *Alternaria* brown spot. Cultural practices which allow air movement and drying-off of trees reduce disease pressure (Whiteside, 1979) to some extent, but effective control of the disease is mainly achieved with fungicidal spray programmes. Fungicides should, therefore, be applied at spraying intervals that ensure sufficient protection of susceptible host tissues throughout the growing season. Spray programmes such as treatment 2, which involved azoxystrobin-difenoconazole mixed with mancozeb in tank mixtures, should therefore be evaluated further and their spray intervals optimised to achieve better control of the disease.

**Table 4.3.4.1.** Application dates, rates and evaluation of fungicides applied in tank mixtures for the control of *Alternaria* brown spot in Nova mandarin orchards in Kirkwood (Eastern Cape) and Buffeljagsrivier (Western Cape), South Africa, for the period between October 2019 and March 2020.

Treatments	Dosage (g/ml per 100L water tank mixture)	Mean % of fruit in each class						
		Mean lesions/fruit <sup>v</sup>						
		Kirkwood			Buffeljagsrivier			
		0 lesions	1-5 lesions	≥6 lesions	0 lesions	1-5 lesions	≥6 lesions	
1	Copper oxychloride/ copper oxychloride/ azoxystrobin + mancozeb + mineral oil/ azoxystrobin + mancozeb + mineral oil/ mancozeb/ mancozeb	200g/200g/20ml+150g+300ml/20ml+150g+300ml/200g/200g	52.8 a	43.2 d	4.0 e	75.0 a	22.8 b	2.2 b
2	Azoxystrobin-difenoconazole +mancozeb+oil/ azoxystrobin-difenoconazole + mancozeb + oil / mancozeb / mancozeb /mancozeb/ mancozeb	30ml+150g+300ml/30ml+150g+300ml /200g/200g/ 200g/200g	33.0 b	50.4 cd	16.6 d	82.5 a	13.3 c	4.2 b
3	Azoxystrobin + mancozeb + mineral oil/ azoxystrobin+mancozeb+mineral oil/mancozeb/ mancozeb/ mancozeb/ mancozeb	20ml+150g+300ml/20ml+150g+300ml /200g/200g/200g/200g	10.2 cd	54.0 bc	35.8 b	76.1 a	22.5 b	1.4 b
4	Dipotassium phosphate+mancozeb/ dipotassium phosphate +mancozeb/ azoxystrobin + mancozeb + dipotassium phosphate + mineral oil + azoxystrobin +mancozeb+ dipotassium phosphate + mineral oil/ dipotassium phosphate +mancozeb/ dipotassium phosphate+mancozeb/ dipotassium phosphate +mancozeb	100ml+100g/100ml+100g/20ml+100g +100ml+300ml/20ml+100g+100ml+300ml/100ml+100g/100ml+100g/100ml+100g	13.4 c	62.6 ab	24.0 cd	81.9 a	16.2 bc	1.9 b
5	Dipotassium phosphate+mancozeb/ dipotassium phosphate + mancozeb /dipotassium phosphate + mancozeb / dipotassium phosphate+ mancozeb/ dipotassium phosphate+mancozeb/ dipotassium phosphate + mancozeb	100ml+100g/100ml+100g/100ml+100g/100ml+100g/100ml+100g/100ml+100g/100ml+100g	9.2 cd	65.2 a	25.6 c	-	-	-
6	Copper oxychloride+silica drench/ Copper oxychloride + silica drench/ azoxystrobin + mancozeb + mineral oil + silica drench/ azoxystrobin + mancozeb + mineral oil + silica drench/ mancozeb + silica drench/ mancozeb + silica drench/ mancozeb	200g + 6ml per tree/200g + 6ml per tree/20ml + 150g + 300ml + 6ml per tree/20ml + 150g + 300ml + 6ml per tree/200g + 6ml per tree/200g + 6ml per tree/200g	-	-	-	76.1 a	22.2 b	1.7 b
7	Grower's standard spray programme		1.2 e	27.8 e	71.0 a	-	-	-
8	Untreated		5.0 de	67.0 a	28.0 c	48.1 b	41.4 a	10.6 a

<sup>v</sup> Means in a column, based on 500 fruit (100 fruit/data tree), followed by the same letter are not significantly different ( $P > 0.05$ ) according to Tukey's least significant difference (LSD) test

## Conclusion to date

All the experimental programmes did not perform well and could not control the *Alternaria* brown spot disease satisfactorily, especially under high disease pressure situations.

## Technology transfer

Moyo, P. and Fourie, P.H. 2019. Epidemiology, prediction and management of *Alternaria*. Presentation at the CRI - IPM and DM workshops, August 2019.

## Future objectives and work plan

Research in future will focus on the inclusion of new chemistry into already existing spray programmes, with the ultimate goal of decreasing the amount of applications during the season. Alternative fungicides will have to be identified because of the withdrawal of mancozeb from the European Union which is South Africa's biggest export market.

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### 4.3.5 PROGRESS REPORT: Development of new spray programmes for the control of citrus black spot

Project 970 (Ongoing) by Providence Moyo and Paul H. Fourie (CRI)

## Summary

There is a constant need to test novel control measures, including the evaluation of new fungicides, against citrus black spot (CBS). Experimental fungicides and adjuvants, from various companies, were tested on 'Valencia' oranges for the control of citrus black spot, according to protocols provided by the different parties. No phytotoxicity and good CBS control (>99% CBS free fruit) was achieved with experimental fungicides such as fenbuconazole as well as with CBS spray programmes using NuFilm 17 as a substitute for mineral oil. Such

products are good candidates for consideration during CBS registration trials. One experimental copper resulted in phytotoxicity and did not produce good CBS control. Testing of novel fungicides and re-evaluation of existing registered fungicides is necessary to improve CBS control.

## Opsomming

Daar is 'n konstante behoefte vir die toets van nuwe beheermaatreëls, insluitend die evaluasie van nuwe fungisiedes, teen sitruswartvlek (SSV). Eksperimentele fungisiedes en bymiddels vanaf verskeie maatskappye, is volgens protokolle soos verskaf deur die verskeie partye, op 'Valencia' lemoene vir die beheer van sitruswartvlek getoets. Geen fitotoksiteit en goeie SSV beheer (>99% SSV skoon vrugte) is met eksperimentele fungisiedes soos fenbukonasool, asook met SSV spuitprogramme waar NuFilm 17 as plaasvervanger vir minerale olie gebruik is, verkry. Sulke produkte is goeie kandidate wat in oorweging gebring kan word gedurende SSV registrasieproewe. Een eksperimentele koper het tot fitotoksiteit gelei en nie goeie SSV beheer gegee nie. Die toets van nuwe fungisiedes en her-evaluering van bestaande geregistreerde fungisiedes is nodig ten einde SSV beheer te verbeter.

## Introduction

Citrus Black Spot (CBS), caused by *Phyllosticta citricarpa* (McAlpine) van der Aa, is a major concern for the South African citrus industry. *Phyllosticta citricarpa* is an A1 quarantine pathogen in the European Union and other CBS sensitive markets where there is a zero tolerance for CBS on fruit.

Citrus black spot holds the potential to reduce the South African competitiveness on the global citrus market, due to its phytosanitary status and the impact thereof on trade of citrus fruit. Hence, research has focussed even more on protecting fruit from infection by the CBS pathogen. Currently, all commercial fungicide applications aimed at protecting fruit from CBS infection begin in mid-October in South Africa, based on research findings from ascospore release and trap data (Kellerman and Kotzé, 1977; Kotzé, 1981). Due to the withdrawal of certain fungicides from the market, as a result of concerns of risks posed to human health and the environment as well as resistance, there is a constant need to evaluate old and new fungicide formulations that may possess activity against CBS, and to structure spray programmes to improve control whilst adhering to permitted maximum residue levels (MRL) and limiting fungicide resistance.

A number of adjuvants are regularly used with systemic fungicide applications in South Africa, to enhance the efficacy of fungicides. However, most systemic fungicides registered for the control of CBS are used in combination with mineral oils. These oils have been shown to enhance the penetration of fungicides into the plant tissues, ultimately increasing the efficacy of the fungicides against CBS (Kellerman and Kotzé 1977). The efficacy of newly developed non-mineral oil adjuvants, as substitutes for mineral oil in standard strobilurin spray programmes, also need to be investigated for the control of *Phyllosticta citricarpa* on citrus in South Africa.

Fungicide manufacturers often develop new fungicide formulations for disease control but they also modify and upgrade old fungicide products to possess new characteristics such as rain fastness and particle size. The evaluation of new fungicide products and re-evaluation of old products for efficacy against CBS remains an integral part of staying globally competitive in the citrus market place.

## Objectives

To evaluate any new potential fungicides and alternative spray programmes for the control of citrus black spot.

## Materials and methods

The trial was carried out in a 1.4 ha commercial orchard (Crocodile Valley Citrus Co.) located in Nelspruit (GPS: 25° 28' 16.38" S and 31° 04' 20.70" E). The orchard was planted in 1986 and consisted of Olinda 'Valencia' orange (*Citrus sinensis*) trees grafted on Rough lemon rootstock (*Citrus jambhiri*), with spacing of 8.3 m x 5.6 m. The orchard consists of sandy-loam soil.

Different fungicides were applied either alone or in tank mixtures with other fungicides. Fungicide spray applications were conducted at intervals and concentrations determined by the different parties whose products were tested. A few treatments were tested as part of a collaboration with the University of Florida in the United States of America. Treatments also involved testing adjuvants as potential replacements for the mineral oil used in CBS spray tank mixtures. The efficacy of the different fungicides in tank mixtures, as well as that of experimental spray programmes, was compared with the registered industry standard CBS treatments.

Commercial fungicide applications against CBS are recommended from fruit set in South Africa and therefore, treatment applications began in mid-October 2019 and were applied using a trailer-mounted, high-volume, high-pressure (2,500-3,000 kPa) sprayer with two hand-held spray guns. Each treatment was replicated four times on single-tree plots arranged in a randomized complete-block design. Fungicide volumes varied according to the size and canopy density of the tree, but all trees were sprayed to the point of runoff.

On 17 August 2020 (a week before harvest), 100 fruit per data tree were evaluated according to a 3-point index: 0 = clean fruit with no CBS lesions; 1 = one to three CBS lesions per fruit; and 2 = four or more CBS lesions per fruit. ANOVA was carried out using XLSTAT, Version 2014.5.03 (Addinsoft, New York, USA) to determine the efficacy of each treatment on CBS severity and incidence. Tukey's least significant difference (LSD) test ( $P = 0.05$ ) was used to compare means. The effect of each treatment or spray programme on the fruit rind (phytotoxicity) was evaluated simultaneously. Phytotoxicity was rated on a scale of 0 to 3: 0 = no dark marks on the rind; 1 = dark marks on one-half of the fruit rind; 2 = dark marks on three-quarters of the fruit rind and 3 = dark marks on the whole fruit rind.

## Results and discussion

Objective / Milestone	Achievement
<ul style="list-style-type: none"> <li>• Evaluation of new fungicides and adjuvants               <ul style="list-style-type: none"> <li>• Applying fungicides at concentrations determined by different parties</li> </ul> </li> </ul>	All treatments were successfully applied on 'Valencia' oranges. The trial was successfully completed and evaluated in August 2020, where after the data was statistically analysed.

The largest export markets for South African citrus have zero tolerance of CBS on fruit, and therefore, only the criteria of fruit without lesions was used in determining the efficacy of the treatments. The trial site was not characterised by a high incidence of citrus black spot, during the 2019-20 season, with the untreated control trees yielding 60.0% CBS free fruit (Table 4.3.5.1).

Most experimental fungicides and spray programmes significantly reduced CBS infection compared to the untreated control (Table 4.3.5.1). The replacement of mineral oil with either NuFilm 17 or Entreé, in spray programmes using mancozeb as a contact fungicide, resulted in good control of CBS which was comparable to that achieved by the industry standard CBS spray programmes using mineral oil as an adjuvant (treatments 13 and 20). The amount of control achieved with NuFilm 17 was always slightly superior to that achieved with Entreé (Table 4.3.5.1). The use of NuFilm 17 or Entreé in the single CBS sprays involving benomyl did not produce good control of CBS as was the registered single benomyl sprays (treatment 21) (Table 4.3.5.1). No phytotoxicity was evident on fruit treated with treatments incorporating NuFilm 17 and Entreé (Table 4.3.5.2). With such good results, NuFilm 17 and Entreé are good candidates for registration as alternative adjuvants to mineral oil in CBS spray programmes.

The experimental copper from Party B did not achieve good CBS control when applied alone (treatment 26) but its performance improved when applied together with mineral oil (treatment 28). Although the experimental fungicide resulted in over 96% CBS free fruit when applied in a tank mixture with strobilurin and mineral oil (treatment 28), its negative effect on the rind cannot be ignored. The fungicide caused severe stippling of the fruit rind (phytotoxicity) (Table 4.3.5.2). Copper fungicides are associated with stippling of fruit tissue (Schutte

*et al.* 1997). Further trials on this experimental copper could investigate the effect of reduced concentrations of the product on CBS control and phytotoxicity.

An increase in the concentration of Adjuvant A, used in the place of mineral oil, in CBS spray programmes resulted in a higher number of CBS free fruit. The high concentration of the Adjuvant A used in CBS spray programs (treatment 34) produced more CBS free fruit compared to other lower concentrations (treatments 35 and 36) (Table 4.3.5.1). The Adjuvant A treatments did not result in any evident phytotoxicity (Table 4.3.5.2). An experimental programme in which the usual first mancozeb spray in normal CBS spray programmes was replaced with a mancozeb-strobilurin-mineral oil tank mixture and a second mancozeb-strobilurin-mineral oil tank mixture was applied before two mancozeb sprays (treatment 37) did not perform well as only 91.5% clean fruit were obtained (Table 4.3.5.1).

Of the fungicides tested as part of a collaboration project with the University of Florida (USA), good control (over 99% CBS free fruit) of CBS was achieved with the application of fenbuconazole (treatment 38) as well as with treatment 39 which alternated azoxystrobin + difenoconazole with copper hydroxide (Table 4.3.5.1). The worst performing product was the fluopyram + tebuconazole mixture (treatment 40) which only produced 67.5% CBS free fruit (Table 4.3.5.1). Some degree of phytotoxicity was observed with treatments involving copper hydroxide (Table 4.3.5.2), which was not unexpected since copper fungicides cause stippling on fruit (Schutte *et al.* 1997). Considering the good results obtained with fenbuconazole in the 2019-2020 season, further trials should be conducted to determine whether good CBS control can be achieved consistently with this fungicide. Due to market pressures on fungicides such as mancozeb, which has been banned on citrus in the EU, rigorous research with the ultimate goal of registration is needed on any promising fungicide for the control of CBS.

**Table 4.3.5.1.** Evaluation of spray programmes for the control of Citrus black spot conducted at Crocodile Valley Co., Nelspruit, Mpumalanga during the 2019-20 season

Company	Treatments	Dosage (g/ml per 100L water tank mixture)	Average number of fruit with CBS lesions <sup>a</sup>		
			0 lesions (%)	1-3 lesions (%)	≥4 lesions (%)
	1 Untreated control		60.0 g	19.8 a	20.2 b
<b>Industry standard</b>	2 Mancozeb <sup>b</sup>	200g	99.8 a	0.2 f	0.0 e
<b>Industry standard</b>	3 Copper oxychloride <sup>c</sup>	200g	99.8 a	0.2 f	0.0 e
<b>Industry standard</b>	20 Mancozeb/mancozeb+azoxystrobin+mineral oil/ mancozeb+azoxystrobin+mineral oil /mancozeb <sup>d</sup>	200g/150g+20ml+250ml/ 150g+20ml+250ml/200g	100.0 a	0.0 f	0.0 e
<b>Industry standard</b>	13 Mancozeb/mancozeb + pyraclostrobin + mineral oil/mancozeb + pyraclostrobin + mineral oil/mancozeb <sup>d</sup>	200g/ 150g + 10ml + 250ml/ 150g + 10ml + 250ml/ 200g	99.8 a	0.2 f	0.0 e
<b>Industry standard</b>	47 Copper oxychloride/ copper oxychloride+azoxystrobin+mineral oil/ copper oxychloride+azoxystrobin+mineral oil/ copper oxychloride <sup>e</sup>	200g/200g+20ml+250ml/ 200g+20ml+250ml/200g	100.00 a	0.00 f	0.00 e
<b>Party A</b>	7 Mancozeb/mancozeb + azoxystrobin + NuFilm 17/ mancozeb + azoxystrobin + NuFilm 17/ mancozeb <sup>d</sup>	200g/ 150g + 20ml + 15ml/ 150g + 20ml + 15ml / 200g	99.8 a	0.0 f	0.2 e
<b>Party A</b>	9 Mancozeb/mancozeb + azoxystrobin + Entreé/ mancozeb + azoxystrobin + Entreé/ mancozeb <sup>d</sup>	200g/ 150g + 20ml + 20ml/ 150g + 20ml + 20ml / 200g	97.5 ab	2.3 ef	0.2 e
<b>Party A</b>	11 Mancozeb/mancozeb + pyraclostrobin + NuFilm 17/ mancozeb + pyraclostrobin + NuFilm 17/ mancozeb <sup>d</sup>	200g/ 150g + 10ml + 15ml/ 150g + 10ml + 15ml / 200g	100.0 a	0.0 f	0.0 e
<b>Party A</b>	15 Mancozeb/mancozeb + pyraclostrobin + Entreé/ mancozeb + pyraclostrobin + Entreé/ mancozeb <sup>f</sup>	200g/ 150g + 10ml + 20ml/ 150g + 10ml + 20ml/ 200g	97.8 a	2.0 ef	0.2 e
<b>Party A</b>	21 Mancozeb+benomyl+mineral oil <sup>g</sup>	200g + 50ml + 250ml	89.8 cde	7.2 bcd	3.0 cde
<b>Party A</b>	23 Mancozeb+benomyl+NuFilm 17 <sup>g</sup>	200g + 50ml + 15ml	84.8 e	9.0 bc	6.2 c
<b>Party A</b>	25 Mancozeb+benomyl+NuFilm 17 <sup>g</sup>	200g + 20ml + 15ml	60.0 g	18.3 a	21.7 b
<b>Party B</b>	26 Experimental copper (x7) <sup>h</sup>	300ml	88.3de	6.7 bcde	5.0 cd
<b>Party B</b>	28 Experimental copper/ experimental copper+azoxystrobin+mineral oil/ experimental copper+azoxystrobin+mineral oil/experimental copper <sup>i</sup>	300ml/300ml+20ml+250ml/ 300ml+20ml+250ml /300ml	96.8 ab	2.5 def	0.7 de
	34 Mancozeb/mancozeb+azoxystrobin+Adjuvant A/ Mancozeb+azoxystrobin+Adjuvant A/mancozeb <sup>f</sup>	200g/150g+20ml+100ml/ 150g+20ml+100ml/200g	95.3 abc	1.5 f	3.2 cde

	35	Mancozeb/mancozeb+azoxystrobin+Adjuvant A/ Mancozeb+azoxystrobin+Adjuvant A/mancozeb <sup>f</sup>	200g/150g+20ml+200ml/ 150g+20ml+100ml/200g	98.5 a	1.3 f	0.2 e
	36	Mancozeb+azoxystrobin+Adjuvant A/ Mancozeb+azoxystrobin+Adjuvant A/mancozeb <sup>f</sup>	200g/150g+20ml+400ml/ 150g+20ml+400ml/200g	99.5 a	0.5 f	0.0 e
	37	Mancozeb+azoxystrobin+mineral oil/ mancozeb+azoxystrobin+mineral oil /mancozeb/mancozeb <sup>j</sup>	150g+20ml+250ml/ 150g+20ml+250ml/200g/200g	91.5 bcd	4.3 cdef	4.2 cde
<b>USA collaboratio n</b>	38	Fenbuconazole (x6) <sup>k</sup>	50 ml	99.3 a	0.5 f	0.2 e
<b>USA collaboratio n</b>	39	Copper hydroxide/azoxystrobin+difenoconazole/ copper hydroxide/azoxystrobin+difenoconazole/ copper hydroxide/azoxystrobin+difenoconazole <sup>k</sup>	335g/96ml/335g/96ml/335g/96 ml	99.8 a	0.2 f	0.0 e
<b>USA collaboratio n</b>	40	Fluopyram+tebuconazole (x6) <sup>k</sup>	50 ml	67.5 f	3.3 def	29.2 a
<b>USA collaboratio n</b>	41	Copper hydroxide/pyraclostrobin/ copper hydroxide/pyraclostrobin/ copper hydroxide/pyraclostrobin <sup>k</sup>	335g/94ml/335g/94ml/335g/94 ml	97.3 ab	2.7 def	0.0 e

<sup>a</sup>Means followed by the same letter in the same column do not differ significantly ( $P = 0.05$ ) according to Tukey's least significant difference test.

<sup>b</sup>Spray dates were: 11 October 2019; 3 November 2019; 29 November 2019; 24 December 2019 and 17 January 2020.

<sup>c</sup>Spray dates were: 13 October 2019; 22 November 2019; 24 December 2019 and 28 January 2020.

<sup>d</sup>Spray dates were: 11 October 2019; 2 November 2019; 15 December 2019 and 27 January 2020.

<sup>e</sup>Spray dates were: 13 October 2019; 22 November 2019; 17 January 2020 and 20 February 2020.

<sup>f</sup>Spray dates were: 12 October 2019; 3 November 2019; 18 December 2019 and 27 January 2020.

<sup>g</sup>Spray dates were: 20 December 2019.

<sup>h</sup>Spray dates were: 12 October 2019; 19 November 2019; 18 December 2019; 12 January 2020 and 07 February 2020.

<sup>i</sup>Spray dates were: 13 October 2019; 18 November 2019; 30 December 2020; 07 February 2020.

<sup>j</sup>Spray dates were: 13 October 2019; 18 November 2019; 30 December 2019; 20 January 2020.

<sup>k</sup>Spray dates were: 12 October 2019; 18 November 2019; 19 December 2019; 15 January 2020 and 12 February 2020.

**Table 4.3.5.2.** Evaluation of the effects of citrus black spot spray programmes on the rind (phytotoxicity) of Valencia oranges at Crocodile Valley Co., Nelspruit, Mpumalanga during the 2019-20 season

Treatments		Dosage (g/ml per 100L water tank mixture)	Mean % of fruit with dark marks (Phytotoxicity)			
			0=no dark marks	1= dark marks on ½ of the fruit	2= dark marks on ¾ of fruit rind	3=dark marks whole fruit
1	Untreated control		100.0 a	0.0 e	0.0 b	0.0 b
2	Mancozeb <sup>t</sup>	200g	100.0 a	0.0 e	0.0 b	0.0 b
3	Copper oxychloride <sup>u</sup>	200g	85.0 c	15.0 c	0.0 b	0.0 b
20	Mancozeb/mancozeb+azoxystrobin+mineral oil/ mancozeb+azoxystrobin+mineral oil /mancozeb <sup>v</sup>	200g/150g+20ml+250ml/ 150g+20ml+250ml/200g	100.0 a	0.0 e	0.0 b	0.0 b
13	Mancozeb/mancozeb + pyraclostrobin + mineral oil/ mancozeb + pyraclostrobin + mineral oil/mancozeb <sup>sz</sup>	200g/ 150g + 10ml + 250ml/ 150g + 10ml + 250ml/ 200g	100.0 a	0.0 e	0.0 b	0.0 b
47	Copper oxychloride/copper oxychloride + azoxystrobin+mineral oil/copper oxychloride +azoxystrobin+mineral oil/ copper oxychloride	200g/200g+20ml+250ml/ 200g+20ml+250ml/200g	87.5 bc	12.2 cd	0.3 ab	0.0 b
7	Mancozeb/mancozeb + azoxystrobin + NuFilm 17/ mancozeb + azoxystrobin + NuFilm 17/ mancozeb <sup>z</sup>	200g/ 150g + 20ml + 15ml/ 150g + 20ml + 15ml / 200g	100.0 a	0.0 e	0.0 b	0.0 b
9	Mancozeb/mancozeb + azoxystrobin + Entreé/ mancozeb + azoxystrobin + Entreé/ mancozeb <sup>sz</sup>	200g/ 150g + 20ml + 20ml/ 150g + 20ml + 20ml / 200g	100.0 a	0.0 e	0.0 b	0.0 b
11	Mancozeb/mancozeb + pyraclostrobin + NuFilm 17/ mancozeb + pyraclostrobin + NuFilm 17/ mancozeb <sup>z</sup>	200g/ 150g + 10ml + 15ml/ 150g + 10ml + 15ml / 200g	100.0 a	0.0 e	0.0 b	0.0 b
15	Mancozeb/mancozeb + pyraclostrobin + Entreé/ mancozeb+ pyraclostrobin+Entreé/ mancozeb <sup>sz</sup>	200g/ 150g + 10ml + 20ml/ 150g + 10ml + 20ml/ 200g	100.0 a	0.0 e	0.0 b	0.0 b
21	Mancozeb+benomyl+mineral oil	200g + 50ml + 250ml	100.0 a	0.0 e	0.0 b	0.0 b
23	Mancozeb+benomyl+NuFilm 17	200g + 50ml + 15ml	100.0 a	0.0 e	0.0 b	0.0 b
25	Mancozeb+benomyl+NuFilm 17	200g + 20ml + 15ml	100.0 a	0.0 e	0.0 b	0.0 b
26	Experimental copper (x7) <sup>sx</sup>	300ml	44.0 e	53.7 a	1.0 a	1.3 a
28	Experimental copper/ experimental copper +azoxystrobin+mineral oil/ experimental copper +azoxystrobin+mineral oil/experimental copper	300ml/300ml+20ml+250ml/ 300ml+20ml+250ml /300ml	66.8 d	32.0 b	1.0 a	0.2 b
34	Mancozeb/mancozeb+azoxystrobin+Adjuvant A/ Mancozeb+azoxystrobin+Adjuvant AA/mancozeb <sup>z</sup>	200g/150g+20ml+100ml/ 150g+20ml+100ml/200g	100.0 a	0.0 e	0.0 b	0.0 b

35	Mancozeb/mancozeb+azoxystrobin+Adjuvant A/ Mancozeb+azoxystrobin+Adjuvant A/mancozeb <sup>z</sup>	200g/150g+20ml+200ml/ 150g+20ml+100ml/200g	100.0 a	0.0 e	0.0 b	0.0 b
36	Mancozeb+azoxystrobin+Adjuvant A/ Mancozeb+azoxystrobin+Adjuvant A/mancozeb <sup>z</sup>	200g/150g+20ml+400ml/ 150g+20ml+400ml/200g	100.0 a	0.0 e	0.0 b	0.0 b
37	Mancozeb+azoxystrobin+mineral oil/ mancozeb+azoxystrobin+mineral oil /mancozeb/mancozeb <sup>sy</sup>	150g+20ml+250ml/ 150g+20ml+250ml/200g/200g	99.8 a	0.2 e	0.0 b	0.0 b
38	Fenbuconazole (x6)	50 ml	100.0 a	0.0 e	0.0 b	0.0 b
39	Copper hydroxide/azoxystrobin+difenoconazole/ copper hydroxide/azoxystrobin+difenoconazole/ copper hydroxide/azoxystrobin+difenoconazole/	335g/96ml/335g/96ml/335g/96ml	97.8 a	2.0 e	0.2 ab	0.0 b
40	Fluopyram+tebuconazole (x6)	50 ml	100.0 a	0.0 e	0.0 b	0.0 b
41	Copper hydroxide/pyraclostrobin/copper hydroxide/pyraclostrobin/copper hydroxide/pyraclostrobin	335g/94ml/335g/94ml/335g/94ml	92.0 b	8.0 d	0.0 b	0.0 b

## Conclusions

A number of fungicides tested during the 2019-2020 season were promising as potential fungicides to control CBS. Such fungicides included indar. NuFilm 17 and Entreé are potential alternative adjuvants to mineral oil in CBS spray programmes.

## Technology transfer

Moyo, P., and Fourie, P.H. 2019. CRI-PhytRisk and citrus black spot management. Presentation at the CRI - IPM and DM workshops, August 2019.

Moyo, P., and Fourie, P.H. 2020. Update on citrus black spot management. Presentation at the CRI - IPM and DM workshops, August 2020.

Moyo, P., van Niekerk, J.M., Carstens, E. and Fourie, P.H. 2020. Updated citrus black spot spray programmes 2020–2021. CRI Cutting Edge 304, October 2020.

## Future objectives and work plan

Research in the future should consist of further evaluation of the promising CBS fungicide programmes as well as other available novel fungicides. There is still a lot of market pressure on several of the most effective fungicides, such as benzimidazoles and dithiocarbamates (mancozeb). Alternative fungicides to replace mancozeb in CBS spray programmes should be identified, tested and developed for CBS control.

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Kotzé, J. M. 1981. Epidemiology and control of citrus black spot in South Africa. Plant Dis. 65:945-950.

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### 4.3.6 PROGRESS REPORT: Susceptibility period of sweet orange fruit to *Phyllosticta citricarpa* in commercial orchards

Project 1186 (2017/04 - 2021/03) by Providence Moyo, T. Nxumalo and Paul H. Fourie (CRI)

## Summary

Citrus black spot is one of the most important fungal diseases of citrus worldwide. The disease is characterized by a long latency period in which symptoms may not appear until fruit ripening and its severity depends on a number of factors including the age of the fruit at the time of infection. It has been demonstrated that fruit becomes resistant to CBS infection with maturity, *i.e.* ontogenic resistance development; however, recent research from Brazil has indicated that fruit is susceptible to infection for longer periods than previously assumed and thus, the need for longer periods of fruit protection have been proposed. To quantitatively and more conclusively demonstrate ontogenic resistance development of citrus fruit to *Phyllosticta citricarpa* infection, fruit in commercial Valencia, Nova and Empress mandarin orchards were inoculated with different concentrations ( $10^1$ ,  $10^3$  and  $10^5$  conidia/mL) of *P. citricarpa* suspensions on a monthly basis or exposed to *P. citricarpa* natural infection at different times through a staggered spray programme. Significant increases in CBS incidence and severity were observed between November and January for both the inoculation and staggered spray trials on Valencia oranges. Although orange fruit were still susceptible before November and after January, CBS incidence and severity were very low and comparable to the un-inoculated control in the inoculation trials. Leaving oranges trees unprotected after January did not significantly increase the incidence and severity of CBS during the staggered spray trials. A trend

in which the susceptibility of fruit decreased with increase in fruit maturity was also demonstrated during trials on mandarins. These findings support the notion of ontogenic resistance by citrus fruits towards *P. citricarpa* infection.

## Opsomming

Sitrus swartvlek is een van die belangrikste swamsiektes van sitrus wêreldwyd. Die siekte word deur 'n lang latente periode gekenmerk, waartydens simptome moontlik nie tot met vrugrypwording verskyn nie, en die siekte se erns hang van 'n aantal faktore af, insluitend die ouderdom van die vrug tydens infeksie. Daar is gedemonstreer dat vrugte met volwassenheid, weerstandbiedend teen SSV infeksie word, nl. ontogeniese weerstandsontwikkeling. Onlangse navorsing uit Brasilië het egter getoon dat vrugte vir langer periodes as voorheen aangeneem, vatbaar vir infeksie bly, gevolglik is die behoefte vir langer periodes van vrugbeskerming voorgestel. Ten einde kwantitatief en meer beslissend ontogeniese weerstandsontwikkeling van sitrusvrugte teen *Phyllosticta citricarpa* infeksie te demonstreer, is vrugte in kommersiële Valencia, Nova en Empress mandaryn boorde met verskillende konsentrasies ( $10^1$ ,  $10^3$  en  $10^5$  konidia/mL) van *P. citricarpa* suspensies op 'n maandelikse basis geïnkuleer, of aan natuurlike infeksie van *P. citricarpa* by verskillende tye deur 'n verspreide spuitprogram, blootgestel. Betekenisvolle toename in SSV voorkoms en erns is tussen November en Januarie vir beide die inokulasie proewe en verspreide spuitproewe op Valencia lemoene waargeneem. Hoewel lemoenvrugte steeds vatbaar vóór November en ná Januarie was, was SSV voorkoms en erns baie laag en vergelykbaar met die nie-geïnkuleerde kontrole in die inokulasieproewe. Deur lemoenbome onbeskermend ná Januarie te laat, het nie die voorkoms en erns van SSV gedurende die verspreide spuitproewe betekenisvol verhoog nie. 'n Neiging waarin die vatbaarheid van vrugte met toename in vrugvolwassenheid afneem, is ook gedurende proewe op mandaryne gedemonstreer. Hierdie bevindinge ondersteun die idee van ontogeniese weerstand deur sitrusvrugte teen *P. citricarpa* infeksie.

### 4.3.7 **PROGRESS REPORT: Influence of shade nets on Alternaria brown spot and citrus black spot: comparing epidemiological model output for covered (under shade nets) and uncovered (normal/open) orchards' weather datasets**

Project 1187 (2017/04 - 2022/03) by Providence Moyo and Paul H. Fourie (CRI)

## Summary

The use of shade/hail nets is increasing in South African orchards. These nets are mainly to mitigate the loss of yield due to detrimental climatic conditions including extreme temperatures, hailstorms and high winds. They also protect crops against insects and birds. The use of hail nets can, however, lead to the modification of the orchard microclimate, in particular humidity and temperature, which are crucial in the growth and development of pathogens such as *Phyllosticta citricarpa*, *Alternaria alternata* and *Botrytis cinerea*, which cause citrus black spot (CBS), Alternaria brown spot (ABS) and Botrytis, respectively. Thus, the use of shade nets can directly or indirectly affect the development of these diseases in citrus orchards. Disease predictions from two trial sites located in two provinces (Mpumalanga and Western Cape) were analysed for the 2019-2020 and 2020-2021 seasons. Pseudothecium maturation was reached earlier in orchards under the net in the two sites during both seasons, where slightly warmer maximum temperatures were experienced compared to the orchards outside the nets. More days suitable for ABS and Botrytis infection (intermediate + high risks) were predicted in the orchard under the net and the orchard outside the net in the Western Cape and Mpumalanga site, respectively, during the 2020-2021 season. These predictions coincided with high relative humidity levels measured in the respective orchards. During the 2019-2020 season, more ABS and Botrytis infection risks were predicted inside and outside the net, respectively. High rainfall and humidity levels were measured in outside and inside the net, respectively, in the 2019-2020 season. Fungicide residue persistence in orchards was analysed from fruit collected from the trial site in Mpumalanga and more fungicides persisted under the net in comparison to the orchard outside the net.

## Opsomming

Die gebruik van skadu- of haelnette neem toe in Suid-Afrikaanse boorde. Hierdie nette het ten doel om hoofsaaklik die verlies aan opbrengs weens nadelige klimaatstoestande, insluitend uiterste temperature, haelstorms en sterk winde, te beperk. Hulle beskerm ook gewasse teen insekte en voëls. Die gebruik van haelnette kan egter tot modifikasie van die boord mikroklimate, veral humiditeit en temperatuur, lei, wat noodsaaklik in die groei en ontwikkeling van patogene soos *Phyllosticta citricarpa*, *Alternaria alternata* en *Botrytis cinerea* is, wat onderskeidelik sitrus swartvlek (CBS), *Alternaria* bruinvlek (ABS) en *Botrytis* veroorsaak. Die gebruik van skadunette kan dus direk of indirek, die ontwikkeling van hierdie siektes in sitrusboorde affekteer. Siektevoorspellings vanaf twee proefpersele wat in twee provinsies (Mpumalanga en Wes-Kaap) geleë is, is vir die 2019-2020 en 2020-2021 seisoene geanaliseer. *Pseudothecium* volwassenheid is vroeër in boorde onder net in die twee persele gedurende beide seisoene bereik, waar effens warmer maksimum temperature ondervind is, in vergelyking met die boorde buite die nette. Meer dae geskik vir ABS en *Botrytis* infeksie (intermediêr + hoë risiko's) is in die boorde onder net en die boord buite die net in onderskeidelik die Wes-Kaap en Mpumalanga perseel, gedurende die 2020-2021 seisoen, voorspel. Hierdie voorspellings het met hoë relatiewe humiditeitsvlakke wat in die onderskeie boorde gemeet is, saamgeval. Gedurende die 2019-2020 seisoen is meer ABS en *Botrytis* infeksie-risiko's onderskeidelik binne en buite die nette voorspel. Hoë reënval en humiditeitsvlakke is onderskeidelik buite en binne die net in die 2019-2020 seisoen gemeet. Fungisiedresidue wat in boorde agtergebly het, is vanaf vrugte geanaliseer wat vanaf die proefperseel in Mpumalanga versamel is, en meer fungisiedes het onder die net agter gebly in vergelyking met die boord buite die net.

#### 4.3.8 **PROGRESS REPORT: Management of pruning debris as part of citrus black spot control strategy** Project 1223 (2019/04 – 2022/03) by Providence Moyo and Paul H. Fourie (CRI)

##### **Summary**

This project aimed to determine to what extent chopped or shredded pruning debris have the potential to contribute to CBS inoculum in citrus orchards. Chopped (shredded) and un-chopped pruning debris were collected from a Eureka lemon orchard in Hoedspruit, placed in wire frames and left under the trees in the orchard to undergo natural decomposition and maturation of *Phyllosticta* fruiting bodies. Naturally abscised leaves were also collected. The decomposing leaves and pruning debris were sampled at monthly intervals from October to March during the 2019-2020 and 2020-2021 growing seasons and sent to DALRRD in Stellenbosch, for analysis for the presence of ascospores of *Phyllosticta* using a Kotzé inoculum monitor (KIM). Generally, a higher number of ascospores were recorded using the KIM in the 2020-2021 season when compared to the 2019-2020 season, with the highest number of ascospores counted from un-chopped pruning debris for both seasons. Shredding pruning debris in the Eureka lemon orchard led to reduced ascospore inoculum load. Results of the KIM analysis were also complimented by the CRI-PhytRisk prediction system which predicted more days with possible ascospore infection in the 2020-2021 in comparison to the 2019-2020 season. A Quest volumetric spore trap was also operated in the same lemon orchard and *Phyllosticta* ascospores were trapped throughout the duration of the trial in the 2020-2021 season.

##### **Opsomming**

Hierdie projek het ten doel gehad om te bepaal tot watter mate opgekapte of gesnipperde snoei-afval tot die CBS-spoorlading in sitrusboorde bydra. Opgekapte en nie-gekapte snoei-afval is uit 'n Eureka-suurlemoenboord in Hoedspruit versamel, in draadrame geplaas en onder die bome in die boord gelaat om natuurlike ontbinding en rypwording van *Phyllosticta* vrugliggampies te ondergaan. Natuurlik gesnyde blare is ook versamel. Die ontbindende blare en snoei-afval is met tussenposes van Oktober tot Maart gedurende die groeiseisoene 2019-2020 en 2020-2021 gemonster en na DALRRD in Stellenbosch gestuur vir ontleding van die aanwesigheid van askospore van *Phyllosticta* met behulp van 'n Kotzé-inokulummonitor (KIM). Oor die algemeen is 'n groter aantal askospore aangeteken met behulp van die KIM in die 2020-2021-seisoen in vergelyking met die 2019-2020-seisoen, met die hoogste aantal askospore wat gedurende beide seisoene van ongekapte blare getel is. Versnippering van snoei-afval in die Eureka-suurlemoenboord lei tot verminderde askospoorlading. Die resultate

van die KIM-analise is ook aangevul deur die CRI-PhytRisk-voorspellingstelsel wat meer dae met moontlike askospoor-infeksie in 2020-2021 in vergelyking met die seisoen 2019-2020 voorspel het. In dieselfde suurlemoenboord is 'n Quest-volumetriese spoorlokval bedryf en *Phyllosticta* askospores is gedurende die duur van die proef in die 2020-2021 seisoen gevang.

#### 4.3.9 PROGRESS REPORT: Further validation and improvements of CRI-PhytRisk

Project RCE-2-12 (1238) (2019/04 – 2022/03) by Providence Moyo and Paul H. Fourie (CRI)

##### Summary

CRI-PhytRisk is a CBS-risk management platform that allows improved decision support to citrus growers in CBS areas, to improve fungicide spray timing as well as indicate CBS-risk on fruit destined for export based on the past season's weather conditions and CBS infection predictions. The CBS models were validated in a collaborative project with Brazilian researchers. The CRI-PhytRisk parameters were more related to CBS severity than incidence, with  $R^2_{adj}$  reaching up to 0.49. Additionally, the CRI-PhytRisk models performed similarly to other models in related research (CRI Project 1149). The CBS pseudothecium maturation model was updated to a recently published model that was developed from a broader dataset. A Botrytis forecast model was successfully programmed into PhytRisk, but requires further calibration of the risk thresholds. Programming has been concluded to port grower-specific data from eCert to CRI-PhytRisk, as well as the functionality to enter CBS fungicide spray records for individual orchards. When predicted CBS infection periods are overlaid with the period of protection from the fungicide sprays, CRI-PhytRisk can easily identify unprotected periods and assign a CBS risk accordingly. This will enable growers to self-assess CBS risk for their orchards and to make informed decisions on market destinations. Once officially accepted, this CBS risk for each orchard can be exported to PhytClean. These latest developments will be incorporated into a general technology upgrade of the CRI-PhytRisk platform, which will also allow analytical functionality of data in CRI-PhytRisk. This general upgrade was initiated late in the RCE-2 funding cycle, and is currently under way. It is planned to relaunch an updated and improved version of CRI-PhytRisk before the new season (September / October 2021).

##### Opsomming

CRI-PhytRisk is 'n SSV-risiko bestuurplatform wat verbeterde besluitneming ondersteuning aan sitrusprodusente in SSV areas verleen, om fungisiedspuit tydsberekening te verbeter, asook om SSV risiko op vrugte wat vir uitvoer bestem is, aan te dui, gebaseer op die vorige seisoen se weerstoestand en SSV infeksie voorspellings. Die SSV-modelle is gevalideer in 'n samewerkingsprojek met Brasiliaanse navorsers. Die CRI-PhytRisk parameters was meer verwant aan CBS-druk as voorkoms, met  $R^2_{adj}$  wat tot 0.49 was. Daarbenewens het die CRI-PhytRisk modelle soortgelyk aan ander modelle in verwante navorsing gepresteer (CRI-projek 1149). Die CBS pseudothecium verouderingsmodel is opgedateer na 'n onlangs gepubliseerde model wat uit 'n breër datastel ontwikkel is. 'n Botrytis-voorspellingsmodel is suksesvol in PhytRisk geprogrammeer, maar vereis verdere kalibrering van die risikodrempels. Die programmering is afgehandel om produsent-spesifieke data van eCert na CRI-PhytRisk oor te dra, asook die funksies om CBS-swamdoderspuitrekords vir individuele boorde in te voer. Wanneer voorspelde CBS-infeksietydperke oorvleuel met die periode van beskerming teen die swamdoderbespuitings, kan CRI-PhytRisk maklik onbeskermdes periodes identifiseer en 'n CBS-risiko daarvolgens toeken. Hierdie sal produsente in staat stel om die CBS-risiko vir hul boorde self te beoordeel en ingeligte besluite oor markbestemmings te neem. Sodra dit amptelik aanvaar is, kan hierdie CBS-risiko vir elke boord na PhytClean uitgevoer word. Hierdie nuutste ontwikkelings sal opgeneem word in 'n algemene tegnologie-opgradering van die CRI-PhytRisk platform, wat ook analitiese funksionaliteit van data in CRI-PhytRisk moontlik maak. Hierdie algemene opgradering is laat in die RCE-2 finansieringsiklus begin en is tans aan die gang. Daar word beplan om 'n opgedateerde en verbeterde weergawe van CRI-PhytRisk voor die nuwe seisoen (September / Oktober 2021) bekend te stel.

#### 4.3.10 **PROGRESS REPORT: Epidemiology, inoculum potential and infection parameters of Citrus Black Spot**

Project 1244 (RCE-2-14) (2019/04 – 2022/03) by Providence Moyo and Paul H. Fourie (CRI)

##### **Summary**

Citrus Black Spot (CBS) is the most important fungal disease of citrus in South Africa, because of its quarantine status in major export countries. Epidemiology of CBS is not fully understood, since there is currently no method to distinguish between ascospores of the CBS pathogen and those of the non-pathogenic *P. capitalensis*. Furthermore, actual infection has not been measured yet, although generic infection models are available. The objectives of this project were aimed at improving our understanding of CBS epidemiology, and to improve spore release and infection models. Many of the technically challenging objectives were not realised due to capacity constraints, mostly as a result of resignations by workers. The project was eventually moved from Stellenbosch University to CRI in Nelspruit. Certain objectives were partially fulfilled. The first pycnidiospore inoculation trial conducted in Nelspruit was evaluated and 21 and 27°C were found to be most suitable for infection of Troyer citrange leaves; lesions were not observed on plants incubated at 15°C. No symptoms developed on the inoculated leaves of the second trial done in Nelspruit as well as the trial conducted in Stellenbosch. A breakthrough with the production of *P. citricarpa* ascospores in culture was achieved in Nelspruit in 2020, but these were produced only in very low numbers that were insufficient to conduct infection studies. Ascospore production is ongoing and being optimised to get enough ascospores for germination and infection studies.

##### **Opsomming**

Sitruswartvlek (SSV) is die belangrikste swamsiekte van sitrus in Suid-Afrika, weens sy kwarantynstatus in belangrike uitvoerlande. Epidemiologie van SSV word nie ten volle verstaan nie, aangesien daar tans geen metode is om tussen askospore van die SSV patoogeen en dié van die nie-patogeniese *P. capitalensis* te onderskei nie. Verder is werklike infeksie nog nie gemeet nie, hoewel generiese infeksie-modelle beskikbaar is. Die doelwitte van hierdie projek was die verbetering van ons begrip van CBS-epidemiologie, en om spoorvrystellings- en infeksie-modelle te verbeter. Baie van die tegniese uitdagende doelwitte is nie gehaal nie weens kapasiteitsbeperkings, meestal as gevolg van bedankings deur werkers. Die projek is uiteindelik van die Universiteit Stellenbosch na die CRI in Nelspruit verskuif. Sekere doelstellings is gedeeltelik bereik. Die eerste piknidiospore-inokulasieproef wat in Nelspruit uitgevoer is, is geëvalueer en daar is gevind dat 21 en 27°C die geskikste is vir infeksie van Troyer citrange blare; letsels is nie waargeneem op plante wat by 15°C geïnkubeer is nie. Geen simptome het ontwikkel in die tweede proef wat in Nelspruit gedoen is nie, sowel as die proef wat in Stellenbosch gedoen is. 'n Deurbraak met die produksie van *P. citricarpa* askospore in kultuur is in Nelspruit in 2020 behaal, maar dit is slegs in baie lae getalle geproduseer wat onvoldoende was om infeksie-studies uit te voer. Die produksie van askospore word voortgesit en word geoptimaliseer om genoeg askospore te kry vir ontkiemings- en infeksie-proewe.

#### 4.3.11 **PROGRESS REPORT: Characterization and management of Valley Bushveld citrus decline**

Project 1068 (RCE 2-07B) (2012/13 – 2020/21) by JM van Niekerk (CRI) and Elodie Stempien (USPP)

##### **Summary**

The Valley Bushveld decline syndrome has been studied extensively in the last five years. Characterization of the pathogens involved identified several, including four new, *Neocosmospora* species as being associated with the internal root and trunk decay symptoms seen in declining trees. As these pathogens are known to infect predisposed hosts, predisposing biotic and abiotic factors were investigated. These investigations revealed that high soil pH and EC probably play a role in predisposing trees planted on Carrizo citrange rootstock, which are sensitive to high soil pH and EC. This could also explain why orchards planted to Rough lemon rootstock do not suffer from decline problems. Finding alternative rootstocks is therefore seen as a long term solution to the decline

syndrome. A rootstock trial, including 10 different rootstocks, were therefore planted in the Sunday's River Valley. However, maintaining tree health in existing young orchards where the decline is expected to appear at some stage, is also important. Several treatment programmes with chemicals and natural plant extract products were therefore tested and it was seen that a benomyl drench treatment or a foliar spray treatment with specific plant extract products, are able to maintain or improve tree health to the same level as trees grown in preplant fumigated soil. These treatments will therefore be evaluated further in the rootstock trial. In the end, management of the Valley Bushveld decline will be a combination of specific rootstocks with treatments such as soil drench or foliar spray treatments.

## Opsomming

“Valley Bushveld Citrus Decline” is in die laaste vyf jaar intensief bestudeer. Karakterisering van die patogene betrokke, het verskeie *Neocosmospora* spesies, insluitend vier nuwe spesies, identifiseer as geassosieer met interne wortel-en stamverrotting simptome. Omdat hierdie patogene bekend is om verswakte gashere te infekteer, is potensiële predisponerende biotiese en abiotiese faktore ondersoek. Hierdie ondersoek het aangedui dat grond pH en EK (elektriese konduktiwiteit) waarskynlik 'n rol speel om bome wat op Carrizo citrange onderstam geplant is, te verswak en predisponeer. Hierdie onderstam is sensitief vir hoë grond pH en EK en dit kan ook verklaar hoekom boorde geplant met Growweskiisuurlemoen onderstam, nie probleme toon nie. 'n Alternatiewe onderstam word dus beskou as die langtermyn oplossing van die probleem. 'n Onderstam proef wat 10 verskillende onderstamme insluit is dus in die Sondagsriviervallei geplant. Die handhawing van boordgesondheid in bestaande jong boorde, waar die “decline” moontlik in die toekoms kan ontwikkel, is egter ook belangrik. Verskeie programme, gebaseer op chemiese en natuurlike plantekstrakprodukte, is dus getoets. Dit is bevind dat 'n benomiël grondbehandling of 'n blaarbespuitingsprogram met spesifieke plantekstrakprodukte, boomgesondheid op dieselfde vlak as bome geplant in berookte grond, kan hou. Hierdie behandelings sal dus verder in die onderstamproef getoets word. Uindelik sal die bestuur van “Valley Bushveld decline” 'n kombinasie van die gebruik van spesifieke onderstamme en boordbehandlings soos 'n grondtoediening of blaarbespuiting wees.

### 4.3.12 PROGRESS REPORT: Unravelling the clonal distribution of *Phyllosticta citricarpa* through a Genotyping-By-Sequencing approach

Project 1235 (2019 - 2022) by Aletta Bester-van der Merwe (SU), Paul Fourie; Elma Carstens (CRI); Megan Dewdney (University of Florida, USA)

## Summary

Knowledge of the population genetic structure of a given pathogen is critical for a comprehensive understanding of disease epidemiology. Molecular markers identified from whole-genome sequence data are a valuable resource to determine the distribution of genetic variation between isolates and populations, and can assist in the understanding of a pathogen's population dynamics. In this study we used high throughput sequencing (HTS) to investigate the genetic diversity within and between *Phyllosticta citricarpa* populations as well as to mine additional SSR markers.

To date, 4 029 SSRs have been identified and *in silico* genotyped in the USA and South African isolates. Seventeen USA isolates have been sequenced of which the genetic diversity has been assessed using *in silico* genotyping and variant calling. The results obtained confirm the clonal nature of the USA *P. citricarpa* isolates and *in silico* detection of mating types confirmed the presence of only one mating type in the USA isolates. A manuscript describing these results has been published: Extending the knowledge of *Phyllosticta citricarpa* population structure in USA with re-sequencing and genome wide analysis: *Physiological and Molecular Plant Pathology*, 113:101591.

We also sequenced and analysed (with the same approaches used for the USA isolates) HTS data from 65 *P. citricarpa* isolates from seven different countries to determine the genetic connectivity between these geographic

locations. In accordance with previously published results (Carstens *et al.* 2017), we found that isolates from China are most genetically distinct from other countries, while there is some degree of genetic connectivity among all other countries. The five South African provinces where citrus black spot is present, also show a genetic overlap between isolates sequenced (Carstens *et al.* 2017). Unfortunately, the DNA from Guarnaccia *et al.* (2017) (DNA from Italy, Malta and Greece) did not pass quality control and could not be sequenced, and no new *P. citricarpa* populations have been collected in South Africa and the USA.

Further work will include the in-depth study of the genetic diversity of *P. citricarpa* in the Eastern Cape, to investigate the clonality and contribution of asexual reproduction in this province. We will sequence six isolates from five orchards each located in the Eastern Cape (Carstens, 2018). Furthermore, the shifts in genetic composition and temporal diversity of two *Phyllosticta citricarpa* populations over two years will also be assessed, to investigate the clonality and contribution of asexual reproduction. A total of 32 isolates will be sequenced from one orchard in the North West and one orchard in Mpumalanga (Carstens, 2018).

The development of primers for the *P. citricarpa* B-tubulin gene and screening of isolates for a genome-wide association study in order to link variation to benzimidazole resistance are in currently progress.

## Opsomming

Kennis van die genetiese struktuur van 'n patoogeenpopulasie is van kritieke belang vir 'n omvattende begrip van siekte-epidemiologie. Molekulêre merkers wat geïdentifiseer word uit heel-genoomvolgorde data is 'n waardevolle bron om die verspreidingspatrone van en variante tussen isolate en populasies te bepaal, en kan help om die populasie dinamika van 'n patoogeen te verstaan. In hierdie studie het ons hoë deurset volgordebepaling (“high throughput sequencing”, HTS) gebruik om die genetiese diversiteit binne en tussen *Phyllosticta citricarpa* populasies te ondersoek, asook om addisionele SSR (“short sequence repeats”) merkers te myn.

Tot op hede is 4029 SSRs (“short sequence repeats”) geïdentifiseer en *in silico* genotipeer in die VSA en Suid-Afrikaanse isolate. Sewentien VSA-isolate se volgorde is bepaal en hul genetiese diversiteit beoordeel deur middel van *in silico* genotiperings en variant opsporing. Die resultate wat verkry is, bevestig die klonaliteit van die VSA *P. citricarpa* isolate en *in silico* genotiperings van paringstipes bevestig dat slegs een paringstipe in die VSA isolate voorkom. 'n Manuskrip wat hierdie resultate beskryf, is gepubliseer: Extending the knowledge of *Phyllosticta citricarpa* population structure in USA with re-sequencing and genome wide analysis: *Physiological and Molecular Plant Pathology*, 113:101591.

Ons het ook HTS-data van 65 *P. citricarpa* isolate vanaf sewe verskillende lande verkry en geanaliseer (met die dieselfde metodes as voorheen gebruik vir die VSA isolate) om die genetiese verbinding tussen hierdie geografiese liggings te bepaal. In ooreenstemming met voorheen gepubliseerde resultate (Carstens *et al.* 2017), het ons gevind dat isolate van China geneties onderskei kan word van ander lande, terwyl daar 'n mate van genetiese konnektiwiteit tussen alle ander lande is. Die vyf Suid-Afrikaanse provinsies waar sitrus swartvlek voorkom, toon ook 'n genetiese oorvleueling tussen die isolate wat bestudeer is (Carstens *et al.* 2017). Ongelukkig het die DNA van Guarnaccia *et al.* (2017) (DNA van Italië, Malta en Griekeland) nie gehaltebeheer geslaag nie en kon dus nie ingesluit word nie. Geen nuwe *P. citricarpa* bevolkings is in Suid-Afrika en die VSA versamel nie.

Verdere werk sal die diepgaande studie insluit van die genetiese diversiteit van *P. citricarpa* in die Oos-Kaap, om die klonaliteit en bydrae van ongeslagtelike voortplanting in hierdie provinsie te ondersoek. Ons sal die volgordes bepaal van ses isolate van vyf boorde wat elk in die Oos-Kaap geleë is (Carstens, 2018). Verder word die verskuiwings in genetiese samestelling en temporale diversiteit van twee *P. citricarpa*-populasies oor twee jaar beoordeel om die klonaliteit en bydrae van ongeslagtelike voortplanting ook in hierdie provinsie te ondersoek. Die volgordes van altesaam 32 isolate sal bepaal word, van een boord in Noordwes en een boord in Mpumalanga (Carstens, 2018).

Die ontwikkeling van inleiers vir die *P. citricarpa* B-tubulien geen en evaluasie van isolate vir 'n genoom wye assosiasie studie om variasie aan bensimidiasool weerstandigheid te koppel, is aan die gang.

#### 4.3.13 **PROGRESS REPORT: The evaluation of different pre-plant products for the control of the citrus nematode, as part of an integrated nematode control approach in citrus replant situations**

Project 762 by Jan van Niekerk and MC Pretorius (CRI)

##### **Summary**

The aim of this project is to find pre-plant treatments that are effective in keeping orchard soils free from citrus nematode and *Phytophthora* spp. for as long as possible after planting. The trial has been going since January 2010. The various treatments were applied prior to planting in January 2010 with some treatments still being applied annually in January and November. Tree stem diameter, tree height, nematode soil and root analysis, *Phytophthora* status in the soil, and a visual tree rating, are the parameters that have been monitored annually since the start of the trial. To date no treatment has stood out in terms of nematode control results. However, based on tree height and trunk diameter measurements, the pre-plant fumigation treatments with 1,3 dichloropropene and metham sodium are starting to stand out. The trees in these treatments are taller with thicker trunks compared to the cadusafos and control treatments. It is therefore becoming clear that pre-plant soil fumigation in a replant situation does improve tree growth in comparison to no treatment or post-plant treatments.

##### **Opsomming**

Die doel van hierdie projek is om vóór-plant behandelings te vind wat effektief is om boordgronde vry van sitrus aalwurm en *Phytophthora* spp. te hou vir so lank as moontlik ná plant. Die proef is al sedert Januarie 2010 aan die gang. Die verskeie behandelings is vóór plant in Januarie 2010 toegedien, terwyl sommige behandelings steeds jaarliks in Januarie en November toegedien word. Boomstamdeursnit, boomhoogte, aalwurm grond- en wortel-analise, *Phytophthora* status in die grond, en 'n visuele boomgradering, is die parameters wat jaarliks sedert die begin van die proef gemonitor word. Tot op datum het geen behandeling in terme van aalwurmbeheerresultate uitgestaan nie. Gebaseer op boomhoogte en stamdeursnitmetings, begin die vóór-plant berokingsbehandelings met 1,3 dichloropropen en metam sodium uitstaan. Die bome in hierdie behandelings is langer met dikker stamme in vergelyking met die cadusafos en kontrole behandelings. Dit word dus duidelik dat vóór-plant grondberoking in 'n herplant situasie boomgroei verbeter in vergelyking met geen behandeling of ná-plant behandelings.

#### 4.3.14 **PROGRESS REPORT: Comparison of the rind phytochemistry and wax composition of CBS resistant and susceptible citrus cultivars.**

Project 1242 / RCE 2-13 (2019/20) by Wilma du Plooy (CRI) and Wilma Augustyn (TUT)

##### **Summary**

This is a study into the volatile and non-volatile secondary metabolites from the rind of lemon (*Citrus limon*), Valencia (*Citrus sinensis*) and Bitter Seville (*Citrus aurantium*). A comprehensive survey through all developmental stages from 2018 and 2019 seasons is being conducted. Two M.Tech. students joined the project in March 2019 and all pre-2019 samples were reanalysed to ensure consistency and continuance of the analytical methods used. Volatile fractions were analysed by GC-MS to identify metabolites as well as variation in metabolomics profiles of the three varieties at different developmental stages of the fruit. Non-volatile fractions were analysed by HPLC and the individual fractions thereafter analysed by UPLC-MS to obtain the metabolomic profiles of the three citrus varieties. The major volatile components identified by gas chromatography were limonene and ethanol. Minor components with relatively higher levels (higher than 1% relative to the major peak) are 2,4-bis(1,1-dimethylethyl)-phenol in Bitter Seville, myrcene and linalool in Valencias,  $\beta$ -pinene,  $\gamma$ -terpinene and citral in lemons. All chromatography data were exported to SIMCA P+ (13.0) software (Umetrics, Sweden) and used to construct chemometric models. Principal component analysis (PCA) scores plot of gas chromatography data demonstrated

that the metabolomics profiles of the volatiles were clustered into three main classes, according to variety. Separation into clusters indicate chemical compositional differences between the clusters. Within variety group separation was seen corresponding to developmental stages of the fruit. Two sets of ripe and colour break fruit were sampled, one set with visible CBS infection and the other with no visible infection. Lemons separated into two groups: one group closely clustering to Valencias contained CBS-infected fruit at both colour break and full ripeness. The second group clustered separately and contained all the other stages of lemon, enabling a clear chemical distinction between infected and uninfected lemons. An orthogonal projection to latent structures-discriminant analysis (OPLS-DA) model was subsequently constructed from HPLC data to identify the variables responsible for class discrimination. The separation can evidently be attributed to differences in the phenolic composition of the various citrus samples. Tentative identification using UPLC-QToF-MS analysis was made of these compounds responsible for the phytochemical separations of the three citrus varieties. Two compounds are very prominent on the Bitter Seville chromatograms, and absent on the chromatograms of Valencias and lemon and may play a role in the observed tolerance of Bitter Seville against CBS infection. The compound identified as a biomarker at 8.89 min (5.04 min in UPLC chromatogram) has tentatively been identified as naringin (naringenin-7-O-neohesperidoside) and the compound with retention time 13.4 min (5.85 min on UPLC chromatogram) as hesperitin-7-O-rhamnoside. However, the identities of these compounds must be confirmed by certified standards.

## Opsomming

Hierdie is 'n studie na die vlugtige en nie-vlugtige sekondêre metaboliete uit die skil van suurlemoen (*Citrus limon*), Valencia (*Citrus sinensis*) en Bitter Seville (*Citrus aurantium*). 'n Volledige reeks verteenwoordigend van alle ontwikkelingstadiums is gedurende die 2018 en 2019 seisoene gebruik. Twee M.Tech. studente het in Maart 2019 aangesluit by die projek en all pre-2019 monsters word oorgewerk om eenvormigheid en kontinuïteit van die analitiese metodes te verseker. Die vlugtige fraksies was deur middel van GC-MS ontleed om sodoende die metaboliete te identifiseer, asook om die variasie in die metabolomiese profiele van die drie tipes teen verskillende ontwikkelingstadiums te bepaal. Die nie-vlugtige fraksies is deur middel van HPLC geanaliseer, waarna die individuele fraksies met UPLC-MS ontleed was om die metabolomiese profiele van die drie tipes te verkry. Die hoof vlugtige komponente soos deur gaschromatografie bepaal, was limoneen en etanol. Mindere komponente met relatiewe hoë vlakke (meer as 1% relatief tot die hoofpiek) was 2,4-bis(1,1-dimetietiel)-fenol in Bitter Seville, mirseen en linalool in Valencia,  $\beta$ -pineen,  $\gamma$ -terpineen en citral in suurlemoene. Die chromatografie data was volledig oorgeplaas na SIMCA P+ (13.0) sagteware (Umetrics, Sweden) en gebruik om die chemometriese modelle mee te bou. Hoofkomponent analiese (PCA) punte grafieke van die gaschromatografiese data het gedemonstreer dat die metabolomika profiele van die vlugtige komponente in drie hoofklasse gegroepeer word, volgens sitrus variëteit. Hierdie skeiding het verder die chemiese samestellingsverskille tussen die groepe bevestig. Binne elke variëteit kon daar verder ook onderskei word tussen ontwikkelingstadiums. Twee stelle vrugte by kleurbreek en volryp was ondersoek: een stel met swartvlekletsels en een stel sonder enige sigbare letsels. Suurlemoene het in twee groepe verdeel: die een groep met swartvlekletsels in beide kleurbreek en volrypheid het naby die Valencias gegroepeer. Die tweede groep het al die ander stadiums van suurlemoene bevat, wat daarop dui dat duidelike chemiese onderskeiding tussen geïnfekteerde en nie-geïnfekteerde suurlemoene getref kan word. Gevolglik was 'n ortogonale projeksie op latente struktuur-diskriminant analiese (OPLS-DA) model gebou vanaf die HPLC data ten einde die veranderlikes wat verantwoordelik is vir die klasonderskeiding, te bepaal. Hieruit was afgelei dat skeiding toegeskyf kan word aan verskille in die fenoliese samestelling van die verskillende sitrusmonsters. Voorlopige identifikasie van die komponente wat lei tot die fitochemiese onderskeiding van die drie sitrustipes, is met behulp van UPLC-QToF-MS analiese gedoen. Twee komponente was prominent op die Bitter Seville chromatogramme, maar afwesig in Valencia en suurlemoene. Hierdie komponente mag 'n rol speel in die weerstandigheid van Bitter Seville teen swartvlek infeksie. Die komponent op die biomerker by 8.89 min (5.04 min in UPLC chromatogram) is voorlopig geïdentifiseer as naringien (naringenin-7-O-neohesperidosied), terwyl die biomerker met 'n retensietyd van 13.4 min (5.85 min op die UPLC chromatogram) geïdentifiseer is as hesperitin-7-O-rhamnosied. Die identiteit van die twee verbindings sal met gesertifiseerde standaard bevestig word.

#### 4.3.15 **PROGRESS REPORT: Epidemiology and management of *Botrytis cinerea* in citrus** Project 1236 (RCE 2-11) (2019/20 – 2020/21) by Dr Cheryl Lennox (SU)

##### **Summary**

When lemon blossoms are infected with *Botrytis cinerea*, rind distortion occurs on juvenile fruitlets. This cosmetic disease has a negative effect on return to producers as fruit get downgraded due to the cork-like scars on the surface of mature fruit. There is currently no way of predicting when blossoms will be infected and there is no fungicide registered to control *B. cinerea* on citrus. Thus, the aim of this study was to validate a risk prediction model for *B. cinerea* on citrus, developed by Citrus Research international, and to evaluate the efficacy of botryticides in controlling *B. cinerea* on lemons.

The fungicides azoxystrobin, fenhexamid, fludioxonil, iprodione, pyrimethanil, and benomyl are highly effective in controlling *B. cinerea* on other crops. Therefore, *B. cinerea* isolates from three lemon orchards not previously exposed to fungicides, were tested *in vitro* against previously determined discriminatory doses of the above-mentioned fungicides in mycelial growth assays. An orchard trial was conducted with recommended dosages of formulated fungicides containing either azoxystrobin, fenhexamid, iprodione, or benomyl. The risk prediction model was evaluated by sampling blossoms and comparing the incidence of blossom colonization to the predicted risk. Weather data from the region was analysed to determine which parameters lead to higher *B. cinerea* blossom colonization. In the mycelial growth assays most isolates from the three regions were highly sensitive at the discriminatory doses. In total, 70% of the isolates were highly sensitive to azoxystrobin, 98% of isolates were highly sensitive to fenhexamid. All 60 isolates were highly sensitive to fludioxonil and iprodione, while 70% of isolates were highly sensitive to pyrimethanil and benomyl, respectively. Isolates from the Citrusdal region had the highest fungicide sensitivity, followed by isolates from the Jonkershoek and Franschoek areas. However, eight isolates from both the Jonkershoek and Franschoek areas were highly resistant to benomyl.

In the orchard trial, benomyl had the highest efficacy and reduced the incidence of blossom infection by 53.85%, compared to that of the untreated control. Azoxystrobin, fenhexamid, and iprodione, reduced the incidence by 33.33%, 30.77%, and 3.21%, compared to that of the untreated control, respectively.

Due to the similar incidence of *B. cinerea* colonization of lemon blossoms during a predicted medium risk of infection and predicted low risk of infection, the prediction model could not be validated. During a predicted low risk in Citrusdal, colonization of 43% was recorded, compared to 47% for a medium risk of infection. In Jonkershoek, the colonization differed between 3%, 39%, and 58%, all for predicted medium risk periods.

Fenhexamid, azoxystrobin, and benomyl were highly effective in controlling *B. cinerea* on blossoms. However, as benomyl residues are not accepted in the EU, future studies should focus on evaluating higher dosages of azoxystrobin and fenhexamid. It is also recommended that the trial be repeated in different regions to evaluate the efficacy of the fungicide under different conditions, as it was seen in the *in vitro* study that the sensitivity of isolates to the fungicides differed between regions. The risk prediction model needs further validation. The model should be validated over several years, as the risk of infection might be medium for the whole flowering period in one year and low or high the next. It is also recommended that the model is tested in conjunction with fungicide applications, after it has been verified. This will give a clearer indication as to whether the occurrence of rind distortion can be reduced when fungicides are applied according to the model.

##### **Opsomming**

Wanneer suurlemoen bloeisels geïnfekteer word deur *Botrytis cinerea* kom skilverwringing voor op jong vrugte. Dit veroorsaak 'n kosmetiese-siekte wat 'n negatiewe finansiële invloed het, omdat die vrugte af gegradeer word weens die kurkagtige letsels op die volwasse vrug. Dit is tans onmoontlik om te voorspel wanneer bloeisels geïnfekteer word en daar is ook geen swamdoder geregistreer vir die beheer van *B. cinerea* op sitrus nie. Die doel

van hierdie studie was dus om 'n siektevoorspelling model wat deur Citrus Research International ontwikkel is vir *B. cinerea* op sitrus, te valideer en om die effektiwiteit van botrytisdoeders te evalueer in die beheer van *B. cinerea* op suurlemoene.

Die swamdoders asoksistrobien, fenheksamied, fludioxonil, iprodioon, pirimetaniel en benomil is hoogs effektief in die beheer *B. cinerea* op ander gewasse. Daarom is *B. cinerea* isolate van drie suurlemoen boorde wat nog nie voorheen aan swamdoders blootgestel is nie, in vitro getoets teen voorheen bepaalde diskriminerende dosisse van die bogenoemde swamdoders, in miselium groei-toetse. 'n Veldproef is ook uitgevoer met die aanbevole dosisse van geformuleerde swamdoders wat asoksistrobien, fenheksamied, iprodioon en benomil as aktiewe bestanddeel bevat. Die risiko voorspellingsmodel is geëvalueer deur bloeisel monsters te neem en die voorkoms van bloeisel kolonisasie te vergelyk met die voorspelde risiko. Weer-data van die streke is ook geanaliseer om die parameters vas te stel wat tot hoër voorkoms van kolonisasie lei.

Die miselium groei-toetse het gewys dat 'n meerderheid van die isolate vanuit die drie streke hoogs sensitief was vir die diskriminerende dosisse. In totaal was 70% van die isolate hoogs sensitief vir asoksistrobien, 98% van die isolate was hoogs sensitief vir fenheksamied, en al 60 isolate was hoogs sensitief vir fludioxonil en iprodioon. Verder was 70% van die isolate hoogs sensitief vir pirimetaniel en benomil. Isolate van die Citrusdal area het die hoogste sensitiwiteit getoon, gevolg deur isolate van die Jonkershoek en Franschoek areas. Agt isolate van die Jonkershoek area, asook agt isolate van die Franschoek area was hoogs weerstandbiedend teen benomil. Benomil het die hoogste effektiwiteit gehad in die veldproef en die voorkoms van geïnfekteerde bloeisels met 53.85% verminder, in vergelyking met die onbehandelde kontrole. Asoksistrobien, fenheksamied en iprodioon het onderskeidelik die voorkoms met 33.33%, 30.77%, en 3.21% verlaag, in vergelyking met die onbehandelde kontrole.

Aangesien die *B. cinerea* bloeisel infeksie dieselfde was tydens 'n voorspelde medium risiko en 'n voorspelde lae risiko infeksieperiode, kon die model nie geverifieer word nie. Tydens 'n voorspelde lae risiko vir infeksie in die Citrusdal area was kolonisasie van 43% waargeneem, in vergelyking met 47% voorkoms van kolonisasie wat tydens 'n medium risiko voorspelling waargeneem is. In die Jonkershoek area het die voorkoms van infeksie tussen 3%, 39%, en 58% verskil vir medium risiko infeksieperiodes.

Fenheksamied, asoksistrobien en benomil het *B. cinerea* op bloeisels beheer. Benomil residue word egter nie aanvaar in die EU nie en toekomstige studies moet daarop fokus om hoër dosisse van asoksistrobien en fenheksamied te evalueer. Dit word ook aanbeveel dat die proef in verskeie streke herhaal word om die effektiwiteit onder verskillende toestande te evalueer, omdat die in vitro studie getoon het dat die sensitiwiteit van isolate verskillend is vir die verskeie streke. Die risiko voorspellingsmodel benodig verdere ontwikkeling. Die model moet ook oor 'n aantal jare geëvalueer word, omdat die risiko van infeksie medium kan wees vir die hele blomtydperk in een jaar, en laag of hoog gedurende die volgende. Dit word ook voorgestel dat die model getoets word te same met swamdoder toedienings, nadat dit geverifieer is. Dit sal aandui of die model die voorkoms van skilverwringing verlaag wanneer swamdoders aangewend word volgens die model.

#### **4.3.16 FINAL REPORT: Evaluation of reduced volume fungicide and pesticide sprays for control of citrus black spot and false codling moth.**

Project 1132 (2014/15 – 2020/21) by Jan van Niekerk (CRI), Gideon van Zyl, Philip Rebel (ProCrop) and Paul H. Fourie (CRI)

#### **Summary**

Citrus trees are susceptible to a wide range of insect pests and fungal diseases. Spray application of plant protection products (PPPs) in citrus production is commonly done using pre-determined application methodologies without taking target pest, orchard and sprayer characteristics into account. This results in the use of common high volume spray application which can be wasteful due to loss of water and PPPs from run-off and drift. High spray

volumes have become an industry norm in South Africa, ranging from 6 000 – 16 000 L/ha; much higher compared with other citrus producing countries. Previous spray deposition research has demonstrated the potential of reduced spray volumes but highlighted the importance of canopy management to improve penetration. The biological efficacy of reduced volume sprays was evaluated in various spray trials in the Limpopo and Western Cape provinces. Trials were done on different citrus types on commercial citrus producing farms. Spray deposition parameters and pest and disease control, following spray programmes applied at reduced volumes (750 to 3 000 L/ha) were compared with the farm's standard spraying volume (4 000 to 9 000 L/ha). Deposition quantity (FPC%) results obtained in these trials, showed that the reduced volume spray applications generally achieved higher deposition quantity values on fruit and leaves in comparison with the high volume applications. However, the higher spray volumes achieved better deposition uniformity results (CV%) and had higher biological control in terms of clean fruit. Therefore, when comparing the biological efficacy and the spray deposition results, it was seen that deposition uniformity strongly influenced the biological efficacy. These results furthermore indicated that lower volume applications should be optimised to improve the deposition uniformity (CV%) and quality (ICD%). Trials were consequently done in mature Nules Clementine and Palmer navel orchards using tree row volume (TRV) to determine spray volumes. In the trial on Nules Clementines, the TRV based volumes were applied at two air volumes of 48 000 m<sup>3</sup>/h and 36 000 m<sup>3</sup>/h using a Rovic & Leers Even Flow machine. These spray volume and air volume combinations were compared with the 12 500 L/ha used by the farm and applied with a Nieuwoudt spray machine. Results from the Nules trial indicated that variation in air volume did not influence deposition parameters on leaves and fruit. The deposition parameters were also not significantly influenced by the range of volumes evaluated. Deposition parameters achieved with the 1/2 and 1/3x spray application (in relation to the 1x TRV application) indicate promise and were therefore tested in the trial on Palmer navel oranges, also including biological efficacy. In the navel trial 2x and 3x TRV, applied with a Rovic & Leers citrus sprayer, was evaluated in comparison to the standard farm application of 10 000 L/ha, applied using a Nieuwoudt spray machine. Results indicated that the 2x and 3x TRV volumes in terms of deposition parameters at the different canopy positions, performed better than the 10 000 L/ha application. Unfortunately, due to low pest pressure, no conclusions could be made on the biological efficacy of the lower volumes versus the 10 000 L/ha application. However, based on results thus far, it is evident that TRV has potential to be used as a method to reduce spray volumes and adapt it better to canopy characteristics.

## Opsomming

Sitrusbome is vir 'n wye reeks insekplae en swamsiektes vatbaar. Spuittoediening van plantbeskermingsprodukte (PBP's) in sitrusproduksie word algemeen gedoen deur gebruik te maak van vooraf-bepaalde toedieningsmetodologieë sonder om teikenplaag, boord en spuitkenmerke in ag te neem. Dit lei tot die gebruik van algemene hoë volume spuittoediening wat tot vermorsing kan lei weens verlies aan water, en PBP's, weens afloop en drywing. Hoë spuitvolumes het 'n norm in die bedryf in Suid-Afrika geword, met reekse van 6 000 – 16 000 L/ha; baie hoër in vergelyking met ander sitrusproduserende lande. Vorige spuitneerlegging navorsing het die potensiaal van verminderde spuitvolumes getoon, maar het die belang van lowerbestuur uitgelig ten einde penetrasie te verbeter. Die biologiese doeltreffendheid van verminderde volume spuite is in verskeie spuitproewe in die Limpopo- en Wes-Kaap-provinsies geëvalueer. Proewe is op verskillende sitrustipes op kommersiële sitrusproduserende plase gedoen. Spuitneerlegging parameters en plaag- en siektebeheer wat op spuitprogramme gevolg het, wat teen verminderde volumes (750 tot 3 000 L/ha) toegedien is, is met die plaas se standaard spuitvolume (4 000 tot 9 000 L/ha) vergelyk. Neerleggingshoeveelheid (FPC%) resultate wat in hierdie proewe verkry is, het getoon dat die verminderde volume spuittoedienings oor die algemeen hoër neerleggingshoeveelheid waardes op vrugte en blare bereik het, in vergelyking met die hoë volume toedienings. Die hoër spuitvolumes het egter beter neerleggingsuniformiteit resultate (CV%) bereik, asook hoër biologiese beheer in terme van skoon vrugte. Wanneer die biologiese doeltreffendheid en die spuitneerlegging resultate egter vergelyk word, is gesien dat neerleggingsuniformiteit sterk die biologiese doeltreffendheid beïnvloed. Hierdie resultate het verder aangedui dat laer volume toedienings geoptimaliseer moet word ten einde die neerleggingsuniformiteit (CV%) en kwaliteit (ICD%) te verbeter. Proewe is gevolglik in volwasse Nules Clementine en Palmer nawelboorde gedoen deur boom-ry-volume (BRV) te gebruik om spuitvolumes te bepaal. In die proef

op Nules Clementines is die BRV-gebaseerde volumes toegedien teen twee lugvolumes van 48 000 m<sup>3</sup>/h en 36 000 m<sup>3</sup>/h deur gebruik te maak van 'n Rovic & Leers Even Flow masjien. Hierdie spuitvolume en lugvolume kombinasies is met die 12 500 L/ha wat deur die plaas gebruik word, en met 'n Nieuwoudt spuitmasjien toegedien word, vergelyk. Resultate vanaf die Nules proef het aangetoon dat variasie in lugvolume nie neerleggingsparameters op blare en vrugte beïnvloed het nie. Die neerleggingsparameters is ook nie betekenisvol beïnvloed deur die reeks volumes wat geëvalueer is nie. Neerleggingsparameters wat met die 1/2 en 1/3x spuittoediening (in verhouding tot die 1x BRV toediening) verkry is, toon belofte, en is dus in die proef op Palmer nawellemoene getoets. Biologiese doeltreffendheid is ook ingesluit. In die nawelproef is 2x en 3x BRV, toegedien met 'n Rovic & Leers sitrusspuit, teenoor die standaard plaas toediening van 10 000 L/ha, toegedien deur gebruik te maak van 'n Nieuwoudt spuitmasjien, geëvalueer. Resultate het aangetoon dat die 2x en 3x BRV volumes in terme van neerleggingsparameters by die verskillende lowerposisies, beter gevaar het as die 10 000 L/ha toediening. Ongelukkig kon geen gevolgtrekkings rakende die biologiese doeltreffendheid van die laer volumes teenoor die 10 000 L/ha toediening gemaak word nie, weens lae plaagdruk. Gebaseer op resultate so ver, is dit egter duidelik dat BRV die potensiaal het om gebruik te word as 'n metode om spuitvolumes te verminder en dit beter by lowerkenmerke aan te pas.

#### 4.4 **PROGRAMME: POSTHARVEST DISEASES**

Programme coordinator: Wilma du Plooy (CRI)

##### 4.4.1 **Programme summary**

In the service project (4.4.5), a total of five newly proposed products were tested to be evaluated against an industry standard (calcium hypochlorite). The most successful of these products were three new technology products, namely Peroxil, a micronised silver suspension, a nano-encapsulated oxygen enriched product called Flowsafe, and a dry PAA in tablet form, called Oxywash. All of these products still have to address regulatory hurdles in 2020 before commercialisation in the citrus industry. The results obtained, however, do encourage further development of the actives. No ring tests were conducted, but the tests for resistance monitoring in collaboration with the Diagnostic Centre were successful. The work done in 2020 was not presented at the online Packhouse Workshop in 2021 and will be included in the presentation at the 2022 workshops, should that be feasible.

The investigation of the fungal contamination on wooden pallet bases used for the export of fresh fruit (4.4.2), was concluded. The apparently unknown source of 2-ortho-phenylphenol contamination was identified as being the pallets themselves. The degradation of pallet bases, when consignments reached their market, raised questions on the quality of the wood preservatives used in the manufacturing of these bases. After the withdrawal of methyl bromide, OPP became the treatment most widely used by pallet manufacturers. This practice, unfortunately, resulted in residue issues on European export markets. We were able to confirm that the contribution of packhouse storage methods to the fungal contamination of the bases were negligible. However, fungal decay was a hazard with the highest priority due to the weakening of the structural strength of the pallets, and the accompanying deterioration of the cartons. Heavily contaminated pallets are considered a point source of fungal spore dissemination that will increase the spore load in containers and cold rooms, and presented a phytosanitary concern. This study looked at the microbiome on contaminated pallet bases, manufacturing aspects of the bases, the role of storage at packhouses, options for wood treatment, as well as possible contribution from the shipping containers and cold storage facilities to the degradation of wooden pallet bases. Three successful replacement products for fungal control were identified, with two fully commercialised.

Evaluation of new postharvest fungicides for the control of *Phytophthora* brown rot (4.4.3) was also concluded. Fungicide management of brown rot in South Africa currently consists only of preharvest strategies and nothing is registered for postharvest management of this disease. In this study the curative and protective efficacy of azoxystrobin (1 125 µg/ml), fludioxonil (598 µg/ml), ammonium and potassium phosphite (1 500 µg/ml) as aqueous dip treatments for the postharvest management of *Phytophthora* brown rot on different citrus types (lemons,

oranges and mandarins), was evaluated. Additionally, wax amended with azoxystrobin (2 500 µg/ml for all three fruit types), and wax amended with fludioxonil (2 300 µg/ml for lemons and 4 600 µg/ml for oranges and mandarins) was evaluated for the prevention of spreading of brown rot (nesting) within cartons during transit. Results indicated that the three tested fungicides have good curative action if applied within 12 h after inoculation. Applications done 24 h after inoculation also provided some curative action but not as effective as earlier applications. Azoxystrobin and potassium phosphite furthermore provided very good protection against infection up to 48 h after application on all three fruit types, but fludioxonil did not fare as well. The protective ability of all three fungicides improved the longer the fungicides remained on the fruit before inoculation. Trials aimed at prevention of nesting during transit indicated that only azoxystrobin amended wax significantly reduced brown rot from spreading to healthy fruit when in contact, compared to the control. The data obtained from this study can add additional value to the already registered postharvest azoxystrobin and fludioxonil fungicides and preharvest registered potassium and ammonium phosphite.

In Project 1250 (4.4.4) imazalil (IMZ) was used as the industry standard against which all other currently registered actives were measured for efficacy in controlling *Penicillium digitatum*. The application of alternative chemicals that are usually considered as resistant management strategies, is being done for use in primary green mould control. The well-known actives are pyrimethanil (PYR), fludioxonil (FLU), 2-orthophenyl phenol (OPP) and thiabendazole (TBZ). Azoxystrobin (AZO) was registered as a postharvest fungicide in 2018, and has had limited use in packhouses since then. It is known that none of these five actives are as effective as IMZ at the conditions that allow optimum action by IMZ. This study is therefore aimed at optimising the packhouse parameters that would allow the most effective action of these alternative chemicals. The actives were tested on lemons, navels and Nadorcotts, at three different temperatures each. Every active has also been tested in combination with every other active. The best combinations are currently being optimised, while an investigation into GRAS chemicals is included.

A pilot study and a semi-commercial study (4.4.6) was conducted in which PYR, FLU and AZO were tested in a wax formulation as alternatives to IMZ. These fungicides were also tested in combination with one another, in the wax. The study focussed on the protective and sporulation inhibition qualities of the amended wax. Azoxystrobin performed best with a high protective control action at the registered concentration on citrus, while sporulation was largely inhibited at the registered concentration of this fungicide. Fludioxonil also had potential in terms of protective control, as well as sporulation inhibition. Fludioxonil performed well with a high protective control and sporulation inhibition at its registered concentration on citrus. Overall, pyrimethanil had the lowest protective control and sporulation inhibition. Maximum residue levels by EU standards were not exceeded.

### **Programopsomming**

In die diensprojek (4.4.5) was vyf nuut-voorgestelde produkte ge-evalueer teen die industrie se standaard saniteermiddel (kalsiumhipochloriet). Drie van die produkte was suksesvol, naamlik Peroxil (’n mikrogeneerde silversuspensie), Flowsafe (nano-geenkapsuleerde, suurstofverrykte produk) en Oxywash (droeë PAA in tabletvorm). Al die produkte moes regulatories struikelblokke oorkom gedurende 2020 voordat hulle kommersieël beskikbaar gestel kon word. Die resultate van die drie produkte reegverdig egter die verdure ontwikkeling van die produkte. Geen ringtoetse was gedoen nie, maar die weerstandsmoitering in samewerking met die diagnostiese sentrum het voortgegaan. Die werk wat in 2020 gedoen was, was nie by die 2021 aanlyn pakhuiswerkswinkel aangebied nie. Indien dit uitvoerbaar is, sal dit saam met die 2022 resultate ingesluit word.

Die ondersoek na die swamkontaminasie van hout palletbassise (4.4.2) is afgehandel. Die oënskynlik onbekende bron van 2-orto-fenielfenol (OPP) was die palette self. Die agteruitgang van die gekontamineerde palletbassise wanneer dit uiteindelik op die uitvoermarkte aangekom het, het vroe laat ontstaan oor die kwaliteit van die houtpreservering wat gebruik was in die vervaardiging van die palette. Metielbromied was vantevore wyd gebruik, maar na die onttrekking van die gebruik daarvan, was OPP die mees algemene chemie wat aangewend was. Hierdie gebruik het egter residue tot gevolg gehad, wat gelei het tot probleem op uitvoermarkte in Europa. Die

studie het vereder bewys dat die bergingspraktyke van die pakhuis nie noemenswaardige bydra lewer tot die swamgroeï nie. Die hoogste prioriteit was egter die gevaar wat swamverrotting inhou, weens die rol wat dit in die strukturele vereswakking van die hout speel, asook die gepaardgaande skade aan die kartonne. Swaar-gekontameneerde pallette word ook beskou as 'n puntbron vir swamspoorvrystelling wat die spoorlading in die behoueringseenhede en kouekamers verhoog, met 'n gepaardgaande fitosanitêre risiko. Hierdie studie het die mikrobiom op gekontameneerde palletbassise, asook vervaardigingsaspekte, die rol van stoorpraktyke by pakhuis, opsies vir houtbehandelings, asook die bydraes van verskepingshouers en kouestoorfasiliteite, bestudeer. Drie produkte wat suksesvolle beheer op swamgroeï kon uitoefen was geïdentifiseer, met twee volledig gekommersialiseer.

Evaluasie van nuwe na-oesfungisiede vir die beheer van *Phytophthora* bruinvrot (4.4.3), is ook afgehandel. Fungisiedbestuur van bruinvrot in Suid-Afrika bestaan tans slegs uit voor-oes strategieë, en niks is vir die na-oes bestuur van hierdie siekte geregistreer nie. Die doelwit van hierdie studie was om die uitwissende en beskermende effektiwiteit van azoxystrobin (1 125 µg/ml), fludioxonil (598 µg/ml), ammonium- en kaliumfosfiet (1 500 µg/ml) as waterige doopbehandelings vir die na-oes bestuur van *Phytophthora* bruinvrot op verskillende sitrustipes (suurlemoene, lemoene en mandaryne) te evalueer. Addisioneel, is waks gewysig met azoxystrobin (2 500 µg/ml vir al drie vrugtipies) en waks gewysig met fludioxonil (2 300 µg/ml vir suurlemoene en 4 600 µg/ml vir lemoene en mandaryne) vir die voorkoming van die verspreiding van bruinvrot kruiskontaminasie binne kartonne gedurende vervoer, geëvalueer. Resultate het aangetoon dat die drie getoetste fungisiedes goeie uitwissende aksie gehad het, wat die voorkoms van bruinvrot betekenisvol verminder het wanneer die fungisied 12 ure ná inokulasie toegedien is. Toedienings wat 24 ure ná inokulasie gedoen is, het ook 'n mate van uitwissende aksie verskaf, maar nie so effektief soos vroeër toedienings nie. Azoxystrobin en kaliumfosfiet het verder baie goeie beskerming teen infeksie op al drie vrugtipies verskaf tot 48 ure ná toediening, maar fludioxonil het nie so goed gevaar nie. Interessantheidshalwe, die beskermende vermoë van al drie fungisiedes het verbeter langer die fungisiedes op die vrug gebly het. Proewe wat die voorkoming van kruiskontaminasie gedurende vervoer ten doel gehad het, het aangedui dat azoxystrobin gewysigde waks die verspreiding van bruinvrot na gesonde vrugte betekenisvol na kontak verminder het, in vergelyking met die kontrole. Die data wat vanuit hierdie studie verkry is, kan addisionele waarde tot die reeds geregistreerde na-oes azoxystrobin en fludioxonil fungisiedes en voor-oes geregistreerde ammonium – en kaliumfosfiet toevoeg.

In Projek 1250 (4.4.4) was imazalil (IMZ) as die industrie-standaard gebruik om die ander geregistreerde aktiewes teen te meet. Die evaluasie van die ander aktiewes wat as alternatiewes in die geval van weerstandbiedendheid gehou was en nooit vir primêre groenskimmelbeheer gebruik was nie, was gedoen. Die bekende aktiewes is pirimetaniel (PYR), fludioksonil (FLU), 2-ortofenielfenol (OPP) en tiabendasool (TBZ). Verder was azoxystrobin in 2018 geregistreer as 'n na-oesfungisied, maar word dit nog nie op groot skaal in pakhuis gebruik nie. Dit is bekend dat nie een van hierdie vyf aktiewes die besondere effektiwiteit het van IMZ onder die toestande waarteen IMZ suksesvol aangewend word nie. Hierdie ondersoek is dus gerig op optimisering van die pakhuisparameters waarteen hierdie alternatiewe wel die mees effektiewe werking sal kan hê, asook moontlike kombinasies om deur sinergisme die effektiwiteit te verbeter. Die aktiewes was getoets op suurlemoene, navel en Nadorcotts, teen drie verskillende temperature. Elkeen van die individuele aktiewes is ook getoets teen elke ander aktief. Die beste kombinasies word tans geoptimeer, en word verdere ondersoek na GRAS kombinasies ook geloods.

In 'n loodsstudie, asook 'n semi-kommersiële studie (4.4.6) was PYR, FLU en AZO in 'n wasformulering getoets as alternatiewe vir IMZ. Hierdie swamdoders was ook in kombinasie met mekaar getoets. Die studie het gefokus op beskermende beheer en teen-sporuleringsbeheer. Azoxystrobin het die meeste potensiaal ten opsigte van beskermende beheer, sowel as sporulasie-remming. Fludioxonil presteer wel met 'n hoë beskermende effek en sporulasie inhibisie teen sy geregistreerde konsentrasie op sitrus. In die algemeen het pyrimethanil die laagste beskermende beheer en sporulasie-remming gehad. Die maksimum residuvlakke volgens die EU-standaarde is nie oorskry nie.

#### 4.4.2 FINAL REPORT: Fungal degradation of wooden pallet bases used in containerised export of citrus fruit

Project 1165 / PHI-8-19 (2019 -2021) by Wilma du Plooy, Muriel Rikhotso and Elaine Basson (CRI)

##### Summary

Recent fungal contamination on wooden pallet bases used for the export of fresh fruit highlighted a problem of which the source was apparently unknown. The degradation of pallet bases, when consignments reached their markets, raised questions on the quality of the wood preservatives used in the manufacturing of these bases. After the withdrawal of methyl bromide, 2-ortho-phenyl-phenol became the treatment most widely used by pallet manufacturers. This practice, unfortunately, resulted in residue issues on European export markets. Further concerns were the contribution of packhouse storage methods to the fungal contamination of the bases, and the possible role of environmental factors (for instance moisture, UV degradation, and insect infestation). However, it was the fungal decay that became the hazard with the highest priority due to the weakening of the structural strength of the pallets and the accompanying deterioration of the cartons. Heavily contaminated pallets are considered a point source of fungal spore dissemination that will increase the spore load in containers and cold rooms, and present a phytosanitary concern. This study looked at the microbiome on contaminated pallet bases, manufacturing aspects of the bases, the role of storage at packhouses, options for wood treatment, as well as possible contribution from the shipping containers and cold storage facilities to the degradation of wooden pallet bases. Three successful replacement products for fungal control were identified, with two fully commercialised.

##### Opsomming

Onlangse swamkontaminasie van houtpalletbassis wat gebruik word vir vars vrugte uitvoere, het 'n probleem met 'n onbekende oorsprong, uitgelig. Die agteruitgang van die gekontameneerde palletbassis wanneer dit uiteindelik op die uitvoermarkte aangekom het, het vroe laat ontstaan oor die kwaliteit van die houtpreservering wat gebruik was in die vervaardiging van die pallette. Metielbromied was vantevore wyd gebruik, maar na die onttrekking van die gebruik daarvan, was 2-ortho-feniel-fenol die mees algemene chemie wat aangewend was. Hierdie gebruik het egter residue tot gevolg gehad, wat gelei het tot probleme op uitvoermarkte in Europa. Verdere bekommernis was dat die bergingspraktyke by pakhuse sou kon bydra tot swamkontaminasie, met vroe oor die rol wat omgewingsfatore soos vog, agteruitgang weens UV en insekbesemting sou kon speel. Die hoogste prioriteit was egter die gevaar wat swamverrotting inhou, weens die rol wat dit in die strukturele verswakking van die hout speel, asook die gepaardgaande skade aan die kartonne. Swaar-gekontameneerde pallette word ook beskou as 'n puntbron vir swamspoorvrystelling wat die spoorlading in die behoueringseenhede en kouekamers verhoog, met 'n gepaardgaande fitosanitêre risiko. Hierdie studie het die mikrobioom op gekontameneerde palletbassis, asook vervaardigingsaspekte, die rol van stoorpraktyke by pakhuse, opsies vir houtbehandelings, asook die bydraes van verskepingshouers en kouestoorfasiliteite, bestudeer. Drie produkte wat suksesvolle beheer op swamgroei kon uitoefen was geïdentifiseer, met twee wat volledig gekommersialiseer is.

##### Introduction

A pallet base is a flat transport structure which supports goods during their storage, stacking, handling and shipping while being lifted by a forklift (Twede and Seike, 2005). In the citrus industry, pallet bases are important for commerce because boxed fruit are shipped over a long distance to international markets as a unit load consisting of an average of 70 cartons per pallet (varies between 45 and 130/pallet). Research shows that worldwide about 1.9 – 2 billion pallet bases are used daily for various purposes (White and Seminar, 2004). The volume of fruit exported by the South African (SA) citrus industry used approximately 2 million pallet bases in 2020 (Sikuka, 2020a), while the export volumes for pome fruit (1 178 000 MT), table grapes (305 000 MT) and stone fruit (67 566 MT), required approximately 1,5 million pallet bases (Sikuka, 2020b).

Since the disuse of methyl bromide (MeBr), an increasing number of fungal degradation outbreaks were seen on wooden pallet bases that are used for exporting citrus fruit. Apart from the cosmetically unacceptable situation, it also has the SA citrus producers very worried about the potential phytosanitary risk. Long markets such as the Far East and Canada comprise about 21% of the SA export market, with these being the markets which are primarily affected negatively by fungal contamination (CRI, PPECB observations). The contamination occurs on the pine wood used for manufacturing of both the slats and corner blocks of the bases. Importantly, Eucalyptus wood, also sometimes in pallet manufacturing, do not experience the same contamination, since the naturally occurring oils in the wood are antifungal by nature. Wood species, density, and thickness plays a pivotal role in the preservation of wood, but it is the nature of the chemical functional groups that finally determines how effective wood preservation will be (Hon, 1995). Wood has an innate ability to withstand attack by initial colonisers, but resulting damage due to transformation of wood structures will have a negative impact on the support structures (Kymäläinen *et al.*, 2014) (Figure 4.4.2.1). The current problem with fungal contamination is severe enough to cause incidental fungal growth on the cartons at the bottom of the pallet. This not only soils the packaging, but also weakens both the wood and cardboard structure (Fackler & Schwanninger, 2012; Hori *et al.*, 2014).

The first major challenge faced in the investigation of the pallets' fungal contamination is that the source of the infection is unknown, which makes it difficult to treat. The second problem is that MeBr was replaced with 2-ortho-phenyl-phenol (OPP). Residues of the chemical were detected in Europe, on fruit that had been declared either chem-free or organically produced, incurring huge financial losses. Although the OPP was discovered at levels close to the limit of detection (LOD = 0,01 ppm), any organically produced or chem-free fruit should have zero residues. In addition, although OPP residues may be allowed on conventionally produced fruit, it adds another chemical residue to an already very limited allowable number of residues to be used. Lastly, it is not a very effective fungicide on the pallet wood, even at the prescribed rate of 6%. The use of OPP therefore poses an unnecessary risk and alternative actives are urgently needed.

Finding suitable and alternative treatments for the wooden pallet bases is important, keeping in mind the following issues that will arise if wooden pallet bases are prohibited:

- Cost of the development of an alternative material
- Currently, plastic cannot carry the weight of a hi-cube pallet
- Due to strength concerns, the current configuration of plastic pallets that has been offered as alternatives to wooden bases, interfere with the cooling of the A-15C cartons used for the majority of exports
- Plastic pallets are very expensive: 8 – 9 times more costly than wood
- Internationally, there is increasing concern about the use of plasticisers, and pressure to recycle and reuse plastic. This is a very costly aspect in terms of plastic pallets.
- To circumvent the previous point, the use of plastic would require a similar system to CHEP, which is huge, cumbersome and to be avoided. According to this system, the original renter is responsible for the base until signed over to the next party. Theft, breakages and negligence have already resulted in the current CHEP system not being supported for wooden pallet bases.

Identifying and characterising the fungi that contribute to the contamination issue will enable decisions regarding suitable control measures for treating the wood, as not all fungi are equally susceptible to a single product. Although a specific product is not currently foreseen, averting a market access risk is imperative in protecting the citrus industry and export opportunities.

The detection of OPP contamination on organically produced and packed citrus was the initial lead to the study. The overall aim of this study therefore is to firstly find the source of the contamination, and evaluate the spread and occurrence of wood fungal contamination. The possibility of the wooden pallets being the source of the issue was included in the investigation. Secondly, the phytosanitary concerns need to be addressed by identifying the fungi isolated from wood pallets. Lastly, various products that claim to prevent fungal growth and subsequent contamination on wood need to be evaluated in a controlled and unbiased trial.



**Figure 4.4.2.1.** Racked pallets in a cold store. Each pallet base slides onto flanged rails, putting a lot of pressure on the wooden structure, obviating the danger of weakened slats when fungal contamination is present.

### **Stated objectives**

#### *Phase 1 – Visiting manufacturers and exporters (exporters)*

Objective 1: Determining the occurrence and spread of the problem - including industries other than citrus.

- Visiting pallet manufacturers
- Liaising with fruit exporters and fresh fruit industries other than citrus
- Questionnaire distributed to exporters and producers

Objective 2: Optimisation of a self-inoculation technique of wood used for pallet base manufacturing.

- Simulated growth on wood used in the manufacturing of export pallet bases.

#### *Phase 2 – Optimising sampling procedures, identification of isolates, and wood preservative investigation.*

Objective 1: Optimising methods by which fungi responsible for the soiling and contamination of the pallets will be isolated from collected wood samples

- Isolation of fungal contaminants

Objective 2: Identification of the fungal specimens isolated from wood pallets

- PCR and ITS sequence comparison in GenBank for confirmation of isolate identities

Objective 3: Investigation of SOPP, as well as alternative products not currently in use for wood preservation of pallets

- Evaluation of OPP treated pallets as a possible source of OPP contamination in export containers
- Evaluation of alternative products using self-inoculated pallet wood.

Objective 4: Determine the contribution of storage practices on the occurrence of fungal contamination on palletising wood.

- Determination of the effect of storage facilities at the packhouse on the development of fungal contamination on pallet bases.
- Quantification of moisture build-up and exposure of pallets to free water in containers.

Objective 5: Finalisation of results and trials, and technology transfer and research papers.

- Feedback at postharvest workshops
- Article, and a research paper at the CRI Research Symposium

## **Materials and methods**

### *Phase 1 – Visiting manufacturers and exporters (exporters)*

Objective 1: Determining the occurrence and spread of the problem - including industries other than citrus.

In this part of the project, physical visits were made to the premises of various pallet manufacturers, exporters of citrus fruit, and exporters of fresh commodities other than citrus (avocados, pome fruit, stone fruit, grapes, and vegetables). The different role-players were interviewed and the extent to which the problem in the citrus industry was shared by other industries was determined.

Samples were collected by the CRI staff from all the sites visited. Several samples were also sent to the CRI postharvest laboratories from packhouses where contaminated pallets were found. All of these samples were used in Objective 1 of Phase 2.

Additional data were collected in the form of anonymous questionnaires disseminated through the CRI mailing lists and at a CRI workshop.

Objective 2: Optimisation of a self-inoculation technique of wood used for pallet base manufacturing.

Wood sections were obtained from different pallet manufacturers (Phase 1). A technique to recreate the fungal growth experienced in the containers had to be developed. Wood slats and blocks such as those used for commercial pallets were obtained from different pallet manufacturers, and a suitable, repeatable self-inoculation method was developed.

### *Phase 2 – Optimising sampling procedures, identification of isolates, and wood preservative investigation.*

Objective 1: Optimising methods by which fungi responsible for the soiling and contamination of the pallets would be isolated from collected wood samples.

Samples with fungi were subjected to the standard mycological techniques for the collection of specimens (Fackler, K. & Schwanninger, M. 2012; Kalawate A. & Mehetre, S. 2015; Ng *et al.*, 2014; Salem *et al.*, 2016):

1. Direct plating was done onto various agar types in order to determine the most useful growth media for isolated specimens.

2. Wood chips were rinsed in ¼ strength Ringers, and following a dilution plate method, the spore suspension was streaked onto the growth media and incubated at 28°C.
3. Swabs were collected from contaminated surfaces, rinsed in ¼ strength Ringers, and following a dilution plate method, streaked onto growth media and incubated at 28°C.

Objective 2: Identification of the fungal specimens isolated from wood pallets

1. Fungal diversity, colony morphology and spore structures were documented using standard visualizing microscopy techniques.
2. Representative fungal cultures obtained for identification and characterisation, were specifically characterised using DNA based molecular techniques.
3. Species representation was determined using ITS sequence comparison in GenBank.
4. A profile of the mycological diversity on the wood types used for palletising, as well as the different areas of production, were drawn up.

Objective 3: Investigation of SOPP, and alternatives not currently in use for wood preservation of pallets

1. The standard application of SOPP as a wood preservative is as a 3% solution. An advisory issued by the CRI after banning of MeBr, was to use a 6% solution, based on information from German markets (pers. comm. Keith Lesar). The efficacy of these two solutions were evaluated, using inoculated wood chips.
2. Alternatives to SOPP and products not currently in use for wood preservation of pallets were investigated (Salem *et al.*, 2016; Zhang *et al.*, 2016).
3. The unidentified source of OPP in export containers causing OPP contamination of organically produced and packed citrus was investigated. The possibility of the wooden pallets being the source of the issue was included in the investigation.

Objective 4: Determine the contribution of storage practices on the occurrence of fungal contamination on palletising wood.

1. Determination of environmental conditions conducive to fungal contamination of wooden pallets. Simulation of environmental and storage conditions to which pallets are subjected were proposed. This included custom-built equipment to enable simulation of the importance of moisture, wood age, and UV.
2. The same equipment was to be used in the determination of the importance of UV and other environmental conditions in the maintenance and efficacy of wood preservatives.
3. The persistence and chemical activity of the wood preservatives were evaluated.

Objective 5: Finalisation of results and trials.

The results from the study were made available to both the pallet manufacturing industry and the citrus producers. This will be done at the annual workshops, as a peer reviewed publication, and as a lecture / poster at the CRI Symposium.

## Results and discussion

### *Phase 1 – Visiting manufacturers and exporters (exporters)*

Objective 1: Determining the occurrence and spread of the problem - including industries other than citrus.

The project started off by dissemination of anonymous questionnaires disseminated through the CRI mailing lists and at a CRI workshop. The results of the investigation are summarised in Table 4.4.2.1 below.

**Table 4.4.2.1.** Results from an anonymous survey done to determine the typical storage conditions used when pallets are stockpiled at packhouses.

	% of total number of respondents	STORAGE CONDITIONS	Mould issues reported
<b>No roof cover</b>	36,54	% of total number of respondents had no roof cover, regardless of floor type	Yes (4%)
	28,19	Has no roof cover, but storage on concrete floors	Yes (4%)
	8,35	Has no roof cover, and stored pallets on bare soil	No
<b>Roof cover</b>	63,46	% of total number of respondents with roof cover, regardless of floor type	Yes (23,10%)
	0,00	Has roof cover with storage on bare soil	NA
	45,36	Has roof cover, but storage area is not completely walled in	Yes (9%)
	18,10	Has roof cover, and also stored pallets indoors	Yes (14,10%)

From these results it was clear that enclosed areas, while seemingly more clean and manageable in terms of dust, created more suitable conditions that resulted in fungal growth on the stored pallets. This immediately negated the claim by some pallet manufacturers that the packhouse storage conditions were to blame for the issue.

Through collaboration with HORTGRO it was determined that the problems and concerns were similar and widespread in the pome and stone fruit industries as well. In addition, they are plagued by reports of unacceptable OPP residues as well. The South African Table Grapes Association reported similar issues with OPP residues, being negatively impacted by detection of the chemical on export markets. The support offered by PPECB was instrumental in gaining access to the cold storage and export container facilities that were visited and sampled.

In a follow-up to the questionnaires, physical visits were made where swabs from different container depots and cold stores were collected. A total of six different cold storage facilities (SAFT, Stellenpark, Melpack, Chiltern, Fruitways and Two-a-Day) were visited as well as three container depots (MSC, UDC and DCP-CPT). The main reason for collecting samples from the various depots and cold stores was because the source of the problem was unknown and these areas are the centres where fruit leave from before they are shipped to international markets. Samples were collected from both washed and unwashed cold stores, and were taken from the wall, floor, drain outlets, coils, pallets and fruit boxes.

A total of 151 swabs were collected and all these were analysed for OPP. Samples of the cleaning materials used at the facilities, were also collected. Lastly, wood shavings from manufactured pallets were collected. All the swabs and collected sample material were sent for OPP analysis at Hearshaw and Kinnes (Cape Town).

Similarly, pine wood samples of the slats used in pallet construction were collected by the CRI staff from all the packhouses and manufacturers visited, and where contaminated pallets were found. These were sent to the CRI postharvest laboratories for further processing.

Results from 150 of the 151 swabs collected from the 15 container depots showed zero detection of OPP. One swab detected 0.09 mg/kg OPP in a single area of one of the cold stores. The results for the chemicals used to wash the containers also showed zero detection of OPP.

From the results of the pallets, however, it was clear that the source of the problem was the pallets on which fruit boxes are stacked during shipping, necessitating determination of the most cost effective and environmentally friendly way to treat the pallets.

Objective 2: Optimisation of a self-inoculation technique of wood used for pallet base manufacturing.

Uncontaminated pine wood sections were obtained from different pallet manufacturers and used in attempts to replicate the development of fungal contamination on pallets (Phase 1). Contaminated wood sections were received from different packhouses throughout the study. Using a permit acquired from DALRDD (previously DAFF), wood sections, originating from pallets coming from South Africa with fruit shipments, were obtained from Malaysia as well. All of these samples were subjected to fungal isolation and species identification.

The self-inoculation technique developed was as follows:

1. Untreated wood with no apparent fungal growth were cut into 20 cm sections and laid in a plastic container (previously washed with 70% ethanol), used as a moisture chamber. The chamber had a layer of paper (sterilised) that was saturated with sterile water at the bottom. The wood was placed on supports to elevate it off the wet paper. These chambers were incubated at 28°C. This procedure was done with untreated wood from five different sources (three packhouses, two lumber mills).
2. Wood chips were prepared by chipping a pine wood slat into small pieces, using a 2.5 cm chisel. The chips were placed into several glass beakers and covered with foil, where after it was sterilised in an autoclave (121°C for 15 minutes). Sterilised wood chips were dried overnight in an oven at 60°C. The dried chips in the glass beakers were measured to 100 g in each container, reclosed with the foil and kept for further use.
3. Uninfected wood was cut into 20 cm sections, wrapped in foil and sterilised at 121°C for 15 minutes. These blocks were sprayed until runoff, using a  $10^4$  spore suspension obtained by scraping spores from previously infected wood, and incubated as described in #1 (about 100 ml spore suspension/block). The suspension of mixed spores was prepared using muslin cloth to trap any wood pieces, and then adding spores until a spectrophotometric absorbance of 1.05 at 425 nm was achieved. The spore count was confirmed at least once, using a haemocytometer.
4. Wood shavings were made of contaminated sections, using a standard planer, with the blade sterilised using 70% alcohol after each cut. The shavings were placed on growth media and incubated at 28°C. Resulting colonies were purified and allowed to sporulate. Spores were collected by rinsing the colonies with  $\frac{1}{4}$  strength Ringers. This method did not yield representative diversities of the fungal growth on the wood, with *Rhizopus* and *Trichoderma* completely overwhelming all other fungal species. Furthermore, the method of shaving wood was difficult to execute with consistently accurate, repetitive motion. It was also very time consuming due to the requirement to sterilise the blade between every cut.
5. Complete, non-identified spore suspensions were collected from the contaminated wood sections by scraping of swathes of fungal spores and mycelium using sterile scalpel blades. The scrapings were suspended in  $\frac{1}{4}$  strength Ringers and vortexed. Scrapings were added until a count of  $10^4$  cfu's per suspension (confirmed using a haemocytometer), were obtained.
6. Five vials of each type of spore suspension prepared above, with  $10^4$  cfu's per suspension (confirmed using a haemocytometer), was re-introduced to the sterilised and oven-dried wood chips by spraying 50 ml of suspension onto the 100 g of chips. The sprayed chips were laid in plastic containers (previously washed with 70% ethanol), and incubated after securely closing the container lids. Incubation was done at 28°C, with the humidity kept at saturation inside the containers (condensation visible). The regrowth of the isolated colonies was slow and unsatisfactory, and the methods were abandoned as an option to infect wood to test alternative products against.).
7. As an alternative method to obtain fungal growth on the pine wood slats, full-length, wet-off-saw slats were placed in polyethylene bags, along with 250 ml water in each bag. The bags were sealed with a cable tie and allowed to incubate at 34°C in an unventilated shipping container.

Of the four methods used, only the last resulted in excellent growth within a reasonable time period (two weeks). Slats with contamination were then used as growth media for testing wood treatments.

**Table 4.4.2.2.** Results from the OPP analyses on wood shaving from various pallets that had visible fungal contamination. The shavings were collected from citrus, pome and avocado exporters.

DATE SAMPLE	PALLET MANUFACTURER	PHC	PUC	CULTIVAR	RESULTS SUMMARY
2019/09/16	ZA-F24 HT	NL6588	NL1241	MIDKNIGHTS	Positive
2019/09/16	ZA-F24 HT	NL6588	NL1241	MIDKNIGHTS	Positive
2019/09/16	ZA-M33 HT	NL1233	X1265	VALENCIAS	Positive
2019/09/16	ZA-W16 HT	NL1233	X1196	MIDKNIGHTS	Positive
2019/09/16	ZA-V01 MB	D7336	D1006	LAMB HASS	Positive
2019/09/16	ZA-F24 HT	X1271	NL0183	VALENCIAS	Positive
2019/09/16	ZA-L01 HT	A6063	A6063	VALENCIAS	Positive
2019/09/16	ZA-G18 HT	L5922	L7422	CAMBRIA NAVELS	Positive
2019/09/16	ZA-M33 HT	E0354	V1021	GOLD DELICIOUS	Positive
2019/07/17	ZA-B31 HT	E2002	E0052	TOPRED	Positive
2019/07/17	ZA-B31 HT	E2002	V0238	CRIPPS PINK	Positive
2019/09/17	ZA-W16 HT	E0048	V0234	GOLD DELICIOUS	Positive
2019/09/18	ZA-M33 HT	X1208	Q0268	MIDKNIGHT	Positive
2019/09/18	ZA-M33 HT	X1208	Q0268	MIDKNIGHT	Positive
2019/09/18	ZA-G18 HT			NADORCOTT	Positive
2019/09/18	ZA-A42 HT	L3999	L7436	MIDKNIGHT	Positive
2019/09/18	ZA-L18 HT	S0014	H2237	NADORCOTT	Positive
2019/09/18	ZA-L18 HT	S0014	S0015	CAMBRIA NAVEL	Positive
2019/09/16	ZA-V01 MB	D7336	D1006	LAMB HASS	Positive

A technique to evaluate product efficacy in controlling the fungal growth experienced in the containers was developed. Pine wood slats and blocks such as those used for commercial pallets were obtained from different pallet manufacturers, and a suitable, repeatable self-inoculation method was developed (see #5 above).

The method is summarised below:

1. Four blocks of pine wood and one long infected wood slat were used for each treatment. Each treatment was done in triplicate, with two treated and two untreated blocks per repeat.
2. Two of the blocks were used as control (Untreated, water submersion only) and two were treated with the chemicals being evaluated.
3. The blocks were immersed into the treatment for 5 minutes and allowed time to dry at room temperature (Figure 4.4.2.2A).
4. Treated blocks were allowed to air dry completely by being placed on a metal rack. The dried blocks were then tied to the infected wood and placed into a clear plastic bag and left in the incubation room with air temperature at 34°C, using the configuration set out below (visualised in Figure 4.4.2.2B):
  - a. Treated

- b. Untreated
- c. Treated
- d. Untreated

Objective 3: Investigation of SOPP, and alternatives not currently in use for wood preservation of pallets

1. The standard application of SOPP as a wood preservative is as a 3% solution. An advisory issued by the CRI after banning of MeBr, was to use a 6% solution, based on information from German markets (pers. comm. Keith Lesar). The efficacy of these two solutions was evaluated, using inoculated wood obtained by incubating pine wood slats [Objective 2(4)]. In this project OPP was initially used as the positive control. However, the results were disappointing at both 3% and 6% application rates, and was eventually abandoned, using only a negative control (water).
2. Alternatives to SOPP and products not currently in use for wood preservation of pallets were investigated. Initial proposed remedies included essential oil, and copper-based chemicals as possible treatments, and various combinations of chemicals and heat. Due to the volatile nature of applied essential oils, these were excluded from the trials. Two copper-based remedies (Anti-Blue and Fungoes) were included. Initially the use of chemicals already applied on fruit (no additional residue) was attempted. No heat treatments could be done in the laboratory environment. Inclusion of products to be tested were dependent on their use in the food industry or previous application on wood pallets.

The treatments used in the first set of trials were

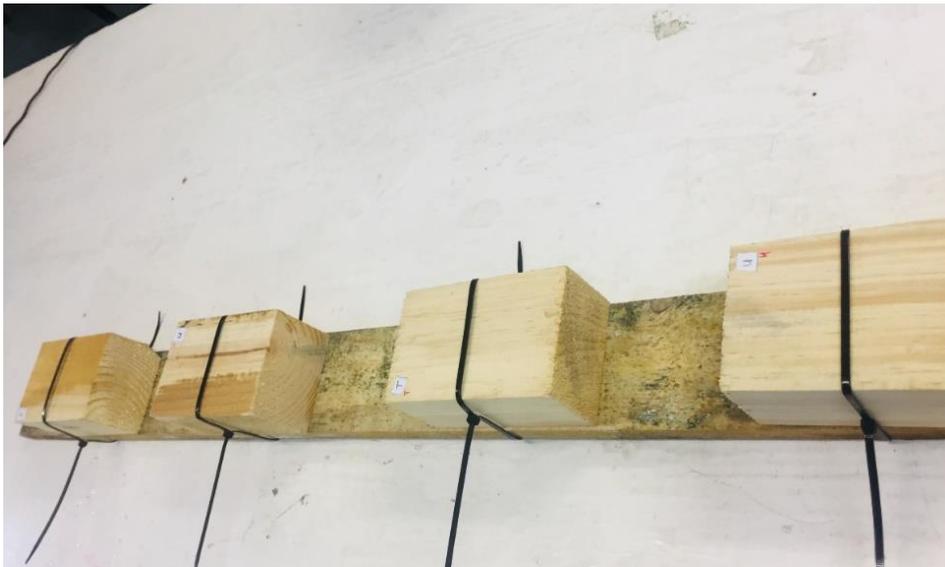
1. Imazalil at 500 ppm dilution
2. TBZ at 500 ppm
3. Supersolve alkaline solvent detergent cleaner @ 100 mL diluted in 5 L hot water
4. Anti-blu @ 150 ml in 5 L water
5. Coverit (Undiluted)
6. Fungoes @ 1:7 dilution
7. 1% Organocide @ 20 mL in 2 L water
8. 5% Organocide @ 100 mL in 2 L water
9. OPP (6%) (positive control) – included in the test for every product
10. Water (negative control) – included in the test for every product

Chemicals in the second set of trials were

1. Diluted Coverit @ 10%, 20% and 50%
2. HD-313 @ 63, 125 and 252 mL in 2.5 L water
3. Sporekill @ 3.75, 7.5 and 15 mL in 2.5 L water
4. Pal-It @ 10%, 20%
5. Etch @ 10%, 20%
6. Water as negative control.



**Figure 4.4.2.2A.** Pine wood corner blocks were kept completely submerged in the test treatment, for 5 minutes each.



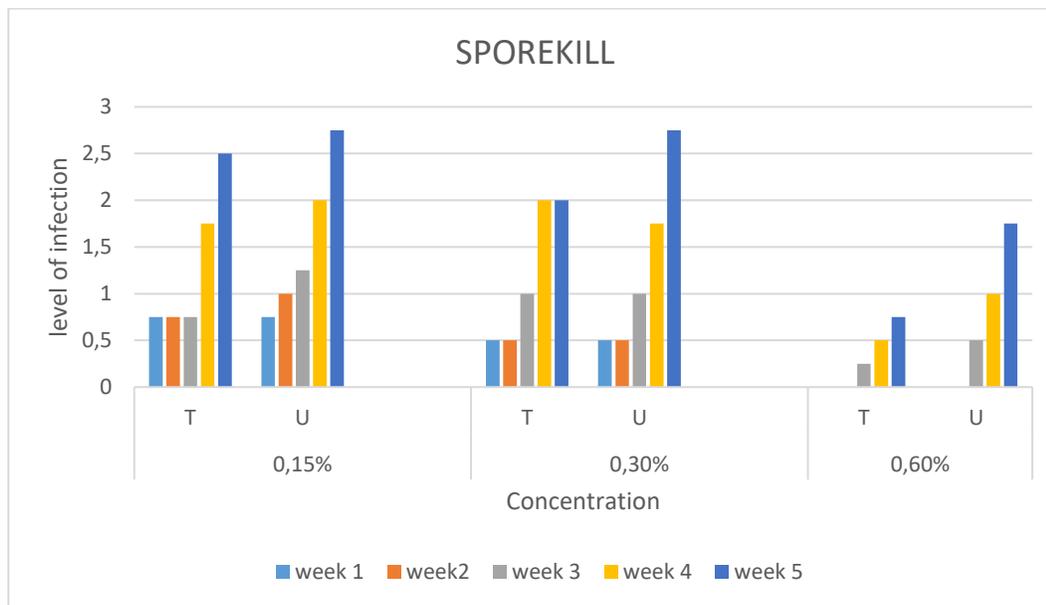
**Figure 4.4.2.2B.** Treated blocks were tied equidistantly to previously infected wood, and placed into a clear plastic bag with 250 ml water to incubate at an air temperature of 34°C.

The level of infection for the wood was visually inspected and scored on a subjective scale from 0 = no infection to 3 = severe infection (Figure 4.4.2.2C).

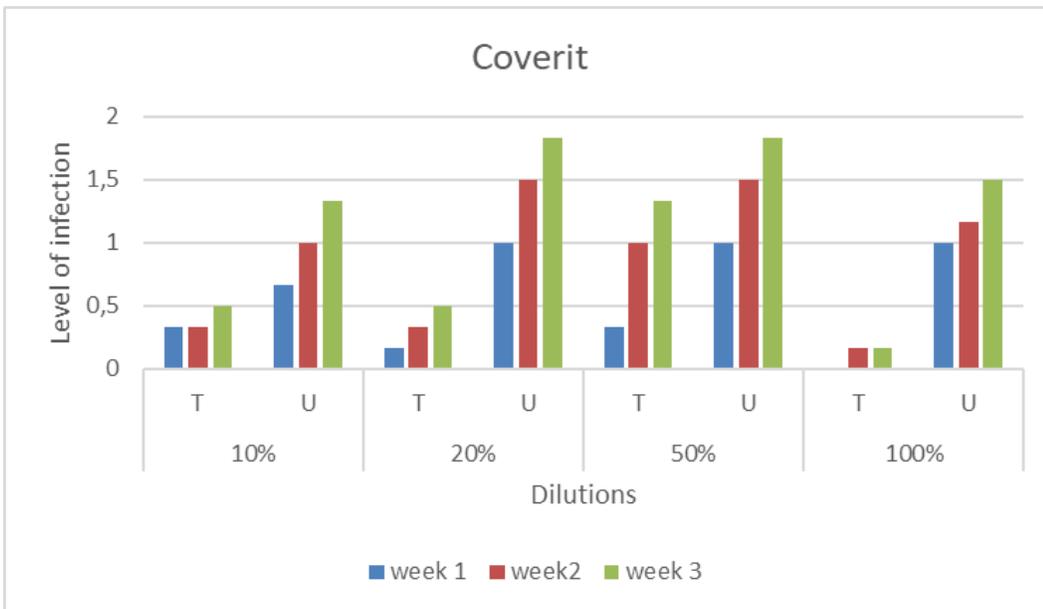


**Figure 4.4.2.2C.** A grading system from 0 – 3 was used in the visual assessment of the efficacy of the products tested, with 0 being effective and 3 denoting no effect in fungal control.

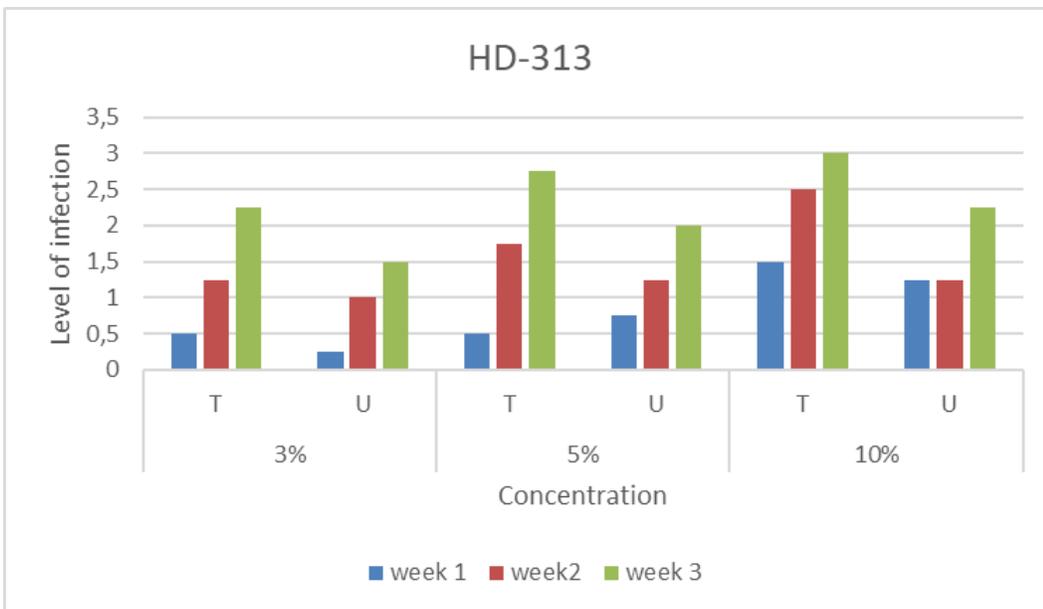
The most effective formulations in preventing fungal degradation of the wood were Etch, Sporekill, Coverit (Pal-It), and HD-313, (Figures 4.4.2.3 - 6). There were concerns about the possible residue transfer due to the use of Sporekill, but the lower concentrations are ineffective after 4-5 weeks, with the higher concentration only moderately successful after 5 weeks. It was considered to be unsuitable for use as a treatment for pallet wood. Although there were no residue alarms, HD-313 suffered inexplicable breakthrough of fungal contamination at all the higher concentrations. This raised some concerns about the consistency of the product when applied to wet-off-saw wood. Coverit, also effective as a control measure against fungal growth, was renamed to Pal-It by the manufacturer.



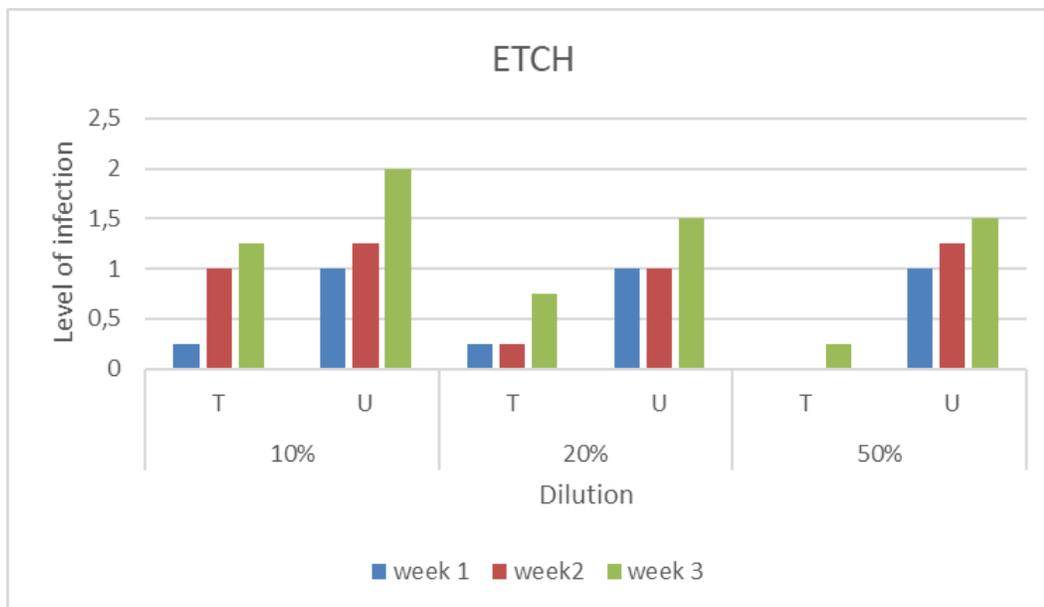
**Figure 4.4.2.3.** Graph of Sporekill efficacy against fungal contamination on a pine wood block after 5 minutes of submersion in the treatment solution.



**Figure 4.4.2.4.** Graph of Coverit efficacy against fungal contamination on a pine wood block after a 5 minutes of submersion in the treatment solution. This product was renamed to Pal-It and retested, yielding similar results.



**Figure 4.4.2.5.** Graph of HD-313 efficacy against fungal contamination on a pine wood block after a 5 minutes of submersion in the treatment solution



**Figure 4.4.2.6.** Graph of Etch efficacy against fungal contamination on a pine wood block after a 5 minutes of submersion in the treatment solution.

*Phase 2 – Optimising sampling procedures, identification of isolates, and wood preservative investigation.*

Objective 1: Optimising methods by which fungi responsible for the soiling and contamination of the pallets were isolated from collected wood samples.

Standard mycological techniques for the collection of contaminating fungal specimens were used:

1. Different techniques for direct plating onto various agar types were done. The media included several basic microbiological substrates. The most useful growth media for isolated specimens was determined to be a standard potato dextrose agar supplemented with 0.05 g of Rose Bengal and 0.25 g of chloramphenicol per 1 L of agar solution. This specific media proved to be the best growth media for cultures obtained from suspensions of both swabs and wood shavings.
2. Wood chips were obtained by chiseling sections off the surface of a contaminated pine wood slat using a 2,5 cm wood chisel. The sections obtained were rinsed in ¼ strength Ringers, and, following a standard microbiological dilution method, a spore suspension of  $10^{-3}$  was streaked onto the growth media from (i) and incubated at 28°C.
3. Swabs were collected from contaminated surfaces, rinsed in ¼ strength Ringers, and, following a standard microbiological dilution method, a spore suspension of  $10^{-3}$  was streaked onto the growth media from (i) and incubated at 28°C.
4. The specimens from the wood chips and the swabs showed very high overlapping incidence, close enough to be regarded as similar.

Objective 2: Identification of the fungal specimens isolated from wood pallets

1. Fungal diversity, colony morphology and spore structures were documented using standard visualizing microscopy techniques. Fungal isolats were purified, and allowed to sporulate. A small amount of mycelial mass with spoulating strucures were collectd and palced on a microscope slide. After treatment with bromophenol blue dye, a coverslip was placed on the mycelium with spores and inspected at 400 x magnification.
2. Representative fungal cultures obtained for identification and characterisation, were specifically characterised using PCR techniques using the following methodology from the Promega Wizard Genomic kit:

- i. Using the pestle, mycelium was macerated in an Eppendorf tube and 200 µl Nuclei Lysis Solution added.
  - ii. The tube was incubated for 30 minutes at 65°C, after which 1µl RNA-ase solution was added.
  - iii. Next, the tubes were placed into a floating holder and gently swirled while being incubated in a 37°C water bath for 15 minutes, then removed and dried using a paper towel.
  - iv. A 100 µl of Protein Precipitation solution was added and the tubes vortexed.
  - v. After vortexing, the solution was centrifuge for 10 minutes while 1.5 ml tubes were prepared and marked for the next step.
  - vi. 200 µl of isopropanol was added to each tube, and from the centrifuged samples, 200 µl of the supernatant was removed and also added into each tube.
  - vii. This formed a milky area, gently inverted in order to mix, and then centrifuged for 5 minutes.
  - viii. The supernatant were carefully decanted and the tubes placed upside up on a paper towel to ensure the pallet was not to lost.
  - ix. This was followed by the addition of 200 µl of 70% ethanol and again gently inverted to mix.
  - x. After centrifuging for 5 minutes, the supernatant was decanted carefully, preserving the pallet, and the tubes upside placed down on a dry paper towel.
  - xi. The tubes were left in an oven for 15 to 30 minutes and then 50 µl DNA Rehydration added and vortexed. Thereafter the samples were ready for PCR analysis.
3. A total of 292 isolates were obtained, with species representation determined using ITS sequence comparison in GenBank (Table 4.4.2.3).
- i. The colony morphology and spore structures are being documented using standard microscopy (n=292).
  - ii. From these representative fungal cultures were obtained for identification and characterisation, using PCR techniques such as β-tubulin sequencing, RFLP, and ITS sequence comparison in GenBank.
  - iii. Fungal DNA was extracted from the mycelia using the Promega Wizard ® Genomic DNA Purification Kit (Promega, Madison, WI, USA).
  - iv. The primer pair, ITS1 (5'- TCC GTA GGT GAA CCT GCG G- 3') and ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC- 3') was used in a 20 µl reaction volume that consisted of 1x Q5® Hot Start High-Fidelity 2x Master Mix (New England Biolabs, Ipswich, Mas., USA), 375nm forward and reverse primer, and 6.5 µl of PCR grade water.
  - v. Samples amplified for the ITS gene were sent for sequencing (n=226).
  - vi. Samples that did not have at least 95% representative certainty when blasting of the ITS primer sequence in GenBank, were regarded as unconfirmed species (n=66).
4. A profile of the mycological diversity on the pine wood used for palletising, as well as the different areas of production, was compiled by source and species (Table 4.4.2.3) and a graphic summarised representation of diversity made (Figure 4.4.2.7) .

**Table 4.4.2.3.** Fungal species isolated from pine wood used for pallet manufacturing, identified using morphology and blasted in GenBank

CRI CODE	SAMPLE	MORPHOLOGICAL ID	GENBANK BLAST
PH 1	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 2	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 3	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 4	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 5	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 6	Lumber	Trichoderma	<i>Trichoderma spirale</i>

PH 7	Lumber	Fusarium	<i>Fusarium striatum</i>
PH 8	Lumber	Fusarium	<i>Fusarium cf. solani</i>
PH 9	Lumber	Trichoderma	
PH 10	Lumber	Fusarium	<i>Fusarium striatum</i>
PH 11	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 12	Lumber	Trichoderma	
PH 13	Lumber	Fusarium	<i>Fusarium cf. solani</i>
PH 14	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 15	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 16	Lumber	Green mould	<i>Penicillium digitatum</i>
PH 17	Lumber	Fusarium	<i>Fusarium solani</i>
PH 18	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 19	Lumber	To be confirmed	<i>Fusarium solani genomic</i>
PH 20	Lumber	Trichoderma	
PH 21	Lumber	To be confirmed	<i>Clonostachys rogersoniana</i>
PH 22	Lumber	Fusarium	<i>Fusarium solani</i>
PH 23	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 24	Lumber	Trichoderma	<i>Fusarium oxysporum</i>
PH 25	Lumber	Fusarium	<i>Fusarium solani</i>
PH 26	Lumber	Fusarium	
PH 27	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 28	Lumber	Fusarium	<i>Fusarium cf. solani</i>
PH 29	Lumber	Trichoderma	
PH 30	Lumber	Fusarium	<i>Fusarium solani</i>
PH 31	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 32	Lumber	Fusarium	<i>Fusarium cf. solani</i>
PH 33	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 34	Lumber	Fusarium	
PH 35	Lumber	Fusarium	<i>Fusarium striatum</i>
PH 36	Lumber	Trichoderma	<i>Trichoderma inhamatum</i>
PH 37	Lumber	Trichoderma	<i>Trichoderma aggressivum</i>
PH 38	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 39	Lumber	Fusarium	<i>Fusarium solani</i>
PH 41	Lumber	Fusarium	<i>Fusarium solani</i>
PH 42	Lumber	Fusarium	<i>Fusarium solani</i>
PH 43	Lumber	Trichoderma	
PH 44	Lumber	Fusarium	<i>Fusarium solani</i>
PH 45	Lumber	Fusarium	<i>Fusarium solani</i>
PH 46	Lumber	Trichoderma	<i>Fusarium solani</i>
PH 47	Lumber	Fusarium	<i>Fusarium solani</i>
PH 48	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 49	Lumber	To be confirmed	<i>Fusarium oxysporum</i>
PH 50	Lumber	Trichoderma	<i>Trichoderma atroviride</i>
PH 51	Lumber	Fusarium	<i>Fusarium solani</i>
PH 52	Lumber	Trichoderma	<i>Trichoderma atroviride</i>

PH 53	Lumber	To be confirmed	<i>Bipolaris cynodontis</i>
PH 54	Lumber	Fusarium	<i>Fusarium striatum</i>
PH 55	Lumber	Fusarium	<i>Fusarium oxysporum</i>
PH 56	Lumber	Fusarium	<i>Fusarium solani</i>
PH 57	Lumber	Trichoderma	<i>Trichoderma harzianum</i>
PH 58	Lumber	To be confirmed	<i>Cladosporium cladosporioides</i>
PH 58	Lumber	To be confirmed	<i>Cladosporium anthropophilum</i>
PH 59	Schenk	Fusarium	<i>Fusarium oxysporum</i>
PH 60	Schenk	Fusarium	<i>Fusarium solani</i>
PH 61	Schenk	Fusarium	<i>Fusarium solani</i>
PH 62	Schenk	Trichoderma	
PH 63	Schenk	Fusarium	<i>Fusarium phaseoli</i>
PH 64	Schenk	Trichoderma	
PH 65	Schenk	Green mould	<i>Penicillium digitatum</i>
PH 67	Schenk	Trichoderma	
PH 68	Marco	Fusarium	<i>Fusarium striatum</i>
PH 69	Marco	Fusarium	<i>Epicoccum sorghinum</i>
PH 70	Marco	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 71	Marco	Rhizopus	<i>Mucor racemosus strain</i>
PH 73	Marco	Fusarium	<i>Fusarium striatum</i>
PH 74	Marco	Fusarium	<i>Fusarium striatum</i>
PH 75	Marco	Blue mould	<i>Penicillium miczynskii</i>
PH 76	Marco	Fusarium	<i>Fusarium oxysporum</i>
PH 77	Marco	Fusarium	<i>Fusarium solani</i>
PH 78	Marco	Fusarium	<i>Lecanicillium fungicola</i>
PH 80	Marco	Green mould	<i>Penicillium cairnsense</i>
PH 81	Marco	Fusarium	<i>Fusarium solani</i>
PH 82	Marco	Trichoderma	
PH 83	Marco	Rhizopus	
PH 84	Sample	Fusarium	<i>Fusarium striatum</i>
PH 85	Sample	Fusarium	<i>Fusarium oxysporum</i>
PH 86	Sample	Trichoderma	
PH 87	Sample	Fusarium	<i>Fusarium oxysporum</i>
PH 88	Sample	Fusarium	<i>Fusarium striatum</i>
PH 89	Sample	Trichoderma	
PH 90	Sample	Green mould	<i>Trichoderma atroviride</i>
PH 91	Sample	Fusarium	<i>Fusarium solani</i>
PH 92	Sample	Trichoderma	
PH 93	Sample	Green mould	<i>Penicillium digitatum</i>
PH 94	Sample	Fusarium	<i>Fusarium oxysporum</i>
PH 95	Sample	Trichoderma	
PH 96	Sample	Green mould	<i>Penicillium digitatum</i>
PH 97	Sample	Fusarium	<i>Fusarium striatum</i>
PH 98	Sample	Trichoderma	
PH 99	Sample	Fusarium	<i>Fusarium striatum</i>

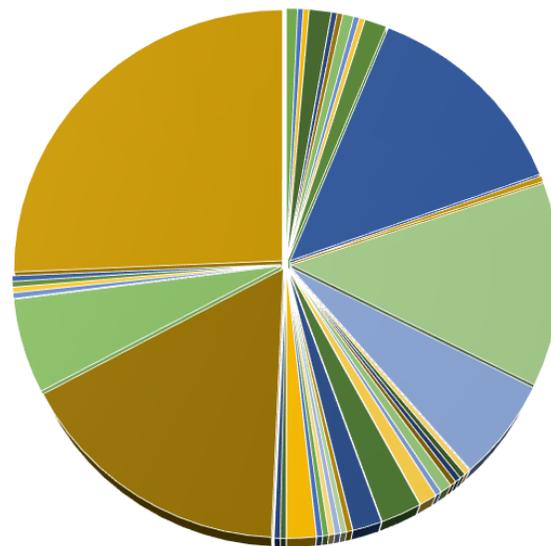
PH 100	Sample	Trichoderma	
PH 101	Sample	Fusarium	<i>Gibberella moniliformis</i>
PH 102	Sample	Fusarium	<i>Fusarium striatum</i>
PH 103	Sample	Trichoderma	
PH 104	Sample	Fusarium	<i>Fusarium striatum</i>
PH 105	Sample	Fusarium	<i>Fusarium oxysporum</i>
PH 106	Sample	Trichoderma	
PH 107	Sample	Fusarium	<i>Fusarium oxysporum</i>
PH 108	Sample	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 109	Sample	Fusarium	<i>Fusarium striatum</i>
PH 110	Sample	Fusarium	
PH 111	Sample	Fusarium	<i>Fusarium striatum</i>
PH 112	Sample	Fusarium	<i>Fusarium striatum</i>
PH 113	Sample	Trichoderma	
PH 114	SRCC	Fusarium	
PH 115	SRCC	Fusarium	
PH 116	SRCC	Fusarium	<i>Alternaria multiformis</i>
PH 117	SRCC	Trichoderma	<i>Trichoderma atroviride</i>
PH 118	SRCC	To be confirmed	<i>Alternaria multiformis</i>
PH 119	SRCC	Trichoderma	
PH 120	SRCC	Fusarium	<i>Fusarium solani</i>
PH 121	SRCC	Trichoderma	
PH 122	Hermitage	Fusarium	<i>Fusarium solani</i>
PH 123	Hermitage	Fusarium	<i>Fusarium solani</i>
PH 124	Hermitage	To be confirmed	<i>Leptosphaerulina chartarum</i>
PH 125	Hermitage	Trichoderma	
PH 126	Hermitage	Fusarium	<i>Fusarium oxysporum</i>
PH 127	Hermitage	Fusarium	<i>Fusarium striatum</i>
PH 128	Hermitage	Trichoderma	
PH 129	Hermitage	Green mould	<i>Penicillium digitatum</i>
PH 130	Manufacture	Fusarium	<i>Fusarium oxysporum</i>
PH 131	Manufacture	Fusarium	<i>Fusarium oxysporum</i>
PH 132	Manufacture	Fusarium	<i>Fusarium solani</i>
PH 133	Manufacture	Fusarium	<i>Fusarium solani</i>
PH 134	Manufacture	Trichoderma	
PH 135	Manufacture	Fusarium	<i>Fusarium oxysporum</i>
PH 136	Manufacture	Trichoderma	<i>Trichoderma atroviride</i>
PH 138	Manufacture	Trichoderma	
PH 139	Manufacture	Fusarium	<i>Fusarium oxysporum</i>
PH 140	Manufacture	Fusarium	<i>Fusarium oxysporum</i>
PH 141	Manufacture	Trichoderma	
PH 142	Manufacture	Fusarium	<i>Fusarium solani</i>
PH 143	Manufacture	Fusarium	<i>Fusarium solani</i>
PH 144	Manufacture	Trichoderma	
PH 145	Patensie	Fusarium	<i>Fusarium solani</i>

PH 146	Patensie	To be confirmed	<i>Pithomyces cynodontis</i>
PH 147	Patensie	Fusarium	<i>Clonostachys solani</i>
PH 148	Patensie	Trichoderma	<i>Trichoderma atroviride</i>
PH 149	Patensie	Green mould	<i>Cladosporium cladosporioides</i>
PH 150	Patensie	Fusarium	
PH 151	Patensie	Trichoderma	<i>Trichoderma atroviride</i>
PH 152	Patensie	Rhizopus	<i>Fusarium solani</i>
PH 153	Patensie	Fusarium	<i>Fusarium oxysporum</i>
PH 154	Patensie	Fusarium	<i>Fusarium solani</i>
PH 155	Patensie	Trichoderma	
PH 156	Patensie	Fusarium	<i>Fusarium oxysporum</i>
PH 157	Patensie	Trichoderma	<i>Trichoderma atroviride</i>
PH 158	Patensie	Green mould	<i>Penicillium digitatum</i>
PH 159	Algoa	Fusarium	
PH 160	Algoa	Trichoderma	<i>Trichoderma atroviride</i>
PH 161	Algoa	Fusarium	<i>Fusarium solani</i>
PH 162	Algoa	Trichoderma	<i>Trichoderma atroviride</i>
PH 163	Algoa	Green mould	<i>Penicillium digitatum</i>
PH 164	Algoa	Fusarium	<i>Fusarium solani</i>
PH 165		To be confirmed	<i>Trichoderma atroviride</i>
PH 166	Summerville	Fusarium	<i>Fusarium oxysporum</i>
PH 167	Summerville	Fusarium	<i>Fusarium solani</i>
PH 169	Summerville	To be confirmed	<i>Cladosporium cladosporioides</i>
PH 170	Summerville	Fusarium	<i>Fusarium striatum</i>
PH 171	Summerville	Fusarium	<i>Fusarium solani</i>
PH 172	Summerville	Trichoderma	<i>Trichoderma atroviride</i>
PH 173	Dicllamarone	Rhizopus	
PH 174	Dicllamarone	Trichoderma	<i>Trichoderma atroviride</i>
PH 175	Dicllamarone	Blue mould	<i>Penicillium cairnsense</i>
PH 176	Midlands	Fusarium	
PH 177	Midlands	Trychoderma	
PH 178	Unknown	Fusarium	<i>Fusarium solani</i>
PH 179	Unknown	Trichoderma	
PH 180	ALG 001	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 181	ALG 001	Trichoderma	
PH 182	Algoa	To be confirmed	
PH 183	Algoa	Trichoderma	<i>Trichoderma atroviride</i>
PH 184	Algoa	To be confirmed	<i>Clonostachys solani f. nigrovirens</i>
PH 185	Algoa	To be confirmed	<i>Clonostachys solani f. nigrovirens</i>
PH 186	Citrus Select 004	Trichoderma	
PH 187	Citrus Select 004	Fusarium	<i>Fusarium oxysporum</i>
PH 188	Citrus Select 004	Trichoderma	<i>Trichoderma atroviride</i>
PH 189	Citrus Select 004	Penicillium?	<i>Penicillium citrinum</i>
PH 190	Citrus Select 004	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 191	Groenkloof Citrus 008	Trichoderma	<i>Trichoderma atroviride</i>

PH 192	Marco 1	Trichoderma	
PH 194	Marco 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 195	Marco 1	To be confirmed	<i>Penicillium lanosum</i>
PH 196	Marco 2	Penicillium	<i>Penicillium glabrum</i>
PH 197	Marco 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 198	Marco 2	Penicillium	<i>Penicillium glabrum</i>
PH 199	Marco 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 200	Marco 3	Penicillium	<i>Penicillium glabrum</i>
PH 201	Marco 3	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 202	Marco 4	Fusarium	<i>Fusarium oxysporum</i>
PH 204	Marco 4	Penicillium	<i>Penicillium brevicompactum</i>
PH 205	Midlands 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 206	Midlands 1	Fusarium	
PH 207	Midlands 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 208	Midlands 2	Fusarium	<i>Fusarium oxysporum</i>
PH 209	Midlands 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 210	Morone 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 212	Mouton Citrus 003	Trichoderma	<i>Trichoderma atroviride</i>
PH 213	Noordhoek 002	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 214	Nu Season	Penicillium	<i>Penicillium citrinum</i>
PH 215	Nu Season 005	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 216	Patensie 1	Fusarium	<i>Fusarium oxysporum</i>
PH 217	Patensie 1	Cladosporium	<i>Cladosporium cladosporioides</i>
PH 218	Patensie 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 219	Patensie 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 220	Patensie 2	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 221	Patensie 3	Trichoderma	<i>Trichoderma atroviride</i>
PH 222	Patensie 4	Fusarium	<i>Fusarium oxysporum</i>
PH 223	Ripcurl 9a	Trichoderma	<i>Talaromyces coalescens</i>
PH 224	Ripcurl 9a	Penicillium	<i>Penicillium chrysogenum</i>
PH 225	Ripcurl 9a	Fusarium	<i>Fusarium oxysporum</i>
PH 226	Ripcurl 9b	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 227	Ripcurl 9b	Fusarium	<i>Fusarium oxysporum</i>
PH 228	Ripcurl 9b	To be confirmed	<i>Trichoderma atroviride</i>
PH 229	Ripcurl 9c	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 230	Ripcurl 9c	Trichoderma	
PH 231	Ripcurl 9d		
PH 232	Schenk 1	Trichoderma	
PH 233	Schenk 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 234	Schenk 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 235	Schenk 3	Trichoderma	<i>Trichoderma atroviride</i>
PH 236	Schenk 4	Trichoderma	<i>Trichoderma atroviride</i>
PH 237	Schenk 4	Penicillium	<i>Penicillium citrinum</i>
PH 238	Sonlia 006	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 239	Sonlia 006	Trichoderma	<i>Trichoderma atroviride</i>

PH 240	SRCC 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 241	SRCC 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 242	SRCC 3	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 243	SRCC 4	Trichoderma	
PH 244	SRCC 5	Trichoderma	
PH 245	SRCC 6	Trichoderma	<i>Trichoderma atroviride</i>
PH 246	SRCC 7	Trichoderma	<i>Trichoderma atroviride</i>
PH 247	SRCC 8	Trichoderma	
PH 248	SRCC 9	Trichoderma	<i>Trichoderma atriviride</i>
PH 249	Sundays River 1	Fusarium	<i>Fusarium oxysporum</i>
PH 250	Sundays River 1	Trichoderma	
PH 251	Sundays River 1	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 252	Sundays River 1	To be confirmed	<i>Fusarium solani</i>
PH 253	Sundays River 1	Fusarium	<i>Fusarium oxysporum</i>
PH 254	Sundays River 1	Penicillium	
PH 255	Sundays River 2	To be confirmed	<i>Fusarium solani</i>
PH 256	Sundays River 3	To be confirmed	<i>Fusarium oxysporum</i>
PH 257	Sundays River 3	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 258	Sundays River 4	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 259	Timber 1		
PH 260	Timber 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 261	Timber 1	Trichoderma	<i>Trichoderma citrinoviride</i>
PH 262	Timber 1	Trichoderma	
PH 263	Timber 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 264	Timber 3	To be confirmed	
PH 265	Timber 4	Trichoderma	<i>Talaromyces amestolkiae</i>
PH 266	Unknown 1	Trichoderma	<i>Trichoderma atroviride</i>
PH 267	Unknown 2	Trichoderma	<i>Talaromyces amestolkiae</i>
PH 268	Unknown 2	Trichoderma	<i>Trichoderma atroviride</i>
PH 269	Unifrutti 2	Mucorales	<i>Peniophora crassitunicata</i>
PH 270	Unifrutti 3	Penicillium	<i>Penicillium glabrum</i>
PH 271	Unifrutti 3	Trichoderma	
PH 272	Unifrutti 3	Mucorales	
PH 273	Unifrutti 4	Mucorales	<i>Rhizopus microsporus</i>
PH 274	Unifrutti 4	Fusarium	<i>Fusarium oxysporum</i>
PH 275	Unifrutti 4	Penicillium	<i>Penicillium glabrum</i>
PH 276	Unifrutti 5	Rhizopus	
PH 277	Twycross	To be confirmed	<i>Trichoderma longibrachiatum</i>
PH 278	Twycross	To be confirmed	<i>Trichoderma citrinoviride</i>
PH 279	Twycross		
PH 280	Twycross		
PH 281	SRCC		
PH 282	SRCC	To be confirmed	<i>Talaromyces amestolkiae</i>
PH 283	SRCC		
PH 284	SRCC		

PH 285	SRCC	To be confirmed	<i>Talaromyces amestolkiae</i>
PH 286	SRCC	To be confirmed	<i>Talaromyces amestolkiae</i>
PH 287	SRCC		
PH 288	SRCC		
PH 289	SRCC		
PH 290	SRCC	To be confirmed	<i>Pithomyces chartarum</i>
PH 291	SRCC		
PH 292	SRCC		
PH 293	SRCC		
PH 294	SRCC		
PH 295	SRCC		
PH 296	SRCC		
PH 297	SRCC		
PH 298	SRCC		
PH 299	SRCC	To be confirmed	<i>Curvularia mebaldsii</i>
PH 300	SRCC		



- *Alternaria multiformis*
- *Cladosporium cladosporioides*
- *Clonostachys solani* f. *nigrovirens*
- *Fusarium* cf. *solani*
- *Fusarium solani*
- *Leptosphaerulina chartarum*
- *Penicillium cairnsense*
- *Penicillium digitatum*
- *Penicillium miczynskii*
- *Pithomyces cynodontis*
- *Talaromyces coalescens*
- *Trichoderma citrinoviride*
- *Trichoderma longibrachiatum*
- *Bipolaris cynodontis*
- *Clonostachys rogersoniana*
- *Curvularia mebaldsii*
- *Fusarium oxysporum*
- *Fusarium striatum*
- *Mucor racemosus* strain
- *Penicillium chrysogenum*
- *Penicillium glabrum*
- *Peniophora crassitunicata*
- *Rhizopus microsporus*
- *Trichoderma aggressivum*
- *Trichoderma harzianum*
- *Trichoderma spirale*
- *Cladosporium anthropophilum*
- *Clonostachys solani*
- *Epicoccum sorghinum*
- *Fusarium phaseoli*
- *Gibberella moniliformis*
- *Penicillium brevicompactum*
- *Penicillium citrinum*
- *Penicillium lanosum*
- *Pithomyces chartarum*
- *Talaromyces amestolkiae*
- *Trichoderma atroviride*
- *Trichoderma inhamatum*
- Unknown

**Figure 4.4.2.8.** Graphical representation of the summarised diversity of the fungal contaminants on the pine wood indicates that individual species hold no phytosanitary threat, with *Fusarium* and *Trichoderma* species dominating.

Objective 4: Determine the contribution of storage practices on the occurrence of fungal contamination on palletising wood.

1. Determination of environmental conditions conducive to fungal contamination of wooden pallets, and simulation of environmental and storage conditions to which pallets are subjected, were proposed. It was also thought that of the importance of UV and other environmental conditions in the maintenance and efficacy of wood preservatives would have been assessed. This would have included the use of custom-built equipment to enable simulation of the importance of moisture, wood age, and UV. However, after the findings of the questionnaires and investigation of the various facilities, this aspect was not pursued.
2. The persistence and chemical activity of the wood preservatives were evaluated by the product owners. Both Etch and Pal-It has excellent wood penetration, and the polymers used as part of the formulations, are UV stable. The actives in Etch has German food safety certification.

## Discussion

The project had a slow start, due to two significant factors:

1. The colonisation of wood was a major challenge due to the extremely slow growth rate of the fungi on the untreated wood.
  - a. Incubation at shipping temperatures yielded zero results, regardless of the fact that the pallets are infected at very low temperatures.
  - b. Uninterrupted, high levels of humidity during incubation is important.
2. Unrealistic timelines which exacerbated the delays due to trouble-shooting the fungal growth rate problem. An intern was appointed in 2019, after which the project made excellent progress.

The OPP issue ran parallel to the fungal contamination study. A meeting was called by the CRI at the 2019 Jeffrey's Bay postharvest workshop, involving CRI, PPECB and a few private packhouses, which resulted in PPECB becoming closely involved in facility sourcing and sampling on the project. From this meeting a plan of action was put forward, whereby products currently used to ensure container hygiene, and products used for wood treatment were investigated. When the pome and stone fruit industries learnt about this initiative through PPECB, they became part of the action team through the involvement of HORTGRO. Since there is certainty of the origin of the OPP residues, all affected industries are installing management policies and guidelines to deal with the issue.

Three alternatives to OPP were identified, with a product based on stabilised glutaraldehyde a promising prospect. The product not only controls fungal growth, but has a sealing action. Due to current travel and movement restrictions, the base materials used for the actives has been hard to obtain, but both products are already fully commercialised, with actives that are food grade and has food safety acceptance.

Isolation and identification of the fungal contaminants were critical in ensuring the issue does not pose a phytosanitary risk. Verification of the identity of the fungal isolates were dependent on time being made available at the molecular facility in Nelspruit. Since biosecurity threats take precedence, this often resulted in extended delays. More regular access to the molecular facility would have allowed for more of the unidentified species to be redone and identified with certainty in the lifetime of the project. The species that have been identified (n=226) are ubiquitous and not a phytosanitary risk.

Objective 5: Finalisation of results and trials. Work to be done at CRI. Technology transfer and research papers.

## Technology transfer

Talks or presentations:

The results from the study were made available to both the manufacturing industry and the citrus producers by means of a Cutting Edge and the industry standards for palletising issued by the CRI.

Presentations that were delivered at the annual workshops:

1. Du Plooy, W. & Basson, E. 2019. Fungal growth on pallets. CRI Postharvest Workshops.
2. Du Plooy, W., Rikhotso, M. & Basson, E. 2020. Pallet treatments – update on SOPP saga. CRI Postharvest Workshops.
3. Rikhotso, M. 2020. Fungal degradation of wooden pallets used in export of citrus fruit. CRI Online Postharvest Workshops.

Technology transfer to be completed in 2021 includes a peer reviewed publication, inclusion in a 2021 CRI Symposium, and an article in the South African Fruit Journal.

Titles for SAFJ articles:

1. The importance of storage facilities on the development of wood saprophytes on pallets.
2. Alternative treatments for wood used in palletising, considering food safety and marketing issues.

Title for refereed paper:

1. Fungal diversity on palletising wood types used in the South African fruit industries.

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#### 4.4.3 FINAL REPORT: Evaluation of new postharvest fungicides for the control of *Phytophthora brown rot*

Project PHI-4-07 (1198) (2018/9 – 2020/21) by Jan van Niekerk (CRI), Lize van der Merwe, Micaela Tobias and Cheryl Lennox (USPP)

##### Summary

Brown rot is a postharvest disease of citrus caused by *Phytophthora* spp. Fungicide management of brown rot in South Africa currently consists only of preharvest strategies and nothing is registered for postharvest management of this disease. The objectives of this study were to evaluate the curative and protective efficacy of azoxystrobin (1 125 µg/ml), fludioxonil (598 µg/ml), ammonium and potassium phosphite (1 500 µg/ml) as aqueous dip treatments for the postharvest management of *Phytophthora brown rot* on different citrus types (lemons, oranges and mandarins). Additionally, azoxystrobin (2 500 µg/ml for all three fruit types) and fludioxonil (2 300 µg/ml for lemons and 4 600 µg/ml for oranges and mandarins) amended wax was evaluated for the prevention of spreading of brown rot (nesting) within cartons during transit. Results indicated that the three tested fungicides have good curative action, reducing brown rot incidence significantly when the fungicide was applied 12 h after inoculation. Applications done 24 h after inoculation also provided some curative action but not as effective as earlier applications. Azoxystrobin and potassium phosphite furthermore provided very good protection against infection if inoculations were done up to 48 h after application on all three fruit types but fludioxonil did not fare as well. Interestingly, the protective ability of all three fungicides was better the longer the fungicides remained on the fruit before inoculation. Trials aimed at prevention of nesting during transit indicated that only azoxystrobin amended wax significantly reduced brown rot from spreading to healthy fruit when in contact, compared to the control. The data obtained from this study can add additional value to the already registered postharvest azoxystrobin and fludioxonil fungicides and preharvest registered potassium and ammonium phosphite.

##### Opsomming

Bruinvrot is 'n na-oes siekte van sitrus, veroorsaak deur *Phytophthora* spp. Fungisiedbestuur van bruinvrot in Suid-Afrika bestaan tans slegs uit voor-oes strategieë, en niks is vir die na-oes bestuur van hierdie siekte geregistreer nie. Die doelwit van hierdie studie was om die uitwissende en beskermende effektiwiteit van azoxystrobin (1 125 µg/ml), fludioxonil (598 µg/ml), ammonium- en kaliumfosfaat (1 500 µg/ml) as waterige doopbehandelings vir die na-oes bestuur van *Phytophthora* bruinvrot op verskillende sitrustipes (suurlemoene, lemoene en mandaryne) te evalueer. Addisioneel, is azoxystrobin (2 500 µg/ml vir al drie vrugtipies) en fludioxonil (2 300 µg/ml vir suurlemoene en 4 600 µg/ml vir lemoene en mandaryne) gewysigde waks, vir die voorkoming van die verspreiding van bruinvrot kruiskontaminasie, binne kartonne gedurende vervoer, geëvalueer. Resultate het aangetoon dat die drie getoetste fungisiedes goeie uitwissende aksie gehad het, wat die voorkoms van bruinvrot betekenisvol verminder het wanneer die fungisied 12 ure ná inokulasie toegedien is. Toedienings wat 24 ure ná inokulasie gedoen is, het ook 'n mate van uitwissende aksie verskaf, maar nie so effektief soos vroeër toedienings nie. Azoxystrobin en kaliumfosfaat het verder baie goeie beskerming teen infeksie op al drie vrugtipies verskaf wanneer inokulasies tot 48 ure ná toediening gedoen is, maar fludioxonil het nie so goed gevaar nie. Interessantheidshalwe, die beskermende vermoë van al drie fungisiedes was beter hoe langer die fungisiedes op die vrug gebly het vóór inokulasie. Proewe wat die voorkoming van kruiskontaminasie gedurende vervoer ten doel gehad het, het aangedui dat azoxystrobin gewysigde waks, betekenisvol die verspreiding van bruinvrot na gesonde vrugte wanneer in kontak gekom het, verminder het, in vergelyking met die kontrole. Die data wat vanuit hierdie studie verkry is, kan addisionele waarde tot die reeds geregistreerde na-oes azoxystrobin en fludioxonil fungisiedes en voor-oes geregistreerde ammonium – en kaliumfosfaat toevoeg.

##### Introduction

*Phytophthora brown rot* outbreaks occur sporadically but when they do happen, can cause substantial economic losses (Adaskaveg *et al.*, 2015 a,b; Graham *et al.*, 1998; Timmer *et al.*, 2000). It is especially severe when rainfall

occur during the late stages of fruit development and ripening (Adaskaveg *et al.*, 2015 a,b). In the orchard, foliar applications with phosphonates have been shown to be an excellent protective control measure for brown rot of fruit, as well as root rot caused by *Phytophthora* spp. (Graham, 2011). It is easily absorbed by the leaves of citrus trees from where it is translocated through the phloem to sinks such as developing fruit and roots (Graham, 2011; Ouimette and Coffee, 1990). At the sites where it accumulates it has been shown to have a direct effect on invading pathogens as well as activating the plant's own defence mechanisms (Afek and Szeijnberg, 1988; 1989; Fenn and Coffee, 1984; 1985; Smillie *et al.*, 1989).

In South Africa two species, *Phytophthora citrophthora* and *P. nicotianae*, are the most prevalent species found in citrus orchards. In South Africa, *P. nicotianae* is the main causal agent of brown rot. However, both have been shown to be involved with brown rot on fruit in other citrus production areas such as Florida and California (Graham *et al.*, 1998; Timmer *et al.*, 2000; Meitz-Hopkins *et al.* 2013). Losses due to brown rot can already take place in the orchard, but the biggest problem is when latent infections from orchards makes its way through the packing process and into a carton. Here the latent infection develops into decay during storage and through nesting many fruit become infected and develop brown rot. These brown rot fruit are then also prone to being infected with secondary decay pathogens such as *Penicillium* spp. (Adaskaveg *et al.*, 2015 a,b; Chitzanidis and Argyri, 1990).

Although abovementioned orchard applications do provide a good level of control of brown rot, additional levels of management in the packing process will reduce losses due to quiescent infections on fruit developing further during storage and transit (Adaskaveg *et al.*, 2015 b). Some studies focussed on postharvest treatments consisting of phosphonate postharvest dips alone or in combination with heat treatments. These studies indicated that postharvest dips with potassium phosphite are effective when done within 4 – 18 hrs after picking and even more effective when solutions were heated up (Adaskaveg *et al.*, 2015 b; Lesar 2004). These findings led to this treatment being registered for use in the USA (Adaskaveg *et al.*, 2015 a). However, limitations on phosphorous acid residues on fruit in certain export markets, have prohibited it being used extensively and led to other options for postharvest control of brown rot being investigated (Adaskaveg *et al.*, 2015 a; Hardman and Hattingh, 2015).

Fludioxonil and azoxystrobin are two fungicides that have been investigated for their ability to control green mould (*Penicillium digitatum*) due to resistance development within *Penicillium digitatum* isolates to imazalil, thiabendazole and other traditional postharvest fungicides (D'Aquino *et al.*, 2013; Kanetis *et al.*, 2007; Schirra *et al.*, 2010). Both of these were found to give good control of imazalil sensitive and resistant isolates of *Penicillium digitatum* (D'Aquino *et al.*, 2013; Kanetis *et al.*, 2007; Schirra *et al.*, 2010). Adaskaveg and Förster (2015) showed that if azoxystrobin was applied at 600 mg/L to Valencia or navel orange fruit it gave very good curative and protective control of *Phytophthora* brown rot. However, they only looked at inoculating fruit with *P. citrophthora* either 15 hr before treatment (curative) or 6 hr after treatment (protective). It would therefore be useful to determine if some curative effect of azoxystrobin could be achieved if inoculations are done less than 15 hr (eg. 12 or 6 hr) before treatment. No data could furthermore be found on the *in vitro* sensitivity of *Phytophthora nicotianae* isolates to azoxystrobin. This fungicide is currently under investigation in South Africa for postharvest registration on citrus.

Fludioxonil was recently registered for postharvest use on citrus in South Africa. It was specifically registered as application in wax aimed at sporulation control of *Penicillium* spp. (Du Plooy and Lesar, 2017). As with azoxystrobin, very little is known about the *in vitro* sensitivity of *P. nicotianae* isolates to fludioxonil, or if the application in wax can have any curative effect on quiescent brown rot infections, or if it could protect fruit against becoming infected from brown rot infected fruit during transit and storage.

It is therefore clear that in terms of postharvest *Phytophthora* brown rot control, very little is known about the potential of fludioxonil and azoxystrobin to provide curative action towards latent *P. nicotianae* infections, or impart a protective effect that will limit brown rot infections spreading within cartons.

## **Stated objectives**

1. Collect *Phytophthora nicotianae* isolates from citrus orchards.
2. Determine *in vitro* baseline sensitivity of *Phytophthora nicotianae* isolates to azoxystrobin.
3. Determine *in vitro* baseline sensitivity of *Phytophthora nicotianae* isolates to fludioxonil.
4. Test various zoospore production protocols to reliably produce zoospores.
5. Develop a fruit inoculation technique for postharvest trials.
6. Determine the curative and preventative ability of fludioxonil and azoxystrobin postharvest treatments against *Phytophthora* infections.
7. Determine whether fludioxonil and azoxystrobin postharvest treatments in wax can prevent transfer of *Phytophthora* brown rot through the nesting effect.
8. Evaluating phosphonate products for their curative and protective action against *Phytophthora* Brown Rot caused by *Phytophthora nicotianae*.

## Materials and methods

### 1. Collection of *Phytophthora nicotianae* isolates from citrus orchards

*P. nicotianae* isolates were collected from both organic and conventional orchards. Isolates were obtained from soil samples taken under citrus trees, halfway between the drip line and the trunk. Weeds, grasses and the top 2 cm of soil were removed before taking samples. Samples were collected at a depth of approximately 20 cm and placed in a plastic bag and kept away from direct sunlight. Soil bating was done according to the technique of Grimm and Alexander (1973) as soon as possible where ice trays were used. Five grams (5g) of soil was placed in each compartment of an ice tray, 14 cubicles per ice tray with one soil sample per ice tray. Sterile distilled water was added to the cubicles but with no overflowing of the water between the cubicles. Two surface sterilized citrus leaf discs of 0.5 cm<sup>2</sup> were left to float in each cubicle and covered with tin foil to prevent light infiltration for 2-3 days at ambient temperature. Four randomly selected leaf discs were plated out on selective PARPH (Kannwischer and Mitchell, 1978) media. Petri dishes were subsequently placed in the dark at 29°C for up to three days and inspected for *Phytophthora* spp. infected leaf discs. *P. nicotianae* isolates morphologically identified from these plates were sub-cultured onto water agar (WA). From the WA plates, the isolates were purified through hyphal tipping. Isolates were stored for further work.

### 2. Determine *in vitro* baseline sensitivity of *Phytophthora nicotianae* isolates to fludioxonil.

For the *in vitro* sensitivity of *P. nicotianae* mycelial growth, based on pilot trials, *P. nicotianae* isolates was grown on CMA for 7 days. Five millimetre diameter plugs were made from the margins of actively growing cultures and plated out onto CMA plates amended with fludioxonil (Teacher, 23% a.i, suspension concentrate, ICA International Chemicals, South Africa) to final concentrations of respectively 0-, 1-, 10-, 100-, 1000-, and 10 000 ppm. All inoculated-amended plates were incubated at 29°C for 7 days before measuring the colony diameter. Two measurements, perpendicular to each other, were done of each colony. For the baseline sensitivity, 50 isolates were used from the organic orchards and citrus nurseries that had no previous exposure to these fungicides or fungicides in the same chemical class. For the non-baseline sensitivity, 60 isolates were used from orchards that had possible previous exposure to these fungicides or fungicides from the same fungicide groups. Each isolate x fungicide concentration combination was replicated twice with the whole trial repeated once. From this data the percentage inhibition was calculated and statistical analyses done.

### 3. Determine *in vitro* baseline sensitivity of *Phytophthora nicotianae* isolates to azoxystrobin.

For the *in vitro* sensitivity of *P. nicotianae* mycelial growth, based on pilot trials, *P. nicotianae* isolates was grown on CMA for 7 days. Five millimetre diameter plugs were made from the margins of actively growing cultures and plated out onto CMA plates amended with azoxystrobin [Obstructo, 25% active ingredient (a.i.), suspension concentrate, ICA International Chemicals, South Africa] to achieve concentrations of 0-, 0.25-, 0.5-, 1-, 10-, 100-, and 2000 ppm. The effect of azoxystrobin was tested with the addition of salicylhydroxamic acid (SHAM, 99 %;

Sigma Aldrich Co.) in the amended CMA plates. SHAM was dissolved in methanol to a final concentration of 100  $\mu\text{g/ml}$  and 1 ml was added to 1 L of CMA. The 0 ppm concentration plates for azoxystrobin had no fungicide but was amended with SHAM. All inoculated-amended plates were incubated at 29°C for 7 days before measuring the colony diameter. Two measurements, perpendicular to each other, were done of each colony. For the baseline sensitivity, 50 isolates were used from the organic orchards and citrus nurseries that had no previous exposure to these fungicides or fungicides in the same chemical class. For the non-baseline sensitivity, 60 isolates were used from orchards that had possible previous exposure to these fungicides or fungicides from the same fungicide groups. The same sets of isolates were used for azoxystrobin and fludioxonil sensitivity testing. Each isolate x fungicide concentration combination was replicated twice with the whole trial repeated once. From this data the percentage inhibition was calculated and statistical analyses done.

4. *Test various zoospore production protocols to reliably produce zoospores.*

Zoospore production was optimized from the Lonsdale *et al* (1988) method. Isolates were hyphal tipped from PARPH and grown on CMA media for 4-7 days in a dark room at 24°C. A damp circular autoclaved miracloth was put on a 90 mm pea agar plate and inoculated with 10 mycelial CMA plugs. The inoculated pea agar plates were incubated in the dark for 4 days at 28°C. After the incubated period, the miracloth was transferred to a sterile 250 ml flask with 1/7 diluted pea broth. The flasks were shaken for 48 hrs at 160 rpm in the dark at 22°C. After the shaking period, the pea broth were poured off and 75 ml salt solution was added and shaken for 30 min at 160 rpm before the salt solution were poured off and replaced with 75 ml fresh solution. This step was repeated that resulted in two salt washes in total. After the last wash, the 75 ml salt solution was again poured off and 20 ml of the salt solution was poured in the flask and placed in the shake incubator at 22°C at 160 rpm in the dark for 24 hrs. After the incubated time, the salt solution was poured off and the miracloth was rinsed twice with 100 ml of sterile distilled water. Forty millilitre of fresh distilled water was added to the miracloth in the flasks and the flasks were incubated for 90 min to three hours at 18°C for release of zoospores. The zoospores were filter through sterile miracloth into a falcon tube. Prior to use, zoospores were kept at 19°C in falcon tubes. Quantification of zoospore was done using a haemocytometer. The zoospore counts were on average  $3 \times 10^5$ . Before fruit inoculations were done, 100  $\mu\text{l}$  of the zoospore solution were plated out on CMA and incubated for 48 hrs at 28°C to determine zoospore viability.

5. *Develop a fruit inoculation technique for postharvest trials.*

In preliminary trials factors such as shallow wounding (to simulate rubbing on orchard soil), incubation time and conditions, and rate of disease development were determined in order to develop a protocol that could be used in the postharvest trials for fruit inoculation. The inoculation technique used in the trials described below are the result of this preliminary work.

6. *Determine the curative and preventative ability of fludioxonil and azoxystrobin postharvest treatments against Phytophthora nicotianae infections.*

Curative ability testing

The fruit were wounded superficially at two locations on the side of the fruit using autoclaved 220 grit sandpaper (only superficial wound). Immediately after wounding, a miracloth (1cm<sup>2</sup>) dipped in a prepared zoospore suspension with concentration of  $3 \times 10^5$ , was placed on the wound. After inoculation, the fruit were incubated at 28°C for 6, 12, 24 and 48 hrs before being dipped in azoxystrobin (450 ml/100 L water; Obstructo, 25% active ingredient (a.i.), suspension concentrate, ICA International Chemicals, South Africa) and fludioxonil (260 ml/100 L water; Teacher 230 SC, ICA International Chemicals, Stellenbosch, South Africa). A separate set of 72 inoculated fruit were used for each time point. After the fungicide dips at ambient temperature, the fruit were left to dry before being placed in cartons with moist paper towel balls. Cartons with fruit and moist paper towel balls were enclosed in plastic bags and taped shut to create conditions of high humidity in the carton. The fruit were incubated in the

dark at 28°C and disease incidence ratings took place four days after inoculations. Each fruit was rated based on brown rot symptom development at the point of inoculation. A rating of 1 was given when one wound showed brown rot symptoms while 2 was given when both wounds became infected and 0 when no wounds became infected. Each treatment time point had three replicates and each replicate consisted of 24 fruits. The trial included positive (fruit were wounded and inoculated) and negative (fruits were wounded and dipped in fungicides) controls. The percentage infected wounds were calculated for each replicate and subjected to statistical analysis.

#### Protective ability testing

All the fruit were wounded as described above before being dipped in azoxystrobin or fludioxonil at the concentrations mentioned above. The dipped fruit were left to dry before being inoculated at 6, 12, 24 and 48 hours after the fungicide dip treatment. Again, for each inoculation time point a separate set of fruit was used. After inoculation, the fruit were placed inside a carton with moist paper towel balls. Cartons were again enclosed in plastic bags and taped shut to create conditions of high humidity. After closing, enclosed cartons were incubated in the dark at 28°C and disease incidence ratings were done four days after inoculation. Disease incidence ratings were done as described above. For each inoculation time point three replicates of 24 fruit each were used. The trial included two control treatments. The first control consisted of fruit that were wounded and inoculated. The second control consisted of fruit that were wounded and dipped in the fungicides. The percentage infected wounds were calculated for each replicate and subjected to statistical analyses.

#### *7. Determine whether fludioxonil and azoxystrobin postharvest treatments in wax can prevent transfer of *Phytophthora brown rot* through the nesting effect.*

Fruit were wounded in the same manner as described above. After wounding, fruit was treated with wax amended with the respective fungicides on a custom-built pack line resembling a line in a commercial packhouse. All fruit types were treated with wax (Endura-Fresh, South Africa) amended with 250 ml/ 25 L of azoxystrobin (2500 µg/ml). For fludioxonil, lemons were treated with wax amended with 250 ml/ 25 L of wax (2300 µg/ml) and the oranges and mandarins were treated with wax amended with 500 ml/ 25 L of wax (4600 µg/ml). The dosages of the different fungicides used on the different fruit types were according to the fungicide label or recommendations of the registration holder. The amended wax was applied at a rate of 1.2 L ton<sup>-1</sup> of fruit. After applying the amended wax coating, fruit were dried in the drying tunnel and at ambient temperature in the laboratory. One-week-old inoculated fruit displaying characteristic brown rot symptoms were packed in crates and surrounded by healthy fruit treated with the fungicide amended wax. Each fungicide amended wax treatment, as well as the wounded, untreated control treatment, was replicated four times with each replicate consisting of 20 fruit. When packing the treated and untreated fruit in the crates, it was ensured that the treated or untreated wounds were in contact with the brown rot symptomatic fruit. Four treated fruit surrounded one symptomatic fruit and three symptomatic fruit were placed in a carton. Moisture balls were again added to ensure high humidity during incubation. Fruit were enclosed with plastic bags and incubated in the dark at 28°C for 7 days. After incubation, the number of newly infected fruit were determined based on whether the wound showed brown rot infestation or not.

#### *8. Evaluating phosphonate products for their curative and protective action against *Phytophthora Brown Rot* caused by *Phytophthora nicotianae*.*

Two phosphonates were evaluated as postharvest dip treatments for the citrus fruit: ammonium phosphite (ICA International Chemicals, South Africa) and potassium phosphite (Fighter, Rolfes Agri, South Africa). The dosage for potassium phosphite and ammonium phosphite was 1500 µg/mL. For both fungicide treatments, a 10 L aqueous solution was prepared using municipal tap water at room temperature.

The postharvest treatment trials were repeated on two citrus types, Eureka lemons and Valencia oranges. At each time point and for each fungicide, three replicates of 24 fruit each were inoculated. After fruit were harvested, a chlorine-wash (H<sub>2</sub>O<sub>2</sub>, 150 µg/mL) was done over rotating brushes. Thereafter, fruit were dried in a drying tunnel of

a mini-packline at room temperature. After 24 hrs, the dried fruit were dipped in a solution of imazalil at a concentration of 500 µg/mL for 1 min (Imazacure, ICA International Chemicals, South Africa) to prevent secondary infection by *Penicillium* spp. Fruits were left to dry at room temperature before being stored at 7°C for no longer than a week. The day before treatment trials began fruits were moved from cold storage to room temperature.

Zoospore inoculum was produced as described above. For both curative and preventative trials, wounding was done at two points on each fruit. Wounding and inoculation was done as described above. For the curative trials, fruits were first wounded and dip treated with the respective fungicides 6, 24, 48 or 72 hrs after inoculation. For the preventative trials, fruits were first dip treated for 15 s in the respective fungicides, left to dry and inoculated, using the method described above at 6, 24, 48 or 72 hrs after fungicide treatment. As described above, each fungicide x time point combination was replicated three times with 24 fruits per replicate. Two controls were used in all trials with three replicates of 24 fruit. The positive control consisted of wounded, inoculated, untreated fruit which were evaluated for brown rot seven to eight days after inoculation. The negative control consisted of wounded, un-inoculated, untreated fruits which were evaluated for brown rot seven to eight days after wounding. After each dip treatment, fruits were dried at room temperature in the laboratory. Once dry, fruits were placed in plastic crates on fruit lining along with wet paper towel balls to ensure high humidity conditions inside the crate. Crates were placed in polyethylene bags and tightly shut with sticky tape before incubating for 7-8 d in the dark at 28°C. After the incubation period, fruit were evaluated for brown rot incidence and given a rating of 0, 1 or 2 based on the number of infected wounds on the fruit. A rating of 0 was an indication of no wounds being infected out of 2 total wounds, a rating of 1 an indication of 1 infected wound out of 2 total wounds and a rating of 2 an indication 2 infected wounds out of 2 total wounds.

### *Statistical analyses*

The percentage (%) of brown rot that developed on fruit during the curative and preventative trials was calculated by expressing the number of infected wounds as a percentage of the total number of wounds per treatment replicate. Analysis of variance (ANOVA) was performed on the percentage brown rot using the GLM procedure of SAS statistical software (Version 9.4, SAS Institute Inc., Cary, NC, USA). To test for deviation from normality, the standardized residuals from the model was subject to a Shapiro-Wilk test (Shapiro and Wilk, 1965). To compare the treatment means, a 5% confidence level was used to calculate Fisher's least significant difference (Ott, 1998). A probability threshold of 5% was considered to be significant for all significance tests.

## **Results and discussion**

### Results

#### 1. *Collect Phytophthora nicotianae isolates from citrus orchards.*

Fifty-one isolates were collected for the baseline study from orchards and citrus nurseries not previously exposed to these fungicides or fungicides in the same chemical class. Sixty-one isolates were collected for the non-baseline study from citrus orchards where previous exposure to these fungicides or fungicides from the same chemical groups possibly occurred.

#### 2. *Determine in vitro baseline sensitivity of Phytophthora nicotianae isolates to azoxystrobin.*

The Ward's Cluster analysis of the percentage inhibition data divided the isolates of the previously unexposed *P. nicotianae* population into 4 azoxystrobin sensitivity groups. The power curve was fitted over the percentage inhibition data of isolates within the different groups to calculate the EC<sub>50</sub> and EC<sub>90</sub> values for each isolate in a group. Analysis of variance (ANOVA) of the EC<sub>50</sub> and EC<sub>90</sub> data indicated a significant difference ( $P < 0.0001$ ) between the mean EC<sub>50</sub> and EC<sub>90</sub> of the different sensitivity groups in both populations.

In this *P. nicotianae* population, groups 3 and 4 had statistically similar EC<sub>50</sub> values that were significantly higher than the EC<sub>50</sub> values of groups 1 and 2. Group 2 had the lowest mean EC<sub>50</sub> value of 0.01 ppm that was significantly lower than the mean of group 1 that was 0.05 ppm. In terms of EC<sub>90</sub> values the differences between the groups was much more pronounced (Table 4.4.3.1). Group 4 had a mean EC<sub>90</sub> of 83.96 that was significantly more than double the mean of group 3 (36.83 ppm). The EC<sub>90</sub> of group 1 was 12.81 ppm that was significantly lower than groups 3 and 4 but higher than group 2. The EC<sub>90</sub> of group 2 was again statistically the lowest at 4.28 ppm (Table 4.4.3.1).

In the pathogen population obtained from orchards potentially exposed to this fungicide, the results look slightly different. The Ward's Cluster analysis resulted in 5 azoxystrobin sensitivity groups. Significant ( $P < 0.0001$ ) differences were again observed between the mean EC<sub>50</sub> and EC<sub>90</sub> values of the different groups (Table 4.4.3.1). The mean EC<sub>50</sub> values of groups 1 and 5 are statistically similar (0.11 and 0.09 ppm respectively). Despite being low, these were statistically higher than that of group 3 at 0.04 ppm. Group 2's EC<sub>50</sub> value was 0.30 that was 2-3 times significantly higher than the 3 groups mentioned above. The highest EC<sub>50</sub> was that of group 4 at 0.46 (Table 4.4.3.1). The EC<sub>90</sub> data indicated that this value for group 3 was 11.45 ppm that was also statistically the lowest EC<sub>90</sub> of all groups. Group 1 had a significantly higher mean EC<sub>90</sub> value (17.06 ppm) compared to group 3. The EC<sub>90</sub> value for group 5 was statistically third highest at 26.83 ppm and that was followed by group 4 (52.59 ppm) and group 2 with the highest mean EC<sub>90</sub> value of 84.85 ppm (Table 4.4.3.1).

**Table 4.4.3.1.** Mean EC<sub>50</sub> and EC<sub>90</sub> values of the different azoxystrobin sensitivity groups within the unexposed and previously exposed *P. nicotianae* populations and calculated from the power curve function fitted to percentage inhibition and fungicide concentration data of isolates within each group.

<i>Phytophthora nicotianae</i> population		Sensitivity group	EC <sub>50</sub> (ppm) <sup>1</sup>	EC <sub>90</sub> (ppm)
Previously unexposed (baseline) n = 51		1	0.05 b	12.81 c
		2	0.01 c	4.28 d
		3	0.18 a	36.83 b
		4	0.19 a	83.96 a
LSD-value			0.013	2.486
SL <sup>2</sup>			<0.0001	<0.0001
Previously exposed (non-baseline) n = 60		1	0.11 y	17.06 z
		2	0.30 x	84.85 w
		3	0.04 z	11.45 e
		4	0.46 w	52.59 x
		5	0.09 y	26.83 y
LSD-value			0.022	2.448

SL

&lt;0.0001

&lt;0.0001

<sup>1</sup>Means followed by the same letter are not significantly different at a 95% confidence level.

<sup>2</sup>SL = significance level

### 3. Determine *in vitro* baseline sensitivity of *Phytophthora nicotianae* isolates to fludioxonil.

A Ward's Cluster analysis was also done on the percentage inhibition per concentration data following *in vitro* sensitivity testing of the previously exposed and unexposed *P. nicotianae* populations described above. The clustering indicated that the isolates from both populations could be divided into 5 different fludioxonil sensitivity groups. Analysis of variance of the EC<sub>50</sub> and EC<sub>90</sub> data showed that there were significant ( $P < 0.0001$ ) differences between groups with regards to these two variables for both populations. In the case of EC<sub>50</sub> values of the previously unexposed population the statistically highest value was seen for group 5 (1613.52 ppm). The second highest mean was that of group 4 at 1111.80. The means for group 1-3 were statistically much lower and ranged between 5.56 ppm (group 1) and 77.63 ppm (group 3; Table 4.4.3.2). In the case of the EC<sub>90</sub> results group 4 had the highest mean at 9929.30 ppm followed by a significantly lower mean for group 5 that were 7718.50 ppm. The lowest mean EC<sub>90</sub> value was recorded for group 3 (1988.50 ppm). The means of groups 1 and 2 were 4907.60 ppm and 4021.80 ppm, respectively. These were statistically the third and fourth highest means (Table 4.4.3.2).

In the previously exposed population group 5 had the significantly highest mean EC<sub>50</sub> value of 84.79 ppm. The mean EC<sub>50</sub> values of the other 4 groups were also statistically different from one another but were in value much lower than that of group 5. The latter values ranged between 3.10 ppm (group 2) and 9.95 ppm (group 4; Table 4.4.3.2). In terms of mean EC<sub>90</sub> values in this population, the ranking of the groups also changed. Group 4 had the statistically highest mean of 6809.90 ppm followed by groups 1 (5748.82 ppm) and 3 (5472.36 ppm). The mean of group 2 was 4987.18 ppm and group 5 had the statistically lowest mean of 1090.50 ppm (Table 4.4.3.2).

**Table 4.4.3.2.** Mean EC<sub>50</sub> and EC<sub>90</sub> values of the different fludioxonil sensitivity groups within the unexposed and previously exposed *P. nicotianae* populations and calculated from the cubic fits function fitted to percentage inhibition and fungicide concentration data of isolates within each group.

<i>Phytophthora nicotianae</i> population	Sensitivity group	EC <sub>50</sub> (ppm) <sup>1</sup>	EC <sub>90</sub> (ppm)
Previously unexposed (baseline) n = 51	1	5.56 z	4907.60 x
	2	25.03 z	4021.80 y
	3	77.63 y	1988.50 z
	4	1111.80 x	9929.30 v
	5	1613.52 w	7718.50 w
LSD-value		25.252	300.73
SL <sup>2</sup>		<0.0001	<0.0001
Previously exposed (non-baseline)	1	5.87 d	5748.82 b

n = 60	2	3.10 e	4987.18 d
	3	8.93 c	5472.36 c
	4	9.95 b	6809.90 a
	5	84.79 a	1090.50 e
LSD-value		0.839	182.97
SL		<0.0001	<0.0001

<sup>1</sup>Means followed by the same letter are not significantly different at a 95% confidence level.

<sup>2</sup>SL = significance level

#### 4. Test various zoospore production protocols to reliably produce zoospores.

Several protocols from literature were combined to develop a protocol that allowed for the constant production of zoospores. The protocol developed is described in the materials and methods section above.

#### 5. Develop a fruit inoculation technique for postharvest trials.

Following some pilot trials, it was established that *Phytophthora* brown rot can be successfully induced on lemons and navel oranges. The final protocol developed is described above and was used in testing the curative and preventative ability of azoxystrobin and fludioxonil in postharvest management of *P. nicotianae* causing brown rot.

#### 6. Determine the curative and protective ability of fludioxonil, azoxystrobin and potassium phosphite postharvest treatments against *Phytophthora nicotianae* infections.

##### Curative ability testing

Analysis of variance (ANOVA) of percentage brown rot data obtained from curative action trials done on lemons, oranges and mandarins indicated that there was a significant ( $P < 0.0001$ ) treatment fungicide x time interaction for all fruit types (ANOVA not shown). The curative results from lemons indicated that if fruit were treated with azoxystrobin 6 hrs after *P. nicotianae* zoospore inoculation, no brown rot developed. When treated 12 h after inoculation, the mean percentage brown rot increased to 5.6% that was statistically the same as at 6 h (Figure 4.4.3.1A). However, when treatment occurred 24 h after inoculation, the percentage brown rot increased significantly to 37.5%. With treatment 48 h after inoculation, the mean further increased significantly to 95.83%. This was significantly more than even the untreated control (76.0% brown rot), indicating the loss of the curative action of azoxystrobin (Figure 4.4.3.1A).

The same trend was seen for the fludioxonil curative treatment. Treatment 6 h after inoculation again resulted in the lowest percentage brown rot (1.4%) that was statistically similar to the mean observed at 12 h (2.8%) (Figure 4.4.3.1A). The percentage brown rot increased significantly to 25.0% with treatment at 24 h. However, all these time points had statistically lower means than were observed in the untreated, inoculated control. Fludioxonil treatment 48 hrs after inoculation resulted in 72.2% brown rot that was statistically the same as the mean of the untreated, inoculated control (80.2% brown rot) (Figure 4.4.3.1A). A slightly different trend was seen for the potassium phosphite curative treatment on lemons. Treatment 6 and 12 h after inoculation led to a mean percentage brown rot that was statistically the same (26.4%). When treated 24 h after inoculation, the mean percentage brown rot increased to 68.1%, which was statistically higher than the means observed at 6 and 12 h

(Figure 4.4.3.1A). This was a statistically lower mean than when treatment occurred 48 h (91.67%) after inoculation, but was statistically similar to the inoculated, untreated control.

Brown rot control results obtained from the curative treatment trials on oranges indicated that fruit treated with azoxystrobin 6 hrs after inoculation, had a mean percentage brown rot of 6.9%, which was statistically similar to the mean observed from fruit treated 12 h after inoculation (1.4%) (Figure 4.4.3.2A). The percentage brown rot increased significantly to 37.5% with treatment at 24 h after inoculation. However, 48 h (52.8%) was statistically the same as the inoculated untreated control (61.5%) (Figure 4.4.3.2A). The same trend was observed for the curative results obtained with fludioxonil on oranges. Treatment 6 hrs after inoculation had the third highest mean percentage brown rot of 20.8%. The percentage brown rot decreased significantly to 0.0% with treatment at 12 h. When treatment occurred 24 h after inoculation, the percentage brown rot increased statistically to 55.6% that was statistically similar to the control. Again, treatment 48 hrs after inoculation, resulted in no control and the mean percentage brown rot that developed was 72.2% which was significantly more than the inoculated untreated control (61.5%) (Figure 4.4.3.2A).

The potassium phosphite treatment on oranges gave overall very good curative control up to 48 hrs after inoculation and had a slightly different trend than for azoxystrobin and fludioxonil. Mean percentage brown rot observed for 6 and 12 h was 1.4% and 2.8% respectively, which was statistically the same. However, the mean percentage brown rot decreased with treatment at 24 h after inoculation and was 0.0%, statistical similar to the fludioxonil treatment 12 h after inoculation. The mean percentage brown rot increased to 15.3% 48 h after inoculation. However, this was still significantly lower than the inoculated untreated control (Figure 4.4.3.2A).

The percentage brown rot data from the curative trial on mandarin fruit showed a similar trend as that observed on the oranges. Data indicated that if fruit was treated with azoxystrobin 6 h after inoculation the mean percentage brown rot was 22.2%. When treated 12 h after inoculation, the mean percentage decreased to 15.3% that was statistically the same as the 6 hrs treatment. However, when treatment occurred 24 hrs after inoculation, the percentage brown rot increased significantly to 48.6%. With treatment 48 hrs after inoculation, the mean percentage brown rot increased again significantly to 58.3%. However, the mean percentage brown rot observed at all four curative treatment time points was statistically lower than the inoculated untreated control, which had a mean of 76.0% (Figure 4.4.3.3A). Fludioxonil treatment had the same trend as observed for azoxystrobin. The highest mean percentage brown rot (43.1%) were seen when treatment was done 6 hrs after inoculation. This was statistically more than the mean percentage brown rot observed at hour 12 treatment point (16.7%) (Figure 4.4.3.3A). The mean brown rot increased again significantly to 47.2%, statistically similar level to that observed with treatment at 6 hrs after inoculation. The mean percentage brown rot for 48 hrs after inoculation (48.6%) was statistically similar to 6 hrs and 24, but again all treatment time points had mean percentages brown rot significantly lower than the control (Figure 4.4.3.3A). Potassium phosphite curative treatment results on mandarins were again slightly different. Hours 6, 12 and 24 were all statistically similar with means of respectively 4.2%, 5.6% and 13.9%. However, potassium phosphite treatment 48 hrs after inoculation of the mandarins resulted in a mean of 62.5% that was statistically the same as the inoculated, untreated control (Figure 4.4.3.3A).

#### Protective ability testing

The ANOVA of percentage brown rot resulting from protective ability trials on lemons, oranges and mandarins indicated again a significant ( $P < 0.0001$ ) fungicide x treatment time interaction (ANOVA not shown). In the case of azoxystrobin, it was seen that when lemon fruit was inoculated 6, 12, 24 or 48 h after fungicide treatment, the mean percentages brown rot developing was statistically the same. The means ranged from 2.8% at 48 hrs to 15.3% at 12 h (Figure 4.4.3.1B). These means were all significantly lower than the untreated, inoculated control with a mean of 80.2%. Interestingly, the means declined with increasing time of inoculation after treatment (Figure 4.4.3.1B). Fludioxonil treatment on lemons had a similar trend but showed over all poorer protective ability in comparison to azoxystrobin and potassium phosphite. Inoculation 6 hrs after treatment resulted in 62.2% brown rot development, which was statistically lower than the control (80.2%). From 6 hrs after treatment to 12 h after

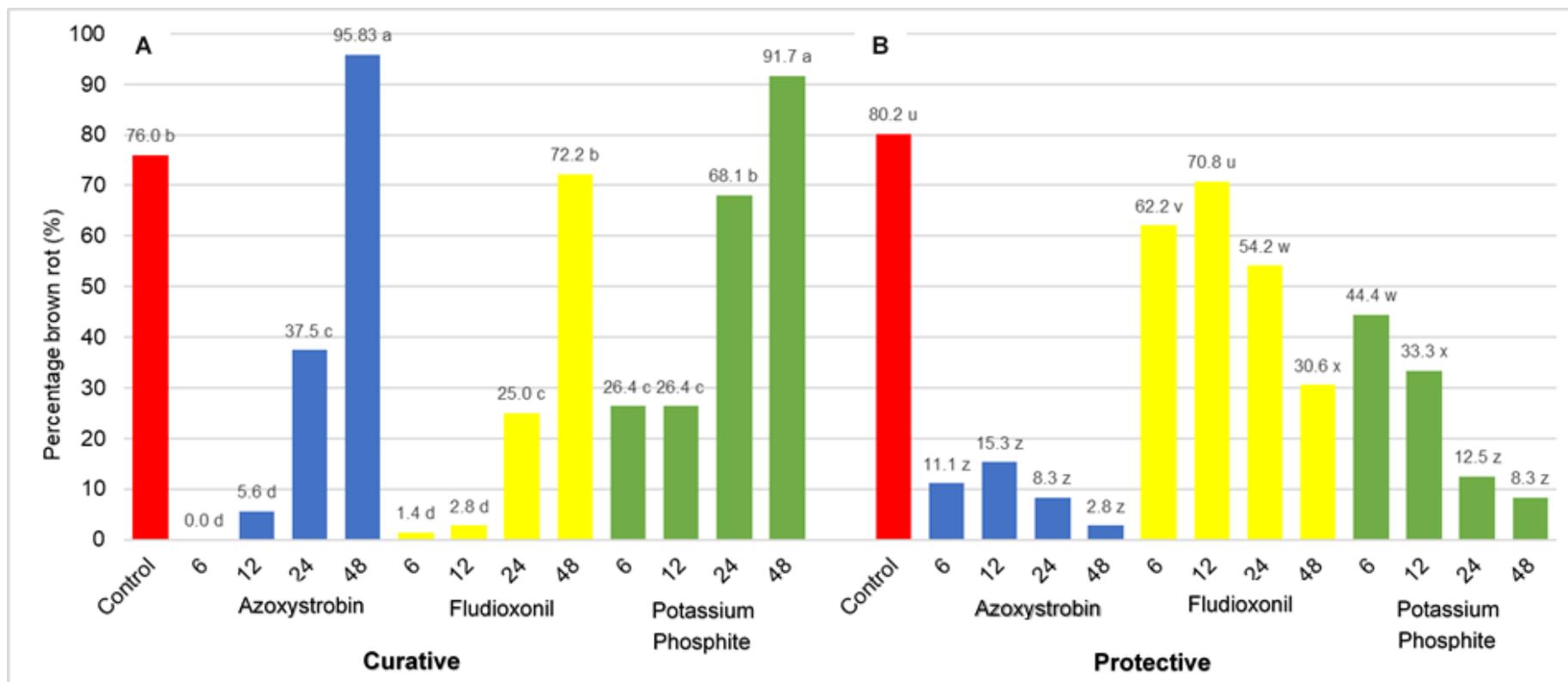
treatment, it increased to 70.8%, which was statistically similar to the inoculated, untreated control (80.2%) (Figure 4.4.3.1B). When inoculation was done 24 h after treatment, the mean percentage brown rot decreased to 54.2% that was statistically lower than the control. The statistically best protective action with fludioxonil was seen when inoculation was done 48 h after treatment. For this inoculation time point, the mean was 30.6% (Figure 4.4.3.1B). It was therefore seen for fludioxonil, that the protective ability improved with increasing time of inoculation after treatment.

The protective ability of potassium phosphite on lemons had a different trend than for azoxystrobin and fludioxonil but the trend to protect the fruit was the same as there where better protection with increased time after the fungicide treatment. Mean percentage brown rot with inoculation 6 hrs after treatment was 44.4% and decreased significantly when inoculation occurred 12 hrs after treatment as the mean brown rot percentage decline to 33.3% (Figure 4.4.3.1B). The percentage brown rot decreased even further at the inoculation time points of 24 (12.5%) and 48 h (8.3%) after treatment that were statistically similar to each other. All the inoculation time points of protective potassium phosphite treatments had means that were statistically lower than the inoculated untreated control (80.2%) (Figure 4.4.3.1B).

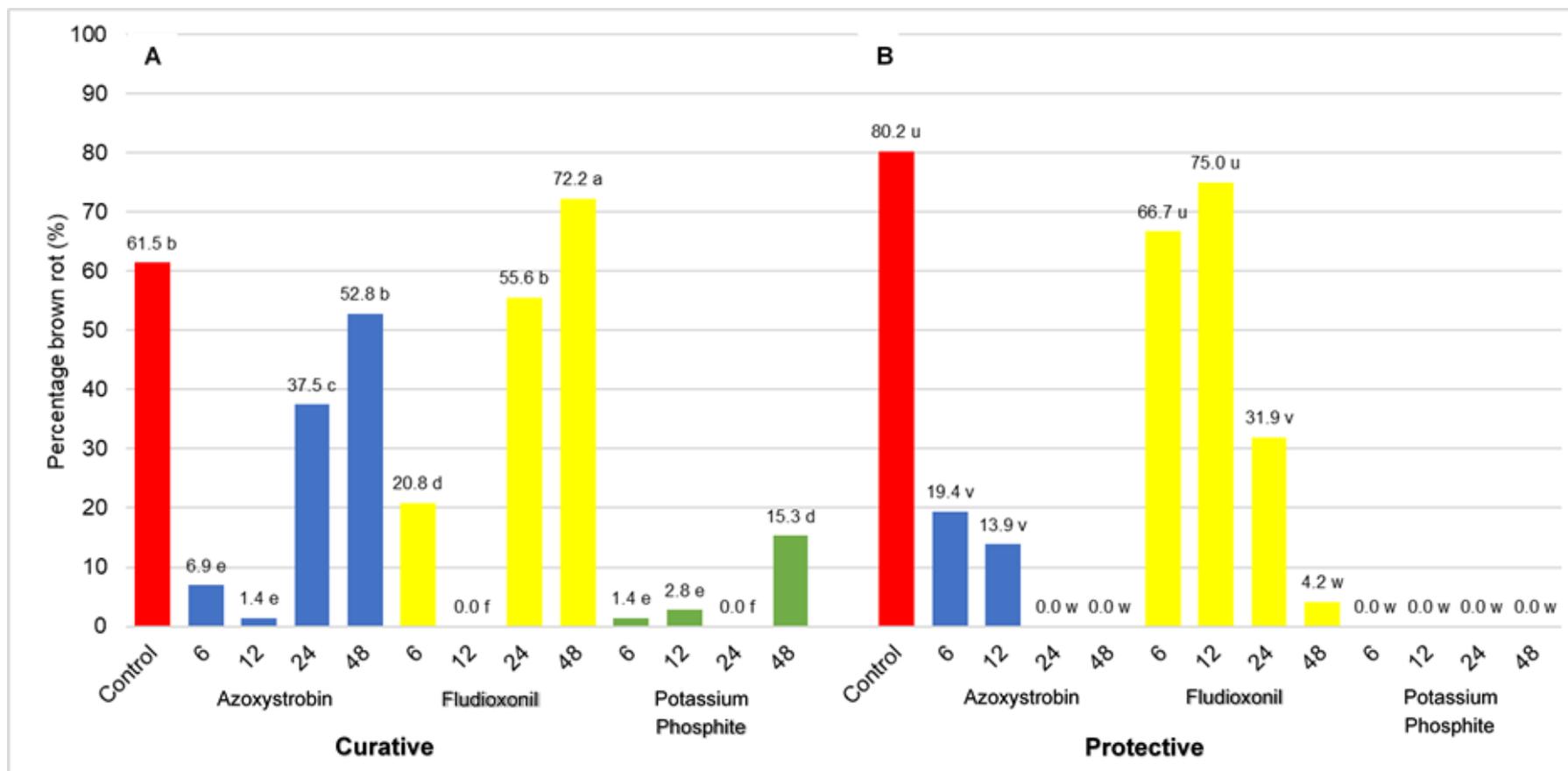
Analysis of data for protective ability on oranges, indicated that if fruit was inoculated with *P. nicotianae* zoospores 6 and 12 h after azoxystrobin treatment, the mean percentage brown rot was statistically the same and respectively 19.4% and 13.9% (Figure 4.4.3.2B). However, when fruit was inoculated 24 and 48 h after treatment, no brown rot occurred (0.0%). This trend is similar to the lemons, as protective ability got better when inoculation was done with increasing time after fungicide treatment (Figure 4.4.3.2B). However, with potassium phosphite, at all four time points (6-48 h), no brown rot was observed (0.0%), which indicated an excellent protective ability on oranges. For both azoxystrobin and potassium phosphite treatment, all inoculation time points had means that were statistically lower than the inoculated, untreated control (80.2%) (Figure 4.4.3.2B).

The percentage brown rot observed on oranges treated with fludioxonil was much higher at all time points than oranges treated with azoxystrobin and potassium phosphite. When inoculation occurred 6 (66.7%) and 12 h (75.0%) after treatment, it was statistically the same to the inoculated untreated control (80.2%) (Figure 4.4.3.2B). Again, the percentage brown rot decreased significantly to 31.9% with inoculation at 24 h. Inoculation 48 hrs after fludioxonil treatment resulted in 4.2% brown rot, which was again a significant decrease. Mean percentages brown rot observed 24 and 48 h after treatment were statistically lower than the control (Figure 4.4.3.2B). Again, better protective action was observed the longer the fungicide was present on the fruit before inoculation.

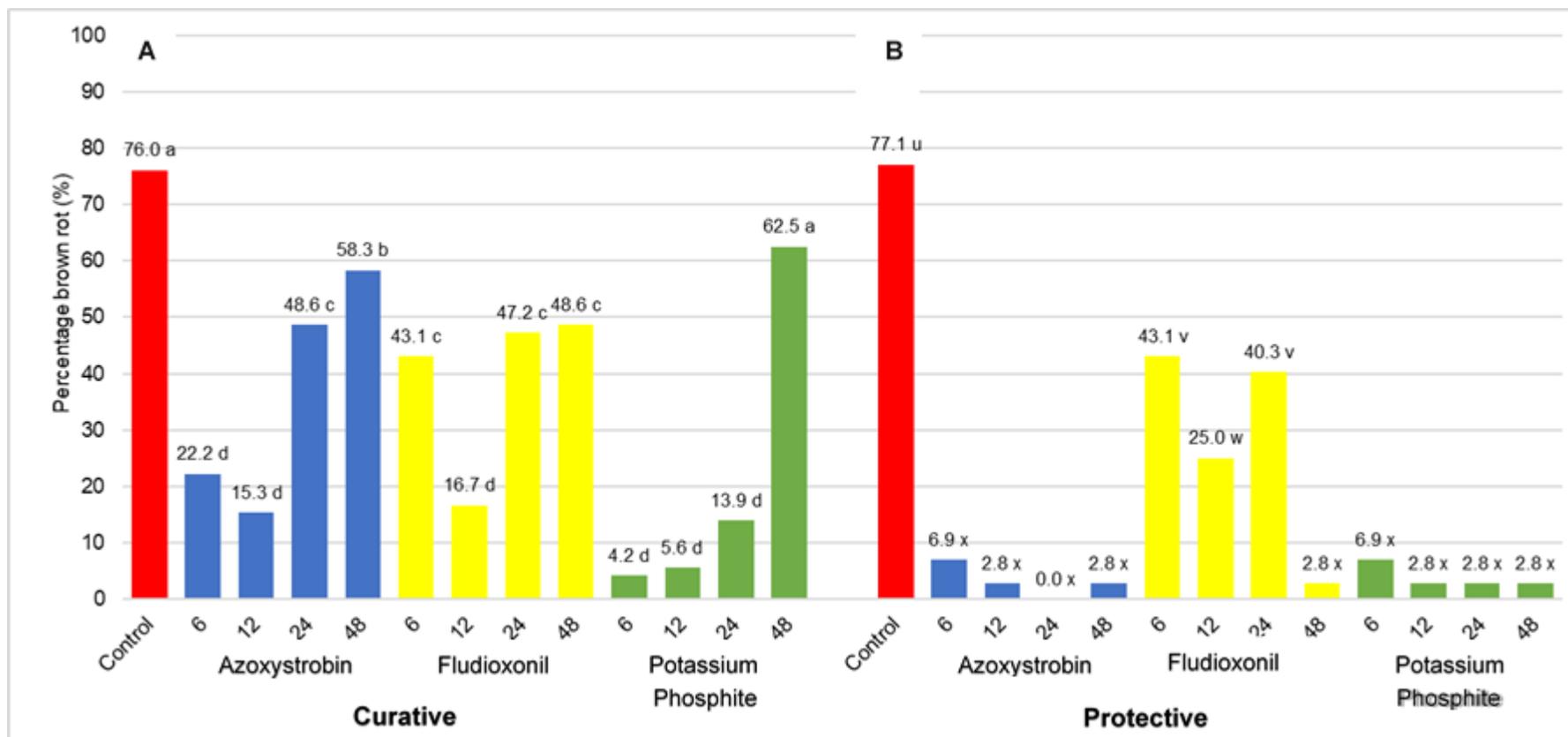
The mean percentage brown rot observed following protective trials on mandarins, were very low for all three fungicides. Treatment with azoxystrobin, showed that when fruit was inoculated 6, 12, 24 or 48 h after fungicide treatment, the mean percentages brown rot that developed was statistically the same, which was the same as the protective lemon data. Again, inoculation 6 h after treatment had mean percentage brown rot of 6.9%, hour 12 and 48 where both 2.8% and fruit inoculated 24 h after treatment had no brown rot lesions (0.0%). Means were furthermore all significantly lower than the untreated, inoculated control (77.1%) (Figure 4.4.3.3B). Protective fludioxonil treatment on mandarins had a different trend than azoxystrobin. Mean percentage brown rot was 43.1% when inoculation was done 6 h after treatment. Inoculation at hour 12 resulted in statistically lower mean brown rot (25.0%) than inoculation at hour 6. In the case of inoculation 24 h after treatment, the mean percentage brown rot increased again to 40.3%, which was statistically similar to the mean of the 6 hrs inoculation time. However, 48 h after fludioxonil treatment, the brown rot percentage decreased significantly to 2.3%, which has the same trend as the other fruit for fludioxonil where hour 48 provided good protective action. Potassium phosphite protective data on mandarins had a statistical similar trend as for azoxystrobin where 6 h after treatment, the mean percentage brown rot was 6.9% and hrs 12-48, 2.8%, and all four time points were statistically the same (Figure 4.4.3.3B). The mandarins showed no phytotoxic damage even with a double dosage of potassium phosphite (3 000 µg/ml).



**Figure 4.4.3.2.** Mean percentage brown rot that developed on lemons, when fruit was treated curatively (A) with azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs after inoculation with *P. nicotianae* zoospores and protectively (B), when fruit was treated with either azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs before inoculation with *P. nicotianae* zoospores.



**Figure 4.4.3.2.** Mean percentage brown rot that developed on oranges when fruit was treated curatively (A) with azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs after inoculation with *P. nicotianae* zoospores and protectively (B), when fruit was treated with either azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs before inoculation with *P. nicotianae* zoospores.



**Figure 4.4.3.3.** Mean percentage brown rot that developed on mandarins when fruit was treated curatively (A) with azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs after inoculation with *P. nicotianae* zoospores and protectively (B), when fruit was treated with either azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs before inoculation with *P. nicotianae* zoospores.

7. Determine whether fludioxonil and azoxystrobin postharvest treatments in wax can prevent transfer of *Phytophthora brown rot* through the nesting effect.

The ANOVA of the percentage brown rot data resulting from nesting prevention trials on lemons, oranges and mandarins indicated that there was a significant fungicide (treatment) effect of respectively 0.0137, 0.0018, on lemons and oranges. However, on mandarins the fungicide effect was not significant ( $P = 0.5320$ ) (ANOVA not shown). Thus, not one of the two tested fungicides were effective on mandarins. In the case of lemons, analysis of the percentage brown rot data for wax amended with azoxystrobin and placed adjacent to rotted fruit, indicated that the mean percentage brown rot that developed was 41.7%, which was significantly lower than the mean observed on fruit from the wax only control (79.2%). The mean percentage brown rot that developed when fruit was covered with fludioxonil amended wax was 70.8% that was statistically similar to the mean of the control (Table 4.4.3.3).

With the oranges, there was a similar trend to that with the lemons, as the mean percentage brown rot of the fruit treated with azoxystrobin amended wax was 56.3%, which was significantly lower than the unamended control mean of 100%. In the case of fruit covered with wax amended with fludioxonil, the percentage brown rot was 89.6% that was statistically the same as the unamended control. The percentage brown rot that developed on mandarins with treatment of azoxystrobin, fludioxonil and the control was respectively 91.7%, 95.8% and 97.9% (Table 4.4.3.3). It was seen that when fruit was covered with wax amended with azoxystrobin and fludioxonil, the mean percentage brown rot that developed were statistically the same as that of the wax only treatment and thus gave no protection.

**Table 4.4.3.3.** Mean percentage brown rot developing on lemons, oranges and mandarins treated with either azoxystrobin, fludioxonil or unamended wax before exposure to *Phytophthora nicotianae* infected fruit in a carton.

Wax treatment	N	Lemons	Oranges	Mandarins
Azoxystrobin	4	41.67 b <sup>1</sup>	56.25 m	91.67 z
Fludioxonil	4	70.83 a	89.58 n	95.83 z
Control (wax)	4	79.17 a	100 n	97.92 z
LSD		23.51	19.62	12.37

<sup>1</sup>Means followed by the same letter are not significantly different at a 95% confidence level.

8. Evaluating phosphonate products for their curative and protective action against *Phytophthora Brown Rot* caused by *Phytophthora nicotianae*.

*Curative ability*

Two citrus fruit types – lemons and oranges – were inoculated with a suspension of *P. nicotianae* zoospores and dip treated with ammonium phosphite or potassium phosphite at 6, 24, 48 and 72 h post-inoculation (hpi). Analysis of variance (ANOVA) of the percentage brown rot data from the curative trials on the two citrus types indicated a significant ( $P < 0.0001$ ) fungicide x time interaction for lemons and oranges (ANOVA not shown).

The results of the curative trial on lemons indicated that if the fruits were treated with ammonium phosphite up to 24 hpi, the brown rot incidence was minimal (1-2% brown rot) (Figure 4.4.3.4). The percentage of brown rot increased to 15% when fruit were treated with ammonium phosphite at 48 hpi. When fruits were treated at 72 hpi, an increase of 28% was observed. The mean percentage brown rot at each time point was statistically lower than the untreated, inoculated control (50% of brown rot).

When the lemons were treated using potassium phosphite, the same trend was observed. Fruit treated at 6 and 24 hpi with *P. nicotianae* zoospores had almost no brown rot (1% of brown rot) (Figure 4.4.3.4). However, the percentage brown rot increased to 23% when fruit were treated at 48 hpi. Interestingly, the brown rot incidence was higher when fruits were treated with ammonium phosphite at 48 hpi (13% brown rot). The percentage of brown rot at 72 hpi for fruit treated with potassium phosphite was 26% which was close to the results observed at the same time point with ammonium phosphite (28%). For lemons, both ammonium phosphite and potassium phosphite had very good curative control (Figure 4) when the treatment was applied up to 24 hpi: the incidence of brown rot did not exceed 2%. Additionally, the overall brown rot incidence on lemons across all post-inoculation time points was significantly lower than the percentage of brown rot for the untreated control.

In the curative trial on oranges, fruit treated with ammonium phosphite at 6 and 24 hpi with *P. nicotianae* zoospores had brown rot percentages of 14% and 5%, respectively (Figure 4.4.3.5). The incidence of brown rot increased significantly to 48% when fruit were treated at 48 hpi. And at 72 hpi, the brown rot incidence increased further to 62%. This was the largest percentage of brown rot noted amongst all the fungicide x time combinations for the curative trial data. For each time point, the brown rot incidence was statistically lower than the brown rot incidence for the untreated, inoculated control (84% of brown rot).

When oranges were treated curatively with potassium phosphite, the brown rot incidence at 6 and 24 hpi was 15% and 10%, respectively (Figure 4.4.3.5). The percentage brown rot increased significantly to 40% when fruit were treated at 48 hpi. However, a decrease of 9% was observed at 72 hpi in comparison to the 48 hpi time point. The percentage brown rot at 48 and 72 hpi were statistically similar. However, the brown rot incidence at all time points were significantly lower than the untreated, inoculated control (84% of brown rot).

Although similar trends were observed in the curative data for both citrus types, the overall brown rot incidence across all time points was higher for oranges than for lemons. While the percentage brown rot at all time points was lower than that for the untreated control, the highest percentage was observed in the curative trial when oranges were treated with ammonium phosphite at 72 hpi with *P. nicotianae* zoospores.

#### *Preventative ability*

Lemons and oranges were dip treated with ammonium phosphite or potassium phosphite and then inoculated with a *P. nicotianae* zoospore suspension at 6, 24, 48 and 72 h post-treatment (hpt). Analysis of variance (ANOVA) of the percentage brown rot data from the preventative trials on the two citrus types indicated a significant fungicide x time interaction for both citrus types (ANOVA not shown). However the interaction for lemons was much weaker ( $P = 0.0435$ ) than for oranges ( $P < 0.0001$ ) (ANOVA not shown).

The data from the preventative trial on lemons showed poor preventative control for both ammonium phosphite and potassium phosphite treatments. The overall percentage of brown rot was furthermore higher for lemons treated with ammonium phosphite (Figure 4.4.3.6). The brown rot incidence on lemons at 6 hrs, 48 hrs and 72 hpt were all statistically similar (Figure 4.4.3.6). At 6 hpt, the percentage of brown rot on lemons was 49%. There was an increase in the brown rot incidence to 56% at 24 hpt. However, a decrease to 51% was observed at the 48 and 72 hpt. For all time points, the percentage of brown rot incidence was lower than that for the untreated control (74% of brown rot), except for the result at 24 hpt (56%) which was statistically similar to the untreated, inoculated control (74% brown rot).

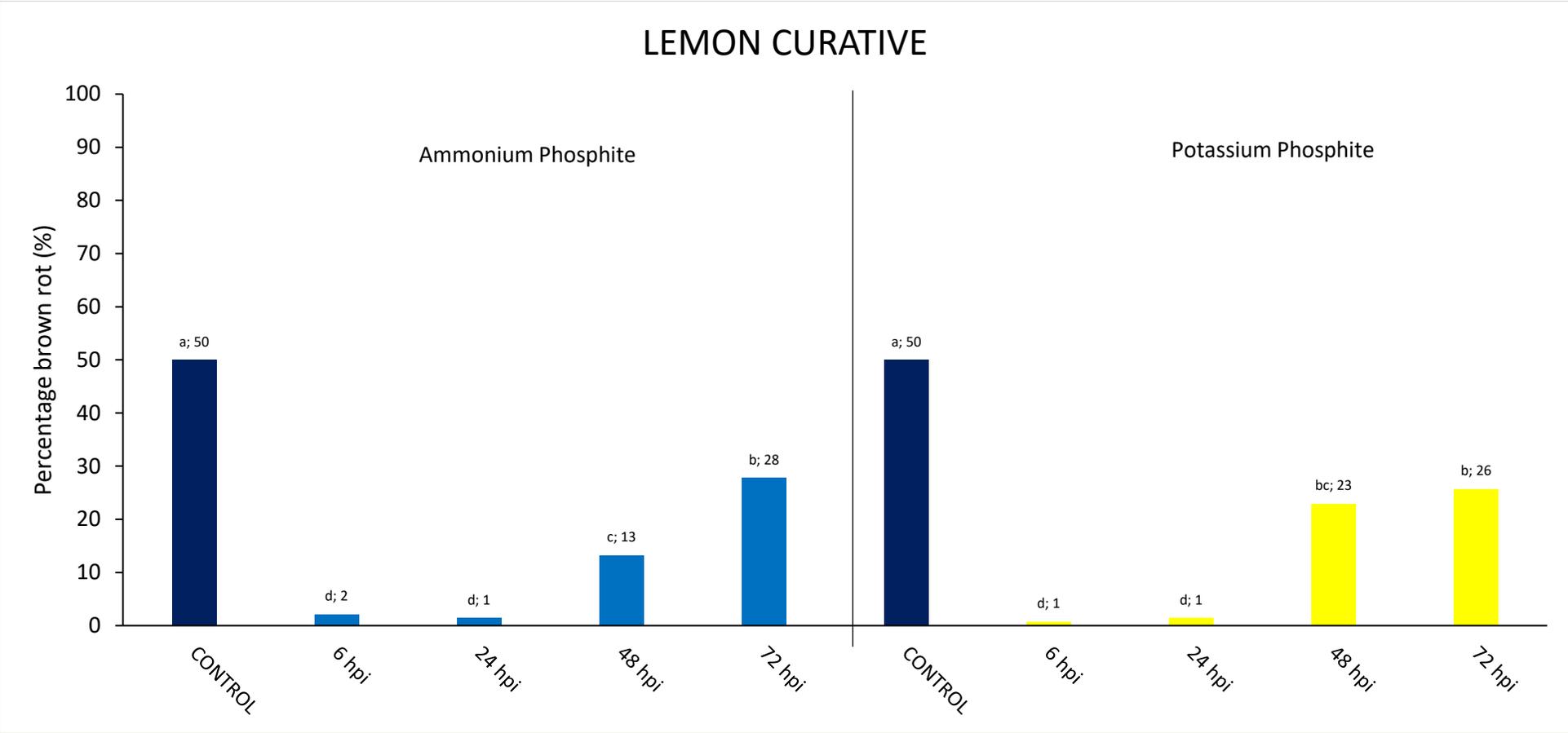
When potassium phosphite was used preventatively on lemons, the incidence of brown rot had more intense fluctuations across all the fungicide x time combinations (Figure 4.4.3.6). The percentage of brown rot at 6 hpt (45%) and 12 hpt (53%) were not statistically different. A decrease in the percentage of brown rot was observed when lemons were inoculated at 48 hpt (36% brown rot). The highest incidence of brown rot was observed at 72 hpt (58%) which was statistically similar to the untreated, inoculated control (74%) and to the 6 (45%) and 24 (53%) hpt time points (Figure 4.4.3.6).

The data from the preventative trial on oranges indicated excellent control by ammonium phosphite and potassium phosphite. For oranges treated preventatively with ammonium phosphite, the brown rot incidence

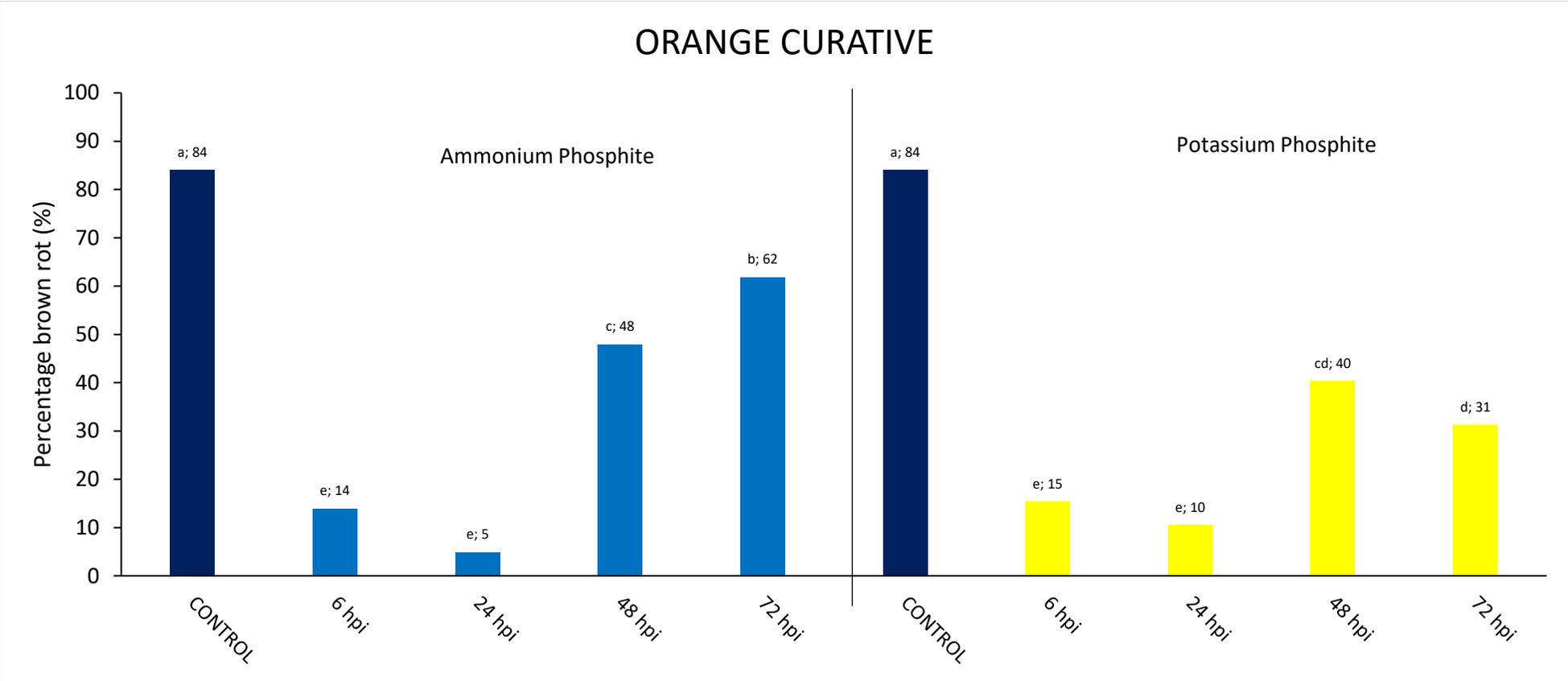
for fruit inoculated at 6 hpt was 19% which was still statistically lower than for the untreated control (48% of brown rot) (Figure 4.4.3.7). When fruits were inoculated at 24 hpt the incidence of brown rot decreased to 1%. This was not statistically different to fruit that were inoculated at 72 hpt when no brown rot developed. At 48 hpt, the percentage of brown rot on oranges was 5% which was statistically similar to the brown rot incidence observed at 6 (13%) hpt.

When oranges were treated preventatively with potassium phosphite, the percentage of brown rot at 6 hpt and 24 hpt was 5% at both time points. There was a further decrease in brown rot incidence to 1% at 48 and 72 hpt with potassium phosphite. The brown rot incidence on oranges was significantly lower than the untreated control (48% of brown rot) for both potassium phosphite and ammonium phosphite.

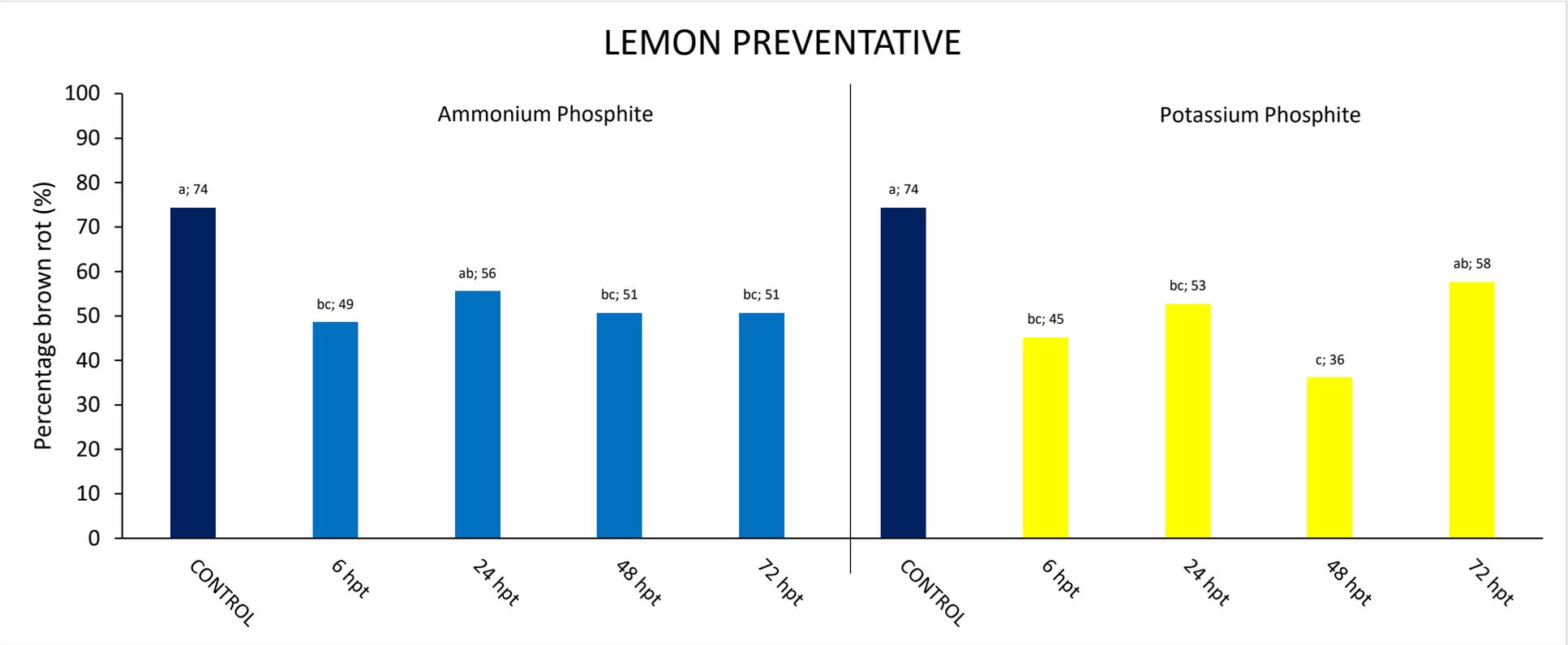
The lowest incidence of brown rot was observed when oranges were treated preventatively with the two fungicide treatments. However, the results for lemons treated preventatively with the two fungicides did not follow the same trend (Figure 4.4.3.6).



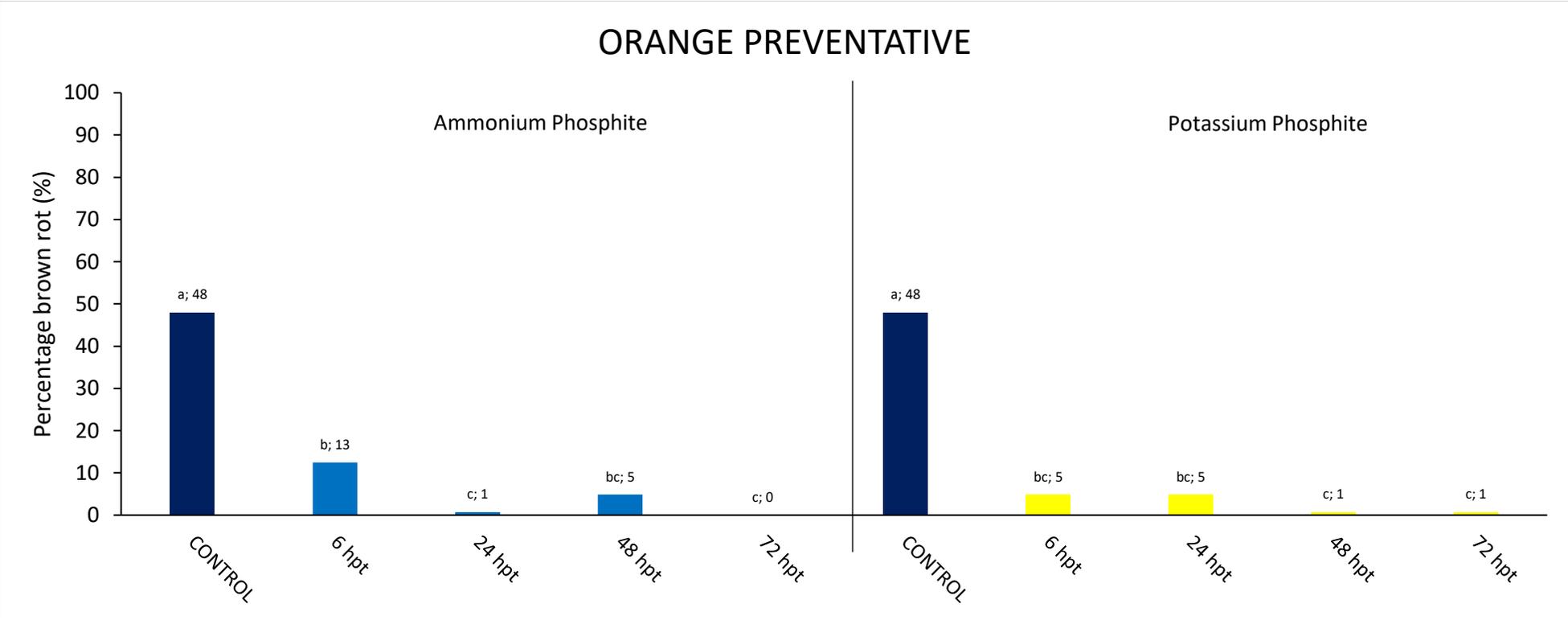
**Figure 4.4.3.4.** Mean percentage brown rot that developed on fruit, when lemons were treated curatively with ammonium phosphite (left) and potassium phosphite (right) at 6, 24, 48 or 72 hpi with *P. nicotianae* zoospores.



**Figure 4.4.3.5.** Mean percentage brown rot that developed on fruit, when navel oranges were treated curatively with ammonium phosphite (left) and potassium phosphite (right) at 6, 24, 48 or 72 hrs hpi with *P. nicotianae* zoospores.



**Figure 4.4.3.6.** Mean percentage brown rot that developed on fruit, when Eureka lemon fruit were treated preventatively with ammonium phosphite (left) and potassium phosphite (right) and inoculated with *P. nicotianae* zoospores at 6, 24, 48 or 72 hpt



**Figure 4.4.3.7.** Mean percentage brown rot that developed on fruit, when navel oranges were treated preventatively with ammonium phosphite (left) and potassium phosphite (right) and inoculated with *P. nicotianae* zoospores at 6, 24, 48 or 72 hpt.

## Discussion

*Phytophthora* spp. pose continuous universal and economical challenges in all citrus production areas and therefore integrated control strategies are needed to manage these pathogens effectively (Adaskaveg *et al.*, 2015a). Previous studies highlighted the importance of alternative treatments for postharvest brown rot. This study constitutes the first evaluation of azoxystrobin and fludioxonil fungicides in South Africa for the control of postharvest *Phytophthora* brown rot in terms of *in vitro* fungicide sensitivity testing and *in vivo* trials on fruit. Along with that, the ability of two phosphonate fungicides to control postharvest brown rot was also tested.

The first main objective of this study was to establish the mean baseline and non-baseline EC<sub>50</sub> and EC<sub>90</sub> values for a previously unexposed *P. nicotianae* population and a population possibly previously exposed to azoxystrobin or fludioxonil or fungicides from the same chemical class.

*In vitro* studies testing strobilurin fungicides, with the use of the pathway inhibitor SHAM, have been used extensively (Kanetis *et al.*, 2008). To effectively evaluate the sensitivity range of azoxystrobin, SHAM was therefore added at 100 µg.mL<sup>-1</sup> to amended media in the current study (Rebollar-Alviter *et al.*, 2007). In the current study, analyses of the EC<sub>50</sub> and EC<sub>90</sub> data led to different azoxystrobin sensitivity groups being identified within the populations based on Ward's clustering analyses. The differences in sensitivity between isolates *in vitro* could be as a result of genetic differences between the isolates. For the QoI chemical class, which includes azoxystrobin, the resistance of the pathogen is considered to be controlled by one mitochondrial gene and resistance risks are considered high (Fabritius *et al.*, 1997; Ziogas *et al.*, 2002). The mean EC<sub>50</sub> values of azoxystrobin for *P. nicotianae* ranged for the different sensitivity groups within the baseline and non-baseline populations, between 0.01 and 0.46 µg.mL<sup>-1</sup>. The EC<sub>90</sub> values for baseline and non-baseline populations of the different sensitivity groups ranged from 4.28 to 84.85 µg.mL<sup>-1</sup>. The EC<sub>50</sub> and EC<sub>90</sub> values of the baseline and non-baseline populations (EC<sub>50</sub> of 0.01 to 0.19 µg.mL<sup>-1</sup> opposed to EC<sub>50</sub> of 0.04 to 0.46 µg.mL<sup>-1</sup>, EC<sub>90</sub> of 4.28 to 83.96 µg.mL<sup>-1</sup> as opposed to 1.46 to 84.85 µg.mL<sup>-1</sup>) were so similar that it could be argued that a sensitivity shift has not taken place between the populations.

In contrast to findings in this study, is a study conducted in 2000, where the efficacy of azoxystrobin, fosetyl-Al, fluazinam, dimethomorph and metalaxyl was tested on three life stages (zoospore germination, sporulation and mycelial growth) of three *Phytophthora* species namely *P. parasitica* (syn *P. nicotianae*), *P. capsici* and *P. citrophthora* (Matheron and Porchas, 2000). The EC<sub>50</sub> of azoxystrobin based on *P. nicotianae* growth inhibition was indicated as >3000 µg.ml<sup>-1</sup>. The authors suggested that the alternative respiration pathway was accessed when growing on nutrient rich agar which led to the low level of sensitivity to azoxystrobin. The EC<sub>50</sub> value in the abovementioned study (>3000 µg/ml) was therefore much higher than found in the current study where SHAM was added to the amended media. Another study where no SHAM was added and azoxystrobin was tested against *P. nicotianae*, the EC<sub>50</sub> values ranged between 56 to 165 µg.mL<sup>-1</sup> (Kuhajek *et al.*, 2003), which was still markedly higher than the EC<sub>50</sub> values in the current study. Azoxystrobin, in combination with SHAM, was tested against *P. cactorum*, collected from strawberries' crown- and leather rot, where the EC<sub>50</sub> values ranged from 0.10 to 15 µg.mL<sup>-1</sup> (Rebollar-Alviter *et al.*, 2007), which was again higher than the current study's *P. nicotianae* EC<sub>50</sub> values. Another study found that azoxystrobin, also amended with SHAM, inhibited *P. capsici* mycelial growth with mean EC<sub>50</sub> values of 1.23 to 86.10 µg.mL<sup>-1</sup> (Qian *et al.*, 2006). This range was very similar to the current study's EC<sub>90</sub> values (4.28 to 84.85 µg.ml<sup>-1</sup>), which could indicate that other *Phytophthora* species are potentially less sensitive towards azoxystrobin compared to *P. nicotianae*. Another recent study conducted with benzothiofuran, another strobilurin fungicide, in the presence of 100 µg/ml SHAM, again towards *P. capsici*, found EC<sub>50</sub> values from different populations ranged from 0.95 to 2.46 µg.mL<sup>-1</sup> (Ma *et al.*, 2018), which were relatively higher than the EC<sub>50</sub> values obtained for azoxystrobin in this study.

A very wide range of EC values was recorded for fludioxonil towards *P. nicotianae* in the current study. The baseline EC<sub>50</sub> values ranged from 5.56 to 1613.52 µg.mL<sup>-1</sup> and non-baseline 3.10 to 84.79 µg.mL<sup>-1</sup>. The EC<sub>90</sub> baseline values ranged from 1988.5 to 9929 µg.mL<sup>-1</sup> and non-baseline values 1090.50 to 6809.90 µg.mL<sup>-1</sup>. The only study that could be found with fludioxonil and *P. nicotianae*, was a study conducted in 2018 where fludioxonil and metalaxyl-M were tested in combination for mycelium growth inhibition of *P. nicotianae*. The EC<sub>50</sub> and EC<sub>90</sub> values obtained in that study were respectively 0.393 µg.mL<sup>-1</sup> and 10.170 µg.mL<sup>-1</sup> (Altin *et al.*, 2018)., However it is important to note that *Phytophthora* spp. are commonly managed with metalaxyl and in

combination with fludioxonil, was therefore very effective. Both EC<sub>50</sub> and EC<sub>90</sub> values are significantly less than what was found in the current study.

Establishing the baseline sensitivity range of any pathogen towards a fungicide is important as it monitors for possible resistance and, to effectively manage it (Qu et al., 2016). Regarding the baseline and non-baseline sensitivity groups of both fungicides in the current study, it was seen that there was little difference in the EC values of the two populations. This indicated that currently there is little or no sensitivity shift between the populations of both fungicides in South Africa. The results of this *in vitro* study also show that the maximum concentrations of azoxystrobin and fludioxonil tested, effectively inhibited mycelial growth of *P. nicotianae*, compared to the growth on unamended control plates. *In vivo* testing of these two fungicides for brown rot control on fruit was therefore warranted.

In following on the *in vitro* sensitivity trials, these fungicides were tested in aqueous solutions for their curative and protective ability in controlling postharvest *Phytophthora* brown rot. Azoxystrobin and fludioxonil amended wax were furthermore evaluated for its ability to prevent the nesting effect, or spreading of brown rot, occurring within export cartons. In these trials, zoospores were chosen as the source of inoculum, as opposed to using mycelial plugs, as it represents more accurately what happens in the field. In orchards, zoospores are splashed upward from the soil to the fruit during raining periods or irrigation. Once on the fruit, the zoospores encyst, germinate and cause characteristic brown rot symptoms (Adaskaveg et al., 2015a).

In some cases, as in the curative trial on oranges, the mean percentage brown rot of the untreated control observed was lower than the mean of other time points. This could be the result of subtle temperature fluctuations in certain areas within the incubation room used for the trials. Nonetheless, the means in the controls were still high enough to make clear conclusions regarding the efficacy of the different fungicides. The two fungicides were tested at four different time points and at specific dosages for each. These dosages were based on the registered label recommendations as well as previous studies. Results from all three fruit types indicated that the fungicides had very good curative action if treatment was done 12 h after *P. nicotianae* zoospore inoculation. The effectiveness of this curative action is noteworthy, as germination of *Phytophthora* zoospores can already occur as little as 3 h after infection (Adaskaveg et al., 2015). Similar curative results were seen when azoxystrobin and fludioxonil were tested against the postharvest citrus pathogen *P. digitatum* and treatment occurred 9 to 21 h after inoculation (Kanetis et al., 2007). In the current study, treatment 24 h after inoculation also resulted in a significant reduction in brown rot development, except for oranges treated with fludioxonil. When citrus fruit are infected in the orchard, it is usually during the raining season and fruits are not picked soon after a rain shower, as the fruit rind can get damaged. Thus, the period between infection in the orchard and the packhouse postharvest treatment, may be longer than 24 h. However, temperatures in the winter are usually much lower than that of *P. nicotianae*'s optimal growth temperature. This can slow down the infection process that can make curative treatments successful (Adaskaveg et al., 2015).

Adaskaveg and Förster (2014) tested both potassium phosphite and azoxystrobin as curative and protective treatments. They concluded that only potassium phosphite displayed good curative action, which contrasts with what was found in the current study, as azoxystrobin in this study was very effective on all citrus types both curatively and protectively. Previously, azoxystrobin (600 µg.mL<sup>-1</sup>) was applied to oranges 12 h after *P. citrophthora* zoospore inoculation that resulted in 81.2 % brown rot incidence, indicating poor curative action (Adaskaveg and Förster, 2014). In contrast, when azoxystrobin was applied to oranges 12 h after inoculation, only 1.4 % brown rot developed, which was significantly lower than observed in the Adaskaveg and Förster (2014) study. However, it is important to note that the active ingredient dosage used in the current study was double of the abovementioned 2014 study and the pathogens were also different in the two studies. Both azoxystrobin and fludioxonil (at 2000 µg.mL<sup>-1</sup>) was tested curatively against other citrus pathogens, *Lasioidiplodia theobromae* and *Diaporthe citri*, causing postharvest stem end rot. In these trials, the fungicides were applied as aqueous dips on lemons 24 h after inoculation, but were found to be ineffective as both resulted in 80 % rot (Cerioni et al., 2017). This contrasted with what was found in the current study, as both azoxystrobin and fludioxonil resulted in good curative action when lemons were treated up to 24 h after inoculations.

As seen from the results, the curative action of azoxystrobin and fludioxonil was very effective up to a certain time point. In contrast, the protective action of the tested fungicides lasted longer than the curative action. Azoxystrobin had very good protective action on all three fruit types and reduced brown rot development significantly (0-44 %) from the inoculated untreated control. On azoxystrobin treated fruit, brown rot incidence did not go above 20% with inoculation up to 48 h after treatment. Adaskaveg and Förster (2014) inoculated fruit 12 h after azoxystrobin treatment (600 µg.mL<sup>-1</sup>) and this resulted in 10.5 % brown rot, which indicated a highly effective treatment. Similarly, in the current study, the percentage brown rot on oranges, when inoculation occurred 12 h after azoxystrobin treatment, was just slightly higher at 13.9 % still indicating an effective treatment in comparison to the untreated control. Fludioxonil did not show the same protective ability as azoxystrobin but resulted in a low brown rot incidence on all three citrus types if inoculation occurred 48 h after treatment. A trend that was seen for the protective action on all three citrus types, was that the longer the fungicide remained on the fruit, the better the action was. This could be due to a rind response, triggered by the treatment of the fruit, such as cell wall changes that lead to mechanical barriers or protective substances being formed (Ramallo *et al.*, 2019).

Previous postharvest studies mentioned had different results compared to the current study. This could be due to several reasons. For one, some of the other studies focussed on other *Phytophthora* spp. such as *P. citrophthora*, which can have different sensitivities to the tested fungicides. Inoculation methods were also different as that used in the current study, for example, zoospore drenched miracloth squares in combination with superficial wounding was used as inoculation technique as opposed to mycelial plugs, with and without wounds used in other studies. Pathogenicity of the isolates used to prepare the zoospore inoculum could also play an important role. Due to the possible variation in virulence between isolates, three isolates were used to produce the zoospore inoculum mixture used in this study.

The last factor that could have led to different results obtained is the different citrus types that were used (lemons, oranges and mandarins). Different trends were observed on the different citrus types (Figs 1-3). This could be due to the differences between the rinds with regard to rind thickness, wax layer thickness or the presence of antifungal constituents in the rinds that play a role in natural resistance (Ben-Yehoshua *et al.*, 1992). In citrus, early maturing varieties (e.g. mandarins) have lower wax levels than later maturing varieties (Petracek, 1997). Mandarin rind also presents a less firm, softer texture and more elasticity than the rinds of oranges and lemons (Petracek., 1997; Nunes, 2008). Lemons furthermore lose their natural antifungal activity after a long time of storage and thus leading to a decrease in the natural defence mechanisms (Ben-Yehoshua *et al.*, 1992). On a phytochemical level it is known that citrus rind does contain antifungal agents to contribute to its natural defence mechanisms and a citrus rind contains a large amount of coumarins, volatile oils and flavonoids which have the potential to resist postharvest fungi (Chen *et al.*, 2019). However, different citrus types produce these different natural defence mechanisms in varying manners in response to pathogen infection (Ben-Yehoshua *et al.*, 1992).

Fungicide applications are also done in a fruit wax coating to improve appearance of fruit for marketing purposes or to prevent the loss of moisture (Kanetis and Adaskaveg, 2007). As postharvest brown rot can spread from infected fruit to healthy fruit during several postharvest stages such as de-greening, storage or transit (Adaskaveg *et al.*, 2015), postharvest treatments should also be able to prevent this. In the current study, it was demonstrated that when azoxystrobin was incorporated in wax and the fruit was coated with it and placed adjacent to one week old brown rot fruit, it gave significant protection from brown rot spreading from infected to healthy fruit, when compared to the control. However, this was only observed in lemons and oranges but not when mandarins were treated with amended wax. Although fludioxonil amended wax treatments did provide a reduction in the spread of infection between infected and healthy fruit, the reduction was not significant when compared to the control.

Based on the results of the current study, the protective action of the two tested fungicides was better than the curative action. This agrees with what Nanni *et al.* (2016) stated that when the active ingredient is already present on the fruit, it will provide better protection when fungal propagules land on the surface as it will interrupt the fungal development (protective). However, the management of diseases gets more complicated when the fungus is already present on the fruit and then treated (curative). Azoxystrobin and fludioxonil are already registered for the control of *Penicillium* spp. in South Africa. They are already in use in the packhouses

and, based on the results of the current study, can add value as they can additionally control brown rot through both curative and protective actions.

Additionally, the ability of potassium and ammonium phosphite to provide postharvest control of Brown rot was tested. Results indicated excellent curative ability for both actives when treatment was done on lemons up to 24 hpi with *P. nicotianae* zoospores. Thereafter, an increase in the mean percentage brown rot was observed. The results from the curative trial on oranges followed the same trend. However, only moderate curative control was achieved when oranges were treated up to 24 hpi. The same trend was observed in a previous study done by van der Merwe (2020) where lemons were inoculated and treated in the same manner. Curative control was lost when fruits were treated at 24 hpi, thereafter a 65% increase in brown rot incidence was observed (van der Merwe, 2020). The results are especially noteworthy due to the epidemiology of brown rot. Fruits usually become infected during the rainy season when inoculum is easily splashed up from the soil. To avoid damage of the fruit rind, fruits are usually not picked soon after a rain shower. As a result, the period between initial infection and postharvest treatment of the fruit may be longer than 24 hrs. However, as indicated above, curative treatments may still be successful due to winter temperatures being much lower than the optimal growth temperature for *Phytophthora* spp. Thus effectively slowing the infection process down (Adaskaveg *et al.*, 2015), making effective curative treatment possible.

In 2019, Romallo *et al.* tested different formulations of potassium phosphite (at 2000 µg/mL) as a postharvest treatment for lemons. Fruits were inoculated, with *P. citrophthora* mycelial plugs, 24 hrs before fungicide treatment. The brown rot incidence was reduced by 20-40% compared to the untreated control (Romallo *et al.*, 2019). In the current study when lemons were treated curatively with potassium phosphite at 24 hpi, a 49% reduction in brown rot was observed. When ammonium phosphite was used as the postharvest treatment, a 49% reduction in brown rot was observed again. Therefore, the efficacy of ammonium phosphite and potassium phosphite in our study was higher compared to the efficacy found by Romallo *et al.* (2019). This may be due to the differences in the pathogen used as well as differences in fruit developmental conditions due to the geographical location of the study.

In our study, very poor preventative control was observed for lemons treated with either of the two phosphite salts. In the preventative study done by van der Merwe (2020) on lemons, a gradual reduction was observed in the mean percentage of brown rot when fruits were inoculated from 6 hpt to 48 hpt. At 48 hpt with potassium phosphite, the author found that the brown rot incidence on lemons was 8.3% (van der Merwe, 2020) compared to the current study where the brown rot incidence on lemons was 36%. The current study concluded with a reduction of 18-25% and 16-38% in brown rot on lemons when ammonium phosphite and potassium phosphite was used, respectively. This was markedly lower than the efficacy recorded by van der Merwe (2020) where potassium phosphite treatments reduced the brown rot incidence by 72%. In the current study, the preventative trial on oranges indicated excellent preventative control for both ammonium phosphite and potassium phosphite. Especially when oranges were inoculated 48 hpt with potassium phosphite, brown rot incidence remained minimal at 1% total brown. When ammonium phosphite was used preventatively and fruits were inoculated at 72 hpt, no brown rot infection was observed. These results were similar to that observed by van der Merwe (2020) where no incidence of brown was observed when oranges were treated preventatively with potassium phosphite for 6 to 48 hpi with *P. nicotianae* zoospores.

Adaskaveg and Förster (2014) tested the curative and protective ability of potassium phosphite on *Phytophthora* brown rot. Oranges were inoculated with *P. citrophthora* zoospores at 12 hrs prior to treatment with potassium phosphite (1260 µg/mL). The author observed a good curative control of brown rot resulting in only 5% of brown rot incidence (Adaskaveg and Förster, 2014). Adaskaveg *et al.* (2015) slightly increased the potassium phosphite dosage in a separate curative study. Fruit were dip treated for 15s in a potassium phosphite solution (1500 µg/mL) at 18 hrs post-inoculation with *P. citrophthora* zoospores. This dosage of potassium phosphite (1500 µg/mL) resulted in 96% brown rot inhibition on oranges (Adaskaveg *et al.* 2015). When fruits were treated at 24 and 30 hpi, the percentage of brown rot was 7.7% and 17.2%, respectively (Adaskaveg *et al.* 2015). This was similar to the results from the current study where the percentage of brown rot at 6 hpi and 24 hpi on oranges treated curatively with potassium phosphite (1500 µg/mL) were 15% and 10% respectively (Figure 4.4.3.7). When ammonium phosphite (1500 µg/mL) was applied as the curative treatment on oranges, the percentage of brown rot at 6 hpi and 24 hpi was 14% and 5% respectively.

The differences between the results of the current study and the results from similar postharvest studies may be due to several reasons. One important difference is the use of *P. nicotianae* zoospores as inoculum instead of *P. citrophthora* mycelia (Adaskaveg and Förster, 2014; Romallo *et al.*, 2019). In the current study, a zoospore suspension served as the inoculum since this closely mimicked the brown rot inoculum source in the field. Additionally, the method for inoculating fruit differed – previous studies made use of mycelial plugs placed onto fruit with or without wounds (Adaskaveg and Förster, 2014; Romallo *et al.*, 2019) whereas the current study inoculated fruit by placing colonized miracloth onto wounds. Since a mixture of *P. nicotianae* isolates were used to produce the zoospore suspension, pathogenicity and variation in virulence are both factors that could influence the results that were obtained. Lastly, citrus fruit used in the current study originated from South Africa whereas previous studies used citrus fruits that were not from South Africa. Physical and chemical differences in the soil and environmental conditions could possibly affect the fruit rind characteristics.

The study done by van der Merwe (2020) and the current study had many similarities including the pathogen used, the inoculum source and the inoculation method. Differences between the results of the two studies may be owed to several reasons. Again, the differences in brown rot incidence for lemons treated preventatively may be due to variation in pathogenicity and virulence of the *P. nicotianae* isolates used. Additionally, the fruits that were used in the two studies were from different production seasons. Therefore, variation in brown rot incidence may be due to chemical and physical differences in the rind of the citrus fruit. Brown rot affects all citrus fruits but is also reported to have an increased impact on lemons (Savita and Avinash, 2012). The difference in trends for the different citrus fruit types (Figs. 4-7) may also be due to physical and chemical differences in the fruit rind. These physical and chemical differences may include rind thickness, fruit rind susceptibility and the natural defence mechanisms of the fruit rind. For citrus fruits, antifungal constituents have been detected which have been shown to contribute to natural resistance (Ben-Yehoshua *et al.*, 1992). The results of the current study indicate good curative control of brown rot on lemons when both ammonium phosphite and potassium phosphite was applied. Interestingly, the results indicated excelled preventative control of brown rot on oranges when both ammonium phosphite and potassium phosphite were applied. Both are registered preharvest fungicides for the management *Phytophthora* spp. on citrus in South Africa.

The results of the current study suggest that both potassium phosphite and ammonium phosphite could be effective in the postharvest management of *Phytophthora* brown rot. If these phosphonate products were to be registered as postharvest treatments for *Phytophthora* brown rot, the impact of brown rot on South African citrus production could be minimized while effectively lowering the economic impact as well. This would add value to the product as it would be used in preharvest and postharvest fungicide programmes.

## **Conclusion**

Prior to the current study, no postharvest treatment was available to control *Phytophthora* Brown Rot. Based on the findings in this study, two fungicides, already registered for postharvest use on citrus, are also effective to manage this disease. Using these two will greatly reduce the postharvest losses due to *Phytophthora* Brown Rot.

## **Future research**

Future studies should further investigate the following aspects

1. The effectivity of a fludioxonil and azoxystrobin combination in controlling postharvest brown rot and if it can act curatively or preventatively for longer than 48 hrs
2. The effectivity of a combined phosphonate and fludioxonil and azoxystrobin treatment in controlling postharvest brown rot
3. Determine the effectivity of different phosphonate concentrations to manage postharvest brown rot

## **Technology transfer**

Results from the research were presented at the 2021 CRI Postharvest Webinar.

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#### 4.4.4 FINAL REPORT: Optimising available alternative postharvest remedies as replacement for imazalil use on citrus exported to Europe

Project 1250 (PHI 27/19) (2019/20 – 2021/22) by Wilma du Plooy, Lindokuhle Mamba, and Paul Fourie (CRI)

### Summary

The particular efficacy of imazalil (IMZ) against the citrus postharvest pathogen *Penicillium digitatum* is of immeasurable importance world-wide. The European Union, has however placed IMZ under pressure with the announcement at the end of 2018 that the use of IMZ on citrus will be recalled from 2020. This drastic announcement was later revised. As a result of the unprecedented threat posed by the withdrawal of IMZ, an urgent investigation into the application of alternative chemicals that are usually considered as resistant management strategies, is being done. The well-known actives are pyrimethanil (PYR), fludioxonil (FLU), 2-

orthophenyl phenol (OPP) and thiabendazole (TBZ). Azoxystrobin (AZO) was registered as a postharvest fungicide in 2018, but has not been used in packhouses as yet. It is known that none of these five actives are as effective as IMZ at the conditions that allow optimum action by IMZ. This study was therefore aimed at optimising the packhouse parameters that would allow the most effective action of these alternative chemicals. The actives were tested on lemons, navels and Nadorcotts, at three different temperatures. Every active was also tested in combination with every other active. The best combinations will be optimised in 2021, while an investigation into GRAS chemicals will also be included.

## Opsomming

Die besondere effektiwiteit van imazalil (IMZ) teen *Penicillium digitatum* as sitrus na-oespatogeen is wêreldwyd van onskatbare belang. Die Europese Unie het egter aan die einde van 2018 IMZ onder druk geplaas met 'n afkondiging dat gebruik daarvan op sitrus herroep sal word teen 2020. Hierdie drastiese aankondiging was wel later hersien. As gevolg van die ongekende bedreiging wat die onttrekking van IMZ inhou, is 'n dringende ondersoek geloods na alternatiewe aanwending van produkte wat tot dusver hoofsaaklik as ondersteuningstrategieë teen weerstandontwikkeling gebruik was. Die bekende aktiewes is pirimetaniel (PYR), fludioksonil (FLU), 2-ortofenielfenol (OPP) en tiabendasool (TBZ). Verder was azoxystrobien in 2018 geregistreer as 'n na-oesfungisied, maar nog nooit in pakhuis gebruik nie. Dit is bekend dat nie een van hierdie vyf aktiewes die besondere effektiwiteit het van IMZ onder die toestande waarteen IMZ suksesvol aangewend word nie. Hierdie ondersoek was dus gerig op die optimisering van die pakhuisparameters waarteen hierdie alternatiewe wel die mees effektiewe werking sal kan hê, asook moontlike kombinasies om deur sinergisme die effektiwiteit te verbeter. Die aktiewes was getoets op suurlemoene, navels en Nadorcotts, teen drie verskillende temperature. Elke een van die individuele aktiewes is ook getoets teen elke ander aktief. Die beste kombinasies moet geoptimeer word in 2021, asook verdere ondersoek na GRAS kombinasie.

## Introduction

World-wide the citrus industry faces similar postharvest decay problems. The two foremost issues are sour rot (caused by *Galactomyces citri-aurantii*) and *Penicillium* decays. Not being able to use highly effective chemical controls is a serious threat to the industry internationally. This was proven after the 2016 season which was the first year of exporting from South Africa without the important sour rot control remedy, guazatine (GZT). During the following export season this resulted in serious decay increasing from 1.9% to 4.17% for Capespan exports alone (Capespan, 2019). In 2018 the company then made a decision to not send any fruit with any sour rot risk to Europe, resulting in decay decreasing to 2.86%. It also resulted in producers in areas with any kind of stressed fruit not exporting to the EU using Capespan. As the company only records "decay", it is impossible to distinguish between sour rot and green mould decay, as all rots received at the markets are observed as *Penicillium*. This is due to the fact that *Penicillium* rot quickly takes over sour rot lesions as a secondary pathogen.

To date, primary *Penicillium* infections have been kept under control using imazalil (IMZ). An impending lowering of this active's residue from 5.0 ppm to limit of detection (0.01 ppm) is one of the most serious threats faced by the South African citrus industry. As a FRAC 3 postharvest chemical, imazalil is a demethylation inhibitor. The importance of this active lies in its unique three-levelled ability to control *Penicillium digitatum*: It has curative, protective as well as excellent anti-sporulating properties. Given the serious increase that was observed in decay when guazatine was lost, it is certain that a similar or worse issue awaits the industry should the threat to withdraw imazalil from use on fruit destined to Europe is eventually be undertaken

There are a small number of other actives registered for postharvest use against *Penicillium* decay: thiabendazole (TBZ), 2-orthophenylphenol (OPP), fludioxonil (FLU), pyrimethanil (PYR), azoxystrobin (AZO). These actives have traditionally been kept for cold damage mitigation and latent pathogen control: (TBZ) (Tomlin, 2005; Eckert & Eaks, 1988), used in the drench before degreening, (PYR) (Tomlin, 2005), or kept on stand-by as counter measures in resistance management (FLU and AZO) (Erasmus *et al.*, 2015). In the case of OPP, the active was seldom used on South African pack lines, as it has low, inconsistent efficacy and added to the number of residues on fruit, leading to an exceedance of the number of residues that were allowed on fruit destined for European supermarket buyers (Hardman, 2018).

With the spectre of no *Penicillium* control, all of these actives had to be revisited and evaluated, not as secondary or supplemental remedies, but as primary control measures against *Penicillium digitatum* (PD).

## Stated objectives

### Phase 1

Objective 1: Evaluation of five registered actives allowed in the European Union, for efficacy against *Penicillium digitatum*.

1. Obtaining the relevant postharvest chemicals.
2. Initial study into the efficacy of individual actives against *Penicillium digitatum*, using a lemon, grapefruit and navel cultivar, and applying them in a fungicide bath, and also evaluating sporulation inhibition, done at three temperatures.
3. Scoring of each active as a stand-alone remedy, evaluating curative and sporulation control.

Objective 2: Finding effective combinations of the currently registered actives.

1. Testing and evaluation of combinations of the actives to increase the efficacy scores, using only a soft citrus cultivar and a navel cultivar, and applying them using a flooder and in a fungicide bath.
2. Scoring of blends of actives to determine if there is synergism between them.
3. Preparation of material for technology transfer.

### Phase 2

Objective 1: Optimising the parameters required to increase the effectivity of the individual actives in the flooder or in fungicide bath application on the packline.

1. Optimisation of exposure time, temperatures and pH to be used for each chemical, and for each different citrus type.
2. Technology transfer: Feedback at postharvest workshops February 2020

Objective 2: Optimisation parameters required for combinations of actives where possible synergism was identified in Phase 1, Objective 2.

1. Optimisation of the effectivity of combinations of the physical conditions for various individual and combinations of the actives, on different commercially important citrus kinds.

### Phase 3

Objective 1 – Updating packhouse managers

1. Preparation of material for final technology transfer January 2022

Objective 2 – Updating the research community

1. Delivering a research paper at the CRI Symposium 2022

Objective 3 – Local and International publications

1. Articles in South African Fruit Journal
2. Articles in peer reviewed journals

## Material and methods

### Phase 1

#### Acquiring and testing registered alternative actives

Objective 1: Evaluation of five registered actives allowed in the European Union, for efficacy against *Penicillium digitatum*.

1. The five permissible chemicals were obtained, while all the work was done at CRI on a mini packline simulating commercial conditions.
2. For each active, the anti-sporulation, curative as well as preventative efficacy thereof against *Penicillium digitatum* were determined using standard inoculation techniques. Each inoculation was followed by using treatments using a fungicide bath, and where applicable, a flooder. Initial investigations were done on 4 commercially important citrus kind at three different temperatures, and

focussed on anti-sporulation and curative control. Once the optimal temperatures per fruit kind were determined, the study was expanded to also evaluate optimal pH and exposure times per active and fruit kind, in South African packhouse conditions.

3. Preventative control of each individual fungicidal active was investigated using only a soft citrus type, as these are particularly prone to diseases during export. For evaluation of preventative treatments, fruit were stored in conditions simulating export conditions after application of the chemicals.
4. For each citrus type or cultivar, aqueous application, and active combination, 5 repetitions consisting of twelve fruit each were done.
5. Each fruit were inoculated four times with equidistant wounds 2 -3 cm from the button end (depending on fruit size), using a standard wounding tool, a  $1 \times 10^6$  PD spore suspension, and aseptic techniques.
6. After inoculation, fruit were placed on pulp trays, placed in a plastic bag and each tray placed in a box, where after it was incubated at 24°C for 4 – 5 days, until 80% of the controls had discernible decay.
7. Evaluations were done using an ultraviolet light source and infections rated from 1 - 4, according to the number of discernible lesions per fruit.
8. Scoring of each active, using a 1 - 4 rating scale of infection, as a stand-alone remedy were done to determine the potential of each active measured against each other.

Objective 2: Finding more effective combinations of the currently registered actives.

1. Testing and evaluation of combinations of all the actives were done using only a soft citrus type and a navel cultivar, and applying it using a flooder, and in a fungicide bath.
2. For each citrus type, aqueous application, and active combination, 5 repetitions consisting of twelve fruit each were done.
3. For each successful combination, the curative as well as preventative efficacy thereof against *Penicillium digitatum* were determined using standard inoculation techniques. Each inoculation was followed by using treatments using a flooder, and a fungicide bath. This were investigated using available, commercially important citrus kinds.
4. Application time in the fungicide bath was 1 minute, and 15 seconds when using the flooder. Each fruit was inoculated four times with equidistant wounds 2 -3 cm from the button end (depending on fruit size), using a standard wounding tool, a  $1 \times 10^6$  PD spore suspension, and aseptic techniques. Fruit were placed on pulp trays, placed in a plastic bag and each tray then placed in a box, where after it was incubated at 24°C for 4 – 5 days, until 80% of the controls had discernible decay.
5. Evaluations were done using an ultraviolet light source and infections rated from 1 - 4, according to the number of discernible lesions per fruit.
6. Scoring of each active, using the same 1 - 4 rated scale of infection, as a stand-alone remedy was done to determine the potential of each active measured against each other.
7. Factsheets aimed at informing packhouses about the use of the alternative actives in the flooder and the fungicide bath will be prepared.

## Phase 2

### Optimising the parameters to be used in aqueous application on the packline

Objective 1: Optimising the parameters required to increase the effectivity of the individual actives in the flooder or in fungicide bath application on the packline.

1. The work was done at CRI, on a mini packline simulating commercial conditions.
2. Optimisation of different parameters applicable to aqueous environments in the packhouse were done evaluating the following parameters:
  - a. temperature (25, 35, 45°C)
  - b. pH (3, 6, 8)
  - c. exposure time (30, 60, 120 seconds)
3. These parameters were investigated for each of the five actives in the study, namely TBZ, OPP, PYR, FLU and AZO. A baseline study was done first, whereby IMZ at the optimal conditions for this active, was the standard against which the other four chemicals were measured for effectivity at three different temperatures. This initial study was done curatively, as well as for sporulation control, and on commercially important soft citrus, lemons, grapefruit and navels. Once the optimal temperature for

each of the chemicals, for each citrus kind were determined, the study were expanded to look at the optimal pH and exposure time at the best temperature.

4. The ability of the various actives to exert preventative control after application at the optimal conditions, and then stored at shipping temperatures, will were determined subsequent to the initial optimisation.
5. For each citrus type, each aqueous application, and active combination, 5 repetitions consisting of twelve fruit each were done.
6. Each fruit was inoculated four times with equidistant wounds 2 -3 cm from the button end (depending on fruit size), using a standard wounding tool, a  $1 \times 10^6$  PD spore suspension, and aseptic techniques.
7. Fruit were placed on pulp trays, placed in a plastic bag and each tray then placed in a box, where after it were incubated at 24°C for 4 – 5 days, until 80% of the controls had discernible decay.
8. Evaluations were done using an ultraviolet light source and infections rated from 1 - 4, according to the number of discernible lesions per fruit.
9. Scoring of each active, using the same 1 - 4 rated scale of infection, as a stand-alone remedy were done to determine the potential of each active measured against each other, using the established parameters.
10. In addition, all of these parameters were investigated for four different citrus kinds: lemons, soft citrus, grapefruit and oranges.

Objective 2: Optimisation parameters required for combinations of actives where possible synergism was identified in Phase 1, Objective 2.

1. Any useful combinations of the actives identified in phase 1, objective 2, were subjected to studies in optimising the parameters to be used for application thereof using a flooder or in a fungicide bath. To this end, therefore, pH, temperature, and exposure time were optimised for the most promising combination of actives, using four different citrus types: lemons, soft citrus, grapefruit and oranges. The same methodology as in objective 1 were followed for the combined actives.

### Phase 3

#### Extension and technology transfer

Objective 1: Updating packhouse managers

1. Feedback about the progress of the work, as well as updates on the working conditions for the actives were delivered at the annual country-wide postharvest workshops at 6 different venues.

Objective 2: Updating the research community

1. At the 2021 online symposium, the work were summarised in a compound talk of the five alternative actives.

Objective 3: Local and International publications

1. The final project finding will be made available to the local and international citrus communities through articles in the South African Fruit Journal
2. At least one peer reviewed journal article will be published.

#### Results and discussion

Objective / Milestone	Achievement
<b>Phase 1</b>	
<b>Objective 1: Evaluation of five registered actives allowed in the European Union, for efficacy against <i>Penicillium digitatum</i>.</b>	
1. Obtaining the relevant postharvest chemicals	All the chemicals have been procured. Fludioxonil and azoxystrobin for postharvest use are manufactured in small quantities due to their limited use in packhouses at the moment, and therefore had longer lead times. The custom-built drenchers have been serviced and recommissioned, but only three were fully functional. In

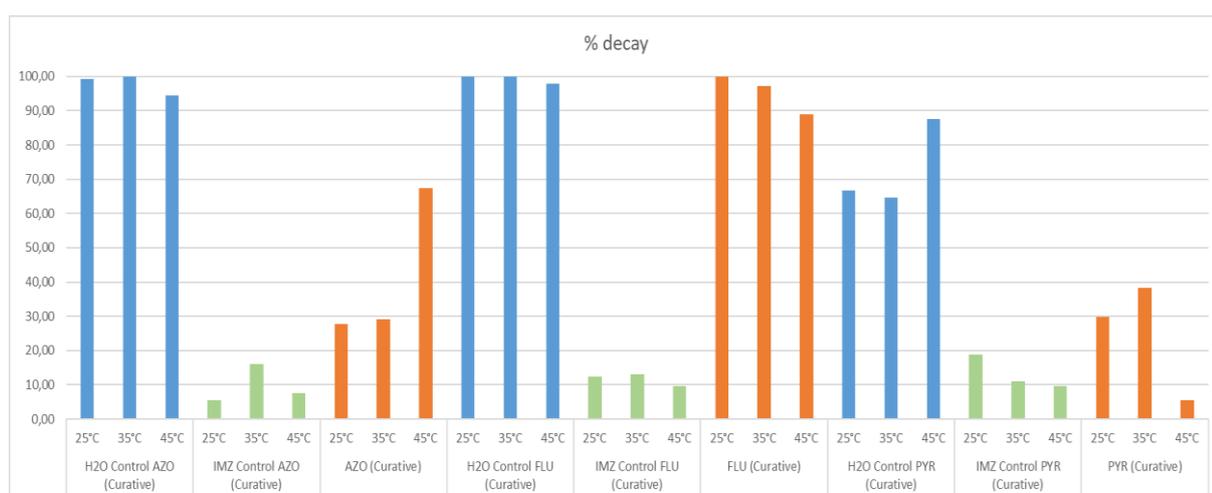
	addition, the wounding tools were extremely blunt and had to be re-sharpened on a toolmakers lathe.
2. Initial study into the efficacy of individual actives against <i>Penicillium digitatum</i> , using a lemon, grapefruit and navel cultivar, and applying them in a fungicide bath, and also evaluating sporulation inhibition, done at three temperatures.	<p>Each of the actives are being evaluated at the moment, using three temperatures and at least three citrus types.</p> <p>All the trials are done in triplicate, with a full second set (i.e. repeat) currently being run on Nadorkott and navel.</p> <p>Due to the size of the trials, only curative treatments and sporulation control is being tested.</p> <p>Grapefruit was not used, after some careful consideration, but instead, soft citrus (Nadorkott) was added.</p> <p>Residue samples are being collected and prepared for analysis (portioned, randomised, pulped and frozen) before dispatch to Hearshaw &amp; Kinnes.</p> <p>Despite some severely slow feedback in the previous 30 months, the analysts did come back with a firm undertaking for improved service, and in addition still offered exceptional rates, which is a considerable relief for the pressure on research funds.</p>
3. Scoring of each active as a stand-alone remedy, evaluating curative and sporulation control.	All the data has been captured, but none has been statistically processed yet, due to the fact that the trial started in March, and has been running non-stop since. This will only be done in September.
<b>Phase 1</b>	
<b>Objective 2: Finding more effective combinations of the currently registered actives.</b>	
1. Obtaining the relevant postharvest chemicals	<p>All the chemicals have been procured on time, but due to firstly, severe pressure on laboratory space for project that required the use of the postharvest laboratory, and secondly, the massive delays due to COVID-19, a total of seven weeks were lost.</p> <p>The custom-built drenchers have been serviced and recommissioned, with new pump ordered, but these could not be installed before lockdown and once the laboratory were operational again, there was simply no time to stop the drenchers for the required time to replace the pumps. This will be done at the end of the 2020 season.</p>
2. Testing and evaluation of combinations of all the actives will be done to attempt to increase the effectivity scores, using only a soft citrus type and a navel cultivar, and applying it using a flooder, and in a fungicide bath.	<p>Due to the serious time constraints, the flooder has been excluded from these trials, as cleaning the brushes between the changes of the chemicals were too difficult and time consuming. The drenchers are being used as simulation of the both the drenching and flooding, as the application methods are the same.</p> <p>The following matrix was applied for the 2020 trials:  AZO+FLU AZO+PYR AZO+OPP AZO+TBZ  FLU+PYR FLU+OPP FLU+TBZ  PYR+OPP PYR+TBZ  OPP+TBZ</p>
3. Scoring of each active, using the same 1 - 4 rated scale of infection, as a stand-alone remedy will be done to determine the potential of each active measured against each other, using the established parameters.	Evaluation of lesion development and scoring of disease control is done by means visual interpretation, using a UV light. The results will be statistically interpreted at the end of the season.

<b>Phase 2</b>	
<b>Objective 1: Optimising the parameters required to increase the effectivity of the individual actives in the flooder or in fungicide bath application on the packline.</b>	
Optimisation of environmental conditions for best combinations	Only one pH is used (incoming water), with a single exposure time (1 minute) was used at three different temperatures, and on three different citrus kinds (lemons, soft citrus, sweet orange). Each trial is run with three repetitions of the treatments, a negative (water) and a positive control (IMZ). The complete trial is then repeated twice. OPP has been put back into the trial, as the possibility of a synergistic effect with some of the other fungicides cannot be dismissed
<b>Phase 2</b>	
<b>Objective 2: Optimisation parameters required for combinations of actives where possible synergism was identified in Phase 1, Objective 2.</b>	
Alternatives and alternative combinations	Combinations that will include the use of GRAS chemicals and food safe natural plant extracts will be included.
<b>Phase 3</b>	
<b>Extension and technology transfer</b>	
Technology transfer.	A presentation was done at the 2021 online workshop. The work will be included in a summarised talk for the 2021 symposium (online).

## Discussion

During phase one, registered, single fungicides were evaluated at the label dose, evaluating their different fungicidal activities side by side. The study focused on the curative ability of individual actives against PD, by applying them in a drench, and also evaluating sporulation inhibition.

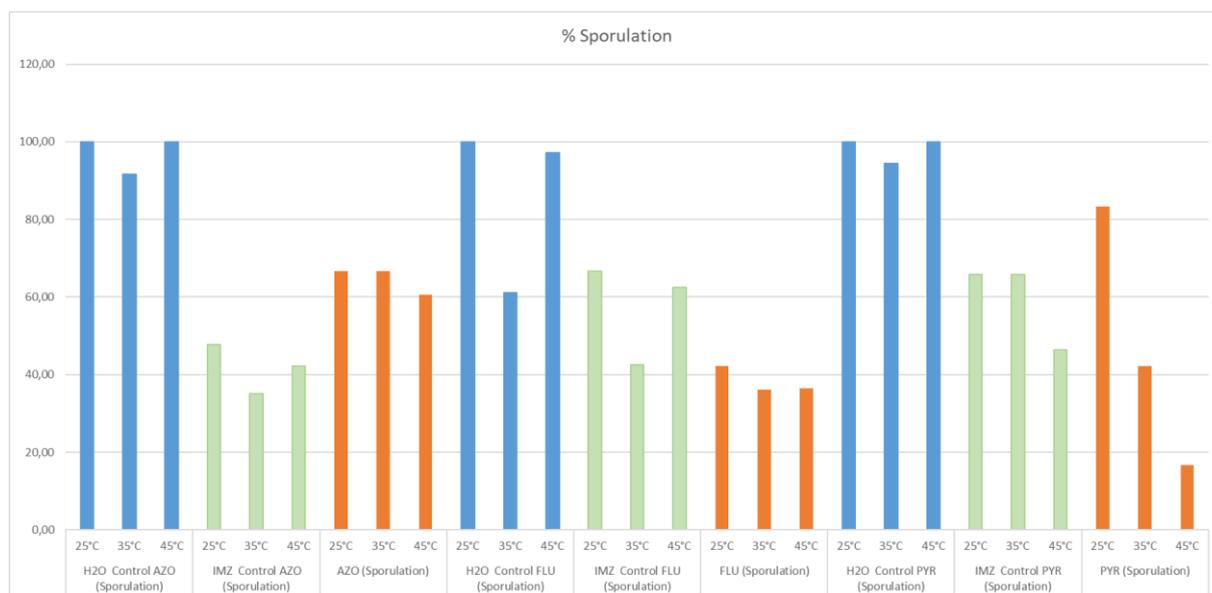
In terms of individual curative disease control, and using lemons as an example, AZO did well at 25 and 35°C, with PYR giving good decay control at 45°C. This, however was due to the residue loading obtained at the higher temperature. The curative disease control exhibited by FLU was unacceptable (Figure 4.4.4.1). Since OPP did not perform well on any of the three citrus varieties tested in terms of both curative control and sporulation control, it was eventually omitted in this part of the trial.



**Figure 4.4.4.1.** Disease control of individual actives on lemons, as percentage decay, at three different temperatures.

None of the chemicals tested were able to give 100% sporulation control, with OPP failing altogether. This failure to impart any disease or sporulation control was the reason OPP was eventually omitted from the trials with the individual chemicals. On lemons, in Figure 4.4.4.2 below, PYR and FLU, gave the most consistent

sporulation control (60 – 63 & 38 - 41% respectively), with PYR anti-sporulation being variable, it was more successful (18 – 82%). Again, PYR was far more successful in sporulation control at the higher residue loading than took place at 45°C.



**Figure 4.4.4.2.** Sporulation inhibition by the individual actives, as % sporulation, at three different temperatures.

The 2020 studies focussed on finding more effective combinations of the currently registered actives. The combination of actives is known to have potentially improved efficacy due to synergistic effects, are being explored. Two registered combinations, namely propiconazole + pyrimethanil (Propirly®), and azoxystrobin and fludioxonil (Evolve®) are available. The first is currently reserved for use in the pre-packhouse drench, with the second considered as a combination product for use in the fungicide bath. Despite these registrations, the trials included them as well, to be able to draw correlations between all the combinations tested. It was also to verify the possible effect that the ratio of the actives in each of the combinations have on one another. All the work in the 2019 and 2020 trials were done at the registered dosages, while an investigation into the ratios of the best non-commercialised combination will be done during the 2021 trials.

Due to a loss of time during the early lockdowns of the COVID-19 pandemic, none of these actives were combined with GRAS chemicals as proposed in 2019. This is, however, being explored in 2021. The investigation into the refinement of the parameters were also postponed to the 2021 season.

## Conclusion

In 2019 it was found that none of the single actives tested, by itself could match the efficacy of imazalil. Due to the successful registration of AZO +FLU and PYR+PPZ combinations, it was believed that other effective combinations may be possible, not excluding the possibility of combining a conventional fungicide with a GRAS chemical or a natural plant extract. The different combinations tested to date highlighted the fact that the actives do have an effect on each other when used in the same solution. No conclusion about the efficacy of the combinations of the various actives can be made at this stage, but the most promising combinations will be explored further. Additionally, alternative disease remedies will be included in the study.

## Technology transfer

The work to date were presented as a technical talk at the 2021 online postharvest workshop. Comprehensive technical feedback will also be given at the 2022 packhouse workshops. A research summary will be delivered at the CRI International Symposium in 2021.

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#### 4.4.5 **PROGRESS REPORT: Provision of an industry service whereby new packhouse treatments are comparatively evaluated, fungicide resistance is monitored and standardised recommendations are provided**

Project 123 (Ongoing) by Wilma du Plooy, Lindokuhle Mamba and Jan van Niekerk (CRI)

#### **Summary**

A total of five newly proposed products were tested to be evaluated against an industry standard (calcium hypochlorite). The most successful of these products were three new technology products, namely Peroxil, a micronised silver suspension, a nano-encapsulated oxygen enriched product called Flowsafe, and a dry PAA in tablet form, called Oxywash. All of these products still have to address regulatory hurdles in 2020 before commercialisation in the citrus industry. The results obtained, however, do encourage further development of the actives. No ring tests were conducted, but the tests for resistance monitoring in collaboration with the Diagnostic Centre were successful. The work done in 2020 was not presented at the online Packhouse Workshop in 2021 and will be included in the presentation at the 2022 workshops, should that be feasible.

#### **Opsomming**

Vyf nuut-voorgestelde produkte was getoets om hulle te evalueer teen die industrie se standaard saniteermiddel (kalsiumhipochloriet). Drie van die produkte was suksesvol, naamlik Peroxil (’n mikro-silversuspensie), Flowsafe (nano-geïnkapsuleerde, suurstofverrykte produk) en Oxywash (droeë PAA in tabletvorm). Al die produkte moes regulatoriese struikelblokke oorkom gedurende 2020 voordat hulle kommersieël beskikbaar gestel kon word. Die resultate van die drie produkte regverdig egter die verdere ontwikkeling van die produkte. Geen ringtoetse was gedoen nie, maar die weerstandsmonitering in samewerking met die diagnostiese sentrum het voortgegaan. Die werk wat in 2020 gedoen was, was nie by die 2021 aanlyn pakhuiswerkswinkel aangebied nie. Indien dit uitvoerbaar is, sal dit saam met die 2021 resultate in 2022 aangebied word.

#### **Introduction**

This ongoing project offers an industry service to evaluate potential new postharvest disease control products or options, as well as to conduct *ad hoc* experiments. Products are mostly submitted from private companies on a voluntary basis, or projects/products are selected by the researchers involved. Given limited time and resources, requests are screened based on industry priorities.

#### **Objectives**

1. Testing new potential products as fungicides, as well as evaluate possible synergistic reactions between chemicals, with specific focus on sour rot.
2. Evaluate available chemistries for use in the heated flooder – the effect of temperature, pH and exposure time, as well as combinations thereof to be evaluated.
3. Introduce and implement the application of GRAS chemicals and sanitizers into the citrus postharvest industry.
4. Analytical lab focus – ring test with the aim to reduce variability.

5. Assessment of fungicide resistance in citrus packhouses.
6. Technology transfer – primarily at the workshops and through collaboration with extension.

## Materials and methods

### Trial protocol for curative control

1. *Ad hoc* tests are performed on products that may be of postharvest use to the citrus industry. Fresh, untreated fruit is collected from a reputable commercial packhouse, sanitised and stored at  $\approx 23^{\circ}\text{C}$  for two days before the trial commences. The fruit is removed from cold storage and allowed to reach ambient temperature before use in a trial.
2. Each treatment has five replicates with 10 -12 fruit in each repeat, except where residue samples are collected, in which case the appropriate repeat has six extra fruit prepared for residue determination.
3. For all trials a sensitive strain of *Penicillium digitatum* (PD) and/or *Galactomyces citri-aurantii* (GCA) are used.
4. A  $10^6$  spore suspension of PD, and a  $10^8$  spore suspension GCA are prepared using the standard in-house laboratory technique.
5. Inoculation are done 6 hours prior to treatment.
6. Fruit are treated according to the label instruction of each the various remedies that are being tested.
7. Lesions are evaluated 4-6 days after inoculation, once  $>80\%$  of the untreated controls show positive lesion development.

### Trial protocol for testing water sanitation products

1. Freshly picked, mature fruit are collected from a commercial packhouse, rinsed in chlorine (150 ppm total chlorine, pH 6 for 60 seconds), and allowed to air dry.
2. Fruit are stored at  $\sim 4^{\circ}\text{C}$  for three days before the trial commences. The day before the trial commences the fruit are moved into ambient temperature ( $22^{\circ}\text{C}$ ) in order for fruit temperature to also reach ambient, and to allow any possible condensation to evaporate.
3. Dip solutions are prepared in 5 L buckets, using tap water corrected to temperature immediately before the fruit are dipped, with a temperature of  $\sim 30^{\circ}\text{C}$ . The temperature at which water enters the laboratory is about  $20^{\circ}\text{C}$ , and adjustments are made to closely resemble typical temperatures found in a postharvest packhouse.
4. Each treatment has 3 replicates with 10 fruit in each repeat.
5. A *Penicillium digitatum* spore suspension is prepared from a virulent, purified culture harvested from fruit, at a  $10^6$  spore concentration in a 5L solution.
6. Control treatments are a standard chlorine treatment and clean water.
7. The product tested is added to the spore suspension and agitated for 3 minutes before fruit dipping.
8. Fruits are wounded by rolling a blunt 9 prong wounding tool over 2 sides of a fruit.
9. After wounding the fruit, they are submerged into the treatment solutions for 1 minute.
10. Fruit are removed and packed into lock back grape cartons, covered with a transparent polyethylene bag with 4 holes to facilitate gaseous exchange and prevent build-up of  $\text{CO}_2$  and ethylene.
11. The treated fruit are incubated until  $>80\%$  decay is visible on the water controls.

## Results

Objective / Milestone	Achievement
1. New potential products will be tested as sanitation agents and/or fungicides.	A total of 5 products were tested. The results are available in the full reports attached herewith.
2. Introduce and implement the application of GRAS chemicals into the citrus postharvest industry	Three products with GRAS actives were tested, with all three being effective as water sanitizing options. Two products with actives derived from natural extracts were not successful.
3. Assist CRI DC with packhouse resistance testing	Swabs are either collected by extensionists visiting packhouses, or sent to the DC. Most do not indicate shifts in sensitivity, however, there were detections. The implicated packhouses were consulted on remedial action.

4. Analytical lab focus – ring test with the aim to reduce variability	No ring tests were conducted in 2020.
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## Discussion

### Alternative products

Two products based on plant extracts, namely Agapan (*Agapanthus* extract), and Sani RC and –RCL were tested.

### Packhouse sanitation

In terms of sanitation chemicals, Peroxil, a nano-encapsulated silver suspension, a nano-encapsulated oxygen enriched product called Flowsafe, and a dry PAA in tablet form, called Oxywash were tested. The results are available in appendices A –E.

### Resistance monitoring

Swabs from actively working packhouses were tested regularly throughout the season. A few incidences of sensitivity shift in pathogen resistance was detected and the packhouses concerned consulted about the issue. Suitable measures to curb this problem was suggested in cooperation with the extensionists. Due to no visitations to the packhouses being allowed, these were all done telephonically or via email. The time needed to meet this objective in the project often results in disruption of the planned workflow, due to the immediacy of processing the swabs sent to the DC.

## Technology transfer

The work done in 2020 was not presented at the online Packhouse Workshop in 2021 and will be included in the presentation at the 2022 workshops, should that be feasible

## Further objectives (milestones) and work plan

1. New potential products will be tested as sanitation agents and/or fungicides; this specifically includes seeking actives for the control of sour rot
2. Introduce and implement the application of GRAS chemicals into the citrus postharvest industry
3. Seek effective products and technologies for water sanitation in citrus packhouses
4. Analytical lab focus – ring test with the aim to reduce variability
5. Assist CRI DC with packhouse resistance testing if required.

## APPENDIX A

### TRIAL: Sanitation of water used in aqueous application in citrus packhouses

On behalf of: Flowsafe Chemicals

BY: Dr Wilma du Plooy, Lindokuhle Mamba and Thabang Mgwenya

#### Objectives

Water sanitizers are believed to enable more effective action of the fungicides and other products used, against postharvest pathogens in the packhouse. Since *Penicillium digitatum* (PD) is the most prevalent of the two major postharvest pathogens, it was decided to use only PD as test organism.

**Crop:** Valencia

**Origin:** Mbombela District

**Trial date:** 05 October 2020

**Trial site:** CRI, 2 Baker Street, Nelspruit, 1201

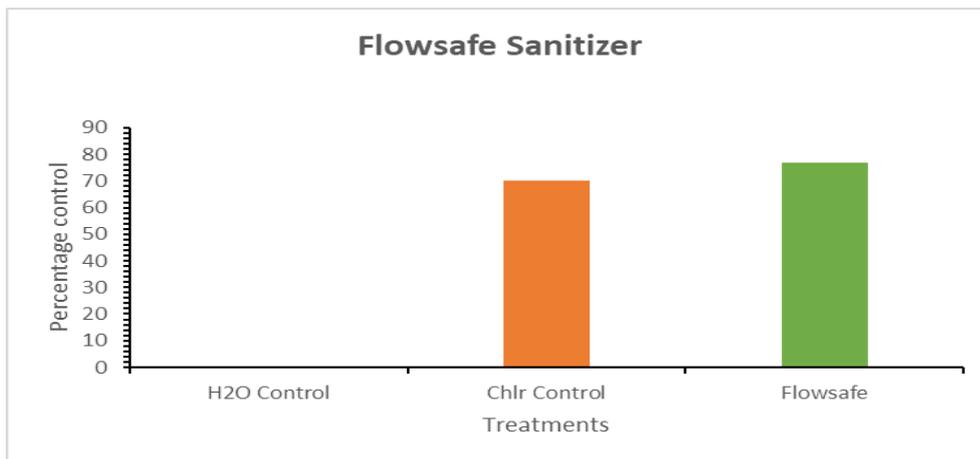
**Report date:** 20 October 2020

#### Materials and methods

1. Freshly picked, mature Valencia fruit were collected from a commercial packhouse, rinsed in chlorine (150 ppm total chlorine, pH 6 for 90 seconds), and allowed to air dry.
2. The fruit were stored at  $-8^{\circ}\text{C}$  for three days before the trial commenced. The day before the trial commenced the fruit were moved into ambient temperature ( $22^{\circ}\text{C}$ ) in order for fruit temperature to also reach ambient, and to allow any possible condensation to evaporate.
3. pH adjustments were made to get to a pH of 6.5.
4. Solutions were prepared with tap water and the temperature corrected immediately before the fruit were dipped ( $\sim 30^{\circ}\text{C}$ ). The temperature at which water enters the laboratory is about  $20^{\circ}\text{C}$ , and adjustments were made to more closely resemble typical temperatures found in a postharvest packhouse.
5. Each treatment had 3 replicates with 10 fruit in each repeat.
6. A *Penicillium digitatum* spore suspension was prepared from a virulent, purified culture harvested from fruit, at a concentration of  $10^6$  spores/ml.
7. Buckets with 50 L of a 2% Peroxil solution were seeded with 50 ml *Penicillium digitatum* spores to get to a final spore count of  $\sim 10^4$ .
8. Control treatments were a standard 150 ppm chlorine solution (FREXUS Chlorine) and clean water to which spore suspensions were added.
9. The solutions were left for 3 minutes to react with the spores.
10. Immediately before treatment, fruit were injured using a metal plate with 9 x 7mm spikes that were rolled across the cheek section of the fruit, on two sides.
11. Injured fruit was placed in the sanitiser solution with the spores, and left in the solution for 3 minutes while slightly agitating it.
12. Treated fruit were removed and dried before placing the 10 fruit from individual replicates in a nectarine liner to separate fruit during incubation. The liners are placed in an open top carton, slipped into a transparent polyethylene bag and closed. The bags were punctured four times on the sides to allow gaseous exchange and prevent excessive moisture build-up.

#### Results and discussion

In comparison to chlorine as the industry standard, Flowsafe gave excellent results. The fact that none of the products had 100% curative value is due to the high inoculum concentration used, which increases the disease pressure as a result of the many visible wounds and prolonged incubation. This is done in order to challenge both the trial product and the standard currently in use.



**Graph 1.** Evaluation of Flowsafe as an alternative postharvest sanitiser towards disease control

### Conclusion

The product has excellent potential as an aqueous sanitiser and further development and registration should be pursued by the product owner.

### DISCLAIMER

This report contains information and results from a confidential trial to determine any combination of product efficacy and/or compatibility and/or phytotoxicity. Results from this trial is indicative of the potential of a product only. By conducting these trials the CRI is assisting the citrus industry in finding postharvest sanitation and disease control options. Any successful interaction still requires that the necessary accreditations be acquired through the relevant regulatory body (Act 5 or Act 36), and does not imply any product endorsement by the CRI.

THIS REPORT MAY NOT BE USED IN ANY WAY AS PART OF THE MARKETING MATERIAL OF THE PRODUCT OWNER OR DISTRIBUTORS.

## APPENDIX B

### **TRIAL: Sanitation of water used in aqueous application in citrus packhouses**

On behalf of: Peroxil – Chris Vorster

BY: Dr Wilma du Plooy, Lindokuhle Mamba and Thabang Mgwenya

#### **Objectives**

A formulation claimed as micronized silver in a peroxide blend, was received. It is believed that it will be effective against *Penicillium digitatum* (PD) and *Galacatomyces citri-aurantii* (GCA) in the packhouse. Since *Penicillium digitatum* is the prevalent of the two organisms, as well as the more readily controlled of the two, it was decided to use only PD as test organism.

**Crop:** Valencia

**Origin:** Ngodwana District

**Trial date:** 1 and 8 September 2020

**Trial site:** CRI, 2 Baker Street, Nelspruit, 1201

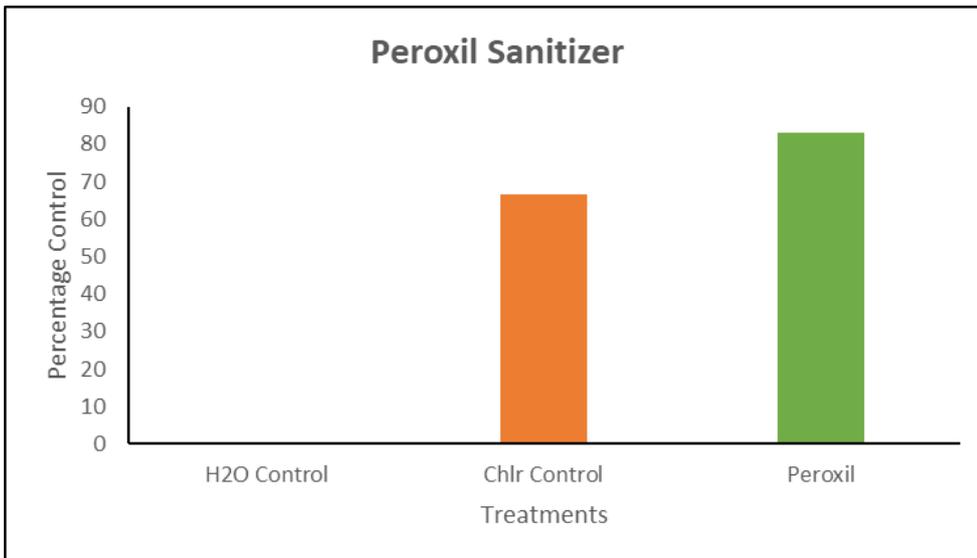
**First report date:** 25 September 2019

**Revised report date:** N/A

#### **Materials and methods**

1. Freshly picked, mature Valencia fruit were collected from a commercial packhouse, rinsed in chlorine (150 ppm total chlorine, pH 6 for 90 seconds), and allowed to air dry.
2. The fruit were stored at ~8°C for three days before the trial commenced. The day before the trial commenced the fruit were moved into ambient temperature (22°C) in order for fruit temperature to also reach ambient, and to allow any possible condensation to evaporate.
3. Water pH adjustments were made to get to a pH of 6.5.
4. Solutions were prepared with tap water and the temperature corrected immediately before the fruit was dipped (~30°C). The temperature at which water enters the laboratory is about 20°C, and adjustments were made to more closely resemble typical temperatures found in a postharvest packhouse.
5. Each treatment had 3 replicates with 10 fruit in each repeat.
6. A *Penicillium digitatum* spore suspension was prepared from a virulent, purified culture harvested from fruit, at a concentration of 10<sup>6</sup> spores/ml.
7. Buckets with 50 L of a 2% Peroxil solution were seeded with 50 ml of the *Penicillium digitatum* spore suspension to get to a final spore count of ~10<sup>4</sup>.
8. Control treatments were a standard 150 ppm chlorine solution (FREXUS Chlorine) and clean water to which the spore suspension were added.
9. The solutions were left for 3 minutes to react with the spores.
10. Immediately before treatment, fruit were injured using a metal plate with 9 x 7mm spikes that were rolled across the cheek section of the fruit, on two sides.
11. Injured fruit were placed in the sanitiser solution with the spores, and left in the solution for 3 minutes while slightly agitating it.
12. Treated fruit were removed and dried before placing the 10 fruit from individual replicates in a nectarine liner to separate fruit during incubation. The liners are placed in an open top carton, slipped into a transparent polyethylene bag and closed. The bags were punctured four times on the sides to allow gaseous exchange and prevent excessive moisture build-up.

#### **Results and discussion**



**Graph 1.** Peroxil, applied as an alternative water sanitizer option, in comparison with chlorine as standard sanitizer

**Table 1.** Table of the levels of control achieved by a 2% Peroxil solution in comparison to standard chlorine as a water sanitiser for use in aqueous applications

Treatment	% Control
Water	0
Chlorine (Frexus)	72
Peroxil 2%	80

The product may be considered for further development as a replacement for chlorine, due to the efficacy found in this pilot trial. There is no concern regarding the silver residues on fruit, since the levels are extremely low and will not contribute to health concern regarding the ADI of Ag+. The lower efficacy of the chlorine was noted, where the usual levels of control is ~80% in these pilot trials.

No phytotoxicity was observed

**Conclusion**

The product tested has potential to be used as a water sanitiser on citrus lines.

**DISCLAIMER**

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THIS REPORT MAY NOT BE USED IN ANY WAY AS PART OF THE MARKTING MATERIAL OF THE PRODUCT OWNER OR DISTRIBUTORS.

## APPENDIX C

### TRIAL: Sanitation of water used in aqueous application in citrus packhouses

On behalf of: Oxywash

BY: Dr Wilma du Plooy, Lindokuhle Mamba and Thabang Mgwenya

#### Objectives

A formulation claimed as a solid PAA /peroxide blend, was received. It is believed that it will be effective against *Penicillium digitatum* (PD) and *Galacatomyces citri-aurantii* (GCA) in the packhouse. Since *Penicillium digitatum* is the prevalent of the two organisms, as well as the more readily controlled of the two, it was decided to use only PD as test organism

**Crop:** Valencia

**Origin:** Mbombela District

**Trial date:** 20 August 2019

**Trial site:** CRI, 2 Baker Street, Nelspruit, 1201

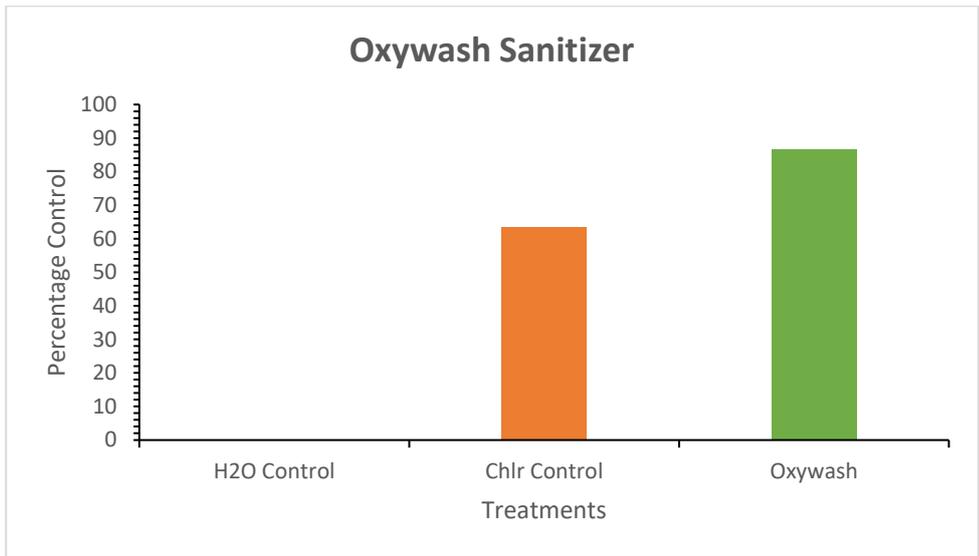
**Report date:** 01 September 2020 & 02 October 2020

#### Materials and methods

1. Freshly picked, mature Valencia fruit were collected from a commercial packhouse, rinsed in chlorine (150 ppm total chlorine, pH 6 for 60 seconds), and allowed to air dry.
2. The fruit were stored at ~4°C for three days before the trial commenced. The day before the trial commenced the fruit were moved into ambient temperature (22°C) in order for fruit temperature to also reach ambient, and to allow any possible condensation to evaporate.
3. Dip solutions were prepared in 5 L buckets, using tap water corrected to temperature immediately before the fruit were dipped, with a temperature of ~30°C. The temperature at which water enters the laboratory is about 20°C, and adjustments were made to closely resemble typical temperatures found in a postharvest packhouse.
4. Each treatment had 3 replicates with 10 fruit in each repeat.
5. A *Penicillium digitatum* spore suspension was prepared from a virulent, purified culture harvested from fruit, at a 10<sup>6</sup> spore concentration in 5L solution.
6. Control treatments were a standard chlorine treatment and clean water.
7. Product tested were added and agitated for 3 minutes before fruit dipping.
8. Fruit were inoculated by rolling a blunt 9 prong wounding tool over 2 sides of a fruit.
9. After wounding the fruit, they were submerged into the treatment solutions for 1 minute.
10. Fruit were removed and packed into lock back grape cartons, covered with transparent polyethylene bag with 4 holes to facilitate gaseous exchange and prevent build-up of CO<sub>2</sub> and ethylene. The treatments were incubated until >80% decay is visible on the water controls.

#### Results and discussion

As a water sanitizer, Oxywash did exceptionally well. The product is easy to work with, as there are no volatiles involved. There is some concern about how many tinfoil covered tablets would be required per water application on a typical packline, as this may impede the workflow. The lower efficacy of the chlorine was noted, where the usual levels of control is 83 - 87% in these pilot trials. This is not due to the chlorine failing, but rather due to the high inoculum that creates significant disease pressure as a result of the many visible wounds and prolonged incubation..



**Graph 1.** Comparison of Oxywash and chlorine, as an as a water sanitizer option

**Conclusion**

The product may be considered for further development and registration as a replacement for chlorine, due to the efficacy found in this pilot trial. There is no concern regarding the PAA residues on fruit, since it breaks down into oxygen and water upon reaction, and will not contribute to health concerns.

No phytotoxicity was observed.

**DISCLAIMER**

This report contains information and results from a confidential trial to determine any combination of product efficacy and/or compatibility and/or phytotoxicity. Results from this trial is indicative of the potential of a product only. By conducting these trials the CRI is assisting the citrus industry in finding postharvest sanitation and disease control options. Any successful interaction still requires that the necessary accreditations be acquired through the relevant regulatory body (Act 5 or Act 36), and does not imply any product endorsement by the CRI.

THIS REPORT MAY NOT BE USED IN ANY WAY AS PART OF THE MARKTING MATERIAL OF THE PRODUCT OWNER OR DISTRIBUTORS.

## APPENDIX D

### TRIAL: Sanitation of water used in aqueous application in citrus packhouses

On behalf of: Corrie Muller, Advantage Chemicals

BY: Dr Wilma du Plooy, Lindokuhle Mamba and Thabang Mgwenya

#### Objectives

The use of sanitizers in the packhouse is believed to increase and enable effective disease control of the postharvest pathogens, *Galactomyces citri-aurantii* (GCA) and *Penicillium digitatum* (PD) in the packhouse. Since *Penicillium digitatum* is the most prevalent of the two postharvest pathogens, it was decided to use only PD as test organism. Previous work with Sani-D had proven that it was very successful against GCA in an aqueous environment. It was not tested against PD at that stage, but was not available for the retest due to COVID restraints on transport. Although Sani-RC and Sani-RCL are formulated for use in wax application, it has the same active and is soluble in water, therefore its possible use outside wax was considered.

**Crop:** Valencia

**Origin:** Mbombela District

**Trial date:** 01 September 2020 & 02 October 2020

**Trial site:** CRI, 2 Baker Street, Nelspruit, 1201

**Report date:** 20 October 2020

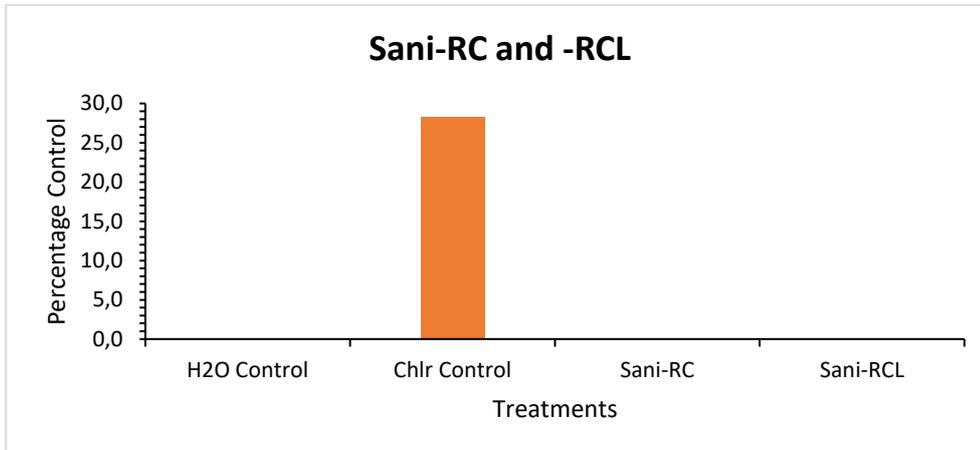
#### Materials and methods

1. Freshly picked, mature Valencia fruit were collected from a commercial packhouse, rinsed in chlorine (150 ppm total chlorine, pH 6 for 90 seconds), and allowed to air dry.
2. The fruit was stored at ~8°C for three days before the trial commenced. The day before the trial commenced the fruit was moved into ambient temperature (22°C) in order for fruit temperature to also reach ambient, and to allow any possible condensation to evaporate.
3. Water pH adjustments were made to get to a pH of 6.5.
4. Solutions were prepared with tap water and the temperature corrected immediately before the fruit was dipped (~30°C). The temperature at which water enters the laboratory is about 20°C, and adjustments were made to more closely resemble typical temperatures found in a postharvest packhouse.
5. Each treatment had 3 replicates with 10 fruit in each repeat.
6. A *Penicillium digitatum* spore suspension was prepared from a virulent, purified culture harvested from fruit, at a concentration of 10<sup>6</sup> spores/ml.
7. Buckets with 50 L of a 2% Peroxil solution were seeded with 50 ml of the *Penicillium digitatum* spore solution to get to a final spore count of ~10<sup>4</sup>.
8. Control treatments were a standard 150 ppm chlorine solution (FREXUS Chlorine) and clean water to which spore suspensions were added.
9. The solutions were left for 3 minutes to react with the spores.
10. Immediately before treatment, fruit were injured using a metal plate with 9 x 7mm spikes that were rolled across the cheek section of the fruit, on two sides.
11. Injured fruit was placed in the sanitiser solution with the spores, and left in the solution for 3 minutes while slightly agitating it.
12. Treated fruit were removed and dried before placing the 10 fruit from individual replicates in a nectarine liner to separate fruit during incubation. The liners are placed in an open top carton, slipped into a transparent polyethylene bag and closed. The bags were punctured four times on the sides to allow gaseous exchange and prevent excessive moisture build-up.

#### Results and discussion

Neither of the two Sani products used in the trial had any effect on PD in an aqueous environment. This is ascribed to the fact that the products are formulated to be used in wax only. For these trials the wounding tool used ensured visible wounds (18 piercings/fruit).

Since the water controls were left to rot to 100% rather than to evaluate it at 80% rot, even the chlorine controls were not as effective usual (~80% control). This is not due to the chlorine failing, but rather due to the high inoculum that creates significant disease pressure as a result of the many visible wounds and prolonged incubation.



**Graph 1.** SANI RC-products, applied as an alternative water sanitizer option, in comparison with chlorine as standard sanitizer

### Conclusion

From the results it is clear that applying a formulation in the right application, is imperative. The Sani-RC and –RCL formulation **were not intended as aqueous sanitisers**, but due to interest in the active and also its possible lateral use, the study was done. These products are supposed to be used in wax, where it acts as an fortifying agent during export. Any other application thereof will be to the detriment of the product. The Sani-D product will be retested against both PD and GCA in 2021.

### DISCLAIMER

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## APPENDIX E

### TRIAL: Sanitation of water used in aqueous application in citrus packhouses

On behalf of: Agapan (trial product from UOFS)

BY: Dr Wilma du Plooy, Lindokuhle Mamba and Thabang Mgwenya

#### Objectives

This sanitizer and postharvest wash were claimed to have been tested against eight phytopathogens (Tegegne *et al.*, 2008), however, this did not include any of the citrus pathogens. The product was offered to CRI for testing on citrus. Agapan seemed to have the best efficacy against hyphomycetes with a more surface bound life cycle. Since *Penicillium digitatum* (PD) is also surface bound, and it is the most prevalent of the two major postharvest pathogens, it was decided to use only PD as test organism.

**Crop:** Valencia

**Origin:** Mbombela District

**Trial date:** 08 September 2020

**Trial site:** CRI, 2 Baker Street, Nelspruit, 1201

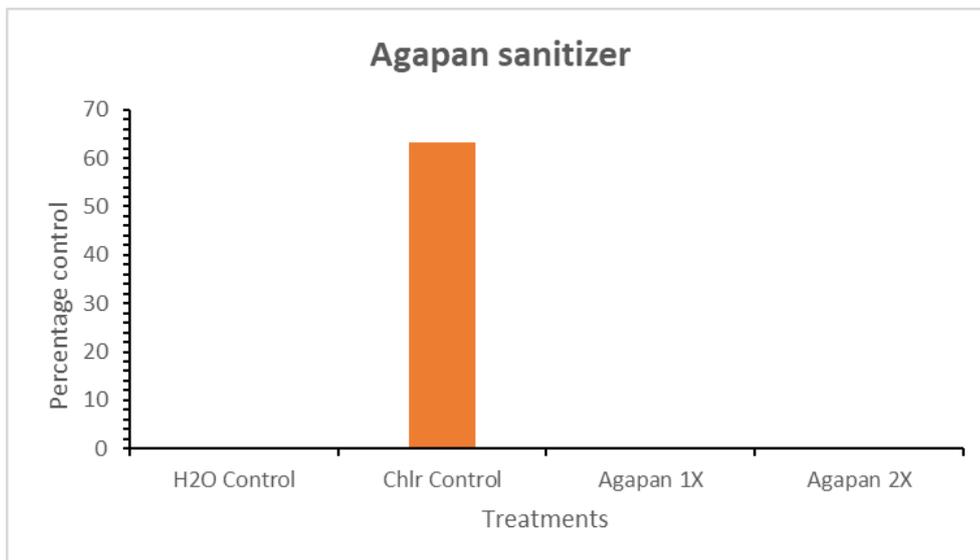
**Report date:** 18 September 2020

#### Materials and methods

1. Freshly picked, mature Valencia fruit were collected from a commercial packhouse, rinsed in chlorine (150 ppm total chlorine, pH 6 for 90 seconds), and allowed to air dry.
2. The fruit were stored at ~8°C for three days before the trial commenced. The day before the trial commenced the fruit were moved into ambient temperature (22°C) in order for fruit temperature to also reach ambient, and to allow any possible condensation to evaporate.
3. Water pH adjustments were made to get to a pH of 6.5.
4. Solutions were prepared with tap water and the temperature corrected immediately before the fruit was dipped (~30°C). The temperature at which water enters the laboratory is about 20°C, and adjustments were made to more closely resemble typical temperatures found in a postharvest packhouse.
5. Each treatment had 3 replicates with 10 fruit in each repeat.
6. A *Penicillium digitatum* spore suspension was prepared from a virulent, purified culture harvested from fruit, at a concentration of 10<sup>6</sup> spores/ml.
7. Buckets with 50 L of a 2% Peroxil solution were seeded with 50 ml of the *Penicillium digitatum* spore solution to get to a final spore count of ~10<sup>4</sup>.
8. Control treatments were a standard 150 ppm chlorine solution (FREXUS Chlorine) and clean water to which spore suspensions were added.
9. The solutions were left for 3 minutes to react with the spores.
10. Immediately before treatment, fruit were injured using a metal plate with 9 x 7mm spikes that were rolled across the cheek section of the fruit, on two sides.
11. Injured fruit were placed in the sanitiser solution with the spores, and left in the solution for 3 minutes while slightly agitating it.
12. Treated fruit were removed and dried before placing the 10 fruit from individual replicates in a nectarine liner to separate fruit during incubation. The liners are placed in an open top carton, slipped into a transparent polyethylene bag and closed. The bags were punctured four times on the sides to allow gaseous exchange and prevent excessive moisture build-up.

#### Results and discussion

Neither of the two concentrations applied had any effect on the *Penicillium digitatum* spores added to the water. If that was the case, there would have been a noticeable effect on the wounded fruit, which there was not. This implies that a significant amount of spores were viable in the water the fruit were dipped into. The lower efficacy of the chlorine was noted, where the usual levels of control is ~80% in these pilot trials. This is not due to the chlorine failing, but rather due to the high inoculum that creates significant disease pressure as a result of the many visible wounds and prolonged incubation.



**Graph 1.** Agapan, applied as an alternative water sanitizer option, in comparison with chlorine as standard sanitizer

### Conclusion

Unfortunately no curative, inhibitory, or other action were displayed by the tested product and in its current format it is not suitable for use as a water sanitiser in the citrus postharvest environment.

### Reference

Tegegne, Pretorius, J. & Swart, W.J. 2008. Antifungal properties of *Agapanthus africanus* L. extracts against plant pathogens. *Crop Protection* 27(7):1052-1060

### DISCLAIMER

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THIS REPORT MAY NOT BE USED IN ANY WAY AS PART OF THE MARKTING MATERIAL OF THE PRODUCT OWNER OR DISTRIBUTORS.

#### 4.4.6 FINAL REPORT: The use of alternative fungicides in wax to control citrus green mould caused by *Penicillium digitatum*.

Project 1251 (2019 – 2020) by Charles Stevens, Cheryl Lennox (SU), Paul H. Fourie, Wilma du Plooy (CRI)

#### Summary

The South African citrus industry depends on the use of synthetic fungicides to control postharvest diseases such as citrus green mould caused by *Penicillium digitatum*. Imazalil (IMZ) is the most reliable fungicide in use for controlling green mould. However, IMZ and its existing maximum residue levels are under review and has the risk of being recalled and therefore alternatives need to be looked at. The project commenced in April 2019. To date, a pilot study and a semi-commercial study was conducted in which pyrimethanil, fludioxonil and azoxystrobin were tested in a wax formulation as alternatives to imazalil. These fungicides were also tested in combination with one another. Azoxystrobin performed best with a high protective control action at registered concentration on citrus, while sporulation was largely inhibited at the registered concentration of this fungicide. Fludioxonil also performed well with a high protective control and sporulation inhibition at its

registered concentration on citrus. Overall, pyrimethanil had the lowest protective control and sporulation inhibition. Maximum residue levels by EU standards were not exceeded.

## Opsomming

Die Suid-Afrikaanse sitrusbedryf is afhanklik van die gebruik van sintetiese swamdoders om na-oes siektes, soos sitrusgroenvrot, wat deur *Penicillium digitatum* veroorsaak word, te beheer. Imazalil (IMZ) is die betroubaarste swamdoder wat gebruik word om groenvrot te beheer. IMZ en sy bestaande maksimum residuvlakke word egter hersien en die risiko bestaan dat dit herroep gaan word en daarom moet alternatiewe na gekyk word. Die projek is in April 2019 begin. Tot op datum is 'n loodsstudie en 'n semi-kommersiële studie uitgevoer waarin pyrimethanil, fludioxonil en azoxystrobien in 'n wasformulering getoets is as alternatiewe vir imazalil. Hierdie swamdoders was ook in kombinasie met mekaar getoets. Azoxystrobin het die meeste potensiaal ten opsigte van beskermende beheer, sowel as sporulasie-remming getoon. Fludioxonil presteer ook goed met 'n hoë beskermende effek en sporulasie inhibisie teen sy geregistreerde konsentrasie op sitrus. In die algemeen het pyrimethanil die laagste beskermende beheer en sporulasie-remming gehad. Die maksimum residuvlakke volgens die EU-standaarde is nie oorskry nie.

## Introduction

South Africa is currently the largest exporter of shipped citrus across the world (Edmonds, 2016). Over the past 10 years most of South Africa's citrus exports went to the European (49%) and Asian (41%) markets (DAFF, 2016). During harvesting processes, micro-wounds occur on the fruit due to inadequate protection and handling. These injuries may function as infection sites for major postharvest pathogens such as green mould, caused by *Penicillium digitatum* (Pers.: Fr.) Sacc (PD). It occurs mostly in tropical and subtropical climatic conditions and causes great economic loss. A site of injury is needed for infection to take place through the flavedo (Kanetis *et al.*, 2008). It is regarded as one of the major causes of decay losses on fruit exported from South Africa (Pelser & Eckert, 1976). Practices used to control green mould includes reducing fruit contamination in the orchard, thereby minimizing pre-harvest infection, reducing fruit contamination in the packhouse, maintaining resistance of fruit to postharvest infection and applying appropriate chemical and physical treatments to the fruit (Smilanick *et al.*, 2006).

The South African citrus industry depends on synthetic fungicides to control these postharvest diseases because of its export distances that are long. There are currently six registered postharvest fungicides that are used for the control of green mould in the citrus industry of South Africa (Erasmus *et al.*, 2015). One of these is Imazalil (IMZ) and is the most reliable and effective fungicide in use at the moment in controlling green mould (Erasmus *et al.*, 2015).

Imazalil is a systemic fungicide that is used for citrus postharvest. It may be applied by the use of seed treatment equipment, drenches, waxing and the use of foaming equipment, sprayers as well as smoke canisters (EPA, 2005). Imazalil is a demethylating inhibiting fungicide and its mode of action acts to inhibit ergosterol biosynthesis. This causes methyl and dimethyl intermediates (Siegel and Ragsdale, 1978). Imazalil is available in different formulations namely; IMZ nitrate salt, IMZ sulphate salt and an emulsifiable concentrate (EC) that may be used for various application techniques, e.g. spraying, dipping or with wax coating application (Kaplan & Dave, 1979). In most European countries as well as in Japan and South Africa, the maximum residue limits (MRLs) for IMZ is 5 ppm for citrus on whole fruit (Brown *et al.*, 1983). The environmental protection agency of the United States has regulated that for citrus fruit the MRLs should be 10 ppm (Watanabe *et al.*, 2000). In a study done by Kaplan and Dave in 1979, a range of 0.6 to 2.0 ppm of Imazalil residues resulted in good decay and sporulation control. This confirmed imazalil's anti-sporulant activity (Eckert and Brown, 1990). Imazalil also has good curative and protective control (Erasmus *et al.*, 2015). However, imazalil and its existing maximum residue levels are under review due to a lack of information/data (particularly on the toxicity of metabolites FK-772 ad FK-284) that was recognized (Brancato *et al.*, 2018). This presents the risk of losing imazalil as a postharvest fungicide and alternatives need to be looked at.

Commonly used fungicides for drenching in most citrus growing countries, including South Africa, are Thiabendazole (TBZ) and imazalil. Azoxystrobin, pyrimethanil (PYR) and fludioxonil are also fungicides that

are active against *P. digitatum* strains that show resistance to TBZ and IMZ (Angioni et al., 2007; Chirra et al., 2008).

The strobilurin fungicide azoxystrobin has been registered for citrus use in the United States (Mondal et al., 2005). Azoxystrobin has both curative and protective activity offering the potential for use as soil, seed and foliar treatments with residual disease control that is long lasting (Schutte et al., 2003). Studies have shown that azoxystrobin is extremely effective against *P. digitatum*, *in vitro*, and that effective concentrations results in reduction of mycelial growth and conidial germination (Zhang et al., 2009).

Pyrimethanil belongs to the anilinopyrimidine class (Leroux, 1996) and has been registered for this use, since resistance against older fungicides are a problem (Kanetis et al., 2008). Previous studies have looked at the use of PYR being applied protectively (before inoculation) as well as curatively (after inoculation) in drench, dip, and wax coating treatments and the fruit were inoculated with or an IMZ-resistant isolate or an imazalil (IMZ)-sensitive isolate of *Penicillium digitatum* (Kellerman et al, 2018). The study found that the IMZ-resistant isolate was effectively controlled by all the PYR treatments, and that in some cases better than the sensitive isolate. They concluded that PYR provides substantial curative control in dip applications, but results in drench and wax treatments were not as good. Following drench treatment of PYR for green mould control, it was found that the control was better on fruit inoculated with smaller spore loads, or when PYR was combined with other fungicides (Kellerman et al., 2018).

Fludioxonil belongs to the class of phenylpyrroles (Leroux, 1996) and is a newly registered fungicide for postharvest use on citrus in South Africa. It was registered to control sporulation of *Penicillium* spp. as a wax application (du Plooy & Lesar, 2017) . Fludioxonil works ideally in a resistance management programme as it belongs to FRAC group 12. It is suggested that the 230 g/l formulation in a wax be applied at a specific rate of 1 – 2 l in 100 l wax. The usual application rate of the wax is 1 l wax/tonne fruit. The final concentration of the fludioxonil is said to vary between 2 300 - 4 600 ppm and this will depend on the rate of addition to wax (du Plooy & Lesar, 2017). In previous studies fludioxonil effectively reduced green mould when it was applied through a simulated commercial packing line system. It was also reported that *P. digitatum* sporulation on citrus was actively suppressed by fludioxonil. However, fludioxonil did show less activity for *P. digitatum* sporulation control when compared to imazalil. The registration of fludioxonil has provided the citrus industry with a new alternative, reduced-risk compound for the control of citrus postharvest disease and the management of fungicide resistance (Zhang, 2007).

One particular study has evaluated the effectiveness of each of these fungicides when they were applied alone or in combination to lemon fruit that were wound-inoculated with imazalil/ thiabendazole (TBZ)-sensitive or -resistant isolates of *P. digitatum*. Azoxystrobin-fludioxonil combinations were meaningfully more effective when compared to fungicide treatments individually. When compared to storage or aqueous fruit coating applications, the effectiveness of all fungicides was much lesser when mixed into a packing fruit coating. Among these fungicides, fludioxonil and azoxystrobin applied in water or storage fruit coating, respectively, gave the best anti-sporulation action. The activity of both of these fungicides were improved through storage fruit coating. Pyrimethanil showed to be least effective fungicide in inhibiting sporulation of the pathogen on decaying fruit. Overall, among the mixtures, azoxystrobin-fludioxonil and TBZ-fludioxonil had the highest anti-sporulation action in aqueous and storage fruit coating applications (Kanetis et al., 2007).

However, the use of synthetic fungicides is gradually being limited and restricted (Dayan et al., 2009) in order to provide safer and biodegradable alternatives (Wilson et al., 2000). Fungicides, including IMZ, used to treat fruit crops such as citrus, are applied and used in large volume dip solutions. Irresponsible disposal of this hazardous waste causes severe environmental issues and may also be costly. In addition, fungi have shown to build up resistance to synthetic fungicides over time with prolonged use, thereby shortening the shelf-life of these protective products (Wild, 1983). In recent years, the emergence of biological control agents has shown promising alternatives to synthetic fungicides (Wilson, 1989). Extracts from plants, including essential oils (EOs), have been researched as substitute methods to protect fruit against postharvest diseases (Klieber et al., 2002; Ahmed et al., 2007). A possible alternative to controlling postharvest pathogens is also the use of essential oils amended coatings on citrus (du Plooy et al., 2009). Thyme, cinnamon and spearmint oils have shown to have good fungicidal potential *in vitro*. Thyme (*Thymus vulgaris*) oil has showed the best inhibition,

inhibiting all of the pathogens tested at a concentration of 1 µl/ml and lower. Cinnamon (*Cinnamomum zeylanicum*) oil is rich in eugenol (81.2%) and also showed good disease control (Combrinck *et al.*, 2011). Spearmint (*Mentha spicata*) oil completely inhibited *P. digitatum* mycelial growth at concentrations of 1 µl/ml and higher (du Plooy *et al.*, 2009).

It is clear that the risk of losing Imazalil as a postharvest fungicide against *P. digitatum* presents potential economic threats to the South African citrus industry and therefore chemical and non-chemical alternatives in a wax formulation need to be looked at, not only to preserve the industry's scarce fungicides, but also to provide fruit that is aesthetically pleasing to exporters and consumers. The aim of this study was to test three possible chemical alternatives i.e azoxystrobin, fludioxonil and pyrimethanil and three non-chemical alternatives i.e thyme, cinnamon and spearmint oils in a wax formulation as a potential control measure (protective control and sporulation inhibition) against citrus green mould.

### **Stated objectives**

1. Pilot trial – Collecting untreated lemons from citrus orchards.
  - Determine the effective concentration of alternative fungicides (azoxystrobin, fludioxonil and pyrimethanil) and essential oils (thyme, cinnamon and spearmint) to prevent pathogen (*P. digitatum*) growth whilst remaining within MRLs
2. Semi-commercial trial – Apply the single effective concentration of alternatives individually to fruit using a commercial packline
3. Semi-commercial trial – Apply single effective concentrations of alternative fungicides in combination with one another
4. Measuring resistance - Determine practical resistance of propiconazole-resistant citrus sour rot and citrus green mould isolates.
  - Determine baseline sensitivities of *P. digitatum* alternative fungicides
  - Determine practical resistance of *P. digitatum* alternative fungicides
5. Evaluate essential oils for their protective control and sporulation inhibition potential.

### **Materials and methods**

Sampling Untreated lemon, navel and mandarin fruits were collected from citrus orchards in the Western Cape and washed in 150 ppm chlorine and allowed to dry overnight at ambient temperature before commencement of the trials. Two-week old cultures of an isolate of *P. digitatum* that is IMZ sensitive (STE-U 6560), plated on potato dextrose agar (PDA) was used to prepare spore suspensions, at a final concentration of  $1 \times 10^6$  spores.mL<sup>-1</sup>.

Protective control Fruits evaluated for protective control were treated with pyrimethanil and fludioxonil at their registered concentrations of 4 000 ppm and 2 600 ppm respectively, in a food-based shellac wax formulation on a commercial waxing applicator. Azoxystrobin concentration was adjusted to a concentration of 4000ppm (As recommended by ICA International Chemicals). These fungicides were also used in combination with one another. Fruits treated with thyme, cinnamon and spearmint was applied at 1/2x, 1x and 2x their recommended concentrations of 48 ppm, 40 ppm and 48 ppm respectively in a food-based shellac wax formulation. The fruit was left to dry for 30 min and then inoculated with spore suspension using a 5 mm wounding tool with four wounds on the shoulder of each fruit.

Sporulation inhibition Fruits used for sporulation inhibition evaluations were treated in the same way but instead inoculated with 0.2 mL of spore suspension using a sterile 0.60 x 25 mm gauge needle. Inoculum was injected beneath the rind of the fruit on the shoulder of each fruit.

Ratings Treated and inoculated fruit from each treatment combination was placed in lock back table grape cartons on trays. Each box was covered with a transparent polyethylene bag and sealed and then incubated at 22°C. Protective control fruit was evaluated after 4 days by recording the number of infected wounds on

each fruit. Sporulation inhibition was evaluated using a rating scale after 10 days. Percentage control and sporulation inhibition was calculated using an untreated 'treatment' as a reference. Imazalil was included as a positive control. A wax only treatment was also included.

Additional tests In addition to the actives tested, two additional products were also tested i.e. Ortosol (orthophenylphenol [OPP]) and Evolve (azoxystrobin + fludioxonil). These products were tested at recommended registered concentrations of 12 500 ppm for OPP and 3 600 ppm (AZX) + 3 600 ppm (FLU). Products were tested in the same method as mentioned above.

Practical resistance of *P. digitatum* and *G. citri-aurantii* was measured through testing the curative abilities of propiconazole by inoculating with a spore suspension adjusted to a concentration of  $1 \times 10^6$  spores.mL<sup>-1</sup>. Three isolates of both pathogens regarded as sensitive and resistant to propiconazole (Mamba, 2019) were tested. For *Galactomyces* spp., isolates GAL313, GAL314 and GAL085 that was previously classified as resistant and GAL103, GAL034 and GAL025 that was previously classified as sensitive, was used. For *Penicillium* spp. isolates, the fruit were inoculated at two points of infection and allowed to dry. After 14h the fruit was dipped in propiconazole for one minute at the registered concentration (600ppm) as recommended for dip or drench treatments. Each box was covered with a transparent polyethylene bag and sealed and incubated in the dark at 28°C.

Baseline sensitivity testing. Pyrimethanil and fludioxonil amended PDA media in 90 mm petri plates were used in these tests. Thirty unexposed isolates of *P. digitatum* were grown on PDA media and incubated for 2 weeks. A 5 mm plug was cut from the edge of pathogen and plated onto growth media amended with fungicide with three replications. Growth media was amended with technical grade pyrimethanil at eight concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.8, 2 and 4 ppm) whereas fludioxonil was tested at 0, 0.003125, 0.00625, 0.0125, 0.025, 0.05, 0.1 and 1 ppm. After 5 days the growth of the pathogen was observed and measured using a digital caliper. Percentage inhibition was subsequently calculated.

## Results and discussion

For single fungicide treatments azoxystrobin had the best protective control with 98% of control across all fruit types. Percentage control was 83% for fludioxonil and 63% for pyrimethanil. Pyrimethanil did not perform well when compared to the registered concentration of imazalil (94%) as well as azoxystrobin and fludioxonil (Figure 4.4.6.1a). Fludioxonil had the best sporulation inhibition of 83% over all fruits, whereas fludioxonil and pyrimethanil has 63% and 54%, respectively (Figure 4.4.6.1b).

For combination fungicide treatments azoxystrobin + fludioxonil performed the best with 98% protective control. Protective control for fludioxonil + pyrimethanil was 92% and 87% for pyrimethanil + azoxystrobin (Figure 4.4.6.2a). For sporulation inhibition, azoxystrobin + fludioxonil delivered the best results with 93% sporulation inhibition. Sporulation inhibition for fludioxonil + pyrimethanil was 72% and 60% for pyrimethanil + azoxystrobin (Figure 4.4.6.2b).

With the use of essential oils, thyme had the best protective control with 18% of control at 2x the suggested concentration. Percentage control was 13% and 12% at the 1x and 1/2x concentrations respectively, across all fruit types. Percentage protective control for cinnamon was 7%, 8% and 9% for 1/2x, 1x and 2x the suggested concentrations, respectively. For spearmint essential oil, protective control was 13%, 5% and 12% at the 1/2x, 1x and 2x the suggested concentrations, respectively. Fruit treated with wax only had the highest level of disease with only 2% protective control, while imazalil had the lowest with 96%. Percentage control was calculated using an untreated fruit as a reference (Figure 4.4.6.3).

Thyme showed the best sporulation inhibition potential of 28% at a 2x concentration. Inhibition was 19% and 11% at a 1x and 1/2x concentration of thyme, respectively. Spearmint showed sporulation inhibition potential of 11%, 13% and 19% at the 1/2x, 1x and 2x concentration, respectively. Cinnamon showed the weakest potential to inhibit spores with 8%, 13% and 21% inhibition at the 1/2x, 1x and 2x the recommended concentration. All averages were calculated over all three fruit types i.e. lemons, oranges and mandarins (Figure 4.4.6.4).

For additional products tested, protective control of Evolve was very good. Evolve did not differ significantly from IMZ treatments on all fruit types. A pooled average of 98.5% protective control over all fruit types was obtained. OPP did not deliver good results as a pooled average over the three fruit types was 31%. For sporulation inhibition, Evolve proved to have good anti-sporulant ability. Evolve inhibited sporulation up to 88%. OPP, however produced, poor results as sporulation inhibition was 16%. Once again, a wax treatment was added to emphasize that protective control and anti-sporulant ability comes from actives used and not wax itself.

For measuring practical resistance of *Galactomyces* spp., three isolates had disease incidence of above 50% when tested against the registered concentration of PPZ of 600ppm. Isolates GAL034 had an infection incidence of 59% while isolates GAL085 and GAL314 both had an incidence 61%. Isolates GAL025 and GAL103 had an infection incidence of 13% for both isolates respectively. Isolate GAL313 had an infection incidence of 15%. The incidence of infection shown here represents the average of both trials for individual isolates (Figure 4.4.6.5).

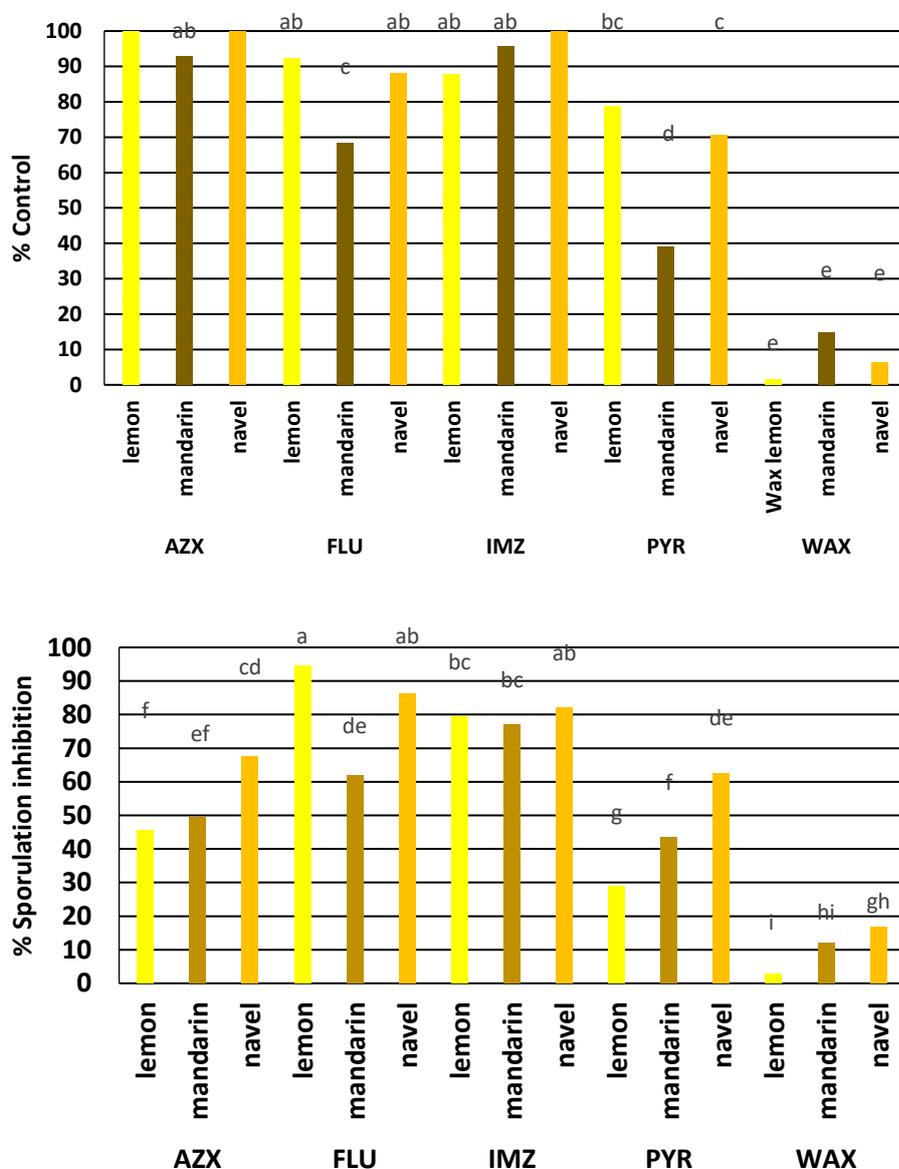


Figure 4.4.6.1 a) Protective control results for use of single fungicides in a wax formulation and b) single use fungicide results for sporulation inhibition.

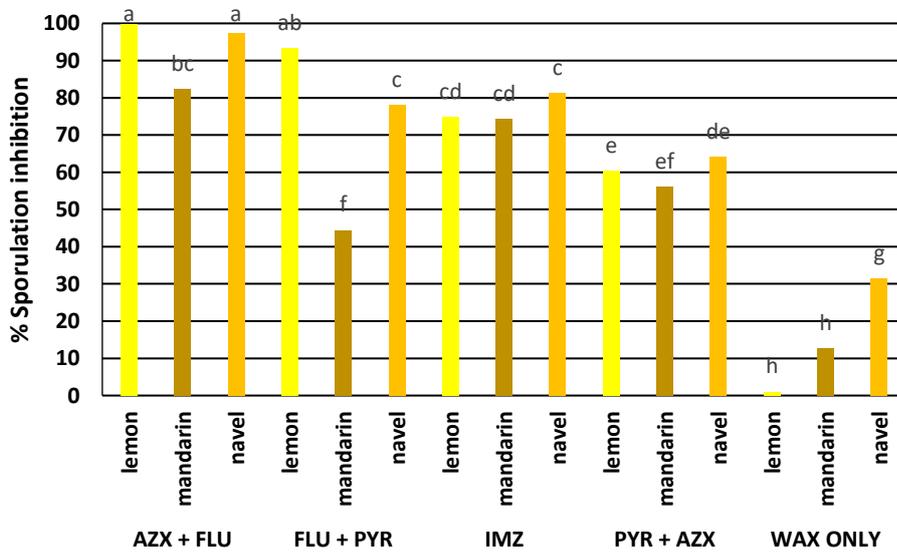
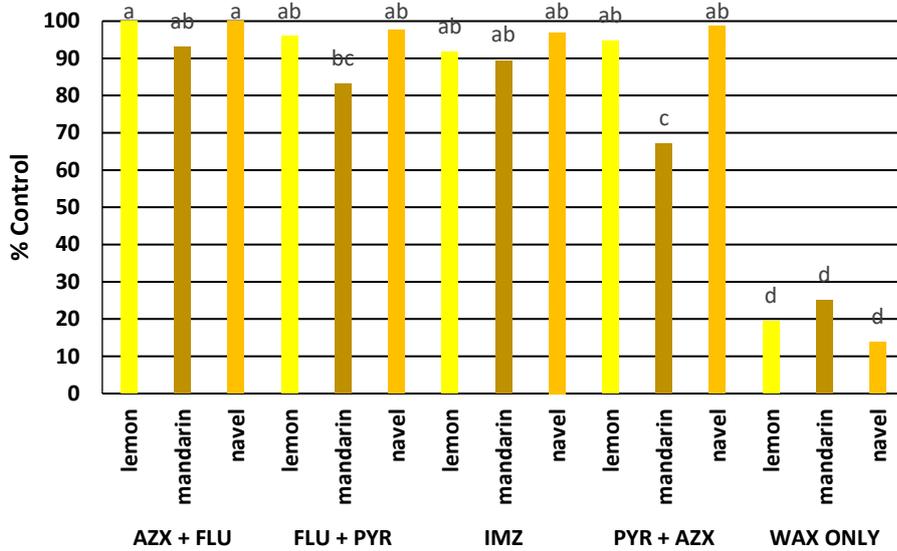
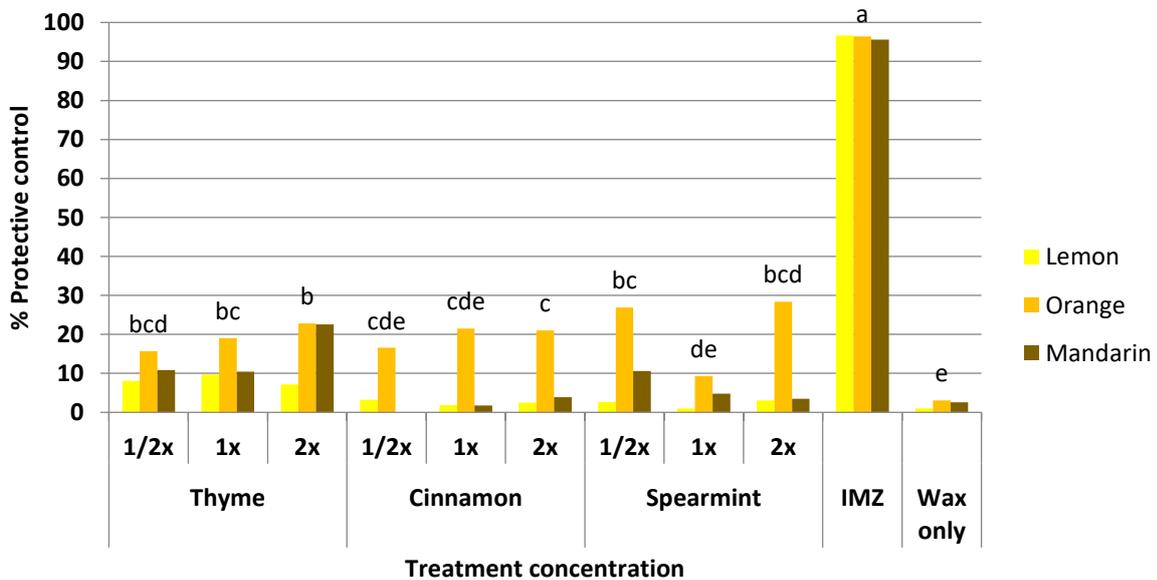
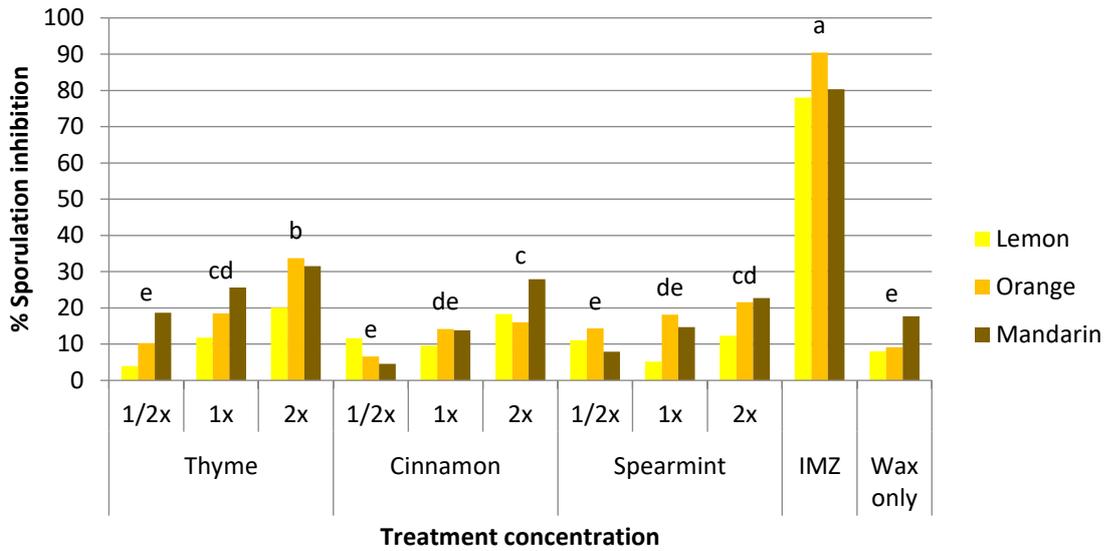


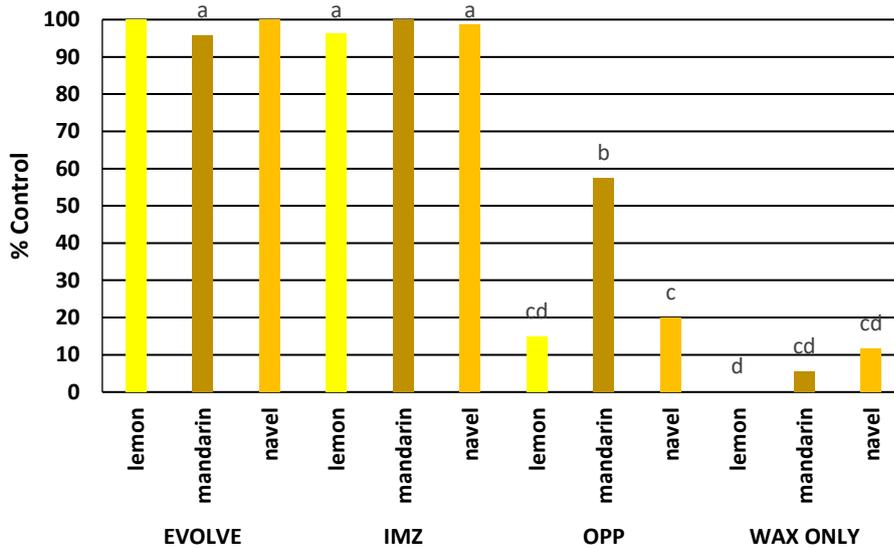
Figure 4.4.6.2 a) Protective control results for use of fungicides in combination in a wax formulation and b) fungicide combination results for sporulation inhibition

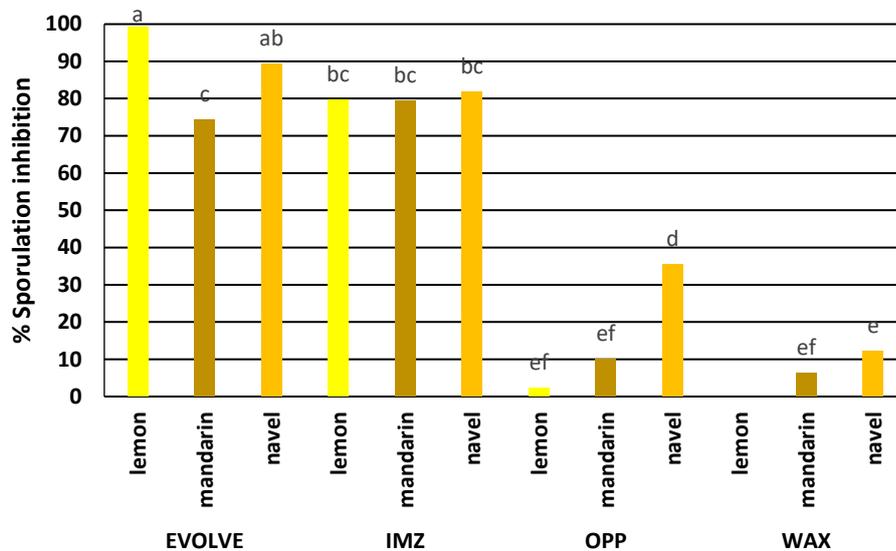


**Figure 4.4.6.3.** Protective control of three essential oils thyme, spearmint and cinnamon, applied in wax in comparison to imazalil applied in wax as well as a wax only treatment

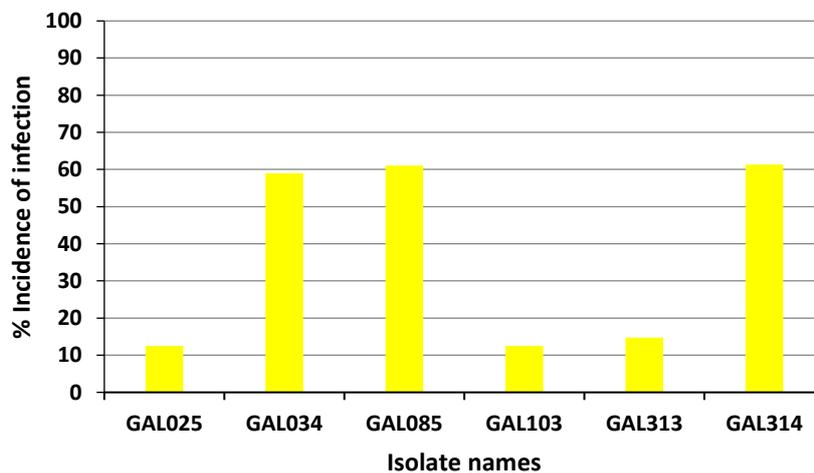


**Figure 4.4.6.5.** Percentage sporulation inhibition of three essential oils thyme (TYM), spearmint (SPM) and cinnamon (CNM), applied in wax in comparison to imazalil applied in wax





**Figure 4.4.6.5** a) Protective control results for use of OPP and Evolve in a wax formulation and b) OPP and Evolve results for sporulation inhibition



**Figure 4.4.6.6** a) Average percentage infection of all isolates of *Galactomyces* spp. tested for practical resistance at the registered concentration of propiconazole (600 ppm)

## Conclusion

Three potential alternative fungicides to be used as replacements for imazalil in wax application were tested to determine their protective control action as well as sporulation inhibition potential. Azoxystrobin performed best with a high protective control action at registered concentration on citrus, while sporulation was largely inhibited at the registered concentration of this fungicide. In 2009, Zhang *et al* showed that *P. digitatum* populations that were never exposed to azoxystrobin pre- or postharvest, were highly sensitive to AZX which suggested that there was a lack of resistant biotypes. Effective concentrations resulted in reduction of mycelial growth and conidial germination (Zhang *et al.*, 2009). For combination fungicide treatments azoxystrobin + fludioxonil performed the best for both protective control as well as sporulation inhibition. Overall, pyrimethanil gave the lowest protective control and sporulation inhibition. Studied fungicides showed to have great potential as alternatives to imazalil in wax formulation, with even greater potential when fungicides were used in combination with another.

The results of this study show the potential of three essential oils in controlling citrus green mould caused by *Penicillium digitatum*. Overall, thyme oil showed the best protective control and the most active inhibitor of spores, however, the use of essential oils still do not show promising results at the suggested concentrations,

especially when compared to the use alternatives fungicides to control the same disease on citrus fruit such as fludioxonil, pyrimethanil and azoxystrobin. Thyme oil's most abundant compound is thymol (63.1%), which has previously shown to be active against *P. digitatum* (Combrinck *et al.*, 2011). Higher concentrations of essential oils could lead to phytotoxic burns on the fruit. High concentrations of essential oils also caused the fruit to smell strongly of the oil in use. Using these essential oils in combination with one another may have potential to control a wider range of pathogens occurring on citrus fruit.

Additional products tested showed Evolve to have good protective control and sporulation inhibition ability, which corresponds with results from combination fungicides of AZX + FLU. Ortosol performed poorly and gave very little protective control and sporulation ability.

Testing for practical resistance, GAL085 was the most resistant isolate towards the registered concentration of propiconazole. All isolates classified as resistant in previous study (Mamba, 2019) was resistant in the *in vivo* trials. However, GAL313 was less resistant in the fruit trials whereas GAL314, which was also classified as a resistant isolate, showed to be more resistant towards PPZ in the fruit trials. Confirming the level of sensitivity of these isolates aids in monitoring these pathogens over time, and observing any changes is important for disease resistance management and fungicide longevity.

Future research may also focus on combining the use of essential oils with less effective fungicides used to control *P. digitatum*. This may potentially increase the protective ability of the fungicide and reduce the prolonged use of effective fungicides, therefore minimizing resistance build-up of pathogens to these effective fungicides and maximizing its shelf-life.

### Current research

- A. *Determine baseline sensitivity of P. digitatum to azoxystrobin.*

### Future research

- A. *The use of essential oils in combination with fungicides as potential control for green mould caused by Penicillium digitatum*

### Technology transfer

Some information and data were presented at CRI workshops in 2021.

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#### 4.5 **PROGRAMME: HLB**

Programme coordinator: H Maree (CRI)

##### 4.5.1 **Programme summary**

The continuous and imminent threat of HLB to our citrus industry warrants all our current efforts to ensure preparedness. The potential impact of the disease makes the management of incursions and the management of the disease a priority. The HLB Research Programme (HLB-RP) was therefore established to increase our current research efforts and to facilitate interdisciplinary research to cover all the different facets of this complex disease.

HLB and Greening disease research was previously mostly part of the Graft Transmissible Disease research programme, within the Disease Management research portfolio. There is also active HLB and Greening related research in the IPM and Citriculture portfolios. The HLB-RP will coordinate research activities across portfolios, which implies that there will be some duplication in the reporting as insect focussed projects on HLB will remain in IPM and plant focussed projects will remain in Citriculture. Interdisciplinary projects that are difficult to sort will fall in the HLB-RP. It is envisioned that there will most likely develop paired or interdependent projects that will remain in their respective portfolios; this will bring unique challenges in terms of coordination and reporting. The HLB-RP will be coordinating all research related to HLB and Greening, as well as coordinate the interface of the programme with the Extension and Biosecurity divisions. The objective is to provide a focal point that enables planning and coordination of the research to best address the industry needs for the Greening and HLB challenges.

## Progress made in HLB/Greening related research 2020/21:

### Diagnostics

The development of cost effective and time efficient assays of the detection of *Liberibacter* species and for the identification of insect species remains a high priority. A new detection assay based on LAMP technology (1265) is close to completion and will be available soon for the in-field detection of HLB (CLas and CLaf). This is a very useful tool to enable surveys in remote regions and other countries without specialized expensive equipment. As part of continuous insect monitoring efforts, a need has arisen to distinguish *Diaphorina citri* and *Trioza erytreae* from other psyllids likely to be caught on yellow traps (1255). Traps are continuously monitored, and insects collected for morphological evaluation. Identified morphological categories will be further characterised by high-throughput sequencing to build a genetic resource that will be used to develop molecular assays for identification. The world authority on psyllid taxonomy Dr Daniel Burckhardt will visit SA in August and September 2021.

### Vector control

Control of the vector is key to the disease management which includes effective monitoring of insect population as well as effective control, chemical or otherwise. The pressure to reduce chemical actives compounds require urgent attention to find alternatives. Most of these trials are conducted in partnership with countries where *Diaphorina citri* is already indigenous. To accelerate outputs and mitigate logistical restrictions associated with partner countries additional partners will be approached. The evaluation of new systemic insecticides for the control of psyllids (1148) is ongoing. Like most projects it was significantly impacted by Covid lockdowns in Kenya. The colony is being bulked up and will be ready for spray trials in 2021. Towards the management of psyllid populations project 1160 developed CTV infectious clones that contain RNAi constructs targeted towards *Trioza erytreae*. Proof-of-concept evaluations will be conducted in project 1315. Project 1315 started in 2021 that will evaluate citrus psyllids monitoring and control techniques in a *Trioza erytreae* colony maintained at University Pretoria (UP). Project 1315 will most likely be expanded to include control measures developed for *Diaphorina citri* internationally, to ensure that these measures are also effective to control *Trioza erytreae*.

### Disease characterisation

To develop an effective disease management strategy a fundamental understanding of the disease is required. In South Africa we have a unique opportunity to study Greening using all the information generated internationally on HLB to increase our preparedness. Two new projects will start in 2021 to determine the genome diversity of citrus infecting *Liberibacter africanus* species and subspecies (1322) and evaluate the influence of CTV infection on CLaf titre (1346).

### Treatment

Treatments developed internationally to modulate disease expression or even “cure” trees from infection will be evaluated for their efficacy using Greening as a model system. A new project will be created to trial various treatments and management practices.

### Optimal plant material

Ultimately we need to prepare for the establishment of HLB in South Africa that require us to start evaluating all the material developed internationally as soon as possible. Rootstocks are critical to tree production. In project 1264, seed production of currently used rootstocks were studied providing cultivar-specific baseline information (i.e. the amount of trees needed per cultivar) that will be required by a seed farms to ensure that we prepare adequately for the potential high demand on seed. In project 1246, potential HLB resistant/tolerant rootstocks developed at UF/CREC will be evaluated seed was imported and phytosanitary screening is close to completion. First orchards will be established summer 2022/23.

## **Programopsomming**

Die voortdurende bedreiging van HLB vir ons sitrusbedryf regverdig al ons huidige aandag om paraatheid te verseker. Die potensiële impak van die siekte maak die bestuur van indringings en die bestuur van die siekte 'n prioriteit. Die HLB Navorsingsprogram (HLB-NP) is dus opgestel om ons huidige navorsingspogings te

versterk en interdisiplinêre navorsing te vergemaklik om al die verskillende fasette van hierdie komplekse siekte te dek.

Navorsing oor HLB en Vergroening-siekte was voorheen meestal deel van die *Graft Transmissible Disease*-navorsingsprogram, binne die *Disease Management* navorsingsportefeuje. Daar is ook aktiewe navorsing oor HLB en Vergroening in die IPM- en Citriculture-portefeuje. Die HLB-NP sal navorsingsaktiwiteit oor die portefeuljes koördineer, wat impliseer dat daar 'n mate van duplisering in die verslaggewing sal wees, aangesien insekgefokusde projekte in IPM sal bly en plantgerigte projekte in Citriculture. Interdisiplinêre projekte wat moeilik is om te sorteer, val in die HLB-RP. Dit word voorsien dat daar waarskynlik gepaarde of interafhanklike projekte sal ontwikkel wat in hul onderskeie portefeuljes sal bly, wat unieke uitdagings in terme van koördinerings en verslagdoening sal meebring. Die HLB-NP sal alle navorsing wat verband hou met HLB en Vergroening koördineer, en die koppelvlak van die program met Voorliging en Biosekuriteit koördineer. Die doel is om 'n fokuspunt te bied wat die beplanning en koördinerings van die navorsing moontlik maak om die bedryfsbehoefte vir die Vergroening- en HLB-uitdagings die beste te bevredig.

#### Vordering in HLB / Vergroening verwante navorsing 2020/21:

##### Diagnostiek

Die ontwikkeling van koste-effektiewe en tyddoeltreffende toetse vir die opsporing van *Liberibacter* spesies en vir die identifisering van insekspesies bly 'n hoë prioriteit. 'n Nuwe opsporing toets gebaseer op LAMP tegnologie (1265) is naby aan voltooiing en sal binnekort beskikbaar wees vir die in-veld opsporing van HLB (CLas en CLaf). Dit is 'n baie nuttige hulpmiddel om opnames in afgeleë streke en ander lande te doen sonder gespesialiseerde, duur toerusting. As deel van deurlopende insekmoniteringspogings het 'n behoefte ontstaan om *Diaphorina citri* en *Trioza erytrae* van ander psylloids te onderskei wat waarskynlik op geel lokvalle (Sticky traps) (1255) vasgevang sal word. Lokvalle word voortdurend gemonitor, en insekte word versamel vir morfologiese evaluering. Geïdentifiseerde morfologiese kategorieë sal verder gekarakteriseer word net hoëdeurset volgorde bepaling om 'n genetiese hulpbron te bou wat gebruik sal word om molekule toetse vir identifikasie te ontwikkel. Die wêreldkenner oor psylloid taksonomie Dr Daniel Burckhardt sal SA in Augustus en September 2021 besoek.

##### Vektorbeheer

Beheer van die vektor is die sleutel tot die siektebestuur wat effektiewe monitering van insekpopulasie asook effektiewe beheer, chemies of andersins insluit. Die druk om chemiese aktiewe verbindings te verminder vereis dringende aandag om alternatiewe te vind. Die meeste van hierdie proewe word uitgevoer in vennootskap met lande waar *Diaphorina citri* reeds inheems is. Om uitsette te versnel en logistieke beperkings wat met vennootlande geassosieer word, te verminder, sal bykomende vennote genader word. Die evaluering van nuwe sistemiese insekdoders vir die beheer van psylloids (1148) is voortdurend. Soos die meeste projekte is dit aansienlik beïnvloed deur Covid-inperkings in Kenia. Die kolonie word op gebou en sal in 2021 gereed wees vir spuitproewe. Vir die bestuur van psyllid bevolkings het projek 1160, CTV klonne, wat RNAi konstrue bevat, gebou wat gerig is op *Trioza erytrae*. Die konsep evaluering sal gedoen word in projek 1315. 'n nuwe projek 1315 begin in 2021 wat sitrus psyllids monitering en beheer tegnieke sal evalueer in 'n *Trioza erytrae* kolonie by Universiteit Pretoria (UP). Projek 1315 sal moontlik uitgebrei word om beheermaatreëls wat vir *Diaphorina citri* internasionaal ontwikkel is, in te sluit om te verseker dat hierdie maatreëls ook effektief is om *Trioza erytrae* te beheer.

##### Siekte karakterisering

Om 'n effektiewe siektebestuurstrategie te ontwikkel, word 'n fundamentele verstaan van die siekte vereis. In Suid-Afrika het ons 'n unieke geleentheid om Vergroening te bestudeer deur al die inligting wat internasionaal op HLB gegenereer word, te gebruik om ons gereedheid te verhoog. Twee nuwe projekte sal in 2021 begin om die genoomdiversiteit van sitrus infekteerende *Liberibacter africanus* spesies en subspecies te bepaal (1322) en die invloed van CTV-infeksie op CLaf titer te evalueer (1346).

##### Behandeling

Behandelings wat internasionaal ontwikkel is om siekteuitdrukking te moduleer óf selfs bome te genees van infeksie, sal geëvalueer word vir hul doeltreffendheid met behulp van Vergroening as 'n model. 'n Nuwe projek sal geskep word om verskeie behandelings en bestuurspraktyke te vergelyk.

#### Optimale plantmateriaal

Uiteindelik moet ons voorberei vir die vestiging van HLB in Suid-Afrika wat vereis dat ons al die materiaal wat internasionaal ontwikkel is, moet begin evalueer, so spoedig moontlik. Onderstamme is van kritieke belang vir boomproduksie. In projek 1264 is saadproduksie van tans gebruikte onderstamme bestudeer wat kultivarspesifieke basislyninligting verskaf (dit wil sê die hoeveelheid bome wat per kultivar benodig word) wat deur 'n saadplase vereis sal word om te verseker dat ons voldoende voorberei vir die potensiële hoë aanvraag op saad. In projek 1246, potensiële HLB bestand / verdraagsame onderstamme ontwikkel by UF / CREC sal geëvalueer word. Saad is ingevoer en fitosanitêre sifting is naby aan voltooiing. Eerste boorde sal in 2022/23 gevestig word.

#### 4.5.2 PROGRESS REPORT: New systemic insecticides for citrus

Project 1148 (RCE-2-25) (2016/7-2021/2) by T G Grout, P R Stephen (CRI), S M Faris and P Nderitu (*icipe*)

##### Summary

In order to prepare for the arrival of *Diaphorina citri* in South Africa, we need to find more systemic insecticides that can be used frequently in nurseries and for non-bearing trees. Early research evaluated several treatments against the brown citrus aphid *Toxoptera citricidus* and gave some promising results. Research is now aimed at evaluating several systemic products against *D. citri* from a culture on potted citrus plants in an insect-proof structure in SE Kenya in collaboration with *icipe*. However, numbers of *D. citri* on citrus in the culture declined, despite the addition of *Murraya koenigii* plants to the culture. It has therefore not yet been possible to conduct the first screening trial of several systemic insecticides against *D. citri* on potted citrus plants.

##### Opsomming

Ten einde vir die aankoms van *Diaphorina citri* in Suid-Afrika voor te berei, moet meer sistemiese insekdoders gevind word wat gereeld in kwekerie en vir nie-draende bome gebruik kan word. Vroeë navorsing het verskeie behandelings teen die bruin sitrus plantluis, *Toxoptera citricidus*, geëvalueer, en belowende resultate is verkry. Navorsing is nou daarop gemik om verskeie sistemiese produkte teen *D. citri* te evalueer, vanaf 'n kultuur op gepotte sitrusplante in 'n insek-bestande struktuur in SO Kenia, in samewerking met *icipe*. Getalle van *D. citri* op sitrus in die kultuur het egter afgeneem, ten spyte van die byvoeg van *Murraya koenigii* plante by die kultuur. Dit was dus nog nie moontlik om die eerste siftingsproef van verskeie sistemiese insekdoders teen *D. citri* op gepotte sitrusplante uit te voer nie.

#### 4.5.3 PROGRESS REPORT: Application of CTV infectious clones to combat HLB

Project 1160 (2016/17 – 2021/2) by R Bester (CRI), D Aldrich (SU), G Cook (CRI), JHJ Breytenbach (CRI), JT Burger (SU), WO Dawson (University of Florida, USA), HJ Maree (CRI)

##### Summary

The confirmed presence of both 'Candidatus' *Liberibacter asiaticus* (CLas), and *Diaphorina citri* in East Africa, requires a proactive approach from the South African citrus industry to prepare for the eventual incursion of HLB. The aim of this project is to establish a suite of citrus tristeza virus (CTV) infectious clones with a range of silencing targets (payloads) that would form part of a management strategy to contain HLB and limit its impact. CTV infectious clones of genotype T36 were imported from collaborator Prof W.O. Dawson (University of Florida) at the start of the project and protocols optimized to successfully infect citrus using the T36 clone. The T36 clone was converted into the local RB (asymptomatic) genotype. However, no systemic infection in *Nicotiana benthamiana* could be observed. Plasmid DNA was sent for low coverage high-throughput sequencing and three mutations in critical open reading frames were identified that can influence virus infectivity

and spread. These mutations will be repaired by replacing fragments of the RB genotype. A dual reporter infectious clone based on the T36 clone that expresses GFP and also contains a PDS silencing cassette was also constructed and evaluated in citrus. The clone proved to be an effective expression and silencing vector. This clone will be used as a test platform to screen different 'payloads' while the RB clone is still under construction. Four RNAi gene targets in *Diaphorina citri* have been identified from literature and homologues for *Trioza erytreae* have been identified using the HTS data generated from *Trioza erytreae* RNA. CTV vectors are being constructed to contain payloads directed towards both *Trioza erytreae* and *Diaphorina citri* for each gene target. CTV vectors targeting all 4 genes per insect will also be constructed.

Additionally, this project also includes the identification of CTV-induced stem pitting determinants. All CTV infectious clone mutants for the stem pitting trials of this study have been successfully assembled and proved to be replication competent in *N. benthamiana*. These clones have also been sent for low-coverage high-throughput sequencing to validate plasmid sequences. Recombinant virions were then purified from systemically infected *N. benthamiana* plants by ultracentrifugation. All available Duncan and Mexican lime test plants for the stem pitting trials have been bark-patch infected with the relevant CTV clones and wild-type CTV sources. These plants will be pruned back as soon as they have been confirmed to be infected with the correct CTV virus/clone. Stem pitting characterisations will be carried out 4 to 6 months after prune-back.

## Opsomming

Die bevestigde teenwoordigheid van beide 'Candidatus' Liberibacter asiaticus (CLAs), en *Diaphorina citri* in Oos-Afrika, vereis 'n proaktiewe benadering van die Suid-Afrikaanse sitrusbedryf om voor te berei op die uiteindelijke indringing van HLB. Die doel van hierdie projek is om 'n panel van CTV infektiewe te vestig met 'n verskeidenheid 'silencing' teikens (payloads) wat sal deel uitmaak van 'n beheerstrategie om die impak van HLB te beperk. Infektiewe klone van die T36 genotipe is ingevoer vanaf medewerker, Prof W.O. Dawson (University of Florida) aan die begin van die projek en protokolle is geoptimaliseer om sitrus suksesvol met die T36-kloon te besmet. Die T36-kloon is omgeskakel in die plaaslike RB (asimptomatiese) genotipe. Geen sistemiese infeksie in *N. benthamiana* kon egter waargeneem word nie. Plasmied-DNA is gestuur vir lae-dekking hoë-deurset-volgordebepaling en drie mutasies in kritiese leesrame is geïdentifiseer wat die infektiwiteit en verspreiding van virusse kan beïnvloed. Hierdie mutasies sal herstel word deur fragmente van die RB-genotipe te vervang. Daar is ook 'n dubbel-rapporteur infektiewe kloon gebou, gebaseer op die T36-kloon (Dawson), wat beide GFP uitdruk asook 'n PDS 'silencing cassette' bevat. Hierdie kloon is reeds geëvalueer in sitrus en het bewys dat die kloon 'n doeltreffende uitdrukking en 'silencing' vektor is. Hierdie kloon sal gebruik word as 'n platform om verskillende 'payloads' te toets terwyl die RB-kloon gefinaliseer word. Vier RNAi-teiken gene in *Diaphorina citri* is uit literatuur geïdentifiseer en homoloë vir *Trioza erytreae* is geïdentifiseer met behulp van die HTS-data wat gegenereer is uit *Trioza erytreae* RNA. CTV-vektore met 'payloads' wat na beide *Trioza erytreae* en *Diaphorina citri* gerig is, word tans gemaak. CTV-vektore wat op al 4 die gene per insek gerig is, sal ook gemaak word.

Verder sluit hierdie projekdoelwitte die bepaling van CTV-geïnduseerde stamgleuf determinante in. Al die CTV-kloonmutante vir die stamgleuf proewe is suksesvol saamgestel en bewys dat dit in *Nicotiana benthamiana* repliseer. Hierdie klone is ook gestuur vir lae-dekking hoë-deurset-volgordebepaling. Rekombinante virions is dan gesuiwer van *N. benthamiana* plante deur ultrasentrifugasie. Die Duncan and Mexican lime proefplante vir die stamgleufproewe is besmet met die relevante CTV-klone en wilde-tipe CTV-bronne. Hierdie plante sal teruggesnoei word sodra daar bevestig is dat hulle met die korrekte CTV-virus/kloon besmet is. Stamgleufkarakterisering sal 4 tot 6 maande na die terugsnoei uitgevoer word.

### 4.5.4 PROGRESS REPORT: Distinguishing *Diaphorina* spp. and *Trioza* spp. from other psylloids likely to be caught on yellow traps

Project 1255 (2021/2) by E Mauda, A Manrakhan, X Sibiya, H Maree, R Bester (CRI), D Burckhardt (Naturhistorisches Museum, Switzerland) & T Grout (CRI)

## Summary

Psyllids are generally host specific leaf phloem feeders and some species transmit bacteria that cause citrus greening (Burckhardt *et al.*, 2020). Recent molecular studies had incorporated findings that resulted in *Diaphorina* being moved from Liviidae to Psyllidae (Burckhardt *et al.*, 2021). Furthermore, this study shows the importance of incorporating morphological identification with molecular studies. As *Diaphorina citri* Kuwayama (Psylloidea: Psyllidae) moves closer to South Africa, citrus growers and nurserymen are starting to use yellow sticky traps to monitor this pest when it arrives. However, identification of *Diaphorina* species in South Africa and other parts of the world is difficult. Two species (*Diaphorina punctulata* and *Diaphorina zebrana*) have been found to feed on citrus as adults, but are not considered vectors of African greening (Catling and Atkinson 1974). Taxonomy of psyllids in the Afrotropical region has been largely ignored. There are currently no morphological keys for identification of these species. *Diaphorina citri* is an important pest of citrus in Asia and America due to its vectoring Asian greening disease (*Candidatus Liberibacter asiaticus*), also known as Huanglongbing or HLB, that can kill citrus trees. The species is currently present in East Africa but has not yet been reported in southern Africa. *Trioza erytrae* Del Guercio (Psylloidea: Triozidae) is a pest of citrus and a known vector of the greening disease. Triozids are difficult to identify, with *T. erytrae* being part of a species complex. The project aims to determine the diversity of *Diaphorina* and *Trioza* species in citrus environments in southern Africa and develop a morphological key for psyllids found on Rutaceae and other indigenous plants near citrus. Sticky yellow traps were set up in citrus orchards and adjacent natural vegetation in the north and east of South Africa to collect psyllids. At least two indigenous *Trioza* species (*Trioza erytrae* and *Trioza afrobsoleta*) and five indigenous *Diaphorina* species (*D. zebrana*, *D. virgata*, *D. punctulata*, *D. clutiae*, and *D. petteyi*) have been morphologically identified from traps and are awaiting confirmation by the taxonomist. Current molecular results revealed that most of the *Diaphorina* species caught in traps matched closely to *Diaphorina lycii* when genetically matched. Morphological specimens of *Diaphorina* and *Trioza* have been pinned for morphological comparison. To date morphological specimens have been collected and photographed for a reference collection and a field survey is planned for active collection and identification of indigenous flora that host *Diaphorina* and *Trioza* species close to citrus orchards.

## Opsomming

Bladvlooi is gewoonlik gasheer-spesifieke blaarfloeëm voeders en sommige spesies dra bakterieë oor wat sitrusvergroening veroorsaak (Burckhardt *et al.*, 2020). Onlangse molekulêre studies het bevindings ingesluit wat daartoe gelei het dat *Diaphorina* vanaf Liviidae na Psyllidae geskuif is (Burckhardt *et al.*, 2021). Hierdie studie dui verder op die belang daarvan om morfologiese identifikasie met molekulêre studies te kombineer. Soos wat *Diaphorina citri* Kuwayama (Psylloidea: Psyllidae) nader aan Suid-Afrika beweeg, begin sitrusprodusente en kwekerybestuurders geel kleeflokvalle gebruik om die plaag te monitor wanneer dit arriveer. Identifikasie van *Diaphorina* spesies in Suid-Afrika en ander dele van die wêreld is egter moeilik. Daar is gevind dat twee spesies (*Diaphorina punctulata* en *Diaphorina zebrana*) as volwassenes op sitrus voed, maar word egter nie as vektore van Afrika Vergroening (Catling & Atkinson 1974) gesien nie. Taksonomie van die bladvlooi familie in die Afro-tropiese streek is sover grootliks geïgnoreer. Daar is tans geen morfologiese sleutels vir identifikasie van hierdie spesies nie. *Diaphorina citri* is 'n belangrike plaag van sitrus in Asië en Amerika omdat dit Asiatiese Vergroeningsiekte oordra (*Candidatus Liberibacter asiaticus*), ook bekend as Huanglongbing of HLB, wat sitrusbome doodmaak. Die spesie is tans teenwoordig in Oos-Afrika maar is nog nie in suidelike Afrika aangeteken nie. *Trioza erytrae* Del Guercio (Psylloidea: Triozidae) is 'n plaag van sitrus en 'n bekende vektor van die vergroeningsiekte. Triozidae is moeilik om te identifiseer aangesien *T. erytrae* deel van 'n spesie-kompleks is. Die projek het ten doel om die diversiteit van *Diaphorina* en *Trioza* spesies in sitrus-omgewings in suidelike Afrika vas te stel, en om 'n morfologiese sleutel vir bladvlooi, wat op Rutaceae en ander inheemse plante naby sitrus voorkom, te ontwikkel. Klewerige geel lokvalle is in sitrusboorde en aangrensende natuurlike vegetasie in die noordelike en oostelike dele van Suid-Afrika opgestel om bladvlooi te versamel. Ten minste twee inheemse *Trioza* spesies (*Trioza erytrae* en *Trioza afrobsoleta*) en vyf inheemse *Diaphorina* spesies (*D. zebrana*, *D. virgata*, *D. punctulata*, *D. clutiae*, en *D. petteyi*) is morfologies vanaf lokvalle geïdentifiseer, en daar word gewag op bevestiging vanaf die taksonoom. Huidige molekulêre resultate het aangedui dat meeste van die *Diaphorina* spesies wat in lokvalle gevang is, met *Diaphorina lycii* ooreenstem wanneer geneties gepas word. Morfologiese monsters van *Diaphorina* en *Trioza* is vasgesteek vir morfologiese vergelyking. Tot op hede is morfologiese monsters vir 'n verwysingsversameling versamel en gefotografeer, en 'n veld-opname is beplan vir aktiewe versameling en identifikasie van inheemse flora wat gashere vir *Diaphorina* en *Trioza* spesies naby aan sitrusboorde is.

#### 4.5.5 **PROGRESS REPORT: Evaluation of new University of Florida (UF) rootstocks**

Project 1246 (2019/20 – 2022/3) by PJR Cronje and J Joubert (CRI)

##### **Summary**

The UF/CREC rootstock programme has been breeding rootstocks for 30 years to address various problems in this production area. In the recent past and due to the massive natural screening as the HLB epidemic spread in Florida, some potential tolerant/resistant rootstocks, with commercial potential, were identified. During 2019, negotiations with the University of Florida were successfully concluded and an MOU signed with CRI. Due to the problematic nature of 2020 no seeds were supplied from UF to CRI. However, early 2021 arrangements resulted in 20 selections of UF seed successfully arriving at CRI-Stellenbosch and taken to DALRRD/Plant Health for pathogen screening. This step is close to being finalised and seed will be released in May to CRI for propagation. Based on the germination percentage and uniformity of the seedlings, a propagation plan will be drawn up to maximise the number of trees made with these rootstocks. It is planned to establish the first orchard in the Nelspruit area in the summer of 2022/23. As plant material becomes available additional evaluation sites will be established in key climatic areas. The initial screening will be based on horticultural performance *viz.* canopy development, flowering, fruit set, size, colour and yield thereafter, Laf susceptibility will also be included.

##### **Opsomming**

Die UF/CREC teel en ontwikkel al vir die laaste 30 jaar onderstamme om verskillende probleme in hierdie area aan te spreek. As gevolg van die massiewe natuurlike sifting wat plaasgevind het soos die HLB-epidemie in Florida versprei het, is enkele potensiële verdraagsame/weerstandige onderstokke geïdentifiseer. Gedurende 2019 is onderhandelinge met die Universiteit van Florida suksesvol afgehandel en 'n MOU met CRI geteken. Gedurende die problematiese 2020 kon geen saad gelewer word nie maar na nuwe beplanning het die besending van saad, wat 20 seleksies insluit, by CRI Stellenbosch opgedaag. Die saad is daarna na DALRRD/PlantHealth geneem vir patogene evaluasies en sal vrygestel word in Mei aan CRI vir verder stappe. Gebaseer op die kiem% en die uniformiteit van die saailinge sal 'n plantvermeerderings plan opgestel word om te verseker dat die maksimum hoeveelheid boompies gemaak kan word met hierdie onderstokke. Die eerste evaluasies boord word beplan om geplant te word in die somer van 2022/23 in Nelspruit area, waarna verder evaluasies blokke in kommersiële aanplanting gevestig gaan word in belangrike produksie areas. Die aanvanklike evaluasies sal fokus op produksies aspekte nl. Blaardak ontwikkeling, blom, vrugset, grotte, kleur asook opbrengs waarna Laf sensitiviteit ook ingesluit sal word.

#### 4.5.6 **PROGRESS REPORT: Studies to improving seed production of rootstock trees**

Project 1264 (RCE 4-2-28) (Apr 2020 – Mar 2021) by Johané Niemann and Paul Cronje (CRI)

##### **Summary**

Citrus rootstocks are a determining factor for the success of commercial citrus plantings. Propagation of rootstocks occur mainly by means of seeds. As a result, high seed quantity and quality are important to ensure a continuous supply. Currently, no clear citriculture guidelines exist to ensure a consistent seed production of rootstock trees. The aim of this study was to obtain information on the phenology of the important rootstock cultivars used in South Africa (SA) to develop production guidelines. Trials were conducted at the Citrus Foundation Block (CFB) on 5-year-old Rough lemon, C-35, X639, Carrizo citrange, Swingle citrumelo, and MxT trees and 10-year-old US-812 trees. The average fruit diameter (mm) differed significantly between cultivars, SC (76), MxT (69), C-35 (63), CC (55) and X639 (46). The average seed number/fruit for SC (19 seeds/fruit) was higher than all cultivars except for CC (18 seeds/fruit), while US-812 had the lowest seed number (3 seeds/fruit). Large fruit produced more mature seeds than small fruit. The average yield/tree varied amongst cultivars. RL had the highest yield (121.61 kg) followed by X639 (81.48 kg), CC (57.30 kg), SC (53.81 kg), MxT (34.09 kg) and C-35 (22.97 kg). X639 produced an average of 2 516 fruit/tree, whereas all other cultivars had below 900 fruit/tree. CC had the highest millilitre viable seed (2 613 ml), followed by X639 (2 133 ml), RL (1 450 ml), and < 1 000 ml for SC, C-35 and MxT. Seed germination was better for fruit sampled at

colour-break compared to green fruit. MxT had the lowest germination percentage at harvest (<59%). Overall, the seedling germination in the greenhouse after 88 d was >83% for all cultivars except MxT (72.8%). GA<sub>3</sub> trials (10, 15, 20 ppm) did not influence the fruit set percentage compared to the untreated control, except for X639, which had a double percentage fruit set (16.83%) at 20 ppm compared to the control. Nitrogen levels were below optimum over both seasons for most of the rootstock cultivars, except for CC and C-35 in 2020. The potassium levels were lower than optimum in 2020 for most cultivars except for CC and US-812. In 2021, the K levels remained below optimum for RL, X639, and MxT. Overall, the tree health of all cultivars was good, with no obvious signs of yellowing. In conclusion, these results provide valuable cultivar-specific information to assist in future planning for rootstock farms, i.e. the amount of trees needed per cultivar to ensure the seed supply is met to assist in HLB management when the turnaround time for commercial citrus trees will be more rapid than it is currently. Therefore, yearly rootstock seed supplies need to be consistent, and cultivation practises on seed farms need to be adapted accordingly.

## Opsomming

Sitrusonderstamme is 'n bepalende faktor vir die sukses van kommersiële sitrusaanplantings. Voortplanting van onderstamme vind hoofsaaklik plaas deur sade en 'n hoë hoeveelheid en kwaliteit saad is dus belangrik om voortdurende voorraad te verseker. Tans bestaan geen duidelike bestuurs praktyk riglyne om sodoende 'n konsekwente saadproduksie van onderstambome te verseker nie. Die doel van hierdie studie was om inligting te verkry oor die fenologie van die belangrike onderstamkultivars wat tans in SA gebruik word om sodoende produksieriglyne te ontwikkel. Die proewe was by die Sitrus grondvesblok (SGB) uitgevoer op 5-jaarige Growweskil suurlemoen (GS), C-35, X639, Carrizo citrange, Swingle citrumelo en MxT bome en 10 jaar oue US-812 bome. Die gemiddelde vrugdeursnee (mm) het beduidend verskil tussen kultivars, SC (76 mm), MxT (69 mm), C-35 (63 mm), CC (55 mm) en X639 (46 mm). Die gemiddelde saadgetal/vrug vir SC (19 sade/vrugte) was hoër as alle kultivars, behalwe vir CC (18 sade/vrug). US-812 het die laagste saadgetal van 3 sade/vrug gehad. Groot vrugte produseer meer sade as klein vrugte. Die gemiddelde opbrengs per boom verskil tussen kultivars. GS het die hoogste opbrengs gehad (121.61 kg) gevolg deur X639 (81.48 kg), CC (57.30 kg), SC (53.81 kg), MxT (34.09 kg) en C-35 (22.97 kg). X639 het gemiddeld 2516 vrugte/boom opgelewer, terwyl die ander kultivars minder as 900 vrugte/boom gehad het. CC het die hoogste hoeveelheid saad (milliliter) gehad, gevolg deur X639 (2133 ml), GS (1450 ml) en <1000 ml vir SC, C-35 en MxT. Saadontkieming was beter vir vrugte wat tydens kleurbreek gepluk was, vergeleke met groen vrugte. MxT het die laagste ontkiemings % by oes gehad (<59%). Oor die algemeen was die saailingontkieming in die kweekhuis na 88d > 83% vir alle kultivars behalwe vir MxT (72.8%). GA<sub>3</sub>proewe (10, 15, 20 ppm) het nie die vrugset% beïnvloed in vergelyking met 'n onbehandelde kontrole nie, behalwe vir X639 wat 'n dubbele persentasie vrugset (16.83%) teen 20 dpm in vergelyking met 'n kontrole gehad het. Daar was 'n neiging dat stikstofvlakke gedurende beide seisoene vir die meeste onderstamkultivars onder die optimale vlak was, behalwe vir CC en C-35 in 2020. Die kaliumvlakke was laer in 2020 as die optimale vir meeste kultivars, behalwe vir CC en US- 812. In 2021 het die vlakke van K onder die optimale vir GS, X639 en MxT gebly. Oor die algemeen was die boomgesondheid van alle kultivars goed, sonder duidelike tekens van vergelying. Ten slotte bied hierdie resultate waardevolle kultivar-spesifieke inligting om dus by te dra tot toekomstige boordaanplantings en beplanning van elke kultivar. Dit sal bydra tot die bestuur van HLB wanneer sitrusbome meer gereeld vervang sal moet word as wat tans die geval is. Daarom moet die jaarlikse voorsiening van onderstok saad konsekwent wees, en die bestuurs praktyke van saadplase moet na gelang aangepas word.

### 4.5.7 PROGRESS REPORT: Validation of LAMP diagnostics for in-field detection of HLB

Project 1265 by Ronel Roberts (ARC-TSC) and Glynnis Cook (CRI)

#### Summary

Both '*Candidatus Liberibacter asiaticus*' (Las), associated with Huanglongbing (HLB) and '*Ca. L. africanus*' (Laf), associated with African Greening are present on the African continent. Due to the significant threat which is posed by HLB to the citrus industry, it is important that rapid diagnostics is available for the detection and differentiation of Las and Laf, from both citrus and insects, which will assist in the monitoring of incursion and following such an event, the spread of HLB in South Africa. Loop-mediated isothermal amplification (LAMP) assays were developed based on *Liberibacter nrdB* gene regions for both Las and Laf as a field diagnostic

tool for screening, prior to laboratory confirmation. Studies conducted to date showed that, when used with positive and healthy plant DNA samples, the LAMP assays were able to detect their respective target *Liberibacter*, with no cross reaction occurring for the other non-target species. The exception to this however, is that the Laf LAMP assay reacts with LafCI bv. citrus, which was identified from citrus in eastern Africa. LafCI bv. Citrus, does however, not react with the Las assay, resulting in no false-positive reaction for Las. Both the Laf and Las assays take 30 min to complete, with further experiments being planned to determine the exact cut-off point at which a low-titre positive sample is no longer detectable by the assay. Additionally, three different plant crude extraction methods were assessed that will enable users to conduct the LAMP assays when doing scouting in orchards for HLB. These extraction methods require further optimisation as a number of healthy samples gave a positive reaction with the Laf LAMP assay.

## Opsomming

Beide '*Candidatus Liberibacter asiaticus*' (Las), wat met HLB geassosieer word, en '*Ca. L. africanus* (Laf), wat met Afrika vergroening geassosieer word is teenwoordig op die Afrika kontinent. Weens die beduidende bedreiging wat HLB vir die Suid-Afrikaanse sitrus industrie inhou, is dit belangrik dat vinnige diagnostiese toetse beskikbaar is wat Las van Laf kan onderskei en korrek identifiseer, in beide insek en plant monsters. 'Loop-mediated isothermal amplification' (LAMP) toetse was ontwikkel wat die *nrdB* geen van beide Las en Laf teiken as 'n veld diagnostiese toets vir die keuring van monsters voor dit in die laboratorium verder geïdentifiseer word. Studies wat gedoen is op plant DNA ekstrakte wat positief vir Laf, of Las was, het daarop gedui dat die twee LAMP toetse die onderskeidelike *Liberibacter* optel en geen reaksie toon met die nie-teiken *Liberibacter*. 'n Uitsondering hierop was wel dat die Laf LAMP toets met LafCI bv. sitrus, wat uit sitrus in oos Afrika geïdentifiseer is, reageer. Ten spyte hiervan, het LafCI bv. sitrus nie met die Las LAMP toets gereageer nie, en geen vals-positiewe reaksies vir Las is aangeteken nie. Beide die Las en Laf toets neem 30 minute om te voltooi, maar verdere eksperimente word beplan om die presiese afsny punt van die toets te bepaal vir lae-titer monsters. Addisioneel was drie verskillende kru ekstraksie metodes geasseseer met die doel dat gebruikers die toets in die veld kan doen tydens veldwerk om die verspreiding van Las te bepaal. Verdere optimisering van hierdie kru-ekstraksie metodes word verlang siende dat gesonde kontroles vals-positiewe reaksies in die Laf LAMP toets gee het.

### 4.6 CRI Diagnostic Centre (Elaine Basson, Charmaine Olivier, Aubrey Metane, Samuel Ndlovu, Nozipho Shabangu and Jan van Niekerk)

Analysis	Citrus nurseries	Commercial samples	Other crops	Research samples
<b>Nematode:Roots</b>	21	1410	0	412
<b>Nematode:Soil</b>	2	813	33	430
<b><i>Phytophthora</i></b>	7 061 <sup>1</sup>	1 451	828	164
<b>Water spore trap</b>	257	0	5	0
<b>Black spot identification (PCR)</b>	0	330	0	130
<b>Black spot benzimidazole resistance</b>	0	90	0	180
<b>Post Harvest Sensitivity</b>	0	66	0	0
<b>Fruit &amp; Foliar identification</b>	1	58	34	34
<b>Soil dilution plating</b>	0	9	24	0
<b>Graft Transmissible Diseases: HLB</b>	0	0	0	274
<b>Graft Transmissible Diseases: Other</b>	0	0	0	6
<b>Biosecurity traps</b>	0	0	0	2 631
<b>SUB-TOTAL</b>	<b>7 342</b>	<b>4 227</b>	<b>924</b>	<b>3 421</b>

<sup>1</sup> Total samples received for citrus nurseries – includes quarterly samples, re-tests and non-certified nurseries

### Citrus Certified Nurseries

It is compulsory for all citrus nurseries participating in the Citrus Improvement Scheme to send samples for *Phytophthora* analysis on a quarterly basis. The irrigation water must also be tested for *Phytophthora* by

making use of the spore trap method. In total, 5 676<sup>2</sup> nursery samples were received by the Diagnostic Centre for *Phytophthora* analyses. Of these samples, 4.84% tested positive. In addition to soil and water samples, nurseries are required to send root samples once a year to test for the presence of *Tylenchulus semipenetrans*. For the nematode root samples, 0% tested positive and for the nematode soil samples 0% tested positive.

Footnote:

<sup>2</sup> Sample number and the percentage positive are only for certified nurseries and only for the quarterly samples received.

### Commercial samples

Samples were received from the following citrus growing areas: Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape, North West, Western Cape and Zimbabwe. Most of the samples received from citrus growers were analysed for *Phytophthora* species including *P. nicotianae* and *P. citrophthora*, and the citrus nematode, *T. semipenetrans*. Fifty eight percent of the 1 410 samples analysed for citrus nematode females had counts above the threshold value of 1 000 females per 10 g of roots, and nematicide treatments were recommended. Forty-six percent of the 1 451 samples analysed for *Phytophthora* tested positive.

### Other crops

Nematode counts were done on soil or root samples of Apple, Avocado, Banana, Granadilla, Kiwi, Macadamia, Peach, Pine, Plum, Potting Mix, and Tomato. Nematodes found present on these crops included: *Hemicycliophora*, *Meloidogyne*, *Mesocriconema*, *Pratylenchus*, and *Scutellonema*. *Phytophthora* and *Pythium* analyses were done on Avocado, Blueberries, Compost, Granadilla, Kiwi, Macadamia, Plum, Pine, Potting Mix, Tomato, and Vegetables. The Diagnostic Centre analysed 26 soil samples from avocado and macadamia nurseries for the presence of *Phytophthora cinnamomi*.

### Research samples

Nematode and *Phytophthora* analysis were done on 1 004 samples from experimental trials and extension samples. The Diagnostic Centre assisted in trials to identify possible citrus black spot lesions using PCR protocols on 130 fruit samples. As part of Biosecurity 2 631 traps were read for the detection of Asian Citrus Psyllid, with 274 samples tested for the detection of African Greening and Huanglongbing.

**CRI Diagnostiese Sentrum** (Elaine Basson, Charmaine Olivier, Aubrey Metane, Samuel Ndlovu, Nozipho Shabangu en Jan van Niekerk)

Ontleding	Sitrus kwekerie	Kommersiële monsters	Ander gewasse	Navorsings- monsters
Aalwurms: Wortels	21	1410	0	412
Aalwurms: Grond	2	813	33	430
<i>Phytophthora</i>	7 061 <sup>1</sup>	1 451	828	164
Water spoorlokval	257	0	5	0
Swartvlek (PKR)	0	330	0	130
Swartvlek benzimidazole bestandheid	0	90	0	180
Na-oes sensitiwiteitstoetse	0	66	0	0
Vrug-en blaar identifikasie	1	58	34	34
Grondverduunning	0	9	24	0
Ent-oordraagbare siektes: Vergroening	0	0	0	274
Ent-oordraagbare siektes: Ander	0	0	0	6
Biosekuriteits lokvalle	0	0	0	2 631
<b>TOTAAL</b>	<b>7 342</b>	<b>4 227</b>	<b>924</b>	<b>3 421</b>

<sup>1</sup> Totale hoeveelheid monsters ontvang van gesertifiseerde kwekerie – sluit in kwartaal monsters, hertoets monsters en nie-gesertifiseerde kwekerie

### **Sitrus Gesertifiseerde Kwekerie**

Dit is verpligtend vir al die sitruskwekerie wat aan die Sitrus Verbeteringskema deelneem om kwartaalike monsters vir *Phytophthora* te laat ontleed. Die besproeiingswater moet ook deur middel van die spoorlokval metode vir *Phytophthora* getoets word. In totaal 5676<sup>2</sup> monsters is deur die diagnostiese sentrum vir *Phytophthora* ontleding ontvang, waarvan 4.84% positief getoets het. Benewens die water en grondmonsters, moet kwekerie een keer per jaar 'n wortelmonster instuur om vir die teenwoordigheid van *Tylenchulus semipenetrans* te toets. Van die wortel- en grondmonsters wat ontvang is, het 0.0% positief getoets vir die teenwoordigheid van *T. semipenetrans*.

Voetnota:

<sup>2</sup> Monster hoeveelheid en die persentasie positief is net vir gesertifiseerde kwekerie en slegs vir die kwartaal monsters ontvang.

### **Kommersiële monsters**

Monsters is uit die volgende sitrusverbouingsareas ontvang: Oos-Kaap, KwaZulu-Natal, Limpopo, Mpumalanga, Noord-Kaap, Noord-Wes, Wes-Kaap en Zimbabwe. Die meeste van die monsters wat van sitrusprodusente ontvang is, is vir *Phytophthora nicotianae* en die sitrusaalwurm, *Tylenchulus semipenetrans*, ontleed. Agt-en-vyftig persent van die 1 410 aalwurmmonsters wat ontleed is, het tellings hoër as die drempelwaarde van 1 000 wyfies per 10 g wortels gehad. Aalwurmdoderbehandelings is in daardie gevalle aanbeveel. Ses-en-veertig persent van die 1451 monsters wat vir *Phytophthora* ontleed is het positief getoets.

### **Ander Gewasse**

Aalwurmtellings is op grond- of wortelmonsters van Appel, Avokado, Piesang, Granadilla, Kiwi, Makadamia, Perske, Denneboom, Pruim, Potmengsel, and Tamaties. Aalwurms teenwoordig gevind op hierdie gewasse sluit in: *Hemicycliphora*, *Meloidogyne*, *Mesocriconema*, *Pratylenchus*, en *Scutellonema*. Avokado, Bloubessies, Kompos, Granadilla, Kiwi, Makadamia, Pruim, Denneboom, Potmengsel, Tamatie, en Groente monsters is vir *Phytophthora* en *Pythium* ontleed. Die diagnostiese sentrum het 26 monsters vanaf avokado en makadamia kwekerie ontvang om vir *Phytophthora cinnamomi* te ontleed.

### **Navorsingsmonsters**

Aalwurm en *Phytophthora* ontledings is op 1004 monsters afkomstig uit navorsingsprojekte gedoen. Die Diagnostiese Sentrum het ook hulp verleen aan navorsingsprojekte in die identifikasie van moontlike sitrus swartvlek letsels deur middel van PKR op 130 vrug monsters. As deel van Biosekuriteit, is 2 631 lokvalle gelees vir die monitering van "Asian Citrus Psyllid", met 274 monsters molekulêr getoets vir die bespeuring van Afrika vergroening en Huanglongbing.

## **5 PORTFOLIO: CITRICULTURE**

### **5.1 PORTFOLIO SUMMARY**

By Paul Cronje (Portfolio Manager: Citriculture, CRI)

The Citriculture portfolio includes a wide range of research themes and producer identified priorities that have a bearing on quality fruit production and export. This includes projects on irrigation, nutrition, crop manipulation, rind condition, cold chain, and cultivar evaluation and rootstock development.

Progress made in evaluating new fertilisers has indicated that in-season regular short-term changes in fertilisation rates are unnecessary and producers should focus on long-term tendencies to adjust fertilisation

programmes. Furthermore, the current autumn sampling time  $\pm 180$  days after full bloom, is sufficient for foliar analysis. In terms of optimising foliar applications Masterlock®, does improve the distribution and uptake of the spray mixture of aerially applied nutrient mixtures. Fruit set of seedless Valencia oranges remains problematic in the warm, dry production areas. However, a study indicated the possible impact of climatic effect in an increased fruit drop. This study also highlights the need to test and use all options available to improve set, including plant growth regulators, but not to neglect adequate soil preparation. The season also saw the publication of the much-awaited Handbook on Fertilisation of Citrus available from CRI. The research output from results on the cold chain has resulted in an improvement in temperature control. The various projects in this focus area try to integrate all the components of the cold chain, i.e., carton, pallet, precooling and shipment, to apply low temperatures without reducing quality effectively. Cold damage is the industry's main external quality aspect, and the complexity of reducing this disorder was evident in the results. Evaluation of new cultivars and selections in all production regions are part of the portfolio's aim to enable long term suitable production of high-quality fruit in all areas for export. In addition, a re-newed focus on rootstock evaluation and development have been implemented to allow a wider selection of rootstocks in the future for all production areas. The portfolio has expanded the scope and depth of research projects to increase the ability to supply timeous and relevant recommendations to producers, packhouse and exporters.

## **PORTEFEULJE OPSOMMING**

Die sitrus produksie-portefeulje sluit 'n wye reeks navorsing temas en produsent-geïdentifiseerde prioriteite in wat 'n direkte invloed uitoefen op die produksie vna vrugte vir die uitvoermark. Dit sluit besproeiing, voeding, gewasmanipulasie, skildefekte, koueketting, en kultivar-evaluering en onderstok-ontwikkeling projekte.

Vordering is gemaak met die evaluering van nuwe bemestingsbestuur opsies en het aangedui dat dit onnodig is op gereelde korttermyn veranderings in bemestingprogramme in die seisoen te maak en dat produsente moet fokus op langtermyn-neigings om programme aan te pas. Verder is die huidige herfs as die tyd om blaarmonsters te neem,  $\pm 180$  dae na volle blom, bevestig as voldoende vir ontleding. Wat die optimering van blaartoedienings betref, het Masterlock®, die verspreiding en opname van die spuitmengsel van voedingstofmengsels wat uit die lug toegedien word verbeter. Die vrugset van pitlose Valencia-lemoene bly problematies in die warm, droë produksie gebiede. 'n Studie het egter die moontlike impak van klimaat stres in blomtyd as moontlik oorsaak aangedui. Hierdie studie beklemtoon ook die behoefte om alle beskikbare boombestuur opsies te toets en gebruik om set te verbeter. Dit sluit in plantgroeireguleerders, maar dis belangrik om nie voldoende grondvoorbereiding af te skeep nie. Die seisoen het ook die publikasie van die langverwagte Handboek oor Bemesting van Sitrus gesien wat beskikbaar by CRI is. Die navorsingsuitsette op die koueketting het gelei tot 'n verbetering in temperatuurbeheer. Die verskillende projekte in hierdie fokus area probeer om al die dele van die koueketting, dit wil sê karton, palet, voorverkoeling en verskeping, te integreer om lae temperature toe te pas sonder om kwaliteit in te boet. Koueskade is die bedryf se vernaamste eksterne kwaliteit probleem, en die kompleksiteit om die voorkoms te verminder van hierdie afwyking was duidelik in die resultate. Die konstante evaluering van nuwe kultivars en seleksies in alle produksie areas is deel van die portefeulje doelwit om langtermyn produksie van hoë kwaliteit vrugte vir uitvoer moontlik te maak. Boonop is 'n nuwe fokus op onderstam-evaluering en -ontwikkeling geïmplementeer om 'n wyer keuse van onderstokke in die toekoms vir alle produksie gebiede moontlik te maak. Die portefeulje het die omvang en diepte van navorsingsprojekte uitgebrei om die vermoë te verhoog om tydige en relevante aanbevelings aan produsente, pakhuisse en uitvoerders te verskaf.

### **5.2 PROGRAMME: RIND CONDITION AND COLD CHAIN**

Programme coordinator: Paul Cronje (CRI)

#### **5.2.1 Programme summary**

The rind condition and cold chain programme aims to directly address relevant factors negating fruit rind quality leading to physiological disorders from the orchards to the cold chain. The dramatic change in the SA cold chain due to reduced shipping temperatures to key markets as well as high volumes of fruit exported has led to a need to systematically address each step in the cold chain in research projects. Ongoing projects on cooling in refrigerated containers have resulted in a considerable improvement in our understanding of the

cooling process and strategies have been developed to improve the cooling terms of uniformity. Two critical aspects feeding into the equation are the re-evaluation of the role of pallet bases as well as carton design. Improvement in cooling efficacy is a requirement to sustainably access cold protocol markets and plays a large part in maintaining rind quality. The chilling injury will be the major rind disorder affecting the SA citrus industry due to the 2°C and below shipping temperature to nearly 60% of our markets. Research on chilling injury has shown that shade netting does not increase chilling injury incidence, which was a concern due to the reduction in light levels. To add to the complexity of fruit's susceptibility to chilling injury, it was shown that rootstocks could affect this disorder. However, the largest source of variation in susceptibility is due to different cultivars and types. Currently, the only successful options to reduce chilling injury are the use of thiabendazole and wax application. Future projects will aim to elucidate the mode of action of these products to improve effective usage in our value chain.

## **Programopsomming**

Die skilkondisie en koueketting-navorsing program is daarop gemik om relevante faktore wat die kwaliteit van die vrugskil benadeel en so kan lei tot fisiologiese afwykings vanaf boordvlak tot in die koueketting aan te spreek. Die dramatiese verandering in die SA sitrus koueketting, as gevolg van die lae temperature waarteen sitrus uitgevoer word na sleutel markte, sowel as die aansienlike hoër uitvoer volumes het daartoe gelei dat elke stap in die koueketting sistematies in navorsingsprojekte aangespreek moet word. Deurlopende projekte oor die oor die verskeping in verkoelde houers het gelei tot 'n aansienlike verbetering in ons begrip van die verkoeling en daaruit is strategieë ontwikkel om meer eenvormige en doeltreffende verkoeling te behaal. Twee kritieke aspekte wat in die vergelyking ingevoer word, is die herevaluering van die rol van paletbasse sowel as karton ontwerp. Verbetering in verkoelingsdoeltreffendheid is nie net 'n vereiste vir volhoubare toegang tot spesiale markte nie, maar speel ook 'n groot rol in die bewaar van skilkwaliteit. Koueskade sal egter die grootste fisiologiese skilafwyking bly vir die SA sitrus bedryf, as gevolg van die verskeping teen 2°C en laer na byna 60% van ons markte. Die resultate toon dat skadunette nie die voorkoms van koueskade verhoog nie, wat kommerwekkend was as gevolg van die afname in lig vlakke onder nette. Om by te dra tot die kompleksiteit wat vrugte se sensitiwiteit vir koueskade beïnvloed, is daar gevind dat onderstokke hierdie defek kan beïnvloed. Die grootste bron van variasie in sensitiwiteit bly egter die uiteenlopende kultivars en tipes. Tans is die enigste suksesvolle opsies om koueskade te verminder die gebruik van tiabendazole en waks behandeling in die paklyn. Toekomstige projekte sal daarop gemik wees om die werkwyse van hierdie produkte te verduidelik om effektiewe gebruik in ons waardeketting te verbeter.

### **5.2.2 FINAL REPORT: Integration of pallet bases and carton designs to improve ventilation of citrus exports**

Project 1237 (February 2019 - March 2021) by Tarl Berry & Paul Cronje (CRI)

#### **Summary**

Ambient loading is an essential component of the South African citrus industry's cold chain, as it reduces strain on the limited cold store facilities. A significant challenge to the cool-down process in containers is the high resistance to airflow of the packaging materials, which do not adequately allow cold airflow to reach the fruit. The objective of this research was thus to quantify the performance of current citrus packaging designs and to identify potential solutions or strategies. The study first performed a survey of the packaging designs being used in the South African citrus industry. From this work, the leading packaging designs in use were evaluated based on resistance to airflow, which can be directly correlated to cooling performance. Characterisations of the packaging systems were evaluated across both the horizontal (emulating precooling) and the vertical axis (emulating container cooling). Next, the effect of additional packaging material (e.g. securing sheets and trays) were also investigated. The survey results showed that the SA citrus industry primarily makes use of A15C, E15D and E10D cartons for export to distant markets. Additionally, the survey showed that the use of trays and securing sheets might have a significant influence on pressure loss across the packaging systems. The results presented include a detailed performance overview of the packaging systems being used in the SA citrus industry. Three main carton designs types were identified. In their current state, the study showed that not all of the designs performed optimally. Two potential solutions for the A15C cartons are a column-stacking approach during palletisation or repositioning vent holes along a 100 mm grid for improved alignment. During

container cooling, the open top cartons cool substantially slower than the A15C. Two strategies for improvement will be the use of larger ventilation openings (the recommendations for the A15C are not applicable for the open top cartons as the openings must be larger). Additionally, the use of non-obstructive securing sheets will be essential over the short term.

## Opsomming

Warmtelaai van verkoelingshouers is 'n noodsaaklike komponent in Suid-Afrikaanse sitrus industrie se koueketting in die, a.g.v. beperkte voorverkoeling fasiliteite. 'n Belangrike uitdaging vir die voorverkoeling proses in houers is die hoë weerstand teen lugvloei wat verpakkingsmateriaal bied en dus nie toelaat dat koue lugvloei die vrugte bereik nie. Die doel van hierdie navorsing was om die vermoë van huidige karton ontwerpe te kwantifiseer en potensiële oplossingstrategieë te identifiseer. Eerstens wa 'n opname gedoen t.o.v. karton en verpakkings ontwerpe gebruik in die Suid-Afrikaanse. Voortspruitend hier uit was die mees gebruikte ontwerpe, geëvalueer op grond van weerstand teen lugvloei a.g.v. die direkte korrelasie met verkoelings effektiwiteit. Die verpakkingstelsels was geëvalueer in beide die horisontale (voorverkoeling) en die vertikale as (houerverkoeling) rigting. Vervolgens was die effek van addisionele verpakkingsmateriaal nl. "Securing sheets" en "trays" ook ondersoek. Die resultate van die opname het getoon dat die sitrusbedryf hoofsaaklik A15C, E15D en E10D kartonne gebruik vir uitvoer. Daarbenewens het die opname getoon dat die gebruik van "trays" en "securing sheets" 'n beduidende negatiewe invloed op drukverlies in die verpakkingstelsel (pallet) kan uitoefen. In die resultate word a 'n gedetailleerde prestasie-oorsig voorgelê van die verpakkingstelsels wat in die SA sitrusbedryf gebruik word. Drie hoof kartonontwerpe was geïdentifiseer, maar daar was gevind dat die huidige toestand van alle ontwerpe nie optimaal presteer nie. Twee moontlike oplossings vir die A15C-kartonne is 'n kolom-stapel benadering van pallette of die her posisionering van ventilasie gate op gelyk met 'n rooster van 100 mm. In die houers verkoel oopvertoon-kartonne aansienlik stadiger af as die A15C. Twee opsies om die ventilasie te verbeter is die gebruik van groter ventilasie-opeeninge (A15C-aanbevelings is nie van toepassing nie oor opeeninge groter moet wees vir oopvertoon-kartonne

## Introduction

The volume of citrus fruit exported from South Africa (SA) under cold sterilization treatments has continued to increase over recent years and has led to pressure on the limited precooling facilities at ports. For instance, in 2019, South Africa exported over 127.5 million cartons (15 kg) to various global markets, making it the second-largest exporter of citrus fruit in the world (CGA, 2019). An interesting factor in this export process is that unlike other producing countries, SA is relatively distant from importing markets (>12 000 km by sea).

The South African citrus cold chain can be summarised into three steps. The first is forced-air cooling (FAC), which is performed after harvest to remove excess field heat from fruit. The technique makes use of a fan system to extract cold airflow horizontally through the palletised fruit. Engineers generally aim to apply about a 100 Pa pressure difference across the pallet for a duration of 2 to 3 days. The second cold chain step typically occurs after FAC, whereby the fruit are stored in a refrigerated room for periods typically less than a week, until loaded into a container. The third step takes place in the refrigerated container, which is used to maintain fruit temperatures during shipping to market. The container delivers refrigerated airflow via a T-bar floor at a relatively low air speed (0.01-0.05 m s<sup>-1</sup>) over roughly 18 to 35 days.

The SA citrus industry makes extensive use of refrigerated containers and reefer vessel shipping. As a consequence, the SA industry has incorporated cold sterilization treatments into the shipping process, which reduces the overall cold chain duration. Recently (since 2018), the SA citrus industry began implementing a new strategy to reduce the risk involved in phytosanitary insect pests during the export process by shipping fruit at various temperatures  $\leq 2$  °C to European Union (EU) markets. This new cold treatment protocol is part of a systems approach, which includes various pre- and postharvest treatments prior to shipping to mitigate the risk of live pest interception in the market.

The large volume of citrus fruit exported to EU, as well as the insufficient precooling infrastructure available, would have result in substantial logistical problems at the ports if started prior to shipping. The practice of ambient loading was thus applied to lessen this challenge and involves loading the fruit warm, i.e. at ambient

temperatures, in the container. The refrigeration unit of the containers thus removes the excess field heat. Past studies (2015-2018) have demonstrated (theoretically and experimentally) that refrigerated containers have sufficient capacity to cool produce to set-point within 2 to 3 days (Defraeye *et al.*, 2015c, 2015b). However, relatively large variations in cooling performance (rate and uniformity) are at times observed, resulting in extended cooling periods (up to 5 days). As a result, ambient loads are not applicable to full cold sterilization markets and can only be applied to longer shipment durations to allow for adequate exposure days from the port to the EU markets.

The citrus cold chain differs from most other fruit in that temperature mismanagement for short durations do not - as a rule - result in a drastic reduction in fruit quality as seen in some climacteric fruit (e.g. stone fruit). However, fruit pulp temperatures that go out of protocol offers two distinct problems for the SA citrus cold chain. Firstly, incorrect temperatures are more often associated with too low air temperatures causing chilling and freeze damage. Secondly, an increase in pulp temperature due to equipment failure or cold chain mismanagement during precooling or shipping could result in failure to comply with cold sterilization protocols, which require costly corrective actions.

The main factors influencing cooling performance of fruit loaded at ambient are the initial pulp temperature and variation in the airflow rates through the pallets. It has further been suggested that current carton designs are insufficiently ventilated to properly utilize a containers air delivery system (Berry *et al.*, 2017; Getahun *et al.*, 2017a, 2017b). Specifically, the heterogeneous airflow distribution observed in refrigerated containers is likely contributing to an increased incidence of chilling injury (in high flow regions) and pallet hot spots (in low flow regions). Packaging systems (carton and pallet designs) with improved ventilation would thus enable more extensive usage of ambient loading, further reducing the pressure on the limited precooling facilities, as well as reducing handling times, which implies faster shipping.

An examination of vent area along the top and bottom surfaces of carton layers in a pallet stack showed that the A15C (Supervent) and E15C (Standard) cartons have 9% and 5% total ventilated area (with no obstruction), respectively. This ventilation is reduced to < 2% when placed on top of a pallet base (standard block or USA orange block pallet). Furthermore, cross-stacking, which is applied to most of the carton layers in a pallet stack, reduces the A15C and E15C designs vent area to 2% and 1%, respectively. The porosity of current carton designs could thus be considerably improved with even slight modifications to vent designs or pallet base slat positioning. This can significantly improve cooling performance during ambient loading, which would make more fruit eligible for ambient loading.

## **Stated objectives**

The aim of this study was to explore the current state of airflow properties in citrus packaging and identify potential solutions. Specific objectives include:

1. Perform a survey of local packhouses to describe the design problem better. The study will also attempt to identify previously unrecognized packaging design solutions that may already be present within the industry.
2. Design novel carton vent hole and pallet base configurations using CAD software based on total unobstructed area.
3. Commercial evaluations of current packaging systems during forced-air cooling and refrigerated container shipping
4. Compare the cooling performance of current designs and identify potential solutions for optimization.

## **Materials and methods**

### **Survey method**

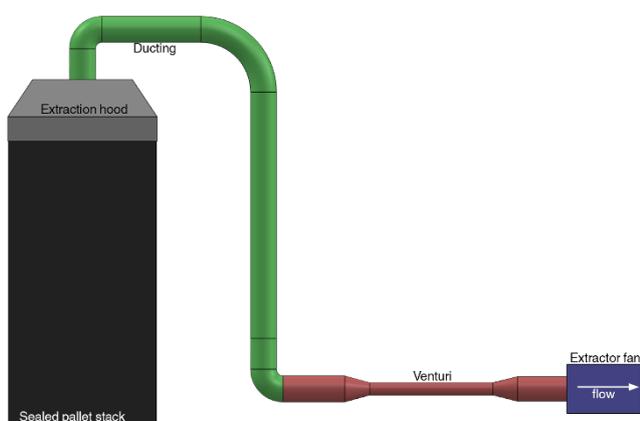
The survey was performed between 2019 and 2020, where cartons were collected and evaluated from multiple cold stores at Durban, Port Elizabeth and Cape Town (South Africa). Aspects of interest included carton dimensions (footprint and height), the vent hole design (size, positions, number) and the palletisation method (Berry *et al.*, 2015). In this case, the palletisation method considers all the additional packaging components

that are also used in a fully stacked pallet. This can consist of pallet bases, securing sheets and pallet caps. (see results for a detailed description of each component).

As a supplement to the detailed survey, industry export data was also compiled over the last two seasons from the Citrus Growers Association (CGA), which is a South African citrus regulatory organisation and lists statistics for each shipment leaving the country. This data included information on the type of packaging being used and the citrus varieties being packed.

### Resistance to airflow experiments

A mobile air extractor device was designed and constructed for this study (Figure 5.2.2.3). The device attaches to either the top or side of a pallet and extracts airflow through the packaged fruit to simulate either a refrigerated container or FAC airflow conditions, respectively. The extractor makes use of calibrated differential pressure sensors (Air Flow Meter Type: A2G-25 air2guide, Wika, Lawrenceville GA 30043, USA) to measure the pressure difference across the pallet stack and across a venturi device for accurate determination of flow rate. A speed-controlled centrifugal fan (Type: K3G250-AV29-B2, Embpapst, Mulfingen, Germany) is used in the system to control the airflow rate. Calibration of the setup showed that the measurement error was <5%.



**Figure 5.2.2.3.** Illustration of the extractor device used to measure air flow rate versus pressure loss curve across pallet stacks.

The pallet stack is first assembled using the respective packaging materials of interest. To simulate container cooling, the extraction hood is attached to the top of the structure, and the sides are sealed with plastic sheeting, so that airflow can only enter the pallet from the bottom (vertically). In each case, a range of airflow rates are applied across the stack and the respective venturi and differential pressure drops are recorded. Forced-air cooling tests (horizontal airflow) was achieved by attaching the hood to the side of the pallet. In this case, it was only necessary to assemble half a pallet stack. Similarly, all sides, except the opposite end of the hood were sealed with plastic liner. All other sides are sealed with plastic sheeting and tape. Each experiment was repeated in triplicate.

### Performance evaluations

The primary factor influencing cooling performance of fruit during FAC or container cooling is the pallet structures resistance to airflow (RTA). Consequently, RTA was used as the primary measure of cooling performance of a pallet stack structure.

The resistance to airflow of the packed cartons was characterised by relating the pressure difference over the stack to the superficial air velocity through the pallet. The superficial air velocity was calculated using the venturi device, which relates pressure loss over a flume to airflow rate (Pozrikidis, 2009, p. 334). This relationship followed a quadratic curve under cold chain cooling conditions, which is characteristic of flow at high Reynolds numbers (>2000) (Berry *et al.*, 2017; Verboven *et al.*, 2006). The Dacy Forchheimer equation (1) is one of the primary approaches used to quantify the pressure loss versus air velocity curve of a system.

$$\nabla p = -\frac{\mu}{K}\mathbf{u} - \frac{C\rho}{K^{1/2}}|\mathbf{u}|\mathbf{u} \quad (1)$$

where  $\nabla p$  is the pressure gradient ( $\text{Pa m}^{-1}$ ),  $\mu$  ( $\text{kg m}^{-1} \text{s}^{-1}$ ) is the dynamic viscosity of the fluid,  $K$  ( $\text{m}^2$ ) is the permeability of the porous medium,  $C$  is the is a dimensionless Forchheimer coefficient,  $\rho$  ( $\text{kg m}^{-3}$ ) is the fluid density,  $u$  ( $\text{m s}^{-1}$ ) is the superficial flow velocity in the porous media.

A linear component in the relationship is also present under very low laminar flow rates and is addressed in detail by Verboven *et al.*, (2006). However, for the benefit of quantifying the resistance to airflow of the packaging systems, it is only necessary to capture the quadratic component of the relationship. Defraeye *et al.* (2015) thus originally proposed the use of a quadratic function with a single coefficient term:

$$\Delta P = \xi G_a^2 \quad (2)$$

where  $\Delta P$  is the pressure drop (Pa),  $\xi$  is the pressure loss coefficient ( $\text{kg m}^{-7}$ ) and  $G_a$  is the airflow rate ( $\text{m}^3 \text{s}^{-1}$ ).

A challenge here is that equation (2) does not account for the depth of the packaging system or the surface area. These factors need to be considered when comparing the various components of packaging systems or multiple orientations of a packaging system. A modification of equation (2) is thus proposed here (eq (3) and eq (4)):

$$\Delta p = \psi_t |\mathbf{u}|\mathbf{u} \quad (3)$$

$$\nabla p = \psi_m |\mathbf{u}|\mathbf{u} \quad (4)$$

where  $\psi_t$  ( $\text{kg m}^{-3} \text{s}^{-2}$ ) is the total quadratic resistance coefficient (tQRC) and  $\psi_m$  ( $\text{kg m}^{-4} \text{s}^{-2}$ ) is the modified quadratic resistance coefficient (mQRC).

Specifically, the tQRC accounts for the total RTA across the whole system and the mQRC accommodates for the depth of the system (i.e. gradient per a unit distance). The (mQRC) for this study was defined as the quadratic relationship between the pressure drop over the distance across which the pressure drop occurred ( $\text{Pa m}^{-1}$ ). Although similar, these two coefficients are used differently in further evaluation and characterisations. Specifically, mQRC is generally more universal understandable and was used as a descriptor in this study.

The other performance parameter examined in this study was the total ventilation area (TVA), which is relevant to the individual carton faces, as well as interfaces within the pallet structures. The TVA was represented as a percentage of the total open (unobstructed) area divided by the total cross-sectional area of the packaging surface area of the interface. This value thus indicates how much ventilation is remaining at an interface after palletisation.

## Results and discussion

### 1. Survey results

The survey identified over 50 unique carton designs that are in active use within the South African citrus industry. Many of these carton designs, however, have a similar design but make use of differing heights to accommodate fruit size or a particular market preference. Despite all these variations, three prominent designs, which account for about 85% of all cartons exported, were isolated.

The three designs, namely the A15C, the E15D and the E10D (Figure 5.2.2.3) and each have a unique footprint and ventilation approach. As these designs represent the majority of the market, they were the primary focus of this study.

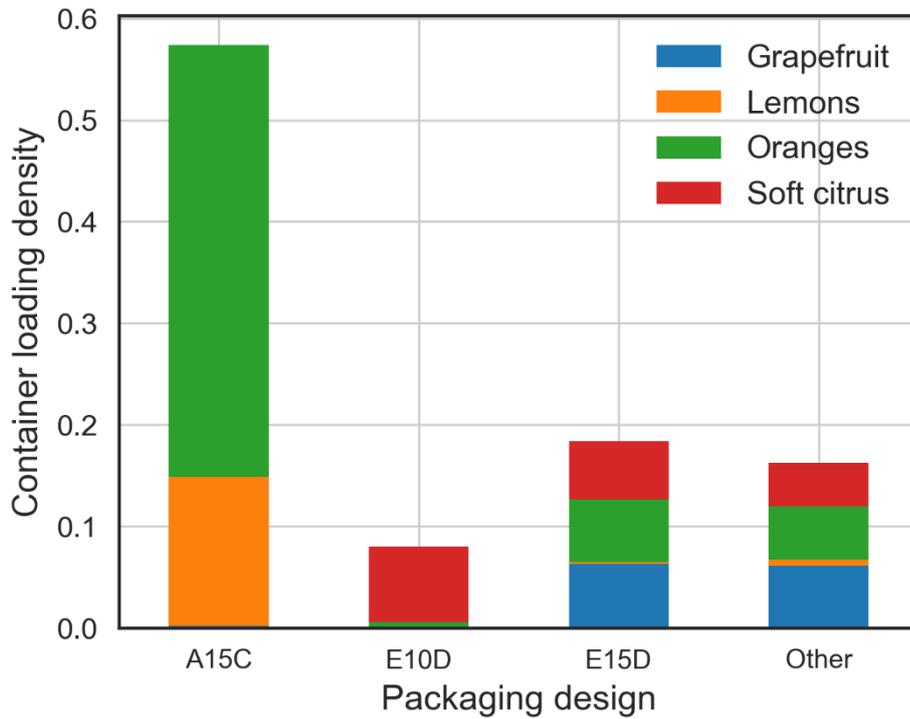


Figure 5.2.2.4. Carton type used for citrus exports during 2019 and 2020 (Data collected from CGA).

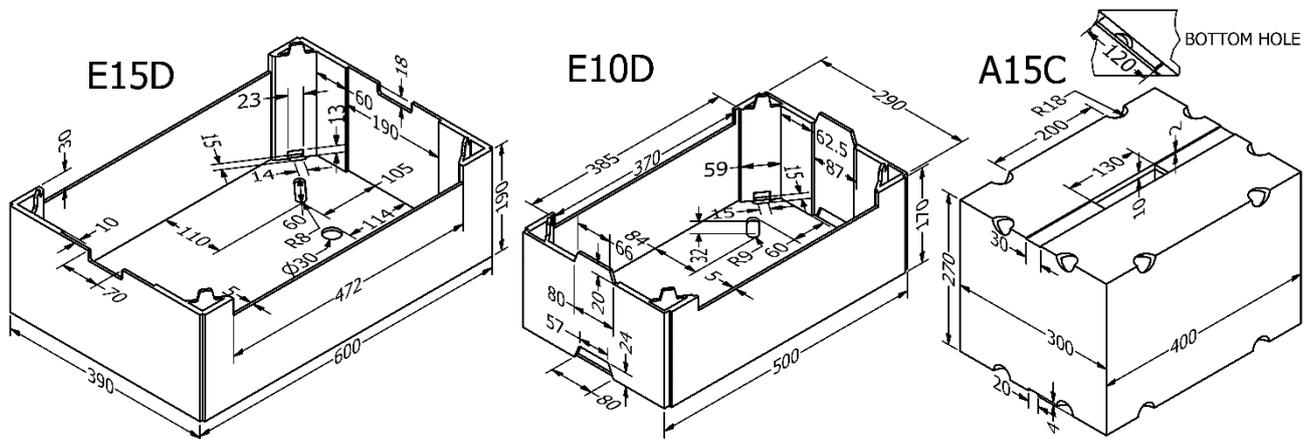
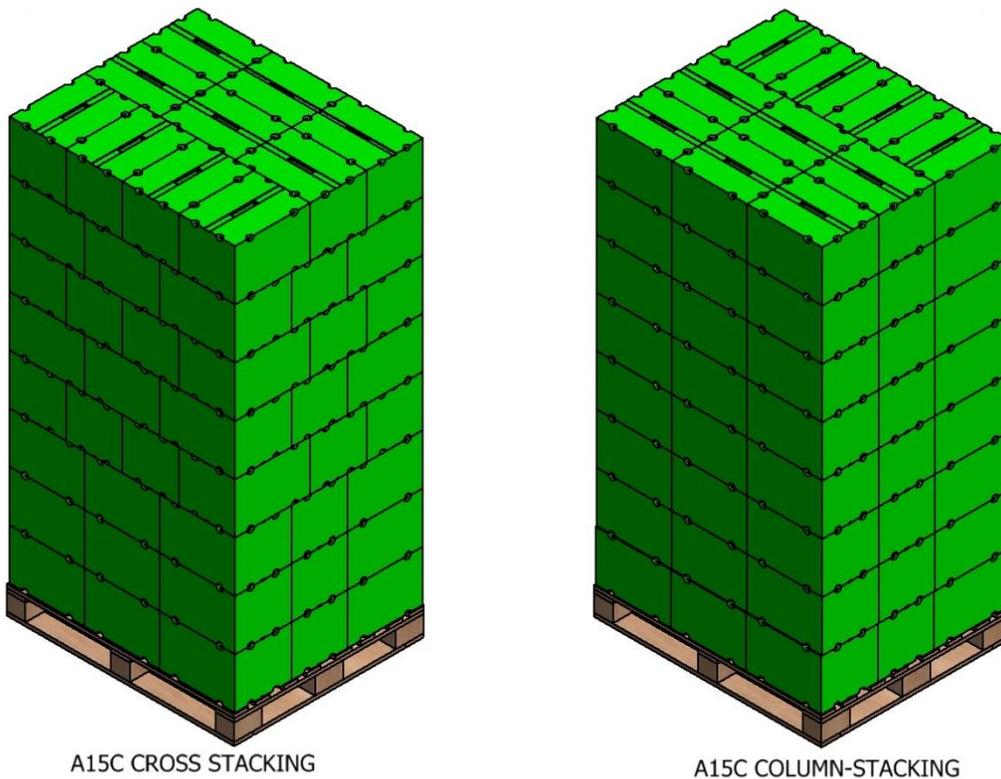


Figure 5.2.2.5. The three most commonly used citrus cartons in the South African export industry. E15D, E10D, A15C (from left to right)

The A15C cartons have two circular holes along each of the horizontal edges of the carton, and a single rectangular opening at both the top and the bottom planes of the carton (Figure 5.2.2.5). A15C cartons are stacked 8 layers high using a semi-alternating cross-stacking stacking pattern, as shown in

Figure 5.2.2.6. In the cross-stacking configuration, the layer orientation alternates from the 4<sup>th</sup> layer onwards (counting from the bottom). The study also investigated a column-stacking configuration, whereby the layer orientation does not alternate or change; the cartons are thus placed directly on top of one another.



**Figure 5.2.2.6.** A15C Stack on a CRI Pallet: Cross-Stacking compared to Column-Stacking

The E15D and E10D display cartons are column-stacked into a 13-layer high pallet with tabs at each corner to resist shearing forces between the individual layers when stacked. The display cartons have rectangular holes on the sides of the carton, circular holes at the carton bottom and bottom holes for the top tabs to slip through ( Figure 5.2.2.5).

The survey identified a plethora of different vent hole designs for both the E15D and E10D. Careful examination of many of these designs showed that the designs examined in this study were similar, if not identical, to what was being used in the majority of exports.

**A comprehensive breakdown of the citrus packaging system structure**

An audit of the components making up a citrus pallet structure identified multiple interacting factors that can influence porosity through the system. Prior studies have generally focused on optimising only the vent hole designs of the cartons to improve cooling uniformity within the individual cartons or throughout the stack (Mukama *et al.*, 2020; O’Sullivan *et al.*, 2016). In contrast, the survey showed a much more complex packaging design problem, indicating the need for a more holistic approach that considered the whole packaging system and each of the respective components. This research field is currently lacking a comprehensive overview of packaging structure as it relates to ventilation.

The approach used in this work, was thus to meticulously model each component in a pallet stack using computer aided design (CAD) software (Autodesk, 2020). This enabled us to quantify the network of vent holes in a pallet structure. This evaluation approach is essential to the identification of the actual aspects influencing cooling performance. CAD models included securing sheet, pallet base, pallet cap and cartons. The CAD model documented the position/number of the pallet bases, carton stacking, and securing sheets. CAD drawings of the E15D, E10D and A15C cartons are shown in Figure 5.2.2.5. Basic dimensions for the securing sheet and pallet cap are available in Figure 5.2.2.7. Three unique pallet base designs were considered for the A15 Cross-stacking pallet stacks ( Figure 5.2.2.8).

The TVA (Total Ventilation Area) was calculated using the unobstructed area by the total area percentage of a packaging's surface area (e.g. the exposed face of a pallet stack). The TVA was calculated at each interface, where carton surfaces make contact with each other during stacking. It is a combination of the TVA of each interface and the number of interfaces that determine the total resistance to airflow of a system.

### Securing sheets and pallet caps

The study identified numerous securing sheets in use. The primary packhouse strategy was to purchase securing sheets with as much robustness as possible. Securing sheets have thus generally been optimised towards designs that make them as compatible with as many carton designs as possible. Most openings on the sheets, therefore, accommodate multiple different tabs (depending on the display carton design) and often do not align with the carton vent holes. Of the many securing sheets identified, the sheets in Figure 5.2.2.7 were representative of the unobstructed vent hole area and were used in this study.

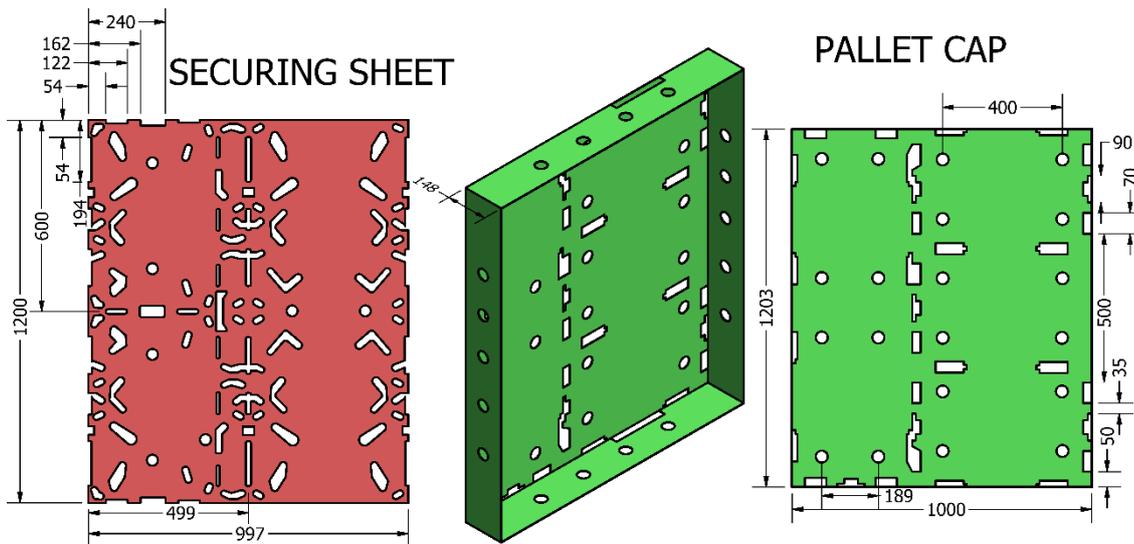


Figure 5.2.2.7. The securing Sheet (red) and pallet Cap (green) used in this study.

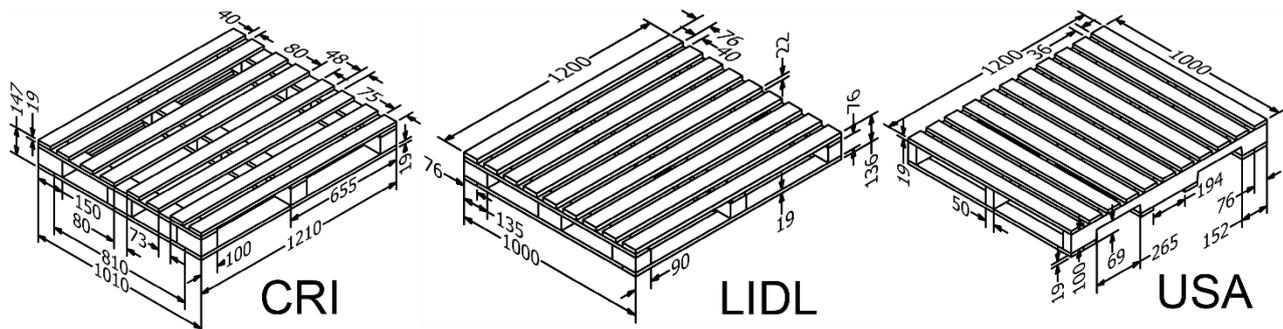


Figure 5.2.2.8. Three main pallet bases in the SA citrus industry.

### A15C carton

The A15C citrus carton is designed to be a high packing density box and is used in more than 55% of all exports (Figure 5.2.2.4).

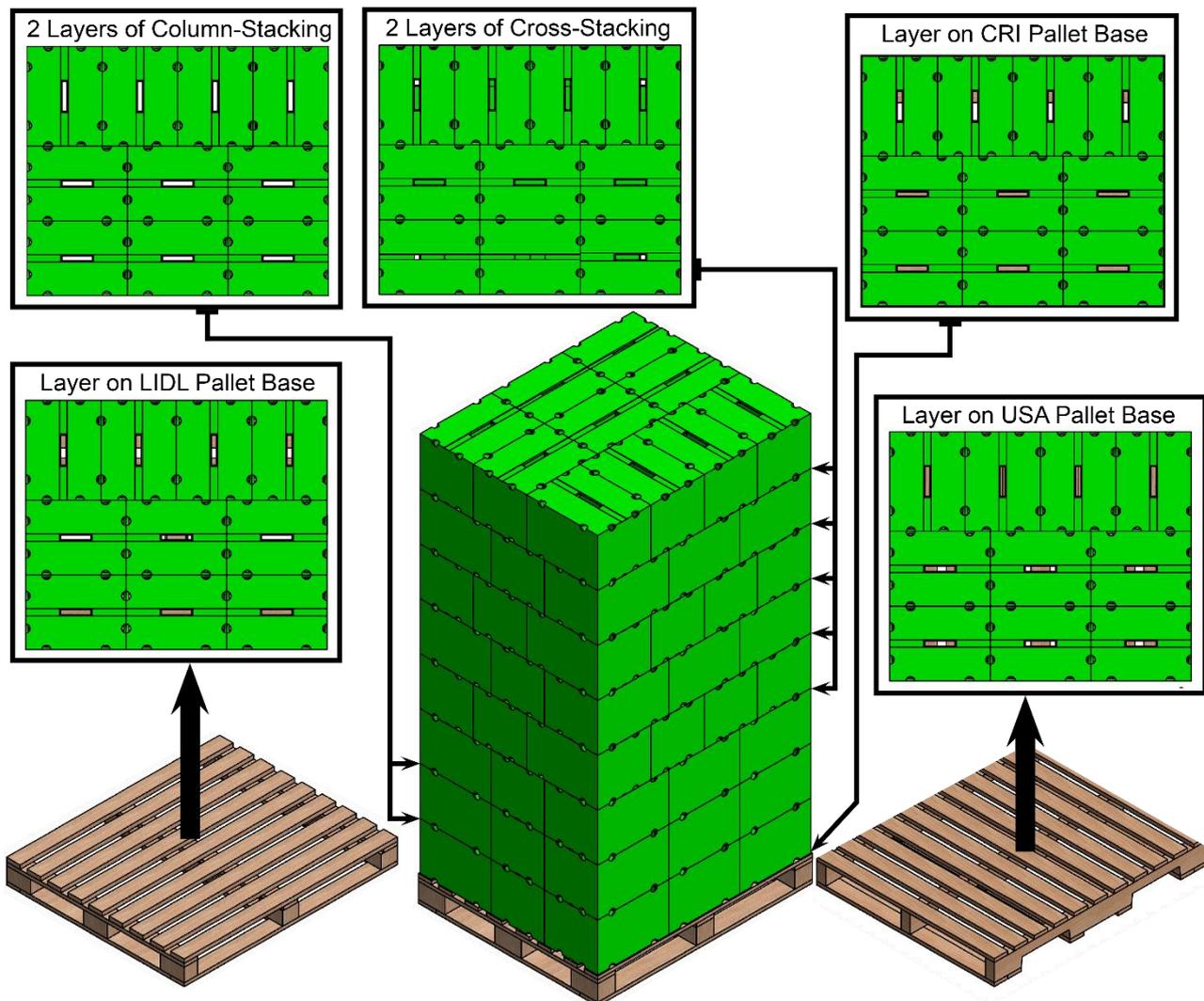
Figure 5.2.2.9 depicts the unobstructed vented areas at each of the unique interfaces along the vertical axis of the carton. The A15C carton is one of the heavier pallet structures due to its higher packing density and is often coupled with alternative pallet bases. The A15C was thus evaluated when using the standard CRI, the LIDL and USA pallet bases.

To improve pallet stability during shipping, the A15C cartons are stacking with a partial cross-stacking pattern. This brickwork lattice helps to stabilise the pallet structure by resisting shearing forces. Column stacking can

result in the pallet tipping as the individual columns of cartons shear past each other. Consequently, the current approach is to column stack the first 3 layers and then cross-stack the remaining layers.

There are several mechanical considerations with a cross-stacking approach. Namely, cartons can support more weight if the forces (i.e. the weight of higher packaging) are directed towards the corners instead of the edges. Cartons manufactured for column-stacking can therefore use less paperboard and are more cost-effective. Cartons along the bottom layers of a pallet stack need to support comparatively more weight. The current approach in the industry is to apply column-stacking for only the first three bottom layers of a pallet structure, which takes advantage of the more robust corner principle.

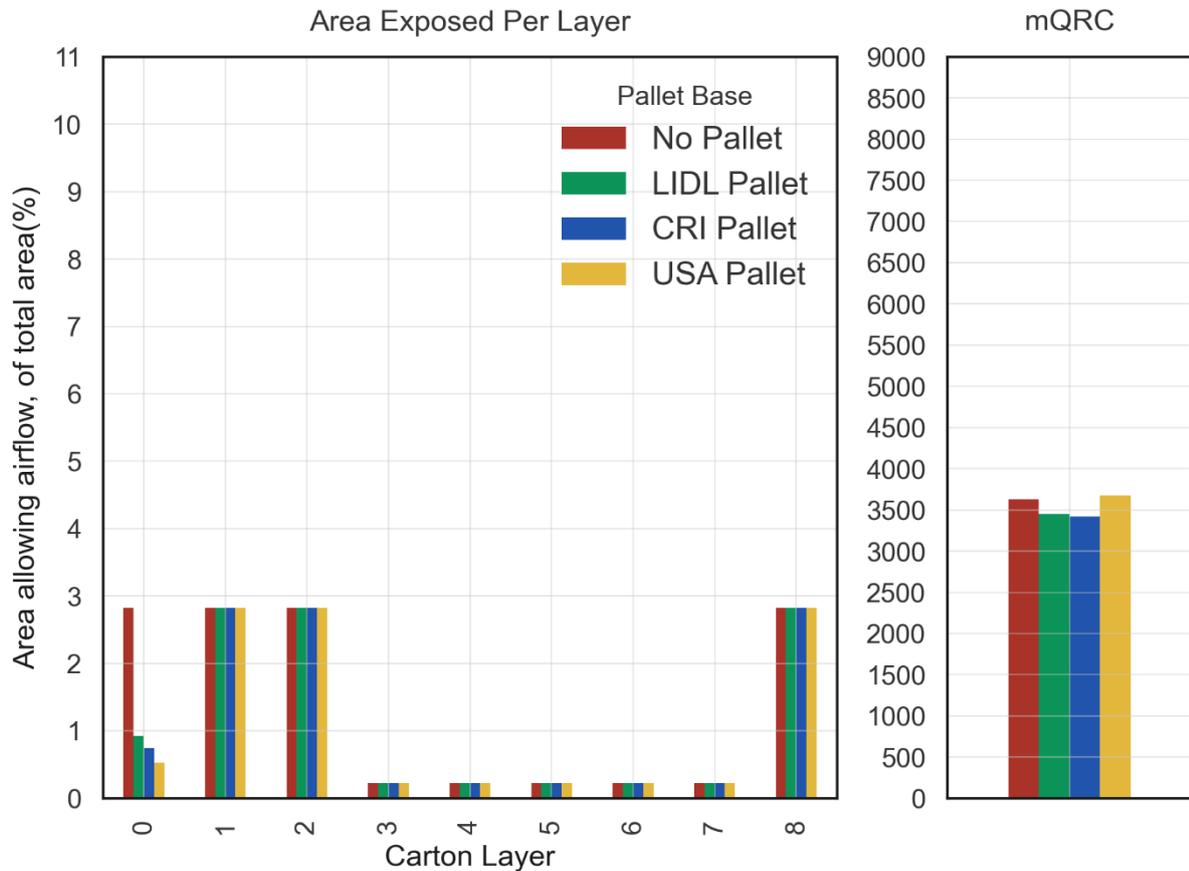
A negative consequence of cross-stacking is that it is not conducive to vent hole alignment and significantly reduces the pallet structure's porosity. Past packaging design approaches prioritised strength versus cooling. However, with an increasing emphasis of the applications of cooling for phytosanitary treatments, this strategy needs to be revisited.



**Figure 5.2.2.9.** A15C Stack on a CRI Pallet Base displaying open area between different interfaces in vertical flow.

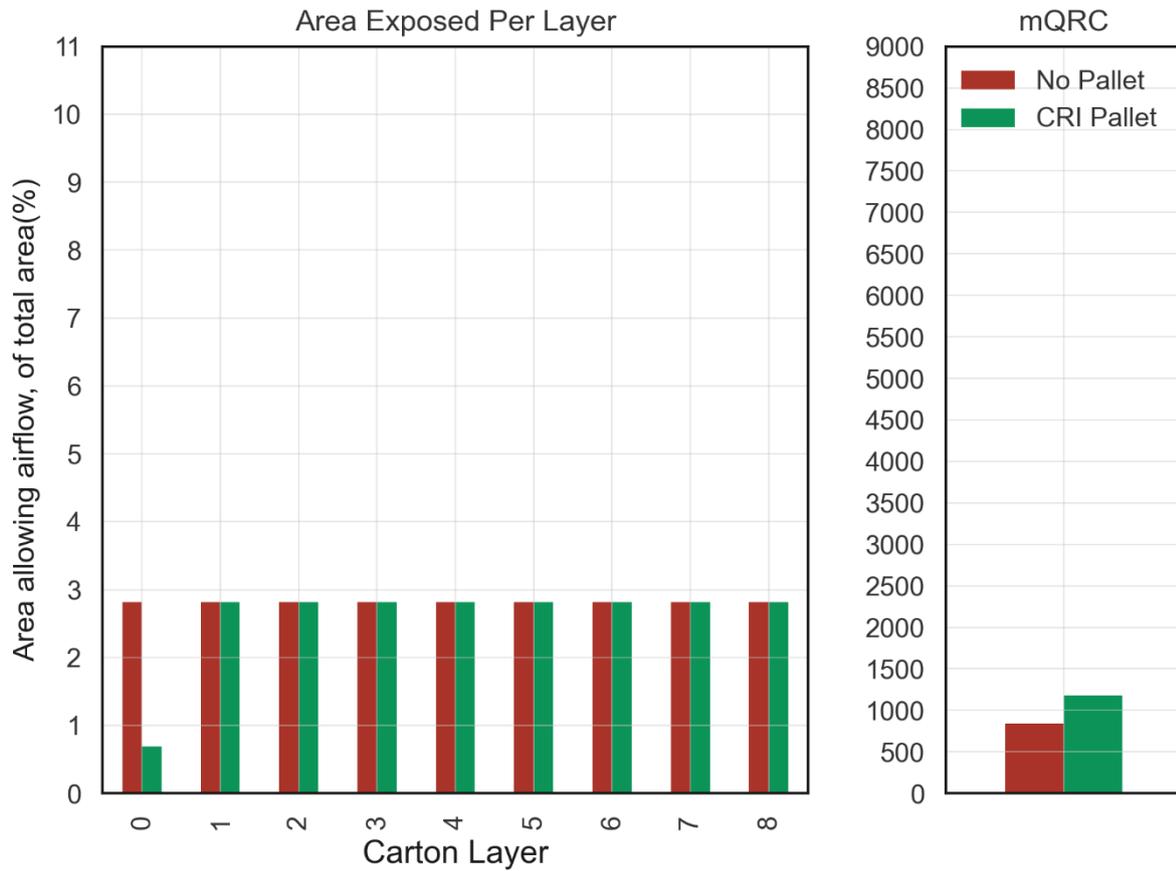
Figure 5.2.2.10 shows the calculated TVA for each interface along the vertical axis and the mQRC, which was measured using the experimental wind tunnel. Carton layer 0 represents the interface between the pallet base and the first carton layer. In a scenario where no pallet base is used, the TVA was 2.9%, which equates to the available, unobstructed vent hole area of the cartons. The LIDL, CRI and USA pallets all reduced the TVA to between 0.5% and 1.0%. However, when examining the effect of the pallets on mQRC, no significant difference

was observable. This is a relatively surprising result, as it has generally be assumed that pallet bases have a more significant effect. A possible reason for this result could be that the cross-stacking layers at 4 of the interfaces was a significantly larger negative factor on air flow through.



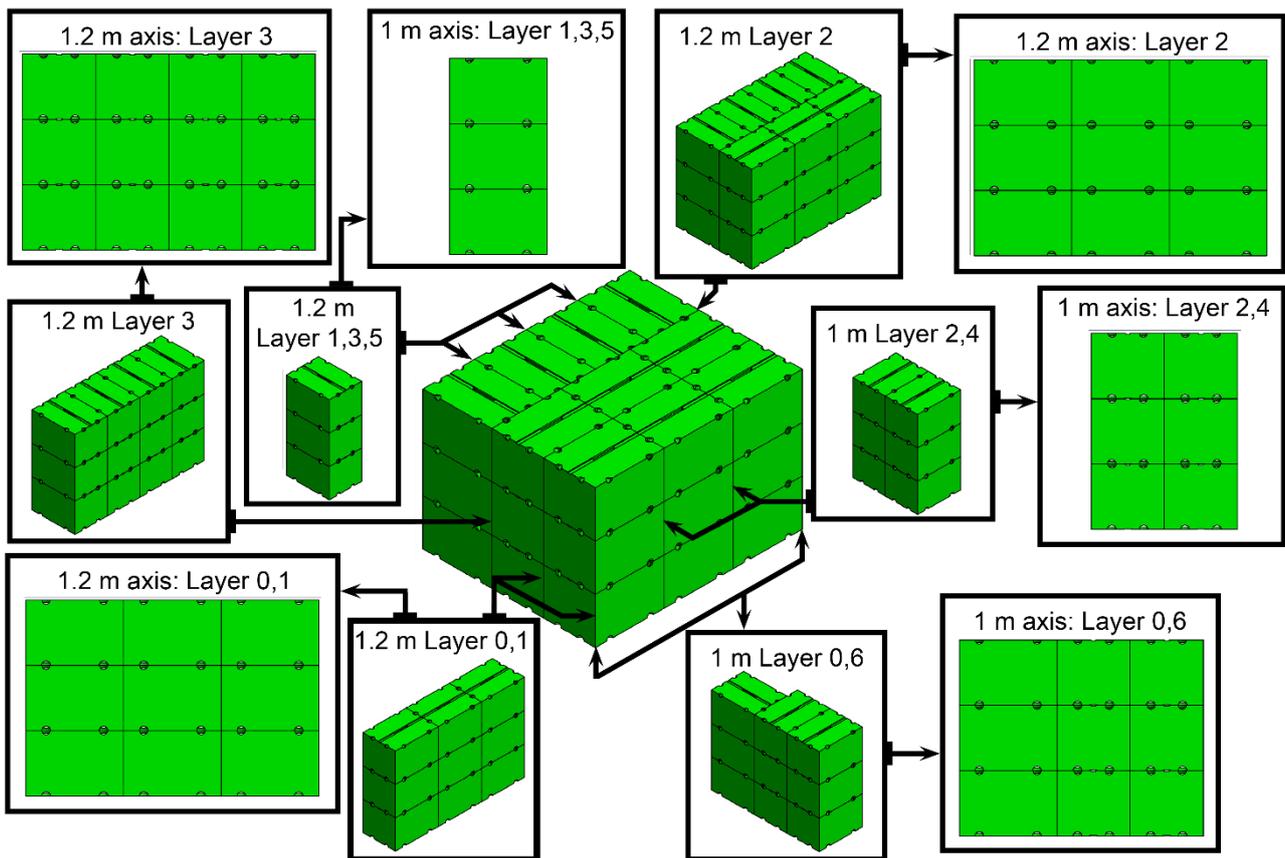
**Figure 5.2.2.10.** Total ventilation area for each layer and the modified QRC: A15C Cross-Stacking pallet (vertical).

Figure 5.2.2.11 depicts the TVA and mQRC values for an A15C pallet using only column-stacking. The results show a 70% reduction in airflow resistance. In contrast to the cross-stacking pallet, the presence of a pallet base increased the mQRC by 50%. The difference here is that the remaining interfaces have larger TVA sizes. The presence of a pallet base thus represents a much more significant point of resistance to the system.



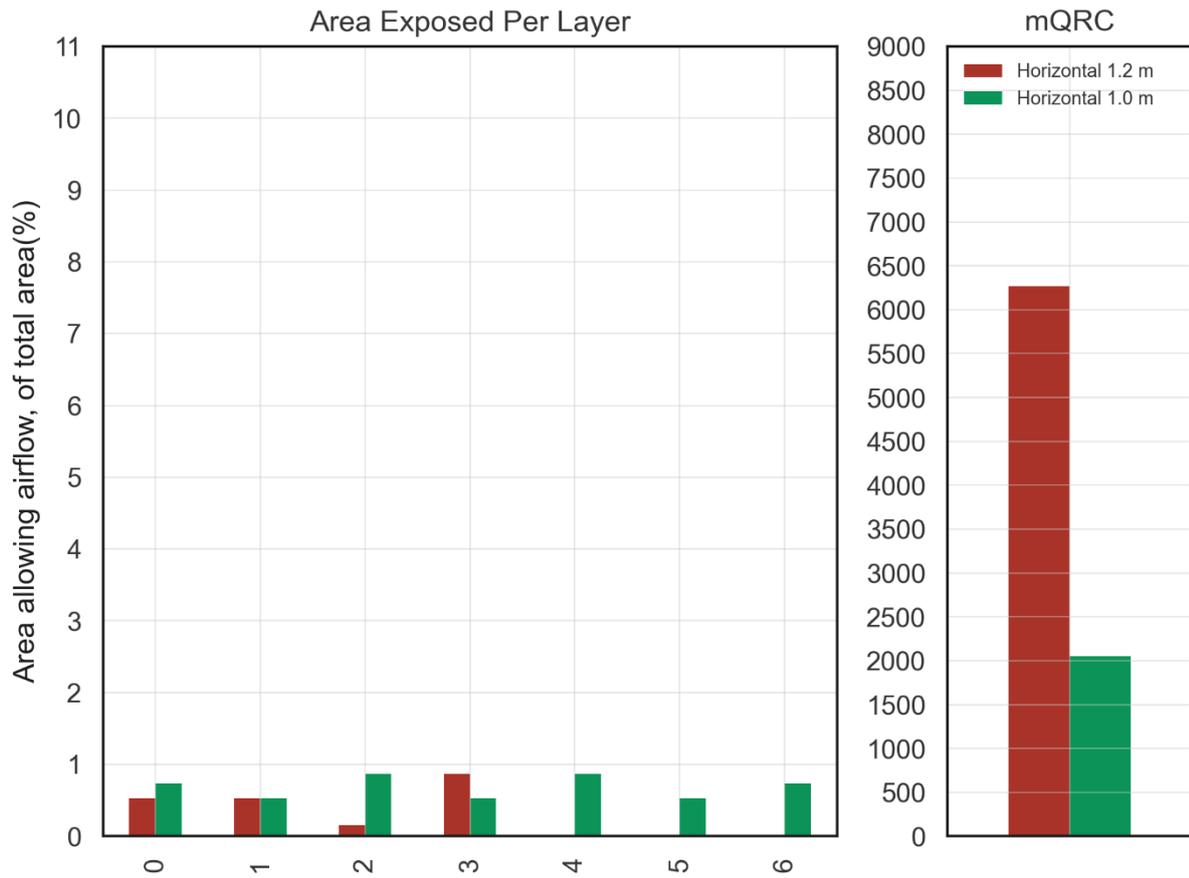
**Figure 5.2.2.11.** Total ventilation area for each layer and the modified QRC: A15C Column-Stacking pallet (vertical).

Figure 5.2.2.12 illustrates the various interfaces along the two (1.0 m and 1.2 m) horizontal axes in an A15C pallet stack. Although there are few interfaces along the horizontal axis, they are more complex as the cartons interlock with each other. Therefore, there is a network of airflow passages in both series and parallel, through which air can flow.



**Figure 5.2.2.12.** A15C Half a Pallet Stack Side View.

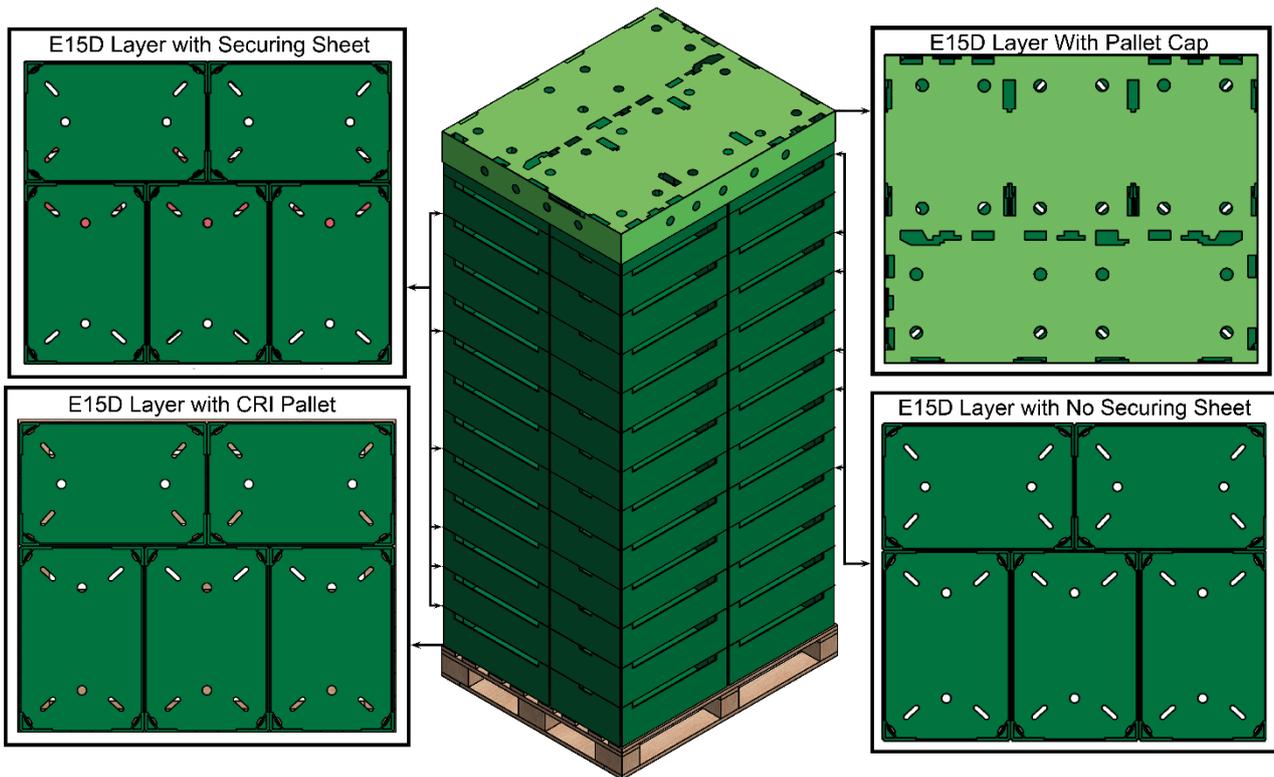
The calculated TVA values and experimentally measured resistance to airflow coefficient (mQRC) are listed in Figure 5.2.2.13. The interfaces against the 1.0 m axis (airflow is perpendicular to the pallet face that is 1.0 m wide) has between 4 and 5 interfaces, while the 1.0 m has 4 interfaces. Vent holes along interface 2 (1.0 m axis) are largely obstructed (0.15%), resulting in an increased airflow resistance of 175%. It should be noted that the mQRC value is adjusted for bed depth and the overall pressure loss (unmodified QRC) for the horizontal axis are, thus, in reality, about 50 less (half the depth) than the vertical axis. Commercially, FAC is performed against the 1.0 m axis (the lower resistance axis), making the high-pressure loss values a minor challenge. However, this may have an effect on the less dominant horizontal flow during container cooling.



**Figure 5.2.2.13.** Total ventilation area for each layer and the modified QRC: A15C Cross-Stacking Carton (Horizontal).

**E15D carton**

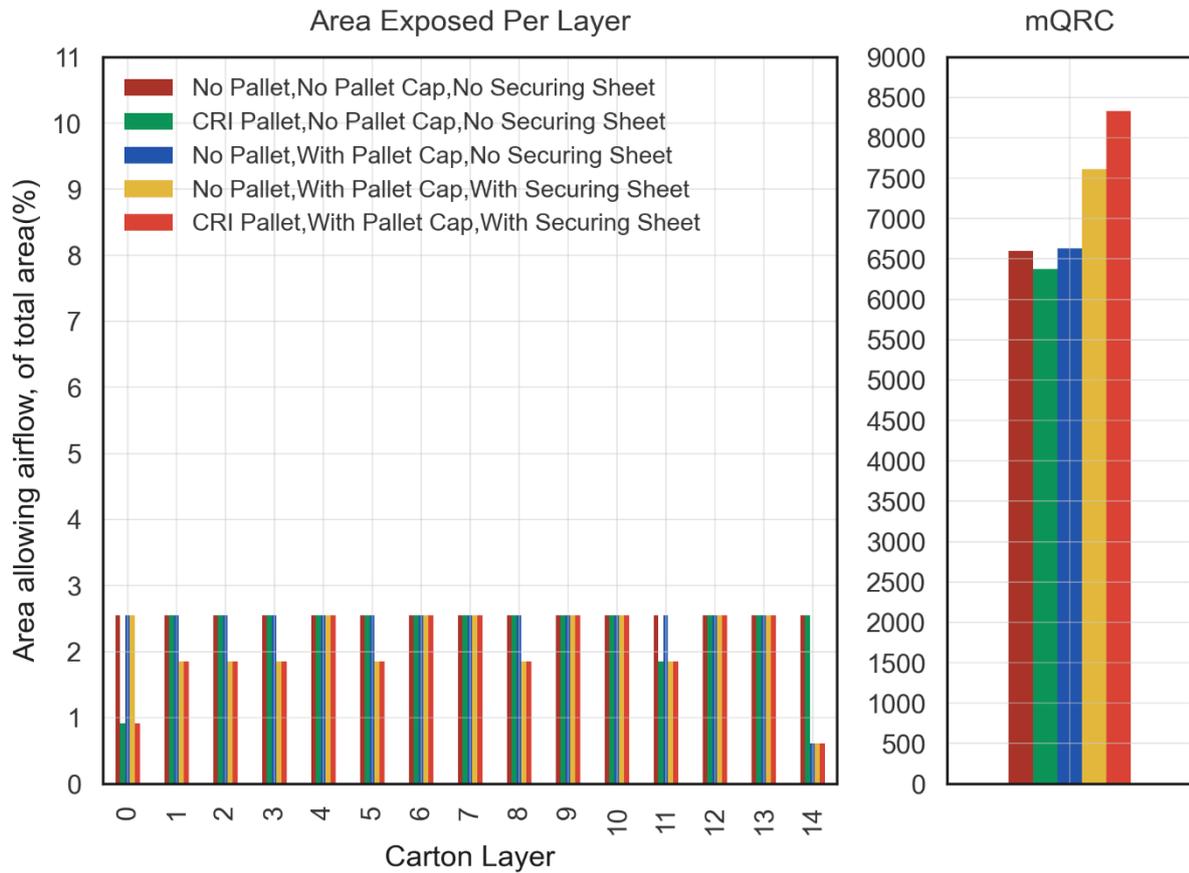
The design of the E15D carton is targeted as a display-ready package to be placed directly on retail displays in the store. The cartons are thus relatively low in height for the cartons to visually display only a single or double layers of fruit. Consequently, the pallet structures have many more carton layers (vertically) than the A15C pallets, resulting in more interfaces and overall higher resistance to airflow. A schematic of the E15D pallet stack, with a pallet cap and securing sheets on a CRI pallet, is shown in Figure 5.2.2.14. The stacks are column-stacked and include the use of securing sheets at specific layers to improve structural stability.



**Figure 5.2.2.14.** E15D Pallet Stack, With Securing Sheets and Pallet Cap on a CRI Pallet

For the E15D, the air travels through the intersection between the CRI pallet base and the first layer of cartons, a series of interfaces between 2 column-stacked layers or a layer and a securing sheet, and through the interface between the top layer and the pallet cap. For pallet stacks without the CRI base, securing sheet or pallet cap, the respective layers are the same.

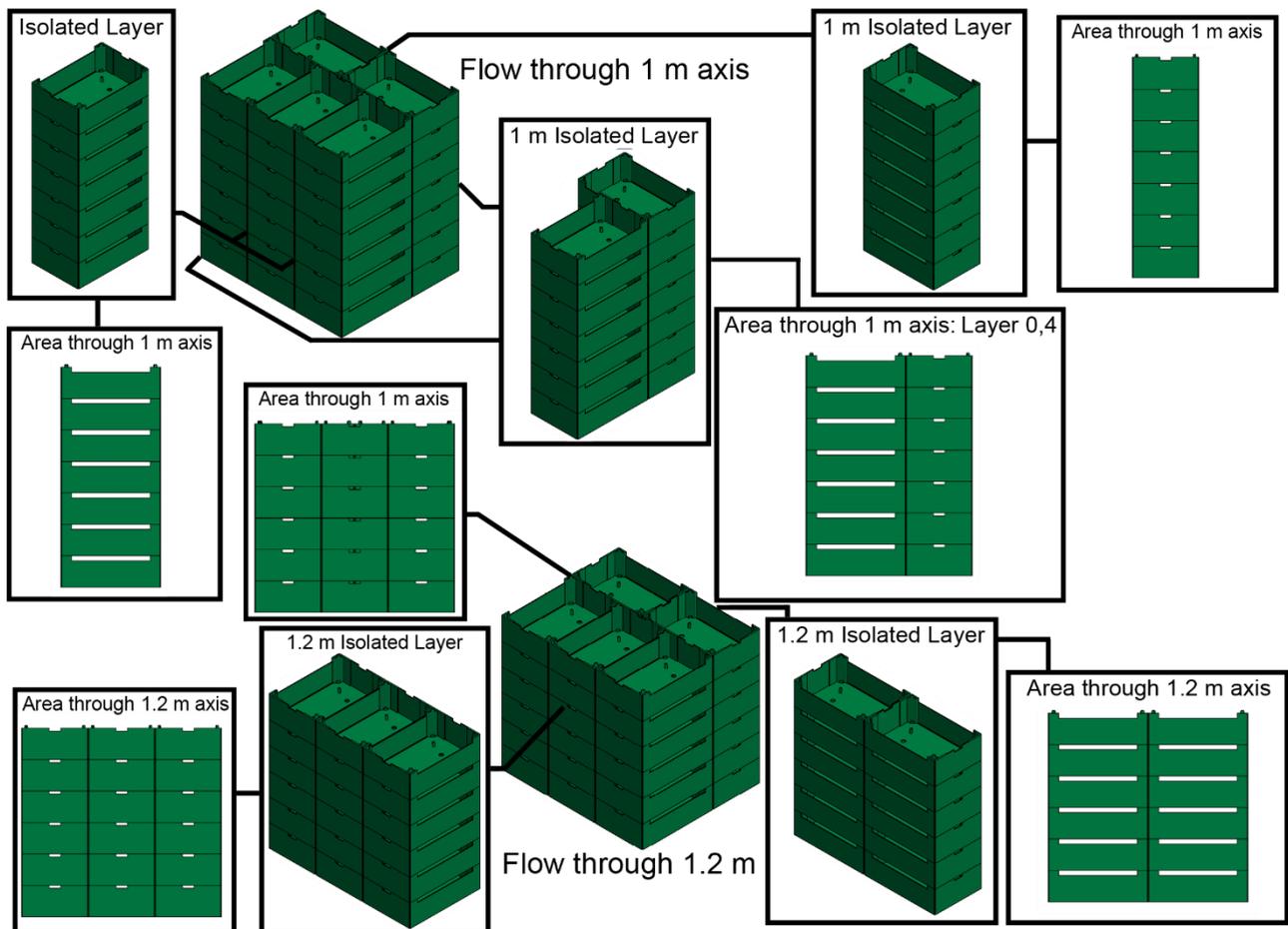
As seen in Figure 5.2.2.15 the E15D Layer with No Securing Sheet (no additional components) has the highest ventilation with the least ventilation obstructions, followed by the E15D Layer with Securing Sheet, E15D Layer with CRI Pallet and lastly, the E15D Layer with Pallet Cap.



**Figure 5.2.2.15.** Total ventilation area for each layer and the modified QRC: E15D Column-Stacking pallet (vertical).

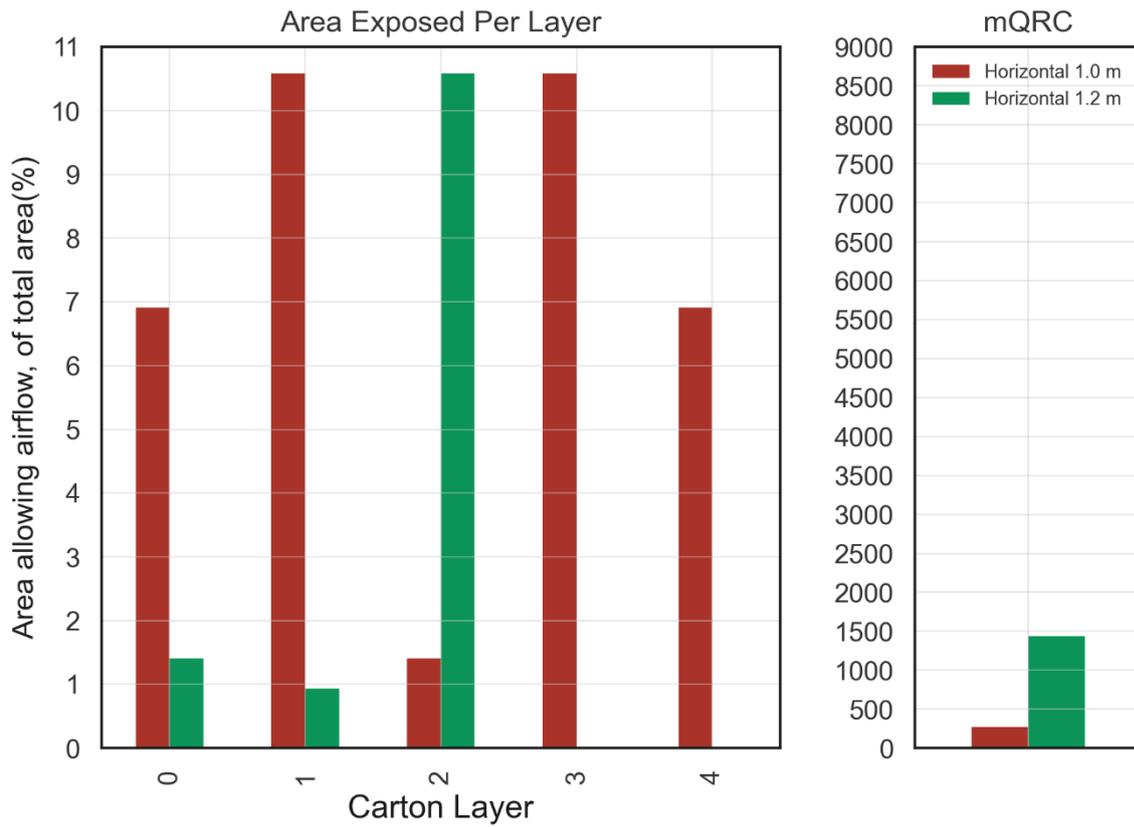
When compared to a standard A15C pallet (9 interfaces), the standard E15D (14 interfaces) carton has considerably more ventilation per layer but also has more interfaces. The net result is a resistance to airflow coefficient that is more than 7 times greater than the A15C. The presence of the CRI pallet, securing sheets, and pallet caps increase the resistance to airflow by about 30%, which is in a similar range of improvement to the column stacked A15C stack.

Figure 5.2.2.16 illustrates the various interfaces along the two (1.0 m and 1.2 m) horizontal axes in an E15D pallet stack. The large footprint of the E15D carton (600 × 400 mm) results in fewer interfaces (carton walls) than other carton designs. Fortunately, FAC is also applied commercially through the 1.0 m axis, which, due to the stacking pattern, has no misalignments of the vent hole.



**Figure 5.2.2.16.** E15D Half a Pallet Stack Side View

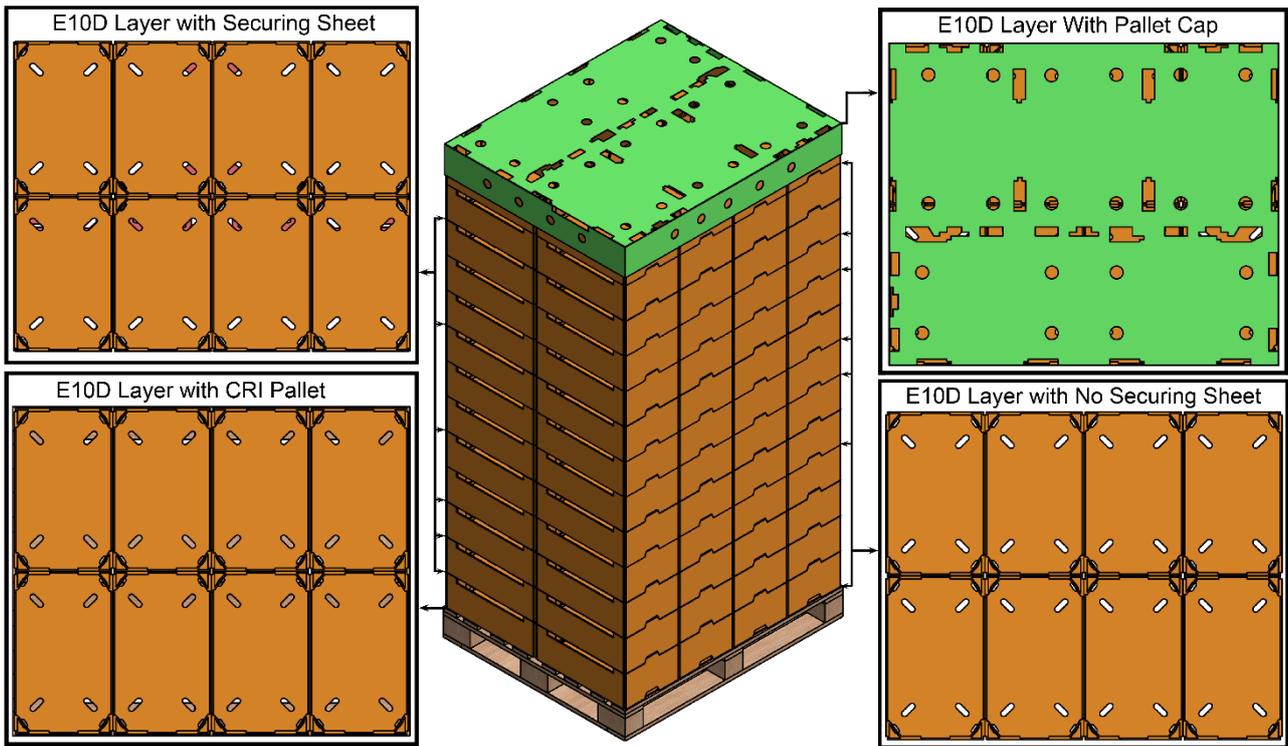
Figure 5.2.2.17 illustrates the high vent hole area along the 1.0 m axis of the pallet, which resulted in a resistance coefficient of about 200. The 1.2 m axis, had considerably higher vent hole misalignments, resulting in a generally lower TVA. Although higher than the 1.0 m axis, the reduced number of interfaces still ensured the mQRC was comparably (A15C) low (~1500).



**Figure 5.2.2.17.** Total ventilation area for each layer and the modified QRC: E10D Column-Stacking Pallet (Horizontal).

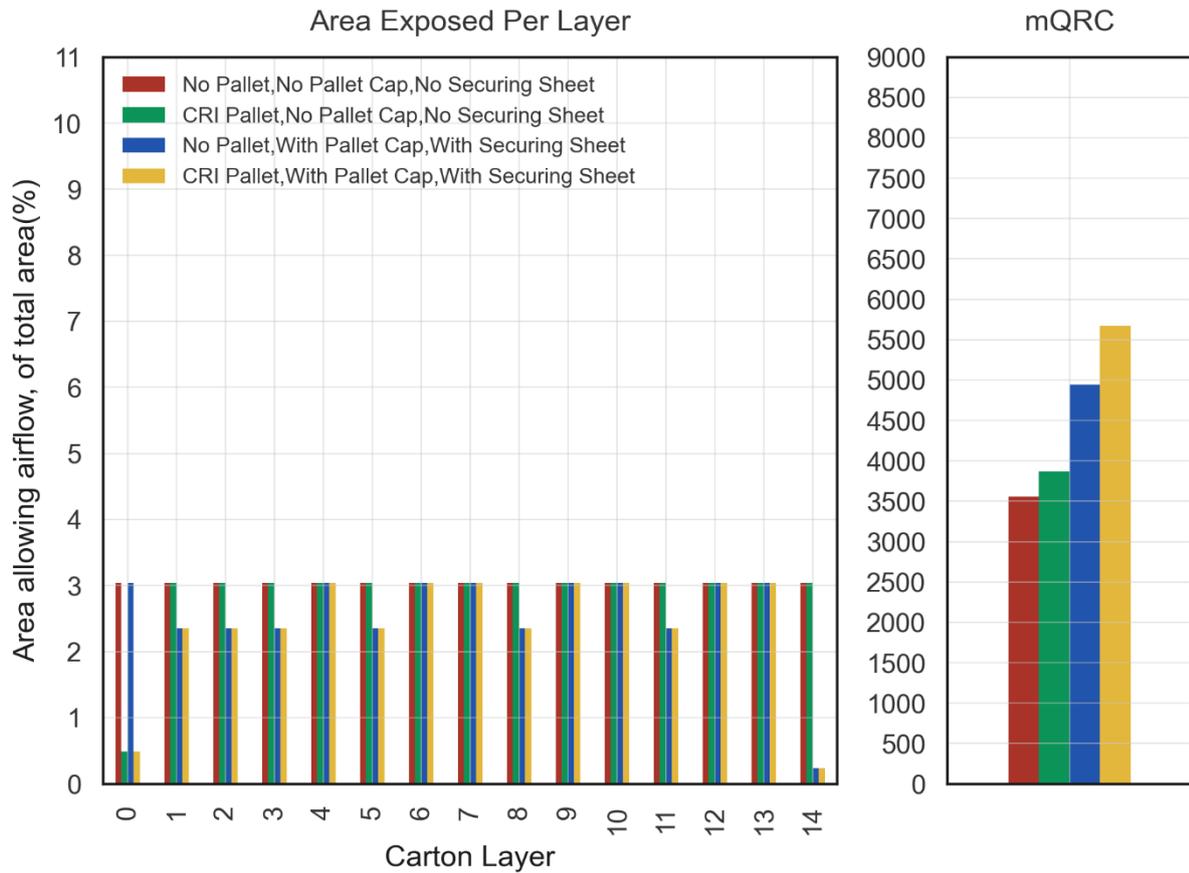
**E10D carton**

Similar to the E15D, the E10D is also a display-ready package with a similar number of interfaces along the vertical axis. Additionally, the E10D stack also makes use of column-stacking and include the use of securing sheets at specific layers to improve structural stability. Figure 5.2.2.18 illustrates the E10D stack and the various unique interfaces at play.



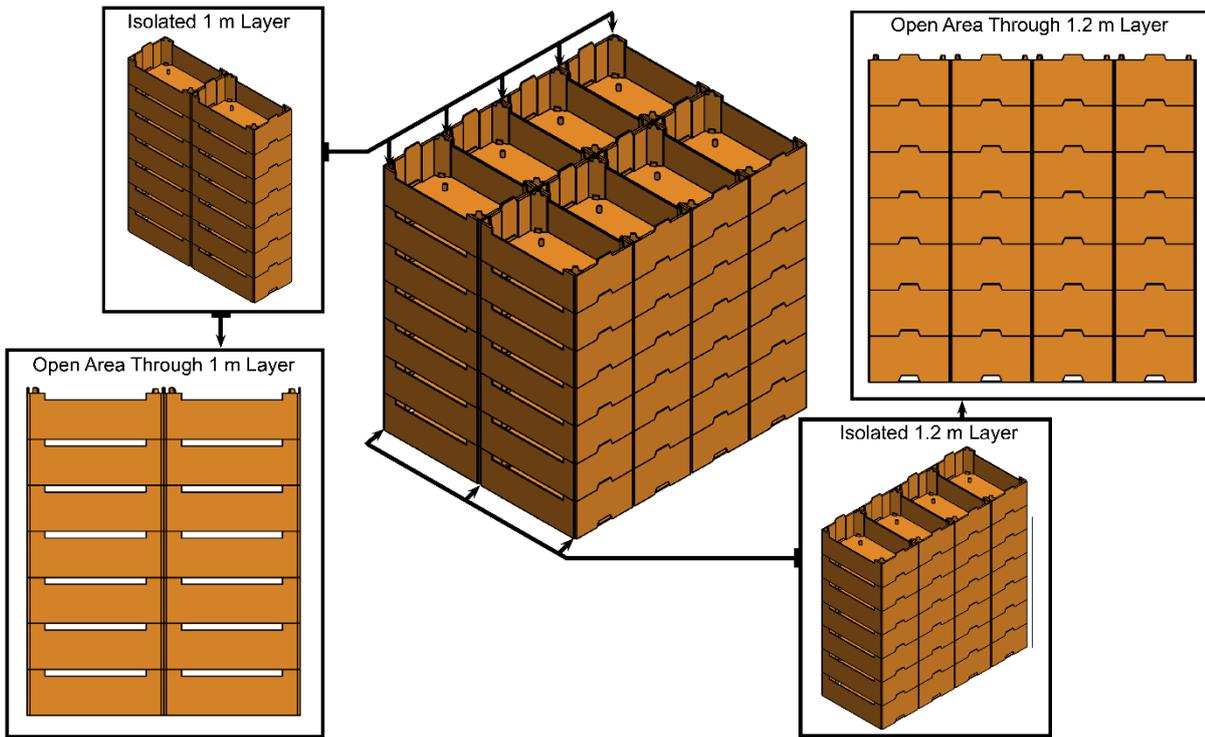
**Figure 5.2.2.18.** E10D Pallet Stack, With Securing Sheets and Pallet Cap on a CRI Pallet

As seen in Figure 5.2.2.19, the E10D Layer with No Securing Sheet (no additional components) has the highest ventilation with the least ventilation obstructions. Substantial benefits concerning pressure loss were observed when removing the pallet cap and securing sheets. The larger TVA (compared to the E15D) resulted in lower airflow resistances (~30% smaller), although the many more ventilation openings may also be a contributing factor (Delele *et al.*, 2013). The results further show a substantial decrease (~45%) in pressure loss when eliminating vent hole obstructions from the securing sheets.

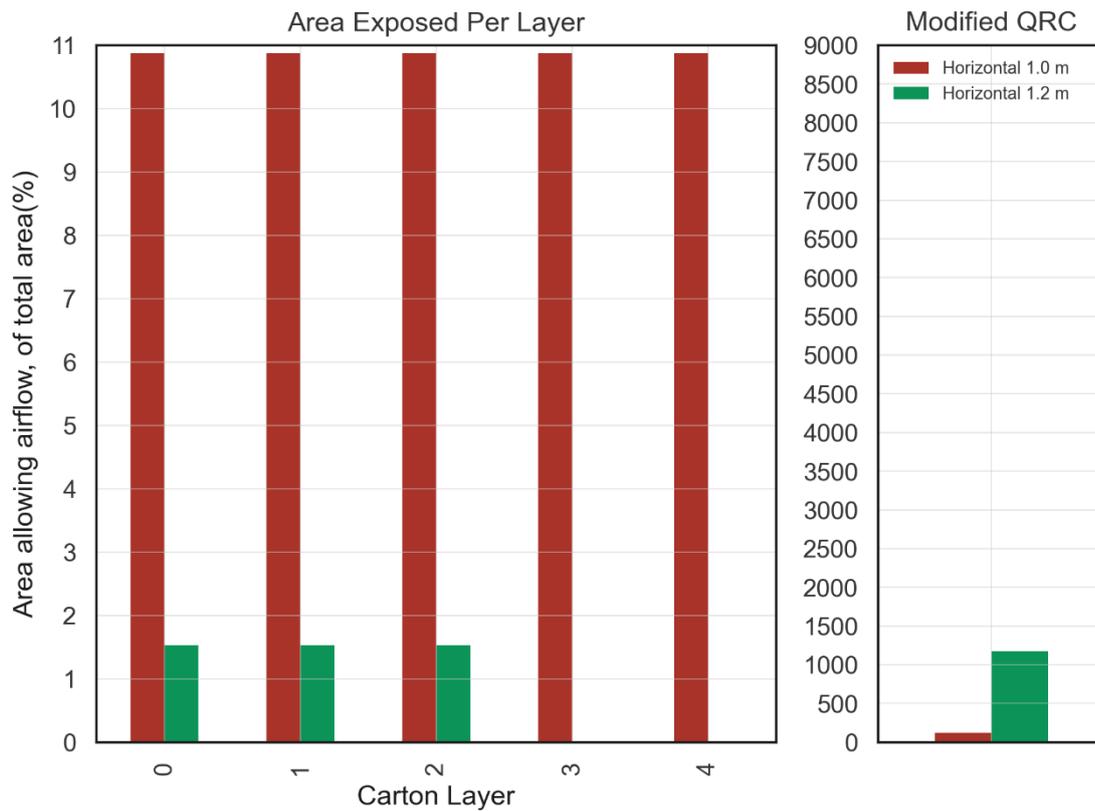


**Figure 5.2.2.19.** Total ventilation area for each layer and the modified QRC: E10D Column-Stacking Pallet (Vertical).

Figure 5.2.2.20 and Figure 5.2.2.21 show the horizontal interfaces and airflow properties of the E10D pallet, respectively. The large TVA along the 1.0 m axis resulted in one of the lowest resistance to airflow coefficient values observed in this study. It should be noted that single large vent holes were positioned at the top of the carton and allowed airflow to bypass the individual fruit lying along the bottom of the carton. However, the lower resistance does increase the overall refresh rate through the carton. The vent holes along the 1.2 m axis, in contrast, were significantly smaller and thus produced a resistance coefficient only slightly lower than the E15D 1.2 m axis.



**Figure 5.2.2.20.** E10D Half a Pallet Stack Side View

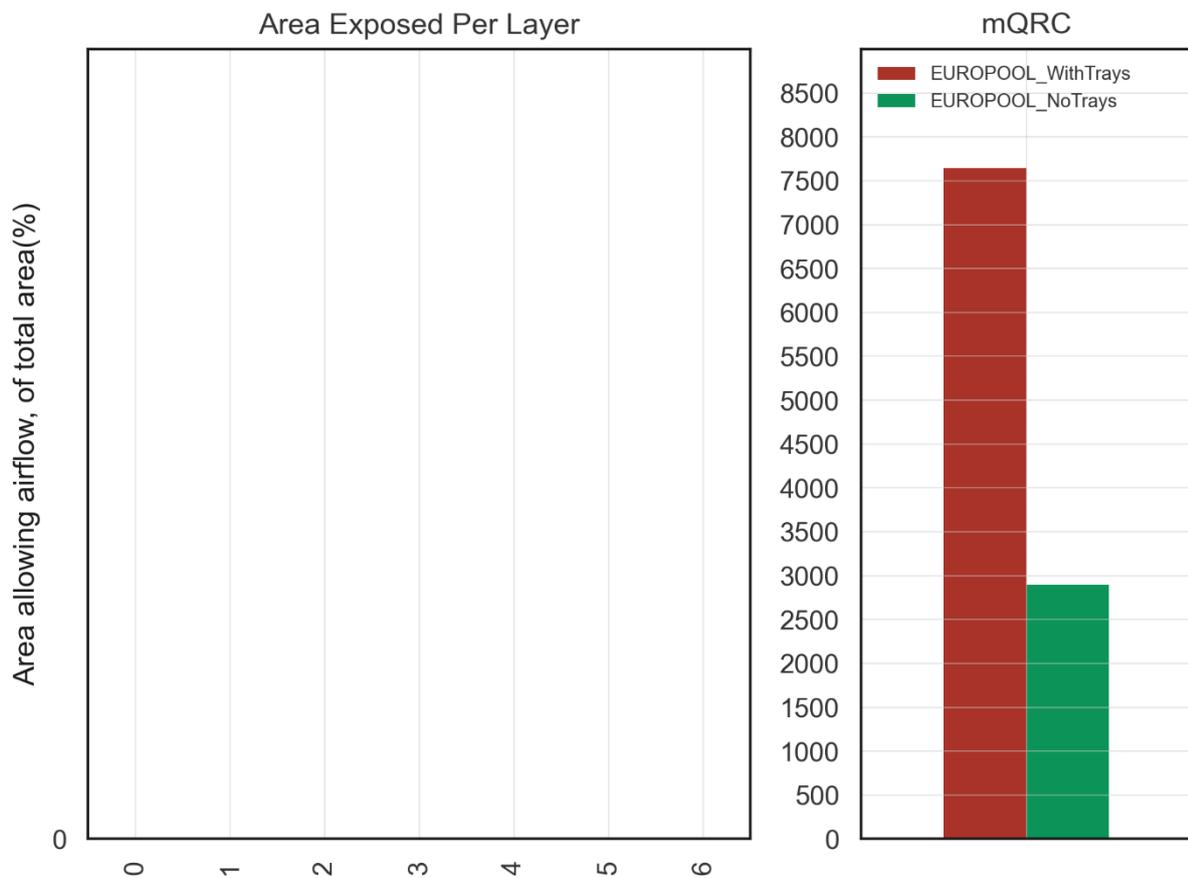


**Figure 5.2.2.21.** Total ventilation area for each layer and the modified QRC: E10D Column-Stacking Pallet (Horizontal).

### Europool crates

Europool crates (600 × 400 mm) are reusable plastic boxes, which are recirculated/reused between the EU and South Africa. Pallet stacks are comprised of about 14 layers. All the walls of the crates are covered with a complex grid of ventilation openings, which is difficult to measure, but that has a TVA larger than the previously discussed cartons. Imports often require the Europool crates to be packaged with poorly ventilated trays, which can restrict airflow.

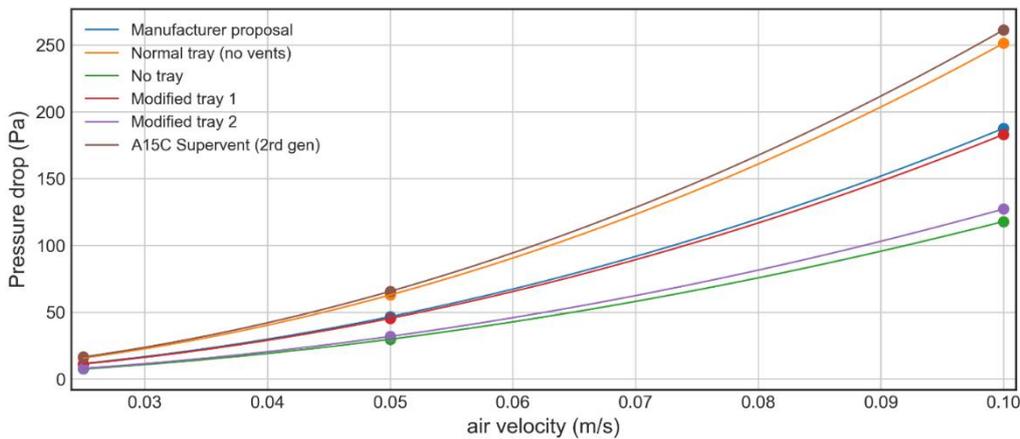
Figure 5.2.2.22 shows the mQRC for Eurocrates packed with and without trays. Without trays, the mQRC was about 2900, which is lower than the standard A15C pallet stack. However, with the use of trays, the pressure loss coefficient increased by 260%. Careful examination of the setup showed that the respective trays were too large for the Europool crates interior. Every gap and opening inside the crate was thus obstructed. Furthermore, the trays were not optimised for citrus fruit, and as a result, all vent holes in the trays were entirely obstructed by the packed fruit.



**Figure 5.2.2.22.** Modified QRC: Europool crates with and without trays (vertical).TVA measurements not included.

### Trays in citrus cartons

Figure 5.2.2.23 shows the pressure loss curves for pallets stacks packed with and without trays, as well as when using various ventilated tray designs. Additionally, the A15C pallet stack was also modelled, which provides a comparative benchmark to the other scenarios. It should be noted, that the pressure loss curves in combination with the refrigeration units, fan system will determine the eventual airflow rate, which relates to fruit cooling rate. Low pressure loss curves are thus desirable and will facilitate improved cooling airflow penetration into the pallet stacks at higher flow rates.



**Figure 5.2.2.23.** Pressure loss curves for the various packaging scenarios.

Results show the A15C Supervent stack has a similar pressure loss to an opentop carton stack using unventilated trays (using a highly ventilated opentop with only 9 layers – high depth). The critical difference here, is that the Supervent cartons direct cool airflow in between the fruit, whereas the unventilated trays redirect airflow around the fruit.

The results (Figure 5.2.2.23) show that the addition of unvented trays more than doubled (110%) the pressure loss across the system (just cartons). In contrast, the inclusion of the ventilated trays increased pressure loss by 56%, compared to packaging without trays. No significant difference was observed between the two ventilated tray designs. The results indicate the vent holes must be in line with the carton vent holes to be beneficial to the system.

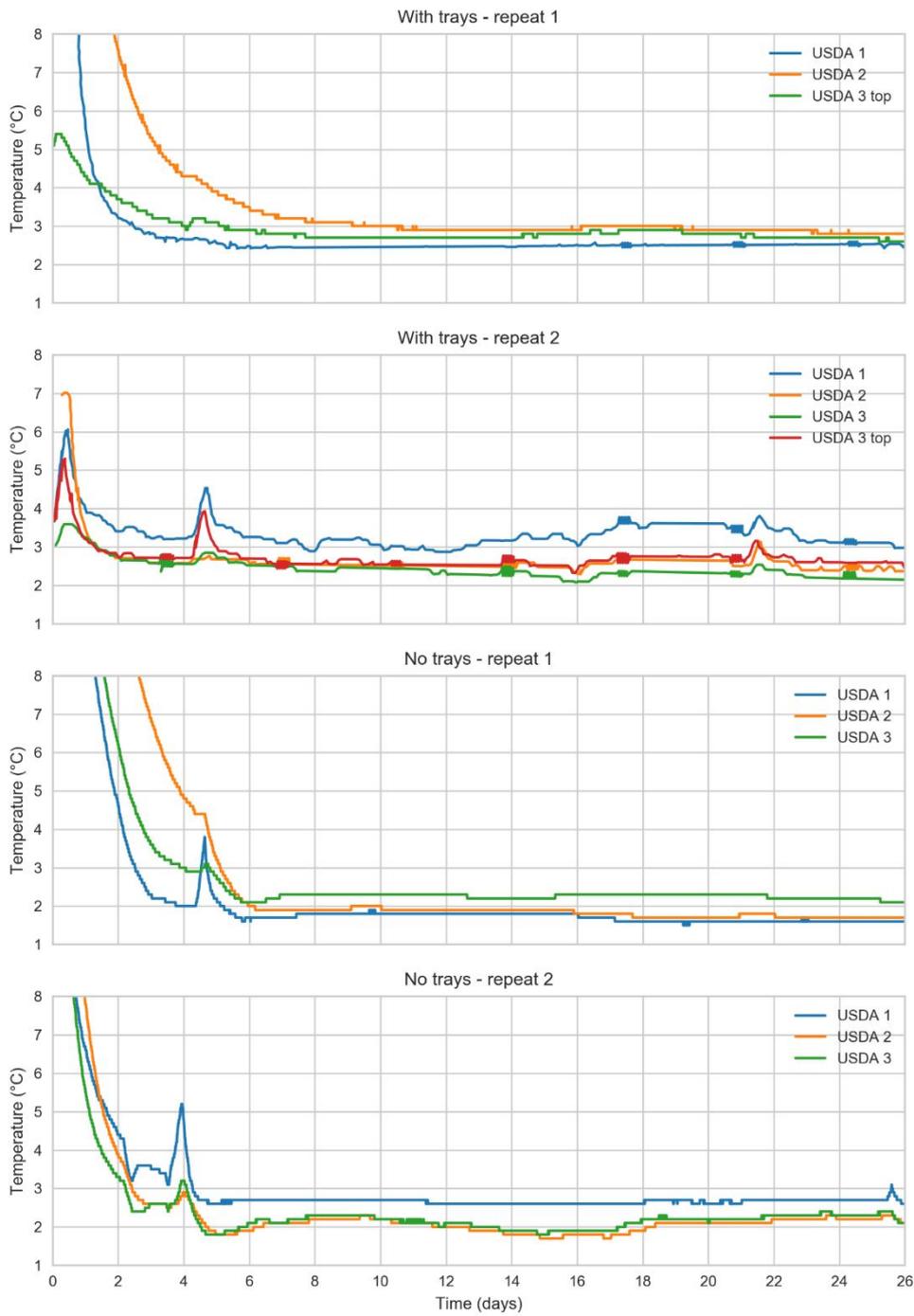
### Container evaluations

Two high-cube containers were stuffed with single layer cartons containing grapefruit packed in trays. Initial fruit temperatures were between 5 and 13°C and container set-point was 2°C. Pulp temperature loggers (TempTale4, Sensitech United Technologies, Prague) were positioned at the USDA 1, 2, and 3 positions. An additional pulp temperature logger was also placed in the top of the USDA 3 pallet; this region was monitored as it is known for its inadequate cooling (i.e. common hotspot).

As a control, USDA 1, 2 and 3 TempTale pulp temperature logger data was recorded for two containers which were stuffed with E15D cartons place-packed with grapefruit (cv. Star Ruby). In this case, container set-point was also 2°C, fruit were loaded at a warmer temperature (~18°C) and the initial cool down occurred under warmer outside temperatures.

Figure 5.2.2.24 shows the USDA cooling curves for the containers packed with and without trays. Table 5.2.2.1 further provides a summary of the average pulp temperatures in 3-day increments. The results show that by day 6, fruit in the control containers had both reached temperatures below 3°C. However, containers packed using trays cooled less rapidly, with all probes only approaching 3°C after about 8 days. Additionally, the containers packed with trays both had one USDA probe that did not cool down effectively and lingered near the 3°C mark throughout the voyage. Both tray-packed containers thus had loggers reporting pulp temperatures above 3°C up until day 18. One of the tray containers further remained above 3°C throughout the voyage. These two loggers suggest the presence of hotspots in the container, which are likely also present in other unmonitored regions of the container.

Average temperatures after cool-down (> 9 days) was about 2.7°C and 2.1°C for the tray-packed and control containers, respectively. The tray-packed containers were thus notably warmer than the control containers (~0.5°C).



**Figure 5.2.2.24.** Temperature curves at the USDA positions for containers packed with and without trays.

**Table 5.2.2.1.** Comparison between the average temperatures in 3-day increments for each of the containers. Red text indicates temperature above 3°C, which is considered inadequate for successful FMS applications.

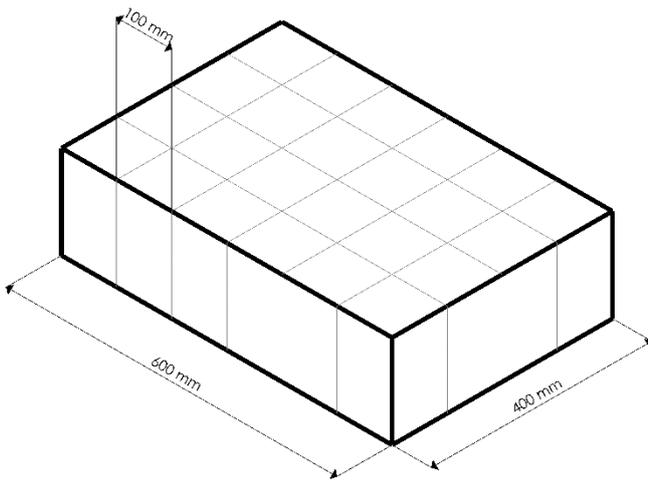
Date range (days)	Logger position		With Trays - repeat 1 (°C)	With Trays - repeat 2 (°C)	No Trays - repeat 1 (°C)	No Trays - repeat 2 (°C)
0-3	USDA (top)	1	4.3	7.7	7.8	6.5
	USDA (mid)	2	4.1	10.3	12.8	7.0
	USDA (mid)	3	3.0		9.5	5.7
	USDA (top)	3	3.6	4.1		
3-6	USDA (top)	1	3.6	2.6	2.1	3.1
	USDA (mid)	2	2.6	4.2	4.2	2.2
	USDA (mid)	3	2.6		2.8	2.3
	USDA (top)	3	3.0	3.1		
6-9	USDA (top)	1	3.1	2.4	1.8	2.7
	USDA (mid)	2	2.6	3.2	1.9	2.1
	USDA (mid)	3	2.4		2.3	2.2
	USDA (top)	3	2.6	2.8		
9-12	USDA (top)	1	3.0	2.5	1.8	2.7
	USDA (mid)	2	2.5	3.0	1.9	2.1
	USDA (mid)	3	2.4		2.3	2.2
	USDA (top)	3	2.6	2.7		
12-15	USDA (top)	1	3.1	2.5	1.8	2.6
	USDA (mid)	2	2.5	2.9	1.9	1.9
	USDA (mid)	3	2.3		2.2	2.0
	USDA (top)	3	2.6	2.7		
15-18	USDA (top)	1	3.4	2.5	1.7	2.6
	USDA (mid)	2	2.6	3.0	1.8	1.8
	USDA (mid)	3	2.2		2.3	1.9

	USDA (top)	3	2.6	2.8		
18-21	USDA (top)	1	3.6	2.5	1.6	2.7
	USDA (mid)	2	2.6	2.9	1.7	2.1
	USDA (mid)	3	2.3		2.3	2.2
	USDA (top)	3	2.7	2.8		

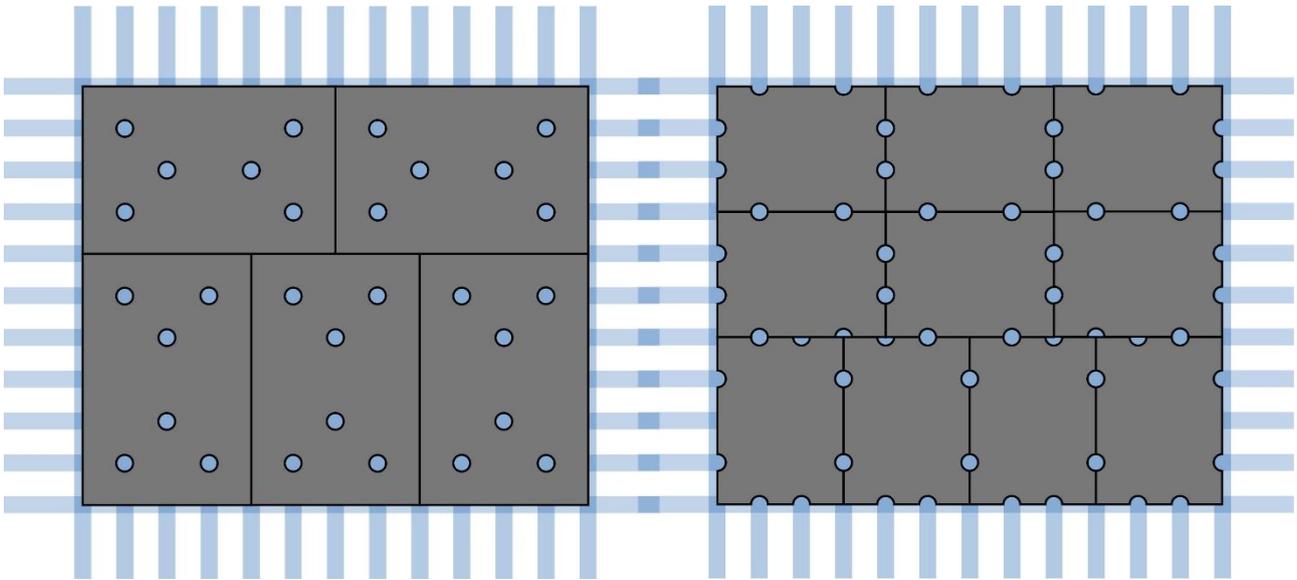
**Conclusions and recommendations**

**1.1. Designing Better Carton Ventilation**

The citrus industry used three stacking approaches, which are represented by the A15C, E15D and E10D cartons. The ratio between the pallet base width and length forces the respective cartons to stack in similar patterns. Interestingly, if a 100 mm grid is projected on top of each carton (Figure 5.2.2.25) in a pallet stack. The grid intersections lines would align when viewed from above. The 100 mm intersection points are thus the most optimal location for ventilation openings. Figure 5.2.2.26 further illustrates these intersection points across a pallet stack.

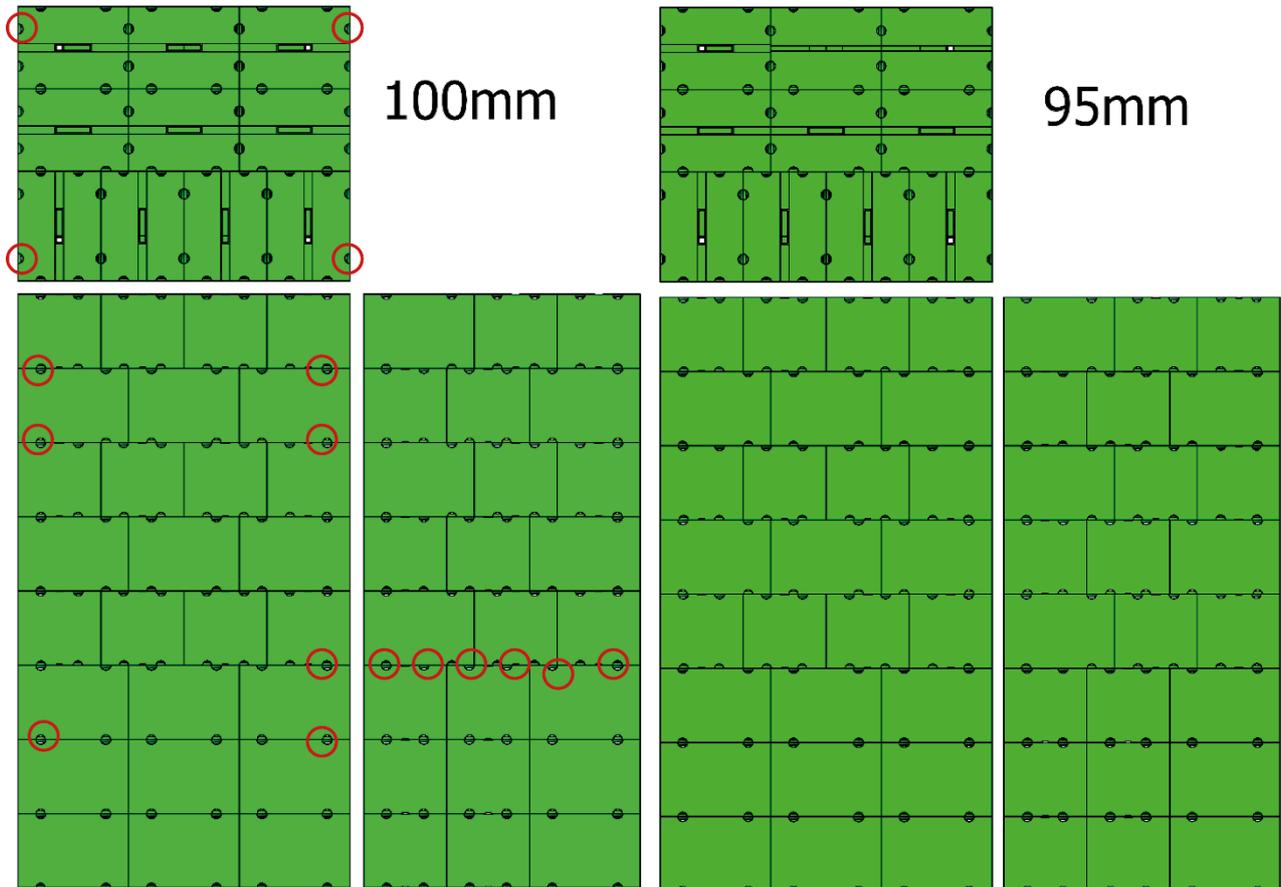


**Figure 5.2.2.25.** Illustration of 100 mm grid projected onto a citrus carton.



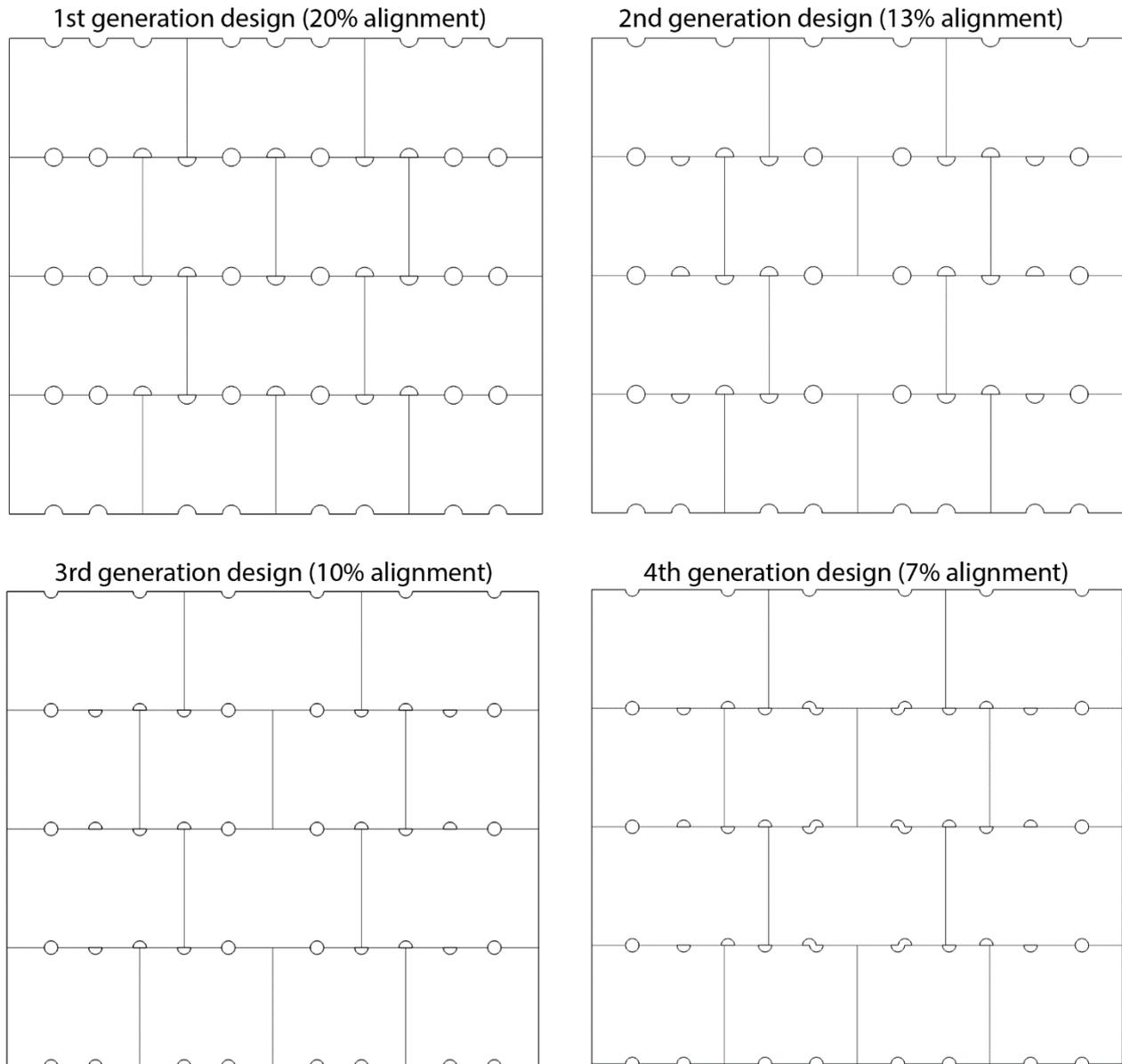
**Figure 5.2.2.26.** Illustration of the 100 mm grid projection on top of a full pallet stack.

Figure 5.2.2.27 is a comparison between the current A15C supervent (95 mm) carton design and a more optimal 100 mm version. As predicted, if a 100 mm approach is applied, there are significantly fewer obstructions in both the vertical and horizontal orientations (indicated by the red circles). This could lead to lower mQRC values as there could be a larger TVA in both the vertical and horizontal directions. In addition, the 100 mm design also has four larger openings in the vertical flow, indicated by the four red circles in the top view.



**Figure 5.2.2.27.** A15C pallet stack with the length between holes on the width set to 100 mm and 95 mm, respectively. Red circles indicate where vent holes better align than in the 95 mm approach.

Literature concerning the A15C Supervent was documented during the survey process. Four designs were identified and are illustrated in Figure 5.2.2.28. No known records have been kept by the industry regarding the actual usage (quantities) of each of these designs. However, the findings illustrate the point that vent hole designs can change and evolve while still being considered by most industry players to be identical. Nevertheless, these changes can potentially have substantial effects on cooling performance.



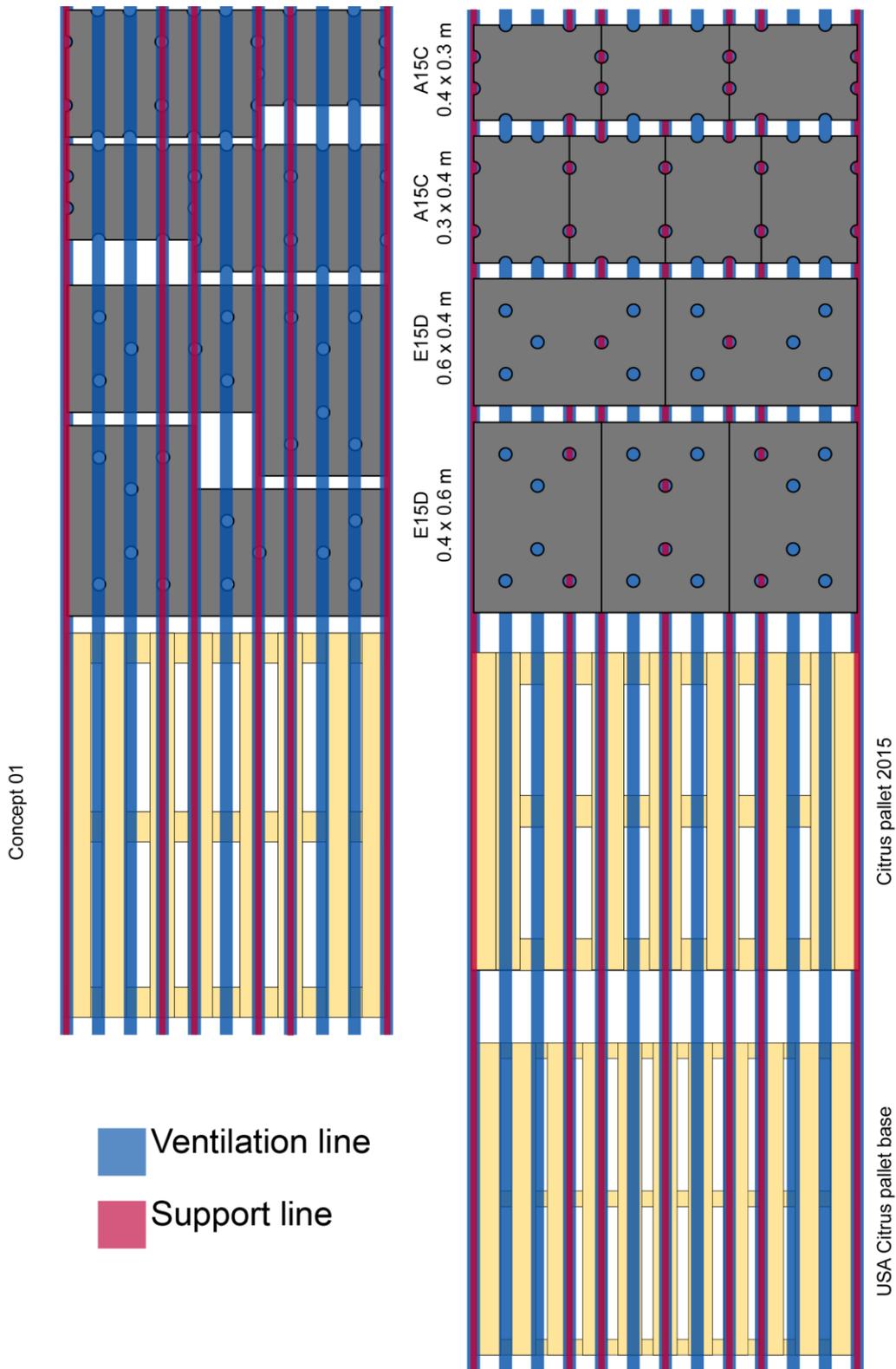
**Figure 5.2.2.28.** Evolution of the supervent citrus carton over the last decade.

### Exploring pallet base designs

Figure 5.2.2.29 shows an illustration of the interaction between pallet base slats and vent holes. In this case, vent holes are positioned along the 100 mm grid (Figure 5.2.2.25 and Figure 5.2.2.26). This consistent design strategy allows the pallet bases to be used for any carton design (as vent holes always align along the same grid). When stacking cartons on the CRI and USA pallet bases, there are 13 potential lines where vent holes could be positioned along. Seven of these (54%) must have slats as they also overlap with carton corners to support the pallet structure adequately. Of the remaining six lines, both pallet bases only have openings along four of the slats (31% ventilation).

The concept 01 pallet base in Figure 5.2.2.29 makes use of slats at a perpendicular angle. There are thus 11 potential lines, 6 of which need to be supported with slats (54%), which is an identical ratio as the other pallet

bases. The concept 01 pallet base leaves the remaining lines open, allowing for a substantially larger, 45% ventilation opening. Although promising, these changes need to be made while also considering the effect on strength and cost.



**Figure 5.2.2.29.** Illustration of vent hole alignment between pallet bases and bottom of cartons during stacks. Red lines indicate support for the corners of cartons, and blue lines show regions that need to be left open for ventilation.

## Conclusions

- The survey identified 3 primary export citrus carton designs, which were representative of 85% of all exports.
- Each of the 3 designs (A15C, E15D and E10D) were modelled in detail using CAD (computer-aided design) software with all the relevant additive/internal packaging. The main factors influencing cooling were found to be:
  - Securing sheets
  - Pallet bases
  - Pallet caps
- Past research has shown that cooling performance is directly related to resistance to airflow. The study thus developed an approach to quantify resistance to airflow using a single metric (mQRC).
- Cross-stacking caused severe restrictions at several interfaces (where carton layers meet), which more than tripled the resistance to airflow along the vertical axis.
  - Column stacking would thus significantly improve cooling performance.
  - However, implementing this approach could negatively influence the overall stability of the pallet. Stability issues thus first need to be addressed.
- Optimising the pallet base to improve ventilation is only beneficial when the rest of the pallet is optimised. Optimised pallet bases should thus not be prioritised unless the other more severe obstruction factors (e.g. cross-stacking, securing sheets) have been optimised.
- Use of non-obstructive securing sheets improved resistance to airflow by 45% and 18% for the E10D and E15D, respectively.
  - The study identified that using correctly designed securing sheets would be one of the most practical methods to reduce resistance to airflow in citrus packaging.
  - Securing sheets are currently being addressed in the industry.
- Along the vertical axis, the opentop pallet structures have airflow resistances twice as large as the A15C (Supervent cross-stacked). This was largely attributed to the many more interfaces (carton layers).
- Opentop cartons thus require much larger vent hole areas than A15C cartons to achieve similar cooling performances.
- Forced-air-cooling is exclusively performed through the 1.0 m axis (through the face of the pallet with a width of 1.0 m), which fortuitously always has perfect vent hole alignment. Obstruction is thus not a concern in this regard.
- Along the horizontal axis, the A15C had a substantially larger (10x) resistance to airflow than the opentop cartons. However, this is more related to the high fruit packing density within the carton.
- Europool pallets usage
  - Without properly ventilated trays resulted in resistance to airflow values equivalent to non-optimised opentop pallets.
  - Europool pallets with trays should preferably not be used for sensitive markets.
- Future carton designs should adopt the 100 mm approach, which drastically improves vent hole alignment during stacking.
  - This approach can, in part, address the vent hole obstruction during cross-stacking.
  - The 100 mm approach also makes it easier to design pallet bases with more optimal slat positions, which could improve ventilation by ~10%
- The use of trays in cartons greatly increases the probability of temperature being higher during shipping in refrigerated containers. The presence of unvented trays increases pressure loss by 110%. Whereas ventilated trays increased pressure loss by 56%. Trays should be avoided at all costs in phytosanitary sensitive markets. However, if trays must be used, they need to be ventilated. Ventilation holes should be positioned between the fruit cups, near to carton vent holes. Vent area should be 6-7%.

## Future research

Cooling is critical for applying cold treatments to phytosanitary sensitive markets, and to a significant extent, this is primarily determined by ventilation holes on the carton. However, there are far too many variables

interacting with the effect of vent hole design, which has made the development of a singular recommendation or design nearly impossible. A more dynamic approach is thus needed.

A significant finding over the course of this study was the observation that the pallet structure primarily operates as an electrical circuit. Specifically, the fan, carton interiors and vent holes function as batteries, wires and resistors, respectively. Flow rate is thus current, pressure is voltage and constriction/obstruction is resistance. A literature review showed that this modelling approach is been applied in the mining industry extensively. Thus, the whole pallet structure can be described as a 3D flow circuit, and with the correct parameters, it could be characterised nearly instantaneously. Furthermore, this perspective model could be used to generate performance indexes for every possible packaging scenario. Hence, providing the industry with a practical guide (i.e. datasheets) for ventilation design. This concept is currently the focus of a Meng research project and is showing promising outputs.

### Technology transfer

- Research outputs from this work have been shared in the technical meetings and 2021 workshops (2020 and 2021).
- This research has been critical in guiding several new research projects:
  - A project to model packaging systems as flow circuits that will allow for the rapid generation of ventilation recommendations (i.e. data sheets).
  - The finding that opentop cartons have much higher airflow resistances vertically, but much lower resistances horizontally. This has substantial implications on cooling in containers. These finding indicates that horizontal airflow may be dominant through packaging during container cooling when using some packaging types. This aspect is now being addressed in another project.

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### 5.2.3 FINAL REPORT: Optimising container loading methods for improved cooling performance Project 1125 (February 2018 - December 2020) by Tarl Berry and Paul Cronje (CRI)

#### Summary

Projections indicate that citrus production will increase significantly, resulting in increased stress on fruit precooling facilities. Additionally, international phytosanitary requirements are expected to become progressively more stringent, further increasing the cooling demand in precooling facilities (e.g. FMS). Ambient loading is a cold chain approach whereby palletised fruit bypass a precooling facility and are loaded warm into the reefer container, after which they are cooled within the container. This approach is a highly valued solution towards reducing stress on facilities, costs and waiting times. The aim of this study was thus to better apply ambient loading strategies. The development of a CFD container model was advanced, which provides improved insights into the cooling processes in citrus loaded containers. This model is an essential tool for the industry as it provides high-resolution insights into the cooling processes in a container and facilitates rapid prototyping of container loading aids. The potential use of an e-void plug as a loading aid was evaluated. The e-void reduced hot spot temperatures by up to 1 °C and showed considerable promise to the industry. Furthermore, pallet spacers were also investigated and showed to reduce the hot spot temperatures by up to 2 °C and therefore have considerable promise as a container loading aid. However, despite these promising loading aids, the results of this study indicate carton ventilation is the most important factor determining cooling performance and solutions to that must be found. Future research will need to focus on a combination of improved ventilation and novel container loading aids.

#### Opsomming

Vooruitskattings dui aan dat sitrus produksie drasties gaan verhoog, dit gaan verhoogde stres op verkoelingsfasiliteite plaas. Daar word ook verwag dat fitosanitêre mark vereistes strenger sal word, wat verder druk op die verkoelingsfasiliteite sal verhoog. Warm-laai is 'n koueketting benadering waar vrugte die voorverkoelingsfasiliteit systap en warm gelaai en verkoel word in die vraghouer. Hierdie metode is 'n waardevolle oplossing om druk op fasiliteite, kostes en wagtye te verminder. Die doel van hierdie studie was om warm laai (ambinet loading) beter toe te pas. Met die vordering in die ontwikkeling van 'n CFD model vir 'n verkoelingshouer, word beter insig gebied in die verkoeling van sitrus in . Hierdie model is 'n noodsaaklike hulpmiddel vir die industrie, aangesien dit 'n hoë resolusie bied oor die verkoelingsprosesse in 'n houer en dus die vinnige ontwikkeling van prototipes van laaihulpmiddels vergemaklik. Die potensiële gebruik van 'n “e-void-plug” ontwikkel via die CFD-model was in 'n houer geëvalueer. Die “e-void” het die temperatuur van die warmste plekke met 1 °C verlaag en is dus belowend vir die bedryf. Daar was ook ondersoek ingestel na die gebruik van paletaf-spasiereders (spacers) en dit was bevind dat die temperatuur in die warmste area met by 2 °C verlaag was. . Daar moet egter gelet word dat ondanks hierdie belowende vordering, dui die resultate aan dat ventilasie van kartonne een van die belangrikste faktore is wat die verkoeling van sitrusvrugte bepaal en oplossing sal gevind moet word hiervoor. Toekomstige navorsing gaan fokus op 'n kombinasie van verhoogde ventilasie en nuwe hulpmiddels vir laai van houters.

#### Introduction

The quantity of citrus fruit exported from South Africa under cold protocols has been steadily increasing over recent years and this trend is expected to continue for the foreseeable future. These growing volumes have led to considerable pressure on the limited pre-cooling facilities at ports. Additionally, in response to stricter phytosanitary requirements by the EU, the SA citrus industry have implemented a strategy that requires fruit to be shipped at temperatures below 2°C. This new protocol is part of a systems approach to mitigate the risk of live pest interceptions in the market, but is placing further pressure on pre-cooling facilities as fruit need to be pre-cooled down to lower temperatures.

The large volume of citrus fruit produced and exported to Europe as well as the insufficient pre-cooling infrastructure available is expected to result in major logistical problems at the ports. The application of ambient loading is helping to mitigate this problem and involves loading fruit warm (at ambient temperature) in the container, and allowing the containers refrigeration unit to remove field heat. Past studies (2015-2018) have

demonstrated (theoretically and experimentally) that refrigerated containers have sufficient capacity to cool produce to set point within about 2 days. However, the cooling performance of ambient loads is often unreliable, due to the relatively large variations in cooling rate and uniformity observed within containers. Subsequent low pulp temperatures can thus cause chilling or freeze damage, whereas pulp temperatures above protocol can result in a failure to comply with cold sterilisation requirements.

This project proposes a two-part approach to improve the accessibility of ambient loading to the citrus industry, thus enabling more fruit to bypass cold stores. The first part of this study (Objective 1) investigates innovative container stuffing approaches to improve airflow distribution within the refrigerated container. Most cooling related challenges occur near the USDA3 position (door region), where fruit are unable to reach or cannot be maintained at the set-point temperature. This region of inadequate cooling can also result in a secondary problem, where the refrigeration unit will deliver air at sub-optimal temperatures, resulting in fruit near the air supply developing chilling/freeze damage. The incidence of these cooling failures varies considerably and their occurrence appears to be independent of container model, packaging type or cold store.

Besides insufficient cooling airflow reaching pallets near the door region, another challenge to maintaining a homogenous fruit temperature in the container is heat energy being conducted through the walls of the container into the sides of the pallets which are in direct contact. This heat gain, in combination with inadequate cooling airflow (usually at the door end of the container) can result in warm fruit temperatures. Pallet spacers, which force the pallets away from the walls of the container were thus examined in this work. Additionally, the study also examined the potential use of extended void plugs and 21 pallet (20 standard pallets + a euro pallet) loading schemes.

The second objective was to further develop a computational fluid dynamics model, to better explain the airflow distribution inside the refrigeration unit. The project thus performed a detailed airflow validation experiment at SRCC.

### **Stated objectives**

The aim of this project is to determine the viability and improve on the large-scale adoption of ambient loading in the FMS without quality loss.

The specific objectives for 2019 are as follows:

1. Improved container loading approaches
2. Validation of the FMS shipment regimes.

### **CFD model development**

One of the main goals over 2020, was to develop a computation fluid dynamics (CFD) model as a tool for this programme (cold chain innovation) to describe the air and heat transfer processes in a refrigerated container loaded with citrus fruit. A first iteration was already developed in 2019, however, several improvements were needed for increased accuracy. For instance, the refrigeration system needed to be modelled individually, which were then included into the CFD as implicit functions. Additionally, the airflow properties of the citrus packaging system needed to be properly characterised, to accurately describe the interaction between cooling airflow and the packed fruit.

### **Refrigerated containers**

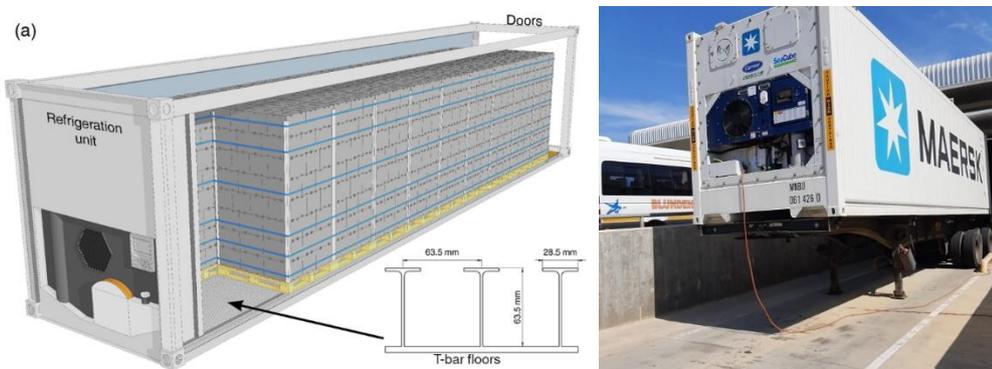
Two refrigerated containers were considered in this study. Both containers were 40-foot, hi-cube (higher than standard design) Maersk models (dimensions listed in

Table 5.2.3.2). These container specifications are representative of what is most commonly used in both the pome and citrus fruit export industries.

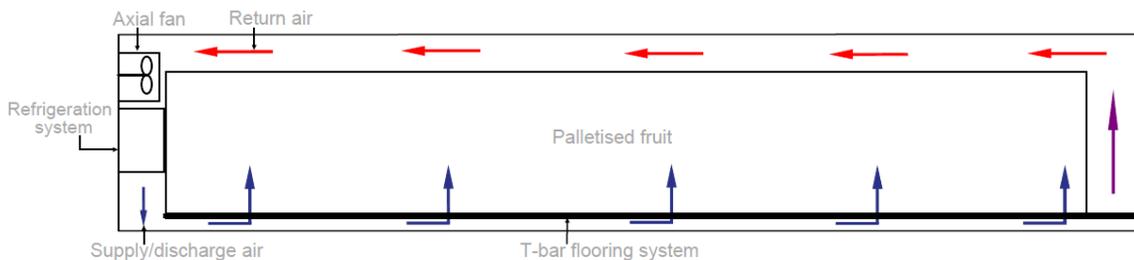
**Table 5.2.3.2.** Geometrical dimensions of container and pallets.

	Number	Dimensions (m) (length × width × height)
Refrigerated container (interior)		11.59 × 2.29 × 2.55
Refrigerated container (exterior)		12.20 × 2.44 × 2.99
Pallet Row 1	11	1.2 × 2.15 × 11
A Pallet stack in Row 1	1	1.2 × 2.15 × 1
Pallet Row 2	9	1 × 2.15 × 10.8
A Pallet stack in Row 2	1	1 × 2.15 × 1.2

The refrigerated containers have a T-bar flooring system, as shown in Figure 5.2.3.30. There are 35 T-bar extruded surfaces which run along the floor from the refrigeration unit up to the door. The container makes use of a bottom air delivery system to circulate cold air through the T-bar floors to the palletised fruit. Figure 5.2.3.31 illustrates the mode of air circulation inside a refrigerated container equipped with a bottom air delivery system.



**Figure 5.2.3.30.** (a) Refrigerated container with bottom air delivery system. (b) Photo of the refrigerated container (Carrier Transicold/Primeline) used in the validation experiments at SRCC.



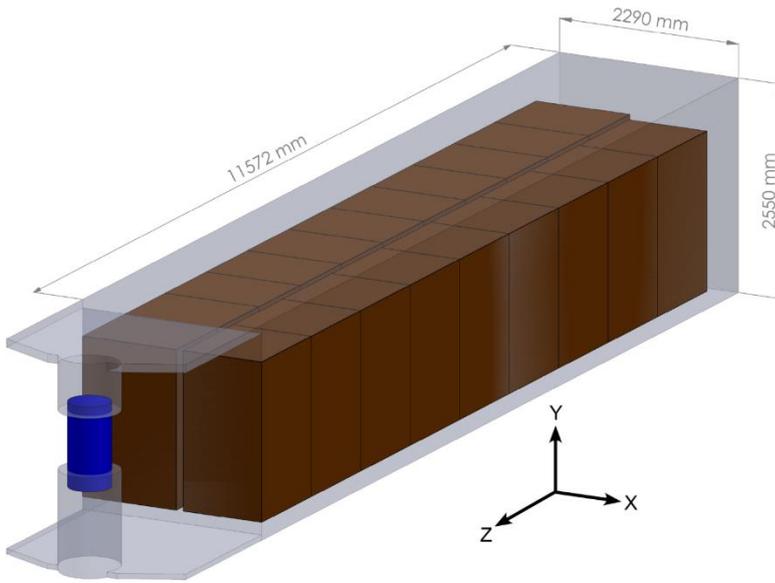
**Figure 5.2.3.31.** Schematic illustration of the airflow circulation inside a refrigerated container when using a bottom air delivery system.

The refrigeration units in Container A and Container B were a Carrier Transicold THINline and Carrier Transicold PrimeLINE systems, respectively. The latter container is rated as more effective performing unit by carrier and both units were thus characterised individually.

### Geometry

The computational domain for the refrigerated container internal dimension is 11.59 m long, 2.29 m wide and 2.55 m high (internal volume: 67.6 m<sup>3</sup>). The computational domain (Figure 5.2.3.32) contains twenty pallets which are arranged in two rows inside the container. Twenty pallet stacks of citrus fruit were loaded inside the refrigerated container. The dimensions of the pallet stack in each row are specified in

Table 5.2.3.2.



**Figure 5.2.3.32.** Fully packed refrigerated container.

**Air domain**

The airflow domain was solved using 3D incompressible Reynolds averaged Navier-stokes equation (RANS) shown below.

Conservation of mass

$$div(\mathbf{u}) = 0 \tag{5}$$

Conservation of Momentum

X-direction

$$div(\rho\mathbf{u}\mathbf{u}) = -\frac{\partial p}{\partial x} + div(\mu grad u) + S_{PM_x} \tag{6}$$

Y-direction

$$div(\rho\mathbf{v}\mathbf{u}) = -\frac{\partial p}{\partial y} + div(\mu grad v) + S_{PM_y} \tag{7}$$

Z-direction

$$div(\rho\mathbf{w}\mathbf{u}) = -\frac{\partial p}{\partial z} + div(\mu grad w) + S_{PM_z} \tag{8}$$

Conservation of Energy

$$\frac{\partial(\rho i)}{\partial t} + div(\rho i \mathbf{u}) = -p div \mathbf{u} + div(k grad T) + S_i \tag{9}$$

where  $p = p(\rho, T)$  and  $i = i(\rho, T)$ , Perfect gas  $p = \rho RT$  and  $i = C_v T$

**Pallet modelling**

The modelling approach for the pallet is an important factor in the airflow characterisation and heat transfer in a refrigerated container. Two major strategies to model pallet stacks in the cold chain include the solid block approach and porous media approach. Hoang *et al.* (2015) examined these approaches and their influence on airflow and heat transfer predictions under cold storage conditions. It was found that the solid block approach requires five times the computational time (cost) compared to the porous media approach.

The pallet stacks were thus assumed as a porous media, and the directional loss properties of stacked fruit are specified in Table 5.2.3.3. Pressure loss across the pallets were characterised using the Darcy-Forchheimer equation (Eqn. (10)) (van der Sman, 2002). The linear component, which represents the viscous resistance was not included, as it provides descriptive benefit and is only relevant at very low flow speeds ( $\sim 0.0001 \text{ m s}^{-1}$ ). The quadratic component (Forchheimer coefficient), represents the inertial resistance and was used to quantify the airflow resistance in the pallets (Defraeye *et al.*, 2015). Flow resistance was included by adding a source term in the momentum equation (Zhao *et al.*, 2016).

$$S_{PM_i} = -F|\mathbf{u}|u \quad (10)$$

The directional loss properties of citrus fruit pallets were experimentally determined in this study using the same approach by Getahun *et al.* (2017) (Table 5.2.3.2; See the packaging project (2020), CRI project 1237).

**Table 5.2.3.3.** Directional loss properties of fruit pallet stack.

Carton	Pallet Flow direction	$\beta \text{ (m}^{-1}\text{)}$	std
A15C (citrus)	Horizontal - 1.00 m depth	6 269	11
	Horizontal - 1.20 m depth	1 663	7
	Horizontal - mean	2 552	30
	Vertical - 2.16 m depth	3 418	7

The thermal and physical properties of the solid and fluid medium are important in modelling heat transfer in a porous domain. Two main heat transfer models are possible, namely: Local thermal equilibrium (LTE) and Local thermal non-equilibrium model (LTNE). In the case of LTE, the solid and liquid phase temperature are assumed to be equal, and no heat transfer is thus considered. In contrast, the LTNE approach considers heat transfer and although it more accurately capture the transfer of heat within the porous domain, is computationally more expensive (Hoang *et al.*, 2015; Laguerre *et al.*, 2008)

The LTE has been applied successfully in prior CFD simulations of refrigerated containers (Getahun *et al.*, 2017). However, the approach limits the robustness of the model, particularly in scenarios where cooling air is penetrating pallets domains with a temperature significantly higher than the set-point. An LTNE approach is thus preferable for scenarios where fruit are loaded warm and have a comparatively low resistance to airflow (i.e., citrus fruit). An LTNE was thus implemented in this study. The thermal properties of the citrus fruit are listed in Table 5.2.3.4. These properties are assumed constant and not dependent on temperature change.

**Table 5.2.3.4.** Thermal properties of Citrus fruit (Defraeye *et al.*, 2014).

Properties	Citrus fruit	Air
Density ( $\text{kg m}^{-3}$ )	960	1.28
Thermal conductivity ( $\text{W m}^{-1}\text{K}^{-1}$ )	0.386	0.024
Specific heat capacity ( $\text{J kg}^{-1}\text{K}^{-1}$ )	3 580	1 004.4

The energy conservation equation of the fluid (air) phase and solid (citrus fruit) phases are solved individually (Eqn. (8) and ((12)).

Conservation of Energy equation for the solid phase (Nield and Bejan, 2017).

$$\left[ (1 - \varphi)(\rho c_p)_s \frac{\delta T_s}{\delta t} \right] = \left[ (1 - \varphi) \nabla \cdot (k_s \nabla T_s) + h(T_f - T_s) \right] \quad (11)$$

Conservation of Energy equation for the fluid phase (air) (Nield and Bejan, 2017)

$$\left[ \varphi(\rho c_p)_f \frac{\delta T_f}{\delta t} + (\rho c_p)_f \mathbf{v} \cdot \nabla T_f \right] = \left[ \varphi \nabla \cdot (k_f \nabla T_f) + h(T_s - T_f) \right] \quad (12)$$

The energy equations for the solid (fruit/packaging) and fluid (air) phase are coupled by the overall heat transfer coefficient (h) and interfacial specific area (Asf) (Gruyters et al., 2018; Lisowa et al., 2001; Nield and Bejan, 2017).

The interfacial specific area (total surface area of fruit per unit volume) (Eqn. (13)) is given by;

$$A_{sf} = 6(1 - \varphi)/d \quad (13)$$

The overall heat transfer coefficient (Eqn. (14)) between the fruit and air is determined using the Nusselt number expression (Eqn. (15)) (Nield and Bejan, 2017).

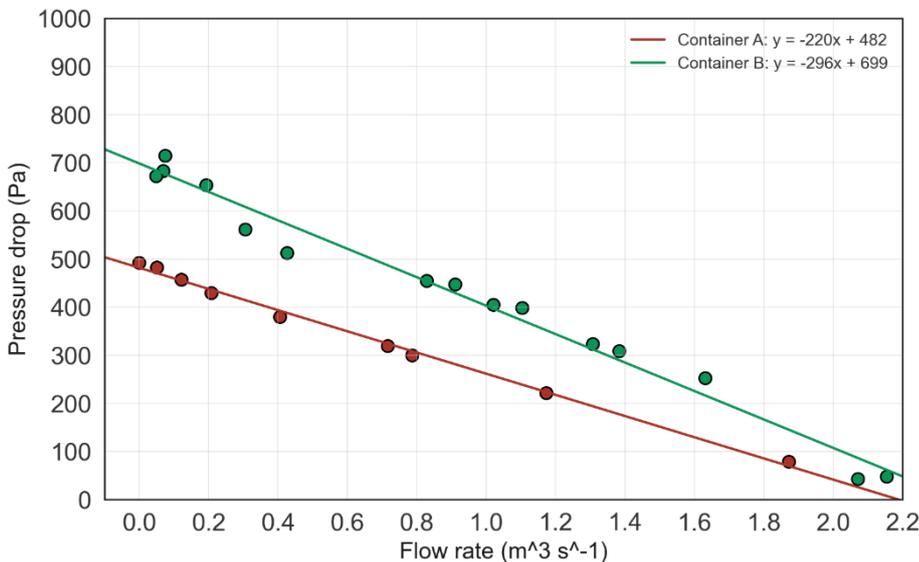
$$h = \frac{d}{Nuk_f} + \frac{d}{\beta k_s} \quad (14)$$

$$Nu = \frac{hd}{k_f} = 2 + 1.1Re^{0.6}Pr^{1/3} \quad (15)$$

where  $\beta$  equals 10 for a porous bed of spherical particles and  $Pr$  is the Prandtl number for air (0.72).

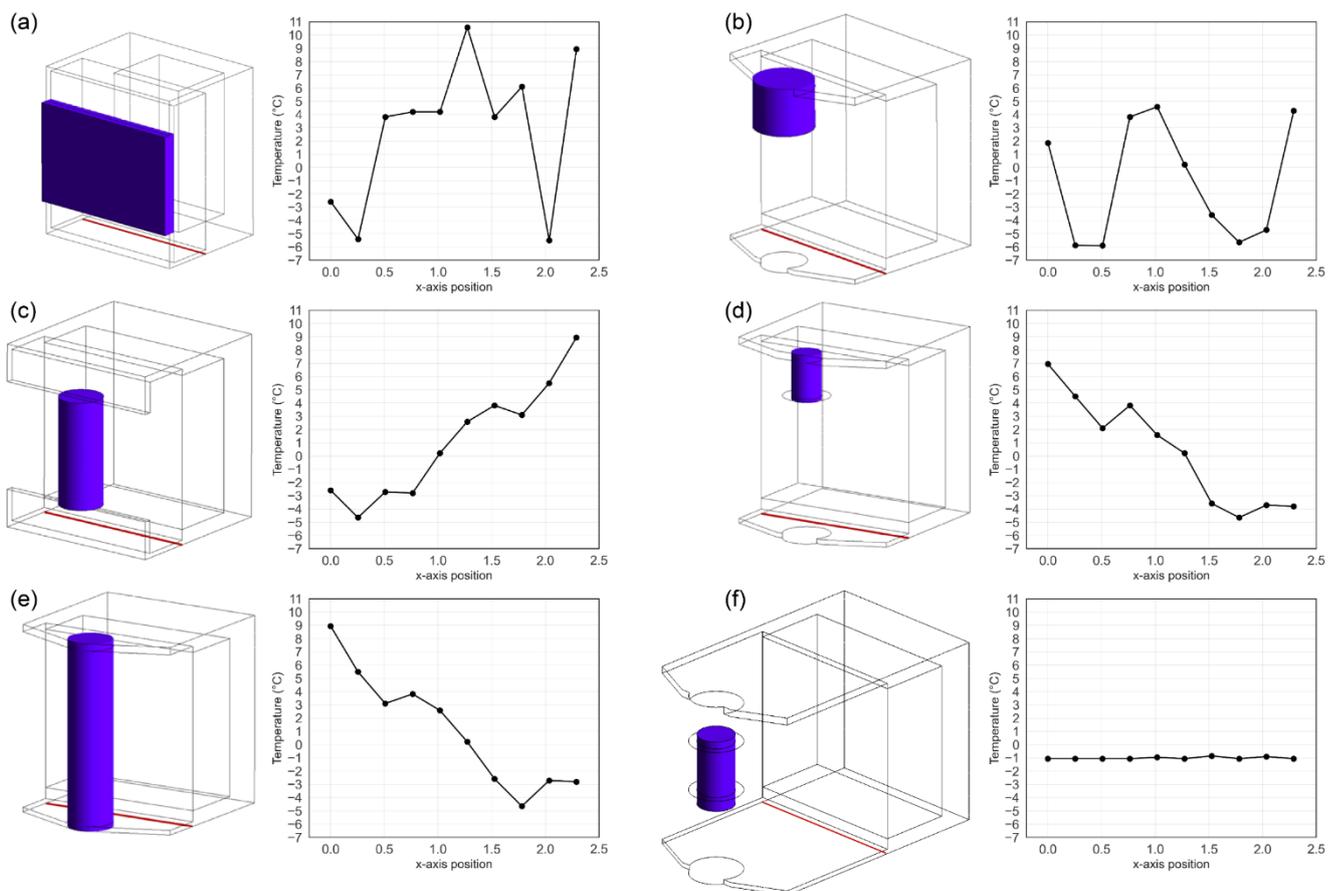
### Refrigeration unit - Momentum source term

The axial fans were implicitly represented in the model by first conducting axial fan performance experiments on both containers. Characterisation of the fans was achieved by iteratively modifying the flow rates at the outlet (through physical obstruction) of the refrigeration unit, and measuring the reciprocal pressure drop across the unit fan using a pressure differential meter (Air Flow Meter Type A2G-50, WIKA, Alexander Wiegand SE & Co. KG, Klingenberg, Germany). Air velocities were measured at the air return region using a RS PRO RS-90 anemometer. The resulting axial performance curve and the corresponding function are shown in Figure 5.2.3.33.



**Figure 5.2.3.33.** Axial performance curve.

An expression using the geometry of the fan duct in the model (length and radius), flow rate of the fan and fan system curve (Figure 5.2.3.33) was coupled as a momentum source using CFX Expression language. A small rotational (swirling) momentum source term was included at the fan region of the RU, to more realistically imitate the mixing action of the axial fan blades on airflow. This feature ensures that air entering the RU with a heterogeneous temperature gradient is first mixed and then exits the simulated refrigerated at a uniform temperature. Figure 5.2.3.34 shows several design iterations that were assessed. The most effective solution found was to apply a small rotational momentum source term in combination with a specific cylindrical geometric design (Figure 5.2.3.34f). The figure depicts the temperature gradient at the RU supply duct while applying a thermal source term to one side of the pallet stack. Substantial temperature gradients were observed in the initial approaches (Figure 5.2.3.34 a, b, c, d, e). However, the final design (Figure 5.2.3.34f) resulted in an acceptably uniform temperature gradient at the supply airflow and the respective geometry was therefore applied in all full-scale simulations.



**Figure 5.2.3.34.** Simulations for the various refrigeration unit geometries that were tested. The blue domain represents a momentum source and energy sink term, so that airflow cycles through the system (top to bottom across blue domain). A heat source was included on one side of the container so that a temperature gradient is present along the x-axis. The red line represents an array of temperature samples, as plotted in the graph on the right. The designs in (a-e) do not effectively mix the air, whereas the design in (f) allowed for a uniform delivery of air.

### Refrigeration unit - Refrigeration capacity

For the transient cooling process of the fruit, the cooling capacity of the refrigerated container and the temperature cooling mode were implicitly represented in the model. Cooling capacity of the refrigeration unit was implemented as an energy sink term. According to Wild (2009), the active cooling capacity (W) of a Thermo King Smart Reefer system ranges between about 6 kW and 9 kW when shipping bananas. Both refrigeration units used in this study were Carrier systems, which are expected to operate in similar cooling capacity ranges. Another factor to consider, is that each refrigeration unit system can make use of multiple

confidential algorithms that regulate the cooling rate that increase power usage efficiency (Lukasse et al., 2011). Consequently, most refrigeration units scale cooling capacity with increasing/decreasing load (Wild, 2009).

Experiments evaluating the cooling capacity of the containers were performed to determine if the parameters provided by Wild (2009) could be applied to the energy sink in the respective model. Experiments were performed by monitoring the airflow rate and temperature difference between the inlet and outlet when empty. The results showed that for temperature differences between 2 °C and 6 °C, the cooling capacity increased from 2 kW to 10 kW, which correlated well with the data provided by Wild (2009). The refrigeration units thermal sink term was set to be dependent on the returning airflow temperature ( $T_{ra}$ ). The energy sink was thus set to linearly increase from 0 kW to 10 kW as  $T_{ra}$  increased from the set-point temperature ( $T_{sp}$ ) to  $T_{sp} + 6$  °C. A sensitivity analysis study showed that at the selected time-step, the model was relatively insensitive to variants in this function.

### Simulation set-up and assumptions

All numerical simulations were run using ANSYS CFX 19.1 CFD code, using high-resolution Advection scheme and high-resolution turbulence for steady-state simulation. The steady-state simulations was first carried out to obtain the airflow characterisation in the refrigerated container and were then used as the initial conditions for the transient simulation.

The turbulence modelling type and boundary layer modelling approach influence to a great deal the accuracy of the simulation (Defraeye *et al.*, 2013). Hoang *et al.* (2015) evaluated the performance of different turbulence models (Standard  $\kappa$ - $\epsilon$ , RNG  $\kappa$ - $\epsilon$ , realisable  $\kappa$ - $\epsilon$ , standard  $\kappa$ - $\omega$  and SST  $\kappa$ - $\omega$ ) in characterising the airflow distribution in a loaded cold store. SST  $\kappa$ - $\omega$  has the lowest mean relative error (MRE) in air velocities with respect to other turbulence models in these types of fresh produce cold systems. A SST  $\kappa$ - $\omega$  turbulence model was thus used to determine the airflow characterisation and temperature distribution in the refrigerated container (Getahun *et al.*, 2017). Various assumptions were used in developing the numerical model of a refrigerated container.

- Volume porosity of the pallet stack was assumed to be uniform spatially and temporally. The volume porosity of the pallet stack is the ratio between the volume of solid (fruit and packaging materials) and the total volume of the porous domain (citrus = 0.45).
- A local thermal non-equilibrium model was used in modelling the transfer of heat from the packaged fruit.
- The pallet stack was modelled as a two-phase porous model, and the appropriate interfacial area density and heat transfer were specified.
- The T-bar floor was modelled as a no-slip boundary wall.
- Second-Order Backward Euler transient scheme was used for transient simulations.

### Mesh Sensitivity analysis and Error calculation

Mesh Sensitivity analysis was performed to determine the best combination of mesh sizes that accurately predict the flow with the minimum computational cost. Steady-state simulations were carried out to show the velocity profile magnitude at the air supply region (Figure 5.2.3.35a) and the middle section of the T-bar floor region (Figure 5.2.3.35b). Fine meshes were made at the region of high computational interest (T-bar floor and refrigeration unit). Figure 5.2.3.36a and Figure 5.2.3.36b show the computational domain geometry and computational mesh generated along XY section.

Table 5.2.3.5 shows the number of cells of different meshing type for sensitivity analysis. The mesh combination for the fine mesh gives approximately the same velocity magnitude profile with less computational load requirements compared to the finer mesh combinations.

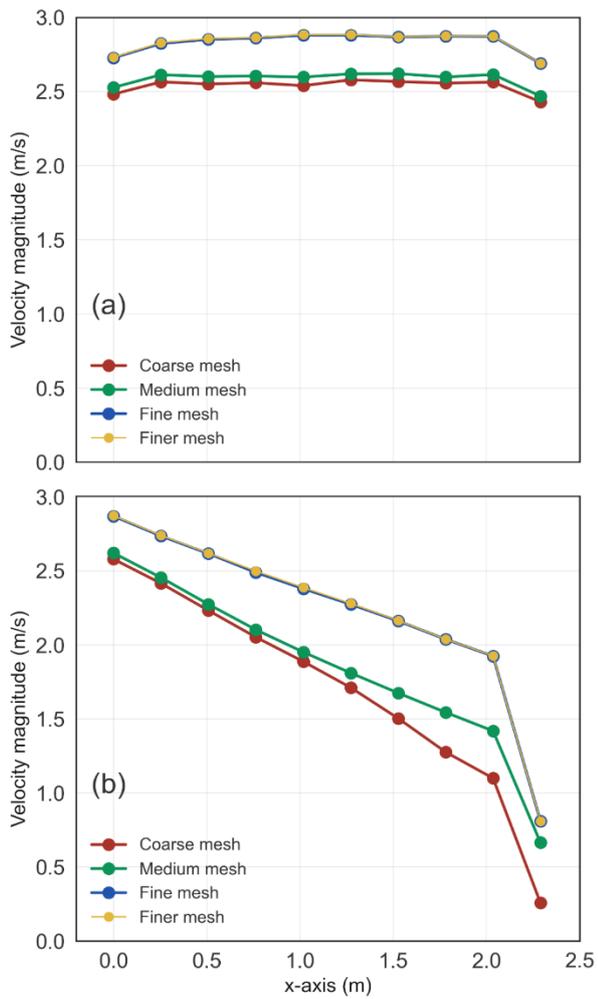


Figure 5.2.3.35. Mesh sensitivity analysis (a) air inlet region (b) T-bar floor region.

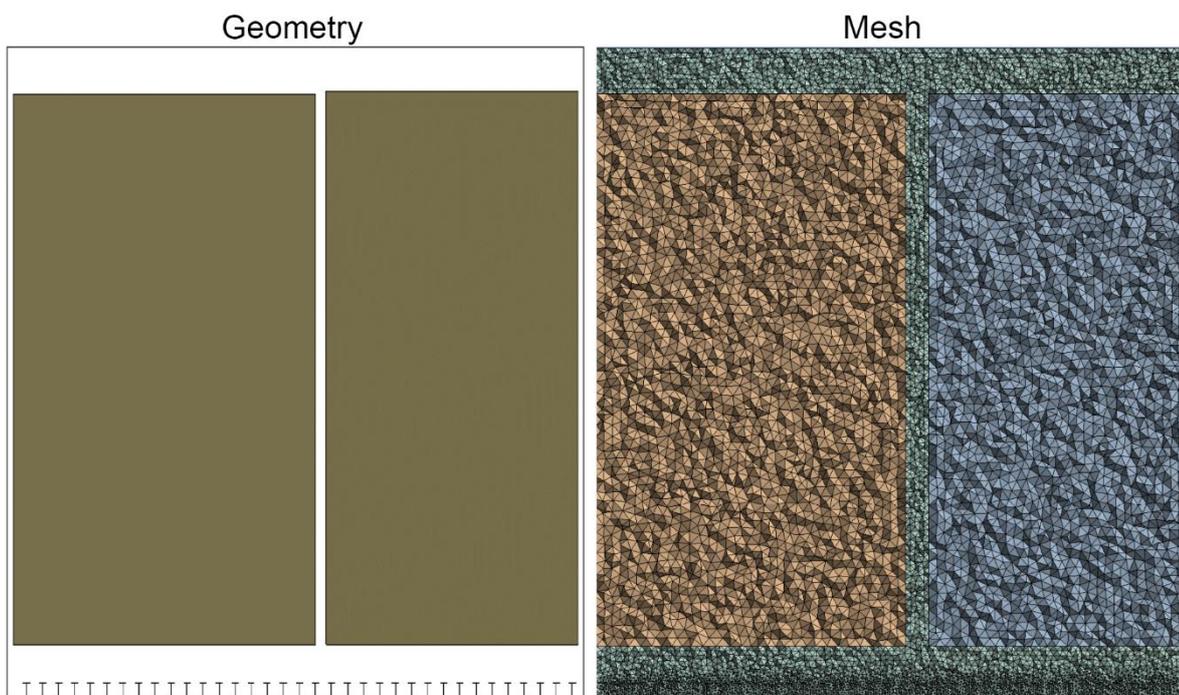


Figure 5.2.3.36. (a) Geometry and (b) mesh of computational domain across XY section ( $Z = 5$  m).

**Table 5.2.3.5.** Number of cells for the various meshing types examined during sensitivity analysis.

Meshing type	Number of cells
Coarse mesh	1 005 967
Medium mesh	5 202 620
Fine mesh	21 426 996
Finer mesh	27 337 776

A modelling prediction error was used to evaluate the accuracy and performance of the model when compared with the experimental data. The average modelling prediction for the airflow characterisation of the refrigerated container and temperature evolution of fruit is obtained, respectively, using equations (16) and (17).

$$E_{velo} = \frac{1}{n} \sum_i^n \frac{||u|^{cf d} - |u|^{exp}|}{|u|^{exp}} \times 100 \quad (16)$$

$$E_{temp} = \frac{1}{n} \sum_i^n \frac{||T|^{cf d} - |T|^{exp}|}{|T|^{exp}} \times 100 \quad (17)$$

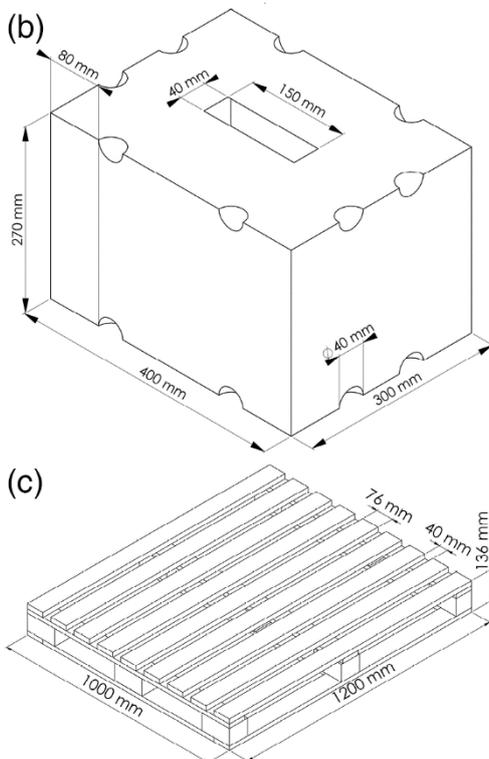
### Validation experiments

#### Materials and methods

Refrigerated container validation experiments were performed on container A at Two-A-Day Group Ltd (Elgin, South Africa, March 2019) and again later on container B at the Sunday river citrus company (Kirkwood, South Africa, May 2020).

#### Fruit and packaging boxes

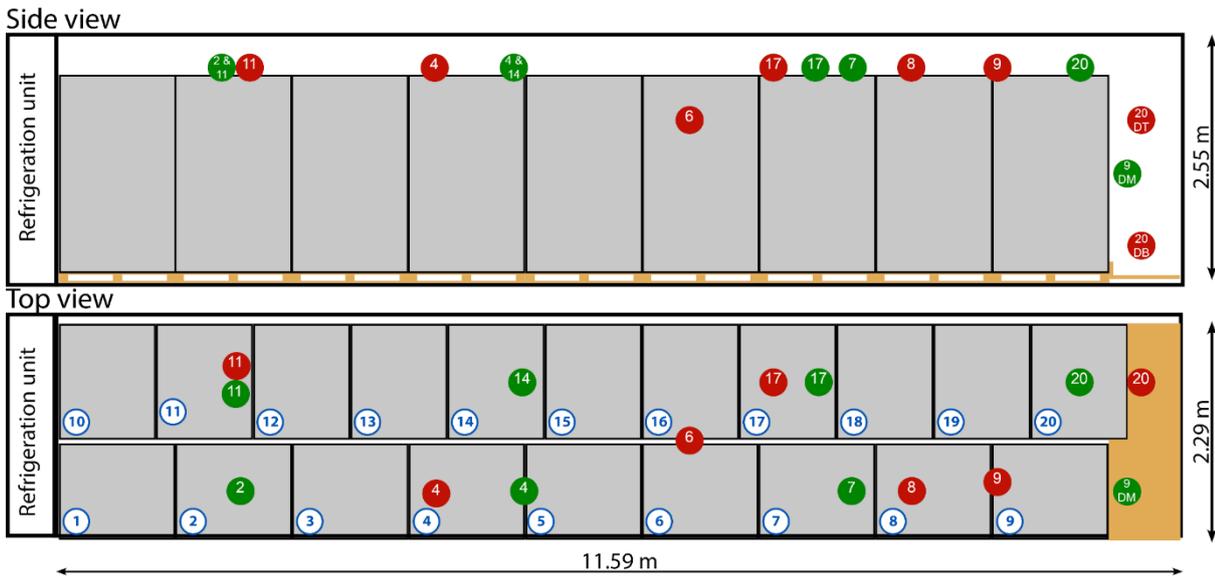
Citrus fruit (cv. Oranges) packed in an A15C carton (Figure 5.2.3.37) were stacked on a standard pallet (1.2 × 1.0 m). Sixty-four fruit with an average diameter of 79 mm, were packed in each carton. The average mass of each pallet stack was 1 200 kg.



**Figure 5.2.3.37.** Schematic diagram of the (b) Supervent carton and the (c) pallet base used in the validation experiments.

### Air-velocity measurement

Candlestick sensors (Advanced Thermal Solutions Inc, Norwood, USA) were used in conjunction with a TVS 1100 Datalogger to measure air-velocities and air temperature at different regions in the refrigerated container. Measurements were taken at multiple regions in the container, with an emphasis at regions between the pallet stacks and container ceiling, and between the pallet stack and the container door (Figure 5.2.3.9).

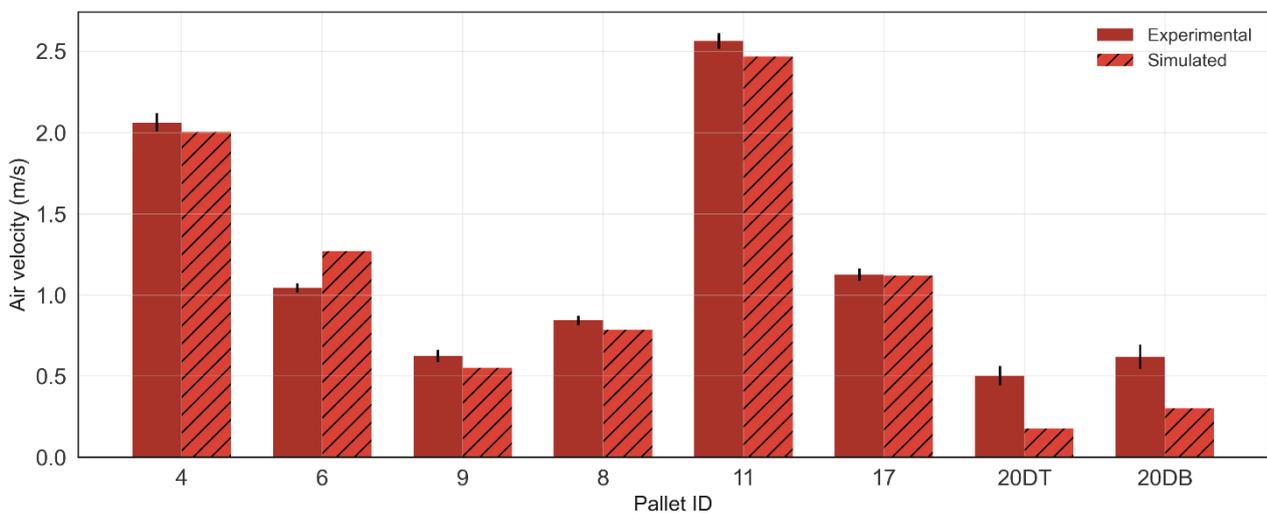


**Figure 5.2.3.38.** Schematic of the refrigerated container showing candlestick sensor placement in both experiments. Blue numbers in white circles show the pallet number; red and green circles indicate the position sensors in container A and B, respectively.

### Results and discussion

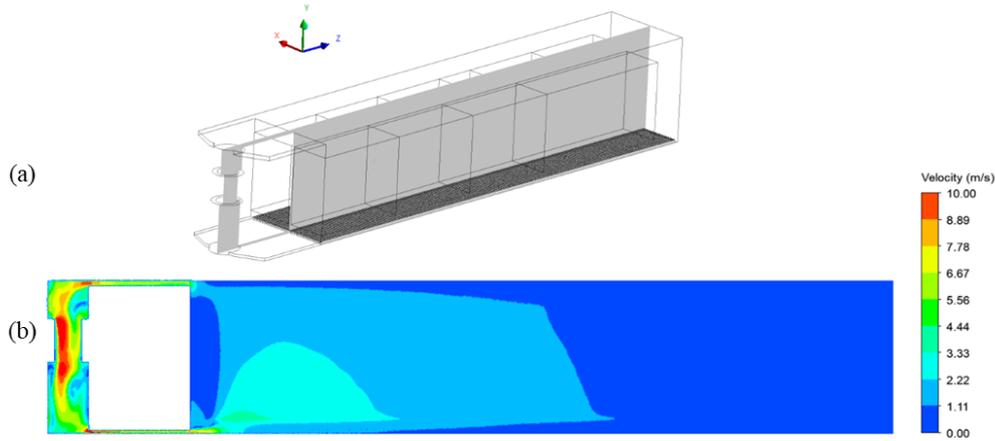
#### Airflow characterisation

The average airflow modelling prediction error for the citrus filled experimental scenarios (container B) was 20% (Figure 5.2.3.39). This shows that the model accurately predicts the velocity profile in the refrigerated container and previous studies have shown that the acceptable prediction error is between 3-30% (Getahun *et al.*, 2017; Hoang *et al.*, 2015).

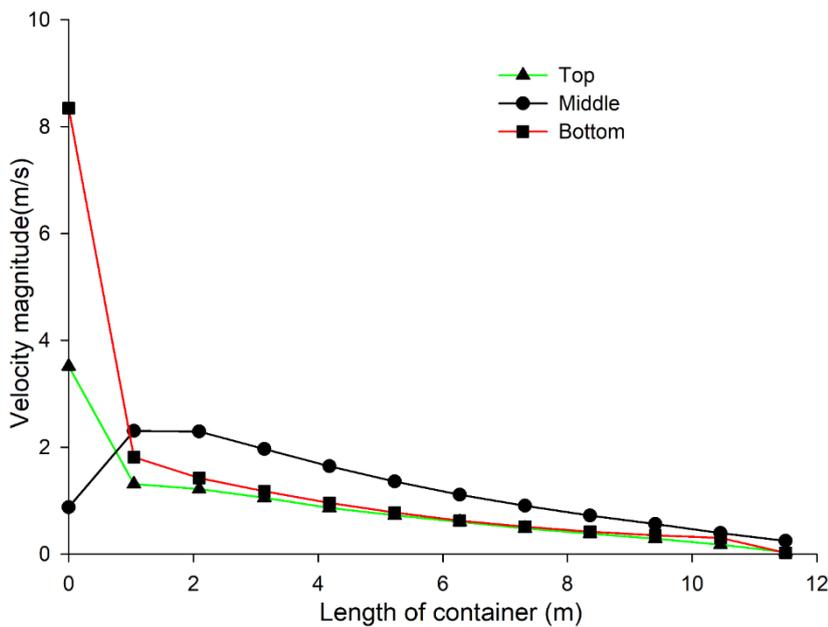


**Figure 5.2.3.39:** Numerical model results and Experimental results for the experimental scenario.

Air gaps between the pallet stack rows cause short-circuiting and large bypass of air in the refrigerated container. This air gap plays a dominant part in the heterogeneity of airflow within the pallet stack. Figure 5.2.3.40 shows the contour and graph of velocity magnitude of airflow in the free region between the pallet stacks. The simulation model was used to evaluate the airflow vertically across (bottom (T-bar), middle, pallet top) the freestream region between the pallet stack ( Figure 5.2.3.41).



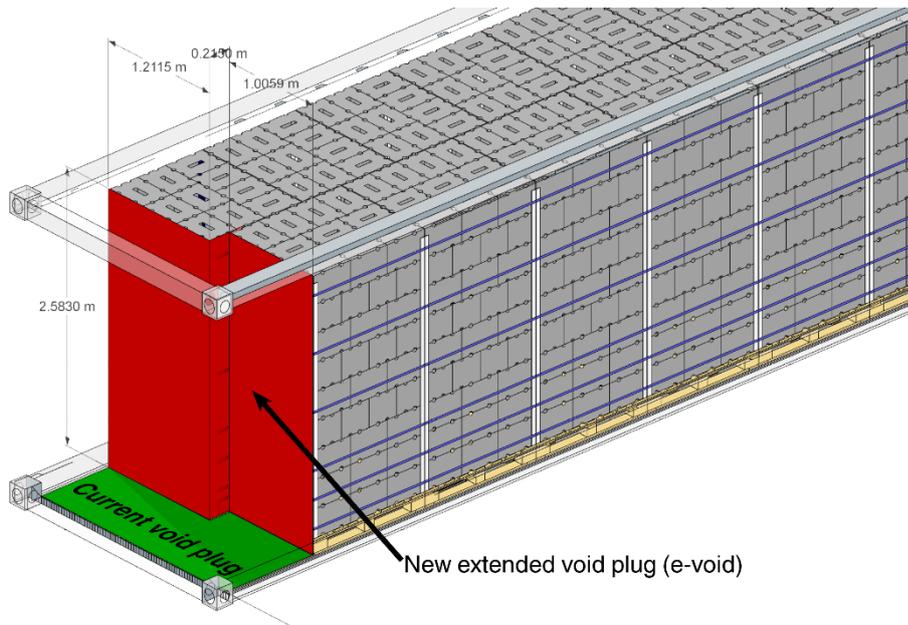
**Figure 5.2.3.40.** Velocity magnitude contour at the freestream region between pallet rows (a) Computational domain YZ plane ( $x=1.04\text{m}$ ) (b) contour plot.



**Figure 5.2.3.41.** Velocity magnitude vertically across the height of the refrigerated container at the freestream region between the pallet stack (YZ plane  $x=1.04\text{m}$ ).

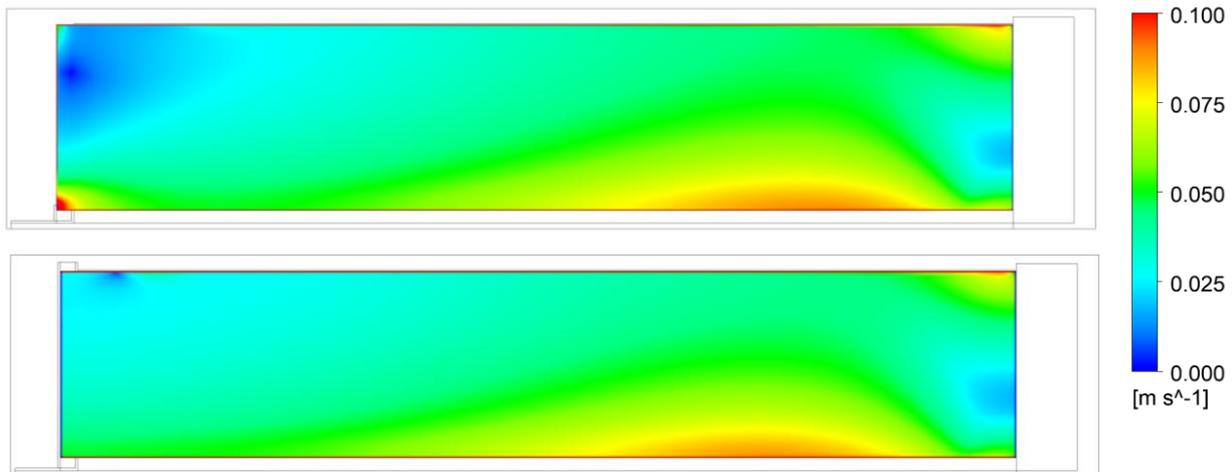
### Exploring extended void plug – loading aid – Simulations

Figure 5.2.3.42 shows the extended void plug (e-void plug) designed to eliminate horizontal airflow into or out of the back sides of the exposed pallet stacks. The flow of air being delivered from the bottom of the pallet is thus determined by the Coandă effect (Reba, 1966), whereby the air ‘sticks’ to the void plug and doesn’t drift toward horizontal flow. The results for both the full and semi void plug showed promising results in simulation and it is expected that the semi-void plug system will be more practical to setup than a full void plug.



**Figure 5.2.3.42.** Illustration of the extended void plug.

Figure 5.2.3.43 show the simulation results for air velocity in a container with and without an e-void plug (A15C cartons). The results indicate promising results that suggest the low air velocity region at the door region of the container (top of pallets) can be eliminated. To test this hypothesis, an experiment was designed.



**Figure 5.2.3.43.** CFD simulation of air velocity in pallets when using a standard container layout (top) and using the e-void plug (bottom). Door side on the left of the figures.

### Exploring extended void plug – loading aid - Experiments

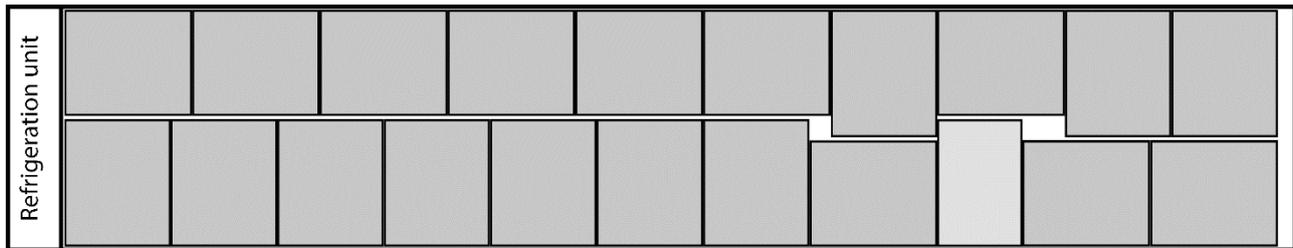
#### Materials and methods

Commercial cooling experiments were applied in 40-foot high-cube refrigerated containers (outer dimensions  $L \times W \times H = 12.2 \times 2.4 \times 2.9$  m). Every container was either stuffed with 20 pallets and an extended void plug or with 21 pallets. Citrus fruit were harvested (near Citrusdal) and taken directly to a packhouse where the fruit received standard commercial treatments. Fruit were then packaged, palletised and transported by road (1 hours) to a nearby cold store (SAFT Kilaney), where the pallets were cooled between 2 and 4 days and then were loaded into a refrigerated container and placed onto a container vessel. The vessel sailed from the Port of Cape Town to an international export market over an 18-day period. The refrigerated container's

temperature set-point was programmed to 0°C (Moore *et al.*, 2016) and vents for fresh air exchange were set to 4.2 L s<sup>-1</sup> as per South African citrus export regulations (PPECB, 2006).

Treatments included a (i) standard container setup, with a void plug. (ii) An extended void plug setup, whereby the vertical wall of the void plug was extended to cover the whole side wall of the last pallet stacks (Figure 5.2.3.42) and (iii) a 21 pallet load-out (Figure 5.2.3.44).

Temperatures were monitored using 4 cellular loggers. Loggers were placed inside a carton for the last four pallets to be loaded. The carton selected from each pallet was from the second (from the top) layer against the container wall. The goal here was to evaluate worst-case temperatures in the container.



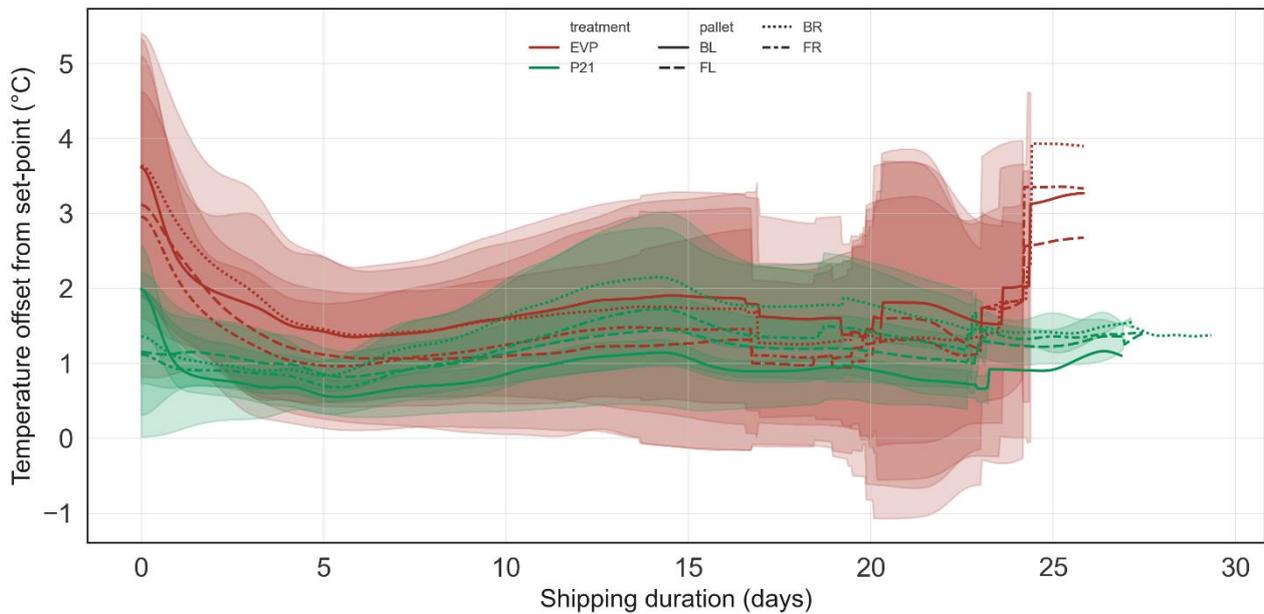
**Figure 5.2.3.44.** Pallet layout using the 21 pallet layout.

Prior detailed studies have showed promising results when using the e-void and an additional set of studies with a larger scope were needed to validate the e-void. The approach of this trial was selected due to COVID, which drastically limited transit (i.e. experiments at Durban or PE) and completely eliminated international flights. This project was initially designed as a full scale, highly controlled trial, where multiple containers would be identically packed and loaded with the respective treatments and then shipped to market. We needed to follow these containers to market to ensure none of the costly equipment is lost during transit. Without following the equipment, there is a high probability of losing equipment and sensors. To ensure we gained some experimental data over 2020, we elected to perform this trial at higher quantities of replicates, but at lower quality (i.e. less sensors per container and less control over each container).

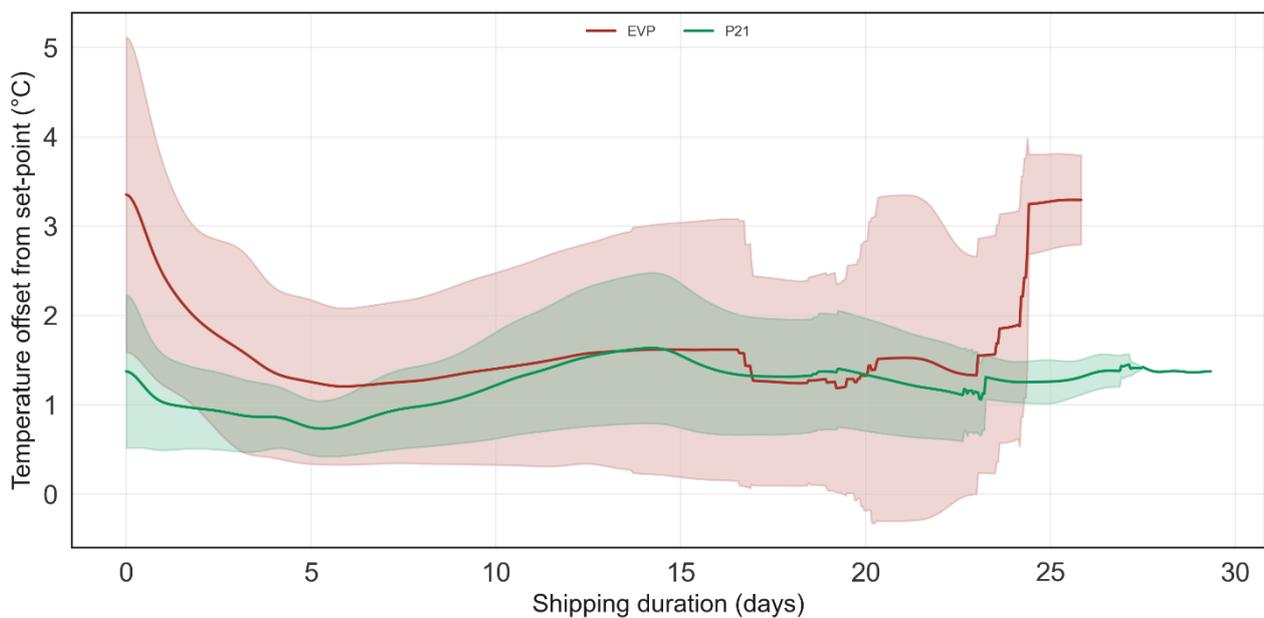
## Results and discussion

Figure 5.2.3.45 and Figure 5.2.3.46 show the mean temperatures (lines) and standard deviation (shaded region) of the temperatures for containers with either 21 pallets (P21) or with e-void plugs (EVP). The plan in this study was to randomly install e-void plugs into containers at the cold store. Experimentally, 25 containers were used, each loaded with 4 cellular loggers. Control data would then be collected from the many other standard container load-outs. The data would be accessed from the prescribed cellular loggers that need to be uploaded by the exporter per container to the CRI. However, it was discovered that the respective cold store was not correctly positioning loggers as per requirements. Fortunately, comparisons are still possible between the P21 containers.

As a side note, the discovery of incorrect logger position at cold stores was addressed over the rest of the year. Cellular logger vendors were contacted and instructed to provide the correct installation information. An extensive programme between the PPECB and CRI was also conducted to reach all cold stores and loading facilities, towards educating role players of their responsibility regarding the correct positioning of loggers. All indications suggest this programme has been successful.



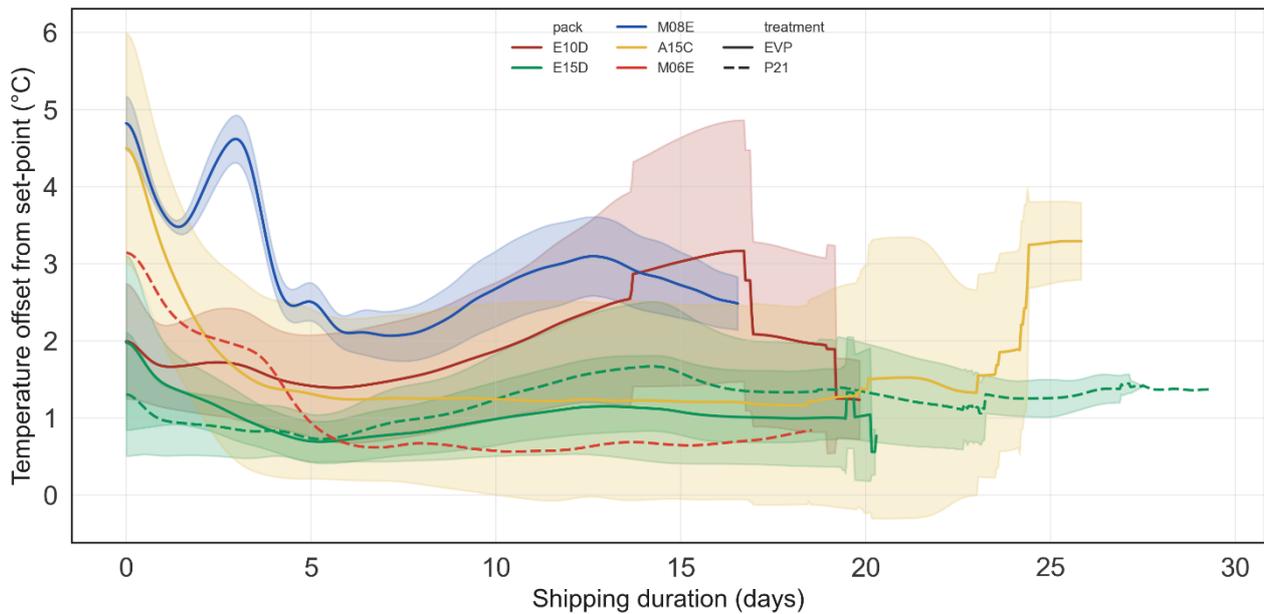
**Figure 5.2.3.45.** Fruit temperatures for containers with 21 pallets (P21) and those with an e-void plugs used, showing distribution per a logger position.



**Figure 5.2.3.46.** Fruit temperatures for P21 and e-void container loads, summary plot. The shaded region showed the standard deviation of the monitored temperatures. Shipments varied in duration, with some being complete after 18 days and others extending beyond 24 days.

Figure 5.2.3.46 indicates similar temperature means but substantially larger temperature variations when using the e-void. However, a closer examination of the data showed that the greater standard deviations were a result of the different packaging designs used. Only the E15D and M06E pallets were used for 21 pallet load-outs, as the containers are too heavy if loaded with 21 pallets of other carton designs. However, comparatively, only the E15D was also tested with the e-void (20 pallet load-out).

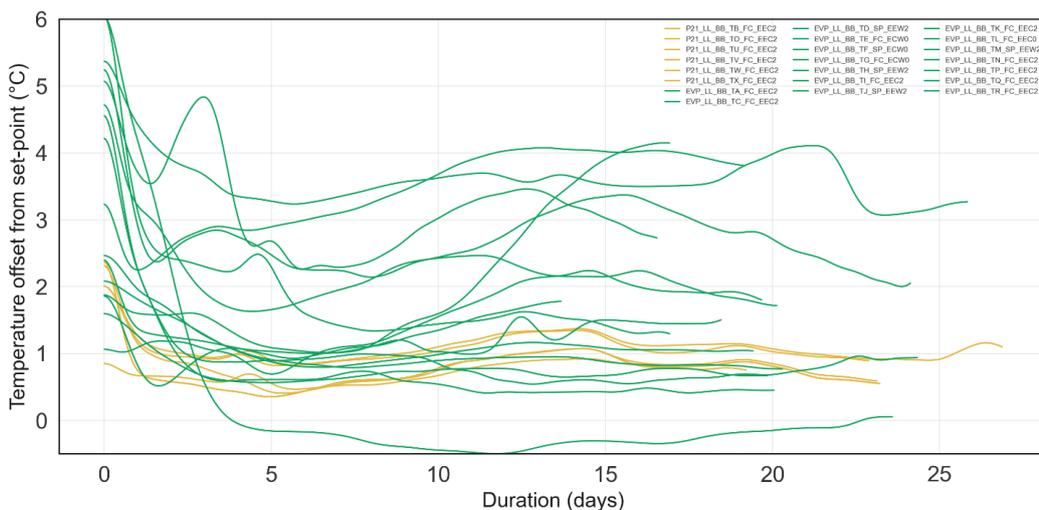
Figure 5.2.3.47 was thus plotted to isolate how the packaging is influencing cooling performance. The results were highly informative and showed a clear trend per packaging type, with respect to the effect of the e-void (green lines). The e-void reduced temperature increases beyond set-point by a maximum of 0.7 °C (at the equator), which corresponds to prior findings.



**Figure 5.2.3.47.** Fruit temperatures for P21 and e-void container loads, for each carton type used in the container. The shaded region showed the standard deviation of the monitored temperatures. Shipments varied in duration, with some being complete after 18 days and others extending beyond 24 days.

The processed temperature results for containers loaded with either 21 pallets (P21) or with e-void plugs (EVP) are shown in Figure 5.2.3.48 to 5.2.3.22. As per Figure 5.2.3.47, the high variability in the e-void containers is attributed to the many different carton designs being used. An important output here is the selection of logger position. In 2019, industry data were used to select the position with the highest probability of being exposed to a warm spot. The left-back position was selected and is now actively used in the industry. The results below and in Figure 5.2.3.45 indicate the left-back position was correctly selected (for the e-void). However, for the P21 containers, the results indicate warmer temperatures at the right-back.

This finding can be attributed to pallet orientation. For 20 pallet container load-outs, pallets on the left side of the container are orientated to be more resistant to airflow than pallets on the right (see project 1237). However, 21 pallet load-outs, the orientation of pallets are reversed at the door end of the container, and the inverse is thus true.



**Figure 5.2.3.48.** Fruit temperatures for P21 and e-void container loads at the back left pallet.

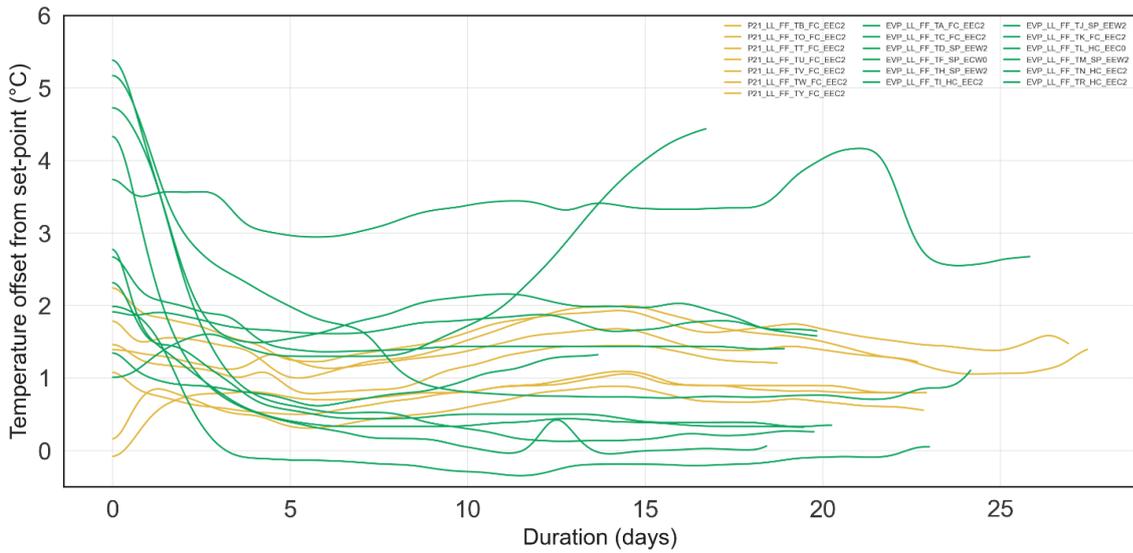


Figure 5.2.3.49. Fruit temperatures for P21 and e-void container loads at the front left pallet.

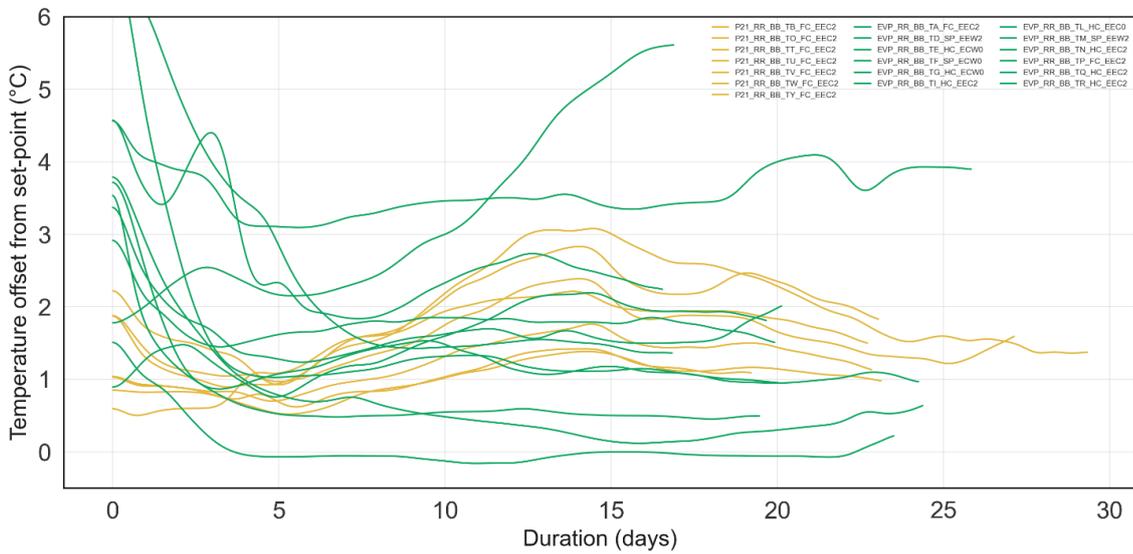


Figure 5.2.3.50. Fruit temperatures for P21 and e-void container loads at the back right pallet.

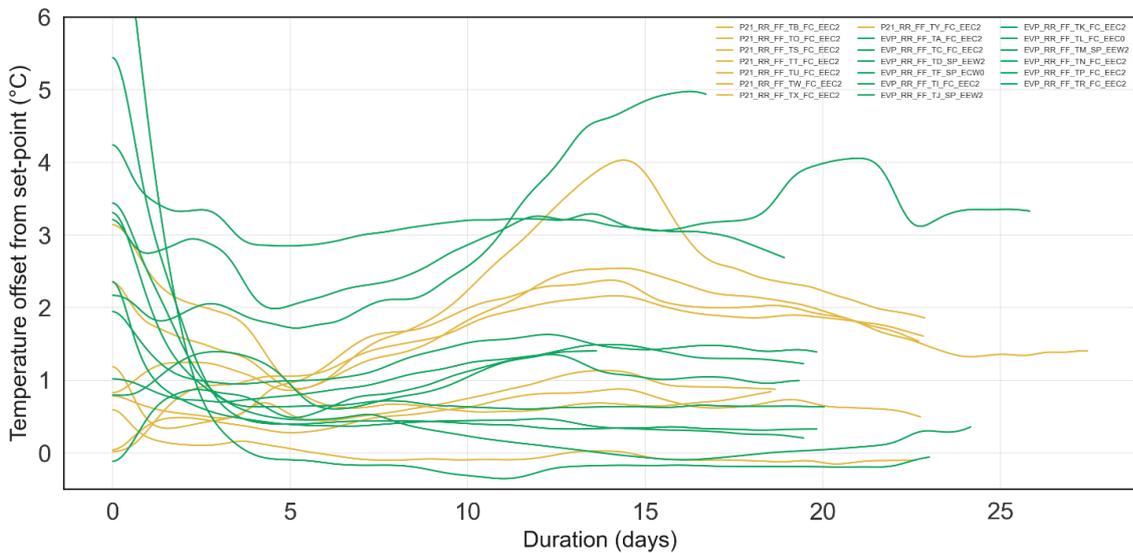


Figure 5.2.3.51. Fruit temperatures for P21 and e-void container loads at the front right pallet.

## Pallet spacers

### Experimental setup

A state-of-the-art Maersk container (MNBU 3995052) was loaded as normal with pallets containing mixed cartons (wrapped and non-wrapped fruit) of lemon fruit of various counts. Four treatments were used in order to see how the temperatures differ when the pallets are in contact with the container wall in contrast to when the pallets are spaced away from the wall.

Spacing between the pallets and container wall was achieved by placing planks of wood under the pallet straps, which provides a relatively unobstructed plenum for airflow. A woodblock was also placed on the floor, against the container wall (between the T-bar flooring). To ensure the actual pallet base was correctly positioned away from the wall.

The sun position with respect to the containers angle is shown in Figure 5.2.3.52. This illustrates that the side of pallet 20 likely received slightly more heat radiation from the sun than the pallet 19. However, there are various factors to take into account, and both sides will thus be considered replications of each other.



**Figure 5.2.3.52.** Sun distribution across the container walls over the experimental period ([www.suncalc.org](http://www.suncalc.org)).

**Control – Pallets against container wall.** The pallets were pre-cooled in the container for three days at 5°C, whereafter tinytag data loggers (View 2, Tinytag, Gemini Data Loggers UK, West Sussex/UK) were inserted into the two respective pallets in positions 19 and 20 of the container. The tinytags were placed inside the selected cartons, against the carton wall, directly opposite the container wall; and also on the door side of the pallet. Loggers were placed at every other layer (top to bottom), and pulp and air temperature was recorded. The pallets were loaded as per usual and data were recorded for three days at a container temperature set to 5 °C.

**TMT. 1 – Both pallets away from wall.** Following the three days, the two pallets were removed from the container and reloaded again. This time an attempt was made to try and create a space between the pallets and the container wall, by placing wooden blocks on the floor, between the pallet and the container wall. The two pallets were placed as far away from the walls as possible (Photo A) and the temperature was set to 4 °C for one day.



**Figure 5.2.3.53.** Photo A. The two pallets spaced away from the wall as far as possible.

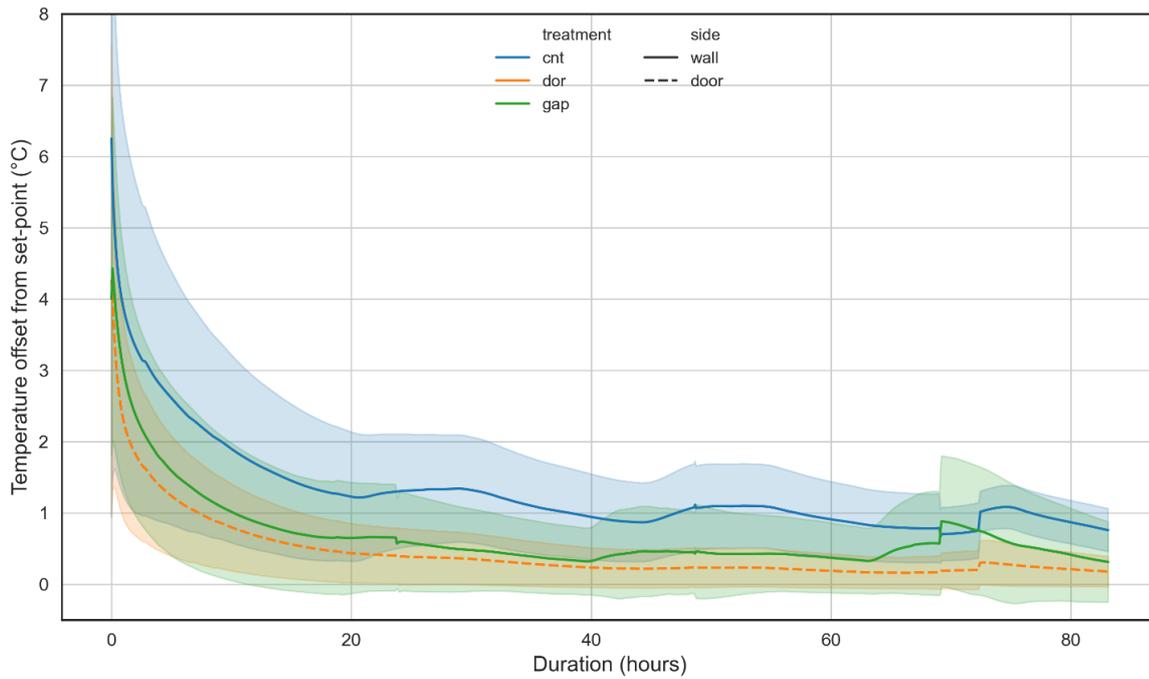
**TMT. 2 – One pallet away from wall.** Either pallet of the last two pallets (20 or 19) were spaced away from the container wall, for several days at 4 °C. Replications were achieved by removing the pallet stacks and shuffling the positions stack or alternating the position of the gap (left or right of the container). Table 5.2.3.6 shows a summary of the treatments and replications.

**Table 5.2.3.6.** Summary of the various treatments, temperature and days.

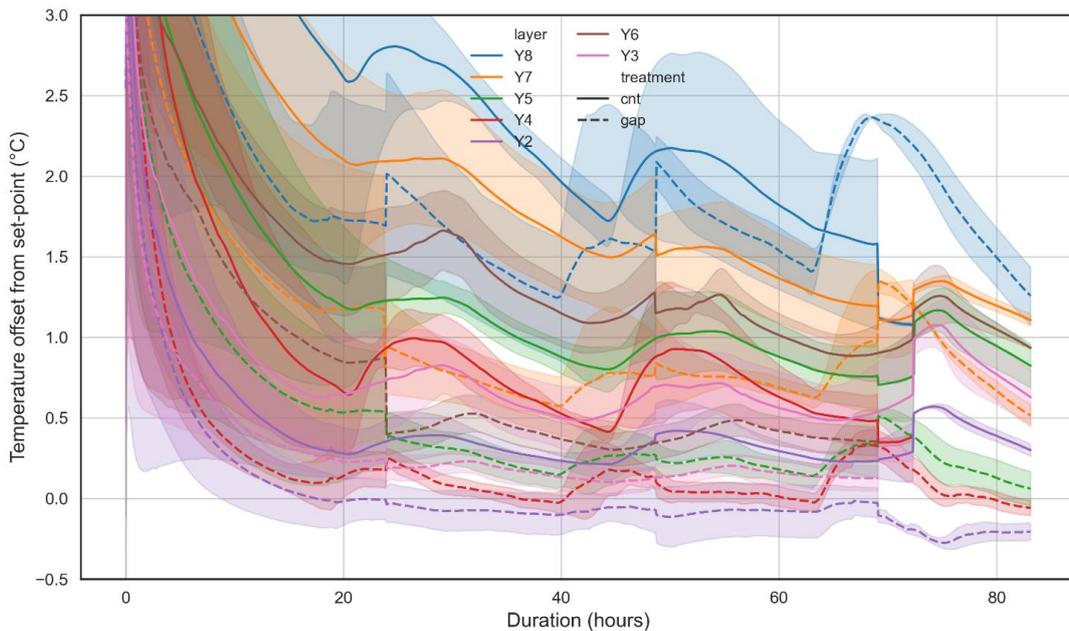
Treatment	Rep	Description	Container Temperature	set	Days in container
Control	1	Normal loading	5 °C		3 days
TMT 1 (both sides)	1	Pallet position R09 & L11 away from container wall	4 °C		1 day
TMT 2 (One side)	1	Pallet position R09: Against wall. Pallet position L11: Away from wall	4 °C		2 days
TMT 2 (One side)	2	Pallet position R09: Against wall. Pallet position L11: Away from wall	4 °C		4 days
TMT 2 (One side)	3	Pallet position R09: Away from wall. Pallet position L11: Against wall	4 °C		3 days

## Results and discussion

The data generated from this study have been summarised in Figure 5.2.3.54 and Figure 5.2.3.55. The results show a reduction in mean fruit temperature of at least 1 °C when including a gap between the pallet and the wall. It should be noted that the pallets used in this trial had undergone extended cooling and had been previously repacked/restacked. The structure of the pallet was thus not as symmetrical and even as a freshly stacked pallet. The gaps between the pallets and wall could therefore be increased under commercial conditions.



**Figure 5.2.3.54.** Mean (line) and standard deviation (shaded) of fruit temperatures in pallets against the container wall (cnt) and with a gap.



**Figure 5.2.3.55.** Mean (line) and standard deviation (shaded) of fruit temperatures in pallets against the container wall (cnt) and with a gap. Focus on carton layer in pallet (Y#), counting from the bottom of pallet. Sharp fluctuations in the profile are a result of the differing cooling durations.

Figure 5.2.3.55 shows the effect of carton height on fruit temperature. This more detailed evaluation indicates that much more significant improvements in cooling may be possible i.e., > 1 °C. Additionally, the results show the temperature offset (from set-point) increases linearly with the height from the t-bar floor. The results show that near the floor, offset temperatures were less than 0.5 °C. At the middle of the pallet, temperature offsets were between 0.5 °C and 1.0 °C; and at the top of the pallet, temperature offsets were between 1.0 °C and 2.0 °C.

It should be noted, that A15C cartons were used in this study. Although the temperature offsets are relatively small for this container, the EVP vs P21 experiments suggest offsets with the A15C range widely between

containers (>3 °C). Factors that can influence this offset include container model, fruit type/size, internal packaging (e.g. wrapping), minor differences in carton/ventilation design and variations in pallet stacking and container loading (i.e. geometry). More work will be required to explore these factors.

## Conclusions

- The CFD model developed in this study showed excellent predictive performance when compared to validation experiments.
  - The model is actively being used to test loading scenarios and phytosanitary cooling applications.
  - The next step will be to include heat transfer through the container wall and better implement airflow resistance parameters.
- CFD simulations of the e-void plug showed increased vertical airflow vectors and reduced horizontal airflow.
  - The e-void eliminated a dead airflow region.
  - It is possible that the e-void is more beneficial when applied in containers with pallets that have a low vertical and high horizontal airflow resistance.
- Experimental evaluations showed the e-void significantly improved cooling performance in containers loaded with E15D cartons.
- Experimental results also confirmed the placement of loggers for the FMS programme. The left-back position pallet is thus confirmed to be ideal and being used in the FMS programme during 2021.
- The effect of packaging type was shown to be a significant influencer of temperature offset from set-point. However, these data do not consider standard layouts. Further research is pending in 2021.
  - The A15C carton generally had the lowest temperature offset mean, but a larger variance was observed with respect to other package designs. The A15C is thus inconsistently effective.
  - Larger temperature offsets were observed for cartons loaded with the E10D and M08E cartons. Although loaded with 21 pallet layout, the high airflow resistances (vertical axis) are consistent with poor cooling airflow penetration.
  - Curiously, the E15D performed in a similar offset range to the A15C, despite having a relatively similar airflow resistance (vertical) to the E10D. More research is needed here.
- Pallet spacers were shown to significantly (> 1 °C) reduce temperature offset.
  - A method to practically apply these devices has, at this time, not been identified. However, several options are being explored.
  - This aspect will be investigated further in 2021.

## Future research

This research project has provided valuable insight into the cooling capacity of refrigerated containers and the potential for loading aids. The following aspects will be investigated in future research:

- Model development.
- Container airflow patterns when using different carton types.
- Matching carton ventilation towards container cooling optimisation.
- Large scale data analysis of container shipments for the identification of trends.
- Further testing of container loading aids:
  - Pallet spaces
  - E-void
  - Floor cover

## Technology transfer

- Research outputs from this work have been shared in technical meetings and 2021 workshops (2020 and 2021).

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## Publications

A publication to an international journal is currently being compiled (final stages) on the model development and validation of the container CFD model.

### 5.2.4 **PROGRESS REPORT: An investigation into aspects affecting chilling injury of citrus** Project 1247 (2019/20-2021/22) by Paul Cronje and Jade North (CRI)

## Summary

Citrus fruit are an important export product for the South African economy, with a significant increase in export volumes forecast. A large portion of this fruit will have to be exported to countries demanding a cold disinfestation protocol of various severities. This project aimed to identify and quantify factors that contribute to CI susceptibility and develop postharvest options to reduce the severity of the susceptibility and severity of chilling injury. In various experiments stretching over two seasons and encompassing all citrus production areas of SA, the impact of preharvest factors such as cultivar, maturity, rootstock, and shade netting were evaluated for impact on CI. In all instances fruit were stored at  $-0.6^{\circ}\text{C}$  for 32 days to simulate shipment of fruit. In the experiments the focus was to use a standard scientific layout, if possible, to allow for statistical analysis. The production area does contribute to susceptibility, which indicates that cultivars from certain regions need additional postharvest focus on their packhouse treatments, as well as optimal logistics in the cold chain to reduce the CI. No consistent pattern could be found in fruit maturity e.g., immature and overmature influence the CI. Rootstocks play an undocumented but significant role in determining fruit susceptibility to CI. These novel results will need follow-up research. Shade netting did not negatively impact on CI of the cultivars evaluated, indicating that this is a viable technology to increase export quality of citrus. Wax remains the primary management tool in the postharvest environment to reduce CI and should be the focus of all packhouse managers to effectively apply it. Without adequate wax application CI could not be addressed in the commercial export market. Chilling injury will be a commercial reality for the SA citrus industry going forward. By taking on these types of multi factorial projects to determine the impact on CI susceptibility, significant contribution to reduce CI incidence was made. This information is already incorporated by producers, exporters and packhouses in their recommendations and practices and has successfully reduced CI during the 2020 season.

## Opsoming

Sitrus is 'n belangrike uitvoerprodukt vir Suid-Afrika met 'n voorspel toename in uitvoer volumes. 'n Groot persentasie vrugte moet egter uitgevoer word na markte wat 'n koue protokol gedurende verskeping vereis. Hierdie projek staan ten doel om faktore wat bydra tot vrugte se koue-sensitiwiteit te identifiseer en kwantifiseer, asook na-oes-opsies te ontwikkel om die voorkoms van koueskade te verlaag. In verskillende eksperimente wat oor twee seisoene gestrek het in die meeste sitrusproduksie streke, was die impak van faktore soos voor-oes, kultivar, vrugouderdom, onderstam en skadunet geëvalueer vir impak op koueskade. Vir die eksperimente was vrugte teen 32 dae by  $-0.6^{\circ}\text{C}$  opgeberg as simulاسie van uitvoer proses en sover moontlike was 'n standaard ewekansige uitleg te gebruik om statistiese analise moontlik te maak. Daar was gevind dat produksiearea wel bydrae tot die sensitiwiteit, wat dui daarop dat kultivars van sekere streke addisionele na-oes-fokus tydens die pakhuisbehandeling benodig, asook optimale logistiek hantering. Geen konsekwente patroon kon egter gevind word in vrugte volwassenheid nie. Onderstam keuse was bevind speel 'n ongedokumenteerde maar beduidende rol in die bepaling van vrugte tov koueskade sensitiwiteit en hierdie nuwe resultaat vereis opvolg navorsings projekte. Skadunet het nie 'n negatiewe uitwerking op die koueskade van die kultivars wat geëvalueer was nie en dui daarop dat die tegnologiese gebruik kan word om uitvoer persentasie te verhoog. Na-oes waksaanwending bly die primêre tegnologie in die na-oes-omgewing om koueskade te verminder en dit behoort die fokus van pakhuis bestuurders te wees om hierdie middel effektief aan te wend. Sonder voldoende waksaanwending sal koueskade nie kommersiële aangespreek word nie. Deur hierdie tipe multi-faktoriale projekte aan te pak om die impak van koueskade te bepaal, is 'n beduidende bydrae gelewer om die voorkoms te verminder. Hierdie inligting word reeds deur produsente, uitvoerders en pakhuis opgeneem in hul aanbevelings en praktyke, en het koueskade gedurende die 2020-seisoen suksesvol verminder.

### 5.2.5 **PROGRESS REPORT: Investigation of factors contributing towards the non-conformance of in-transit citrus container shipments to cold protocol markets.**

Project 1240 (PHI 4-11) (2019/2 – 2020/21) by Paul Cronje, Tarl Berry (CRI) and Leila Gebers-Goedhals and Gculi Khumalo (SU-Logistics)

## Summary

The cold chain is vital in the reduction of negative physiological developments in citrus fruit to prolong the shelf-life and influence market rates. It is also important for some niche export markets, which have specified

time-temperature cold treatment protocols as phytosanitary risk mitigation measures for pests. Preliminary findings suggest a framework for successful engagement with the cold-treatment process. This framework was adapted from a South African citrus industry assessment conducted and it comprises three components: the pre-cooling phase, the container-packing phase, and the pulp/probe-stabilisation phase. The results of the study serve as a basis to further develop the research area by presenting recommendations. One aspect indicating a possible advantage to manage the complex landside process of the cold chain is the use of cellular based temperature monitoring devices making continuous access to temperatures possible, at this stage. The results of a commercial scale trial in Durban port provided real-time product temperature visibility, mostly in the cold store segment rather than during the shipping leg. In addition, the loggers are easier to insert and allow automated retrieval for temperature than the onboard probes. Their drawback lies in their signal strength and loss of connectivity during the seagoing stage (loss of line of sight to cellular network). This aspect of the project will be further evaluated in 2021 and final recommendations made.

## **Opsomming**

Die koueketting is noodsaaklik om die negatiewe fisiologiese prosesse van 'n sitrusvrugte se veroudering te verminder gedurende uitvoer en so die rakleefyd te verleng en die markwaarde te handhaaf. Dit is ook belangrik vir sommige uitvoermarkte wat kouebehandelingsprotokolle vir seker fitosanitêre plaes verlang. Voorlopige bevindings dui op 'n raamwerk vir suksesvolle betrokkenheid by die kouebehandelingsprotokol. Hierdie raamwerk is aangepas uit 'n evaluering van die Suid-Afrikaanse sitrusbedryf en bestaan uit drie komponente: die voorverkoeling-, laai- en die stabilisering fase. Die uitvloeïing van die studie dien as basis om die navorsing projek verder te ontwikkel. Een aspek wat dui op 'n potensieële voordeel is die gebruik van sellulêre gebaseerde temperatuur moniterings toestelle wat tydens die land gebaseerde gedeelte van die koueketting deurlopende toegang tot vrugtemperatuur moontlik kan maak. Die resultate van 'n kommersiële skaal proef in die Durbanse hawe, wys daarop dat 'n duidelik verhoogde sigbaarheid van die produk moontlik is voor verskeping plaasvind. Daarbenewens is die loggers makliker in te plaas en word die temperatuur outomatiese herwin tydens aankoms in die mark sonder 'n persoon wat betrokke raak. Die nadeel lê in die toerusting se seinsterkte en die verlies aan verbinding tydens die seevaart (verlies van siglyn na 'n mobiele netwerk). Hierdie aspek van die projek sal in 2021 verder geëvalueer word en finale aanbevelings gemaak word.

## **5.3 PROGRAMME: PRODUCTION AND QUALITY**

Programme coordinator: Pieter Raath (CRI)

### **5.3.1 Programme summary**

Research in this programme aims to provide practical recommendations to optimise citrus (1) fertilisation, (2) irrigation and (3) tree manipulation for production of maximum yields of quality fruit, with the least possible use of resources. During the 2020/2021 research period, two new projects were initiated on irrigation and one on root growth restricting soil management practices to reduce water consumption and restrict tree vigour, while one project was ongoing and three were completed. Directly below a summary of the results of the three completed projects, as well as the ongoing project, is provided. For elaborated summaries, see sections 5.3.2 to 5.3.5 below.

In the project that evaluated fruit set strategies for seedless 'Valencia' in the Letsitele region, possible factors that may influence the amount and quality of seedless Valencia produced in South Africa were investigated. Climatic conditions and production trends in Kirkwood and Letsitele were reviewed and showed that tracking weather extremes can assist in explaining variation in yields between seasons. The study also highlighted the unfortunate lack of targeted climatic studies in the various citrus producing regions to be able to identify climatic factors that cause inconsistent fruit set in each region. The study also revealed (i) that the use of uniconazole applications can increase the yield, and if high N applications are made, uniconazole should be applied to counteract the possible excessive vegetative growth that it may cause; and (ii) that higher potential soils improved the fruit size distribution, improving the orchards' profitability. Furthermore, by using the orchard level budget model it was shown that high fruit prices are the main factor ensuring profitability – high fruit prices,

however, are only obtained for high-quality fruit, meaning that producers must use innovative horticultural practices in the orchard.

Evaluation of the viability of aerial application and of adjuvants of micronutrients indicated that aerial application of micro-nutrients increased the concentration of the applied nutrient but should only be used to maintain the micronutrient nutrition status of trees. The different foliar nutrition products showed no difference in efficacy. Masterlock®, a product used to enhance spray droplet size and distribution, dramatically increased the droplet distribution, droplet size and total wetted surface area throughout the tree canopy. It will significantly improve the distribution and uptake of the spray mixture of aerially applied nutrient mixtures.

The response of Midnight Valencia in Nelspruit and Orri mandarins in De Wet to excessive fertilisation with N, P, K and Mg was investigated – with a specific focus on the changes in the trees' nutritional status as expressed in leaf and fruit analysis, vegetative growth responses, fruit set and effects on fruit quality. Significant insight regarding the lack of responsiveness - with the exception of nitrogen (N) - of both Valencia and mandarin trees to excessive mineral nutrition was gained. Consequently, it was concluded that in-season regular short-term changes in fertilisation rates in orchards that were applied have little value. Long-term tendencies should rather be used to establish the effect of over-fertilisation, and to adjust fertilisation programmes. The autumn sampling time ( $\pm 180$  days after full bloom) for foliar analysis was shown to sufficiently reveal the trees' overall nutritional response to a season's fertilisation and there is very limited value in using other sampling times. Furthermore, the sufficiency ranges should be regarded as secondary to observation of long-term trends in the foliar analysis, and foliar analysis should not be the only indication of whether the fertilisation programme followed is appropriate, especially in conditions of ample to high rates of fertilisation. Finally, the lack of response in fruit quality in these situations of excessive fertilisation highlighted the senselessness of over-fertilisation - the ability to manipulate tree performance or fruit quality is completely lost.

Lastly, in line with Citrus Research International's (CRI) mandate to maximise the long-term global competitiveness of the Southern African Citrus Growers, the Handbook on Fertilisation of Citrus was published to supplement the Fertilisation Guidelines, as in the CRI Production Guidelines. The Handbook is central to the CRI mandate to empower both citrus growers and advisors to obtain a thorough understanding of the theoretical and practical elements that need to be considered for optimal citrus nutrition management.

Outputs of research within this programme remain diverse, and some of them novel. Insights into complex production problems are being obtained, with simultaneously practical, readily-applicable outputs for cost-effective production.

### **Programopsomming**

Navorsing in hierdie program is daarop gemik om praktiese aanbevelings te genereer waardeur sitrus (1) bemesting, (2) besproeiing en (3) boom manipulasie verbeter kan word ten einde die hoogs moontlike oeste van goeie vruggehalte verkry kan word; terwyl die impak op hulpbronne verminder. Gedurende die 2020/21 navorsingsperiode was twee nuwe projekte van stapel gestuur wat fokus op besproeiing en een wat die waarde van inperking van wortels om waterverbruik en boomgroei te verminder ondersoek. Een projek bly lopend en drie projekte is afgehandel. Direk hieronder word 'n opsomming van die dire afgehandelde projekte asook die lopende projek se resultate gegee. Vir uitgebreide opsommings, sien afdelings 5.3.2 tot 5.3.5.

In die projek wat vrugset strategieë vir saadlose Valencia in Letsitele evalueer het, is moontlike faktore wat die aantal en kwaliteit van Valencia wat in Suid-Afrika produseer word ondersoek. Klimatologiese toestande en produksie neigings in Kirkwood en Letsitele is beoordeel en het aangetoon dat naspur van ekstreme weerstoestande kan help om variasie in opbrengs tussen seisoene te verklaar. Hierdie studie het ook die tekort aan gefokusde klimatologiese studies in die verskillende produksie streke uitgelig, waardeur klimaatsfaktore wat wisselvallige vrugset in die verskillende streke verklaar kan word. Voorts is ook bevind dat die gebruik van uniconazole toedienings opbrengs kan verhoog, en as groot N toedienings gemaak word, behoort uniconazole toedienings gedoen te word om die vegetatiewe groei in toom te hou. In hierdie studie is ook gevind dat op hoë potensiaal gronde 'n beter vrugrootteverspreiding verkry word – met die gepaargaande verhoging in

die boord se winsgewendheid. Deur middle van die “boordvlak begrotingsmodel” is daar getoon dat hoë vrugpryse die dominante faktor is wat winsgewendheid verseker. Hoë vrugpryse word egter net vir hoë kwaliteit vrugte verkry, wat beteken dat produsente innoverende praktyke moet aanwend tot hierdie doel.

Evaluasie van die werkbaarheid van lugbespuitings en bymiddels tot mikro-element blaarvoedingsprodukte het aangetoon dat lugbespuitings met mikro-elemente die konsentrasie van die betrokke element in die blare verhoog, maar net tot die mate dat dit vir handhawing van die blare se voedingstatus gebruik kan word. Die verskillende produkte wat in die projek evalueer is het nie in effektiwiteit verskil nie. Masterlock®, 'n produk wat gebruik word om druppelgrootte te verhoog en verspreiding, het die druppelgrootte, verspreiding en totale benatte oppervlak suksesvol regdeur die boom vergroot. Dit sal die verspreiding en opname van die spuitmengsel van lug bespuitte blaarvoedings verbeter.

Die reaksie van Midnight Valencia in Nelspruit en Orri mandaryne in De Wet op oormatige bemesting met N, P, K en Mg is ondersoek – met 'n spesifieke fokus op die veranderinge in die bome se voedingstatus (blaar en vrug analise), vegetatiewe groei, vrugset en vrug kwaliteit. Insiggewende data wat 'n tekort aan reaksie na beide Valencia en Orri mandaryne op oormatige bemesting toon – met uitsondering van stikstof (N) is verkry. Die gevolglike afleiding is dus dat in-seisoen gereelde kort-termyn aanpassings in bemesting van oorbemesting bome nie waarde het nie. Lang-termyn tendense moet eerder gebruik word om die effek van oorbemesting te evalueer, en om bemestingsprogramme mee aan te pas. Die herfs monsternemingstyd ( $\pm 180$  dae na volblom) is ook gevind om die algemene reaksie op die seisoen se bemesting effektief weer te gee en daar is beperkte waarde om ander tye te blaarmonsters te neem. Verder is ook gevind dat die algemene norme wat gebruik word as sekondêr tot lang-termyn neigings in blare se voedingstatus gesien te word – dit moet slegs as 'n aanduiding gebruik word om vas te stel of die bemestingsprogram wat gevolg is voldoende is, veral in toestande van gunstige tot oormatige vlakke van bemesting. Die tekort aan reaksie in vrugkwaliteit op oorbemesting het dan ook nie sinneloosheid van oorbemesting uitgelig – die vermoë om boomprestasie en vrugkwaliteit te manipuleer gaan in geheel verlore.

Laastens, in ooreenstemming met CRI se mandaat om die langtermyn globale kompetendheid van Suid-Afrikaanse sitrusprodusente te verhoog, is die Handboek vir Bemesting van Sitrus uitgegee – aanvullend tot die Bemestingsriglyne in die CRI produksiehandleiding. Die Handboek is sentraal tot CRI mandaat om sitrus produsente en adviseurs te bemagtig om 'n deeglike begrip van die teoretiese en praktiese elemente wat oorweeg moet word vir optimale plantvoeding te kan ontwikkel.

Uitsette van navorsing in hierdie program is uiteenlopend, nuut en innoverend. Insig rakende komplekse produksie kwessies word verkry, terwyl praktiese, toepasbare en koste-effektiewe oplossings voortdurend gesoek word.

### 5.3.2 **FINAL REPORT: Evaluation of aerial application and adjuvants of micronutrients** Project 1230 (2019/20 – 2021/22) by P.J. Raath (CRI), P. Strydom (Villa Crop) and V. White (CRI)

#### **Summary**

A trial to evaluate aerial application of micronutrients, and to test the value of an added adjuvant (MasterLock®) to improve application efficacy and nutrient uptake, was initiated in collaboration with Villa Crop. During September 2019 and 2020 an experimental orchard was selected at Letaba Estates (Tzaneen) and foliar B and Zn products were applied, as well as a patented product (MasterLock®) that enhances droplet distribution through the tree canopy. Leaf analyses to quantify total B and concentrations were conducted on samples before and two weeks after application. Droplet distribution and size was measured throughout the canopy to ascertain the efficacy of MasterLock® to improve distribution and wetting levels of the spray mixture.

Aerial application of micronutrients increased the total foliar nutrient concentration and can be used to maintain the micronutrient nutrition status of trees. Compared to the producer's prescribed programme, (present farm practice at a much lower application concentration), the Max-In products were not more effective to increase the foliar total B and Zn concentration when applied either aerially or conventionally. MasterLock® dramatically

increased the droplet distribution and total wetted surface area throughout the tree canopy. Droplet size was also increased which explains the better distribution through the canopy.

This study conclusively established that aerial application of foliar nutrients is effective and can be used successfully – even at the conventionally prescribed rates. MasterLock® will significantly improve the distribution and uptake of the spray mixture of aerially applied nutrient mixtures.

## Opsomming

'n Eksperiment om toediening van spoorlemente deur middel van lugbespuiting te evalueer, asook om die waarde van 'n bymiddel se vermoë om toedienings- en opname-effektiwiteit te verhoog, is in samewerking met Villa Crop geïnisieer. 'n Eksperimentele boord op Letaba Estates, Tzaneen, is gekies. Die eerste eksperimentele toediening van B en Zn blaarvoedingsprodukte, asook 'n gepatenteerde produk (MasterLock®) wat druppelverspreiding deur die lower verbeter, is in September 2019 en 2020 gedoen. Blaarontledings om die totale B en Zn konsentrasies te kwantifiseer is op blare gedoen wat voor, en twee weke na toediening, gemonster is. Druppelverspreiding en grootte is reg deur die boom se lower gemeet sodat die effektiwiteit van MasterLock®, om die verspreiding en benatting van die spuitmengsel te bevorder, bepaal kan word.

Lugbespuiting van spoorelemente het die totale konsentrasie van B en Zn in die blare verhoog en kan gebruik word om die spoorlementvoedingstatus van die bome te handhaaf. In vergelyking met die produsent se normaal voorgeskrewe praktyk (huidge praktyk waar toediening teen 'n veel laer konsentrasie is), was die Max-In produkte nie meer effektief om die blare se totale B en Zn konsentrasies te verhoog nie – nie met lugbespuitings of konvensioneel toegedien nie. MasterLock® het die druppelverspreiding en totale benatte blaaroppervlak reg deur die lower dramaties verhoog. Druppelgrootte is ook verhoog, wat die beter verspreiding verklaar.

Hierdie proef het data opgelewer wat oortuigend toon dat lugbespuitings met blaarvoedingsprodukte effektief is en suksesvol gebruik kan word – selfs teen die konvensioneel voorgeskrewe konsentrasies. MasterLock® sal die verspreiding en opname van spoorelemente wat met lugbespuitings toegedien word betekenisvol verhoog.

## Introduction

Large farming operations, where large blocks of similar, early ripening varieties are produced in early regions are under pressure to apply foliar nutrients and plant growth regulators (PGRs) in a short time-frame. The parallel need to simultaneously apply pest and disease control products makes application of foliar nutrients a practical challenge (personal communication: Ben Vorster). Aerial application of foliar nutrients is a novel approach to reduce tractor traffic in orchards and application time at critical phenological stages. Not only can vast areas be sprayed in a very short period, but also it is regarded as less costly and reduces pressure on the labour force. How effective penetration into the canopy, wetting of leaf surfaces and uptake of nutrients are, is unclear due to the small droplet size generated. Furthermore, mature citrus trees can present formidable application challenges due to their tall, broad and dense canopies, and application in this manner does not create the turbulence required to penetrate the canopy of the trees, so it is unclear whether wetting of the full leaf surface can be achieved (Salyani & Cromwell, 1992; Hoffmann & Salyani, 1996).

With these challenges in mind, one product is of particular interest, MasterLock®, a unique utility adjuvant that improves droplet adhesion, reduces bounce and increases droplet spreading to improve contact and coverage of spray mixtures. It also assists with penetration of droplets into the canopy (Winfield United, 2017), making it ideal to improve aerial application of foliar nutrients. In addition, the Villa MAX-IN micronutrient range has CornSorb™ technology included into the formulation. It is a combination of high fructose corn syrup and alkyl polyglucosides (APG). The high fructose corn syrup increases humectancy (leaf stays wet for longer) and the APG (sugar head with carbon tail) increases element/nutrient absorption. It is claimed by Villa that the unique combination of activities within CornSorb™ increase nutrient uptake by 25-50% compared to non-chelated micro-nutrients (tested against sulphates, boric acid and Fe-EDTA), as stated in the patent registration obtained in Feb. 2010 by WinField Solutions, LLC (Dr. Dale Blevins contract support data, Univ. of Missouri).

This project was therefore initiated in collaboration with the Nutrisolutions team of Villa (originally headed up by Teunis Vahrmeijer) with the main intention to establish whether the use of aerial application of foliar micronutrients is viable and effective. Since initiation of the project, Teunis Vahrmeijer has left the services of Villa Crop, leaving his colleague (Pierre Strydom) to manage and conduct the trial on his own. He subsequently enrolled at University of Pretoria for an MSc degree in February 2020, using this project as the topic for his dissertation. Doctor Nicky Taylor (UP) is his study leader, with Pieter Raath (CRI) the co-study leader.

### Stated objectives

To establish whether:

- Aerial application of foliar micronutrient spray tank mixtures is viable and effective.
- To what extent MasterLock® improves the penetration of spray tank mixtures into the tree canopy, and nutrient uptake for both conventional and aerial application of foliar nutrient spray mixtures.
- To what extent uptake is improved by the MAX-IN range of micronutrients for both conventional and aerial application of foliar nutrient spray mixtures.

### Materials and methods

#### Trial plot:

A field experiment was conducted in a mature Valencia orchard (block H75) at Letaba Estates, Letsitele (23.8667° S, 30.3167° E). Temperature, relative humidity and wind speed were recorded using a weather station present at the farm.

#### Treatments and experimental design:

Nine treatments, as indicated in Table 5.3.2.1, were applied in triplicate in a completely randomised block design. The treatments applied aim to compare aerial sprays (per aeroplane as indicated in Figure 5.3.2.1) with conventional spraying, as well as the efficacy of MasterLock® and the Max-IN products to improve foliar nutrition compared to the farm's normal practices (using Ecklomag®).

The experiment consisted of 27 experimental plots/sections, broken up in two sectors, namely:

- One sector of 24 plots, viz. seven treatments (1-7) replicated three times;
- Another sector of six plots, viz. treatments 8 & 9 also replicated three times.

Treatments 8 and 9 assess the efficacy of MasterLock® alone, which was sprayed at a later stage; these treatments were applied in a separate sector of the block.

Each experimental plot/section consisted of four rows that were separated on one side by four buffer rows, and on the other side by a road, as well as one buffer row on each side of the road (Figure 5.3.2.2). Within each experimental plot/section, sampling was done from the two middle rows, from six trees with similar vigour and canopy structure.

**Table 5.3.2.1.** Foliar nutrition treatments applied to a mature Valencia block on Letaba Estates to evaluate the efficacy of aerial foliar nutrition sprays as well as two commercial products.

TREATMENT	Description of treatments						
	Products and volumes used	Volume water Used (L/ha)	Total applied/ha (g)		Concentration in spray mixture (mg/L)		
			Zn	B	Zn	B	
1 Aerial Max-in	Max-in Boron 3.40L/ha + Max-in Zinc 4.5L/ha	100	329.4	326.4	3294	3264	

2	Conventional Max-In	Max-in Boron 3.40L/ha + Max-in Zinc 4.5L/ha	2 000	329.4	326.4	164.7	163.2
3	Aerial Max-In + MasterLock	MasterLock Rate 1% + Max-in Boron 3.40L/ha + Max-in Zinc 4.5L/ha	100	329.4	326.4	3294	3264
4	Conventional Max-In + MasterLock	MasterLock Rate 0.1% + Max-in Boron 3.40L/ha + Max-in Zinc 4.5L/ha	2 000	329.4	326.4	164.7	163.2
5	UTC	Untreated Control, only water applied	2 000	0	0	0	0
6	Aerial Farm Practice	Citrus to Grow® + Organotech®	100	5.2	6.8	52	68
7	Conventional Farm Practice	Citrus to Grow® + Organotech®	2 000	5.2	6.8	2.6	3.4
8	Aerial MasterLock +	MasterLock Rate 1%	100	N/A			
9	Conventional + MasterLock	MasterLock Rate 0.1%	2 000	N/A			



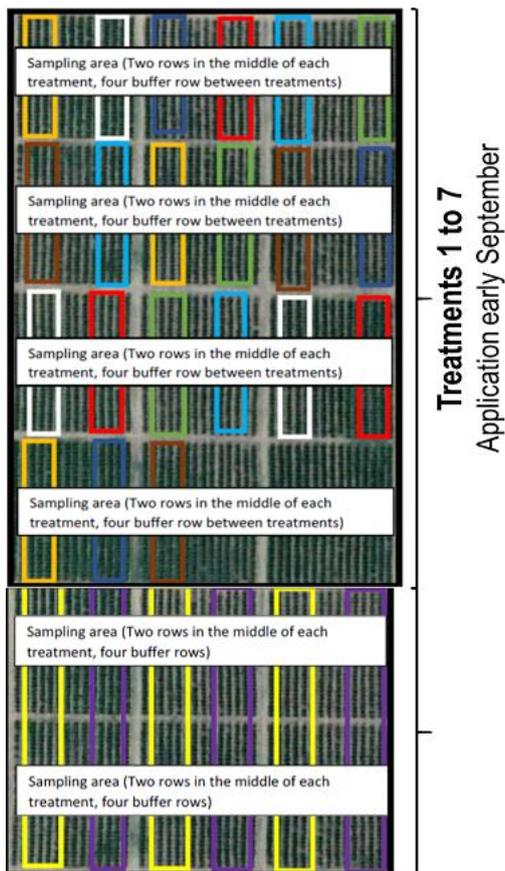
**Figure 5.3.2.1.** Evaluation of aerial application of foliar nutrients was conducted when products were applied by aeroplane.

The following actions/measurements were conducted on the selected trees:

- Sampling for foliar analysis before, and seven to fourteen days after application of spray mixture.
- Sampling of leaves at the same time for quantification of nutrient content in leaf tissue (quantification of Zn and B that that passed the cuticle and was taken up), using Scanning Electron Microscopy (SEM).
- Establish wetting pattern of spray applications on the tree, with an emphasis on the inside of the canopy, using water sensitive paper and by dissecting the canopy in three sections, placing the targets at three different heights within the tree.
- Measurement of droplet sizes using fluorescent dye and imaging software on water sensitive paper.
- Fruit set evaluations.
- Yield and fruit quality.

Timeline:

The trial ran over two seasons (summer 2019/2020 & 2020/2021). The second season was used to verify the results.



**Figure 5.3.2.2.** Trial layout indicating the treatments, experimental plots and sampling locations of the foliar nutrition trial conducted on Letaba Estates in a mature Valencia block.

## Results and discussion

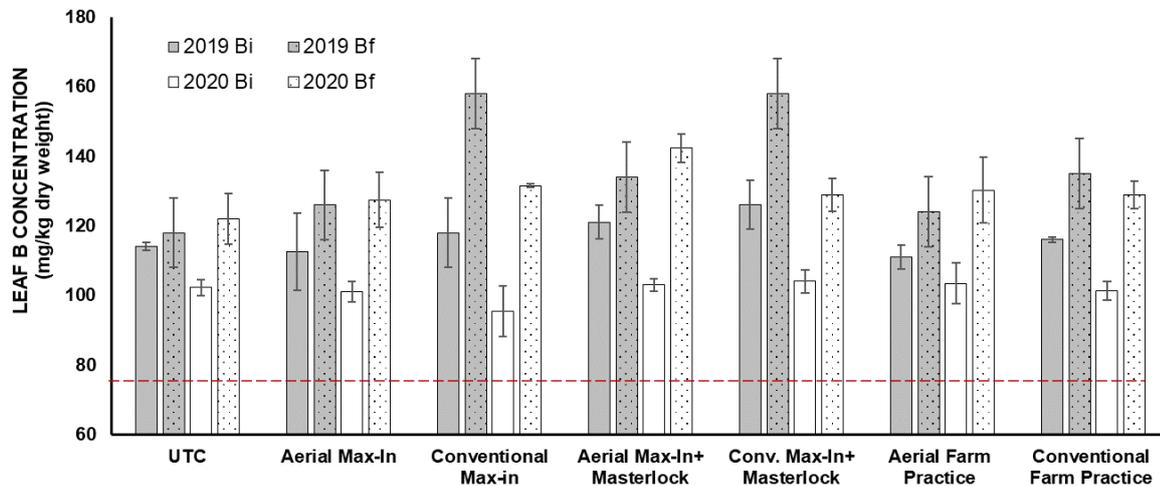
*Efficacy of method of application:* Pre-application boron (B) and zinc (Zn) leaf concentrations were within the accepted local norms proposed by Raath (2021) for both seasons (Figures 5.3.2.3 & 5.3.2.4). Although not statistical, an increase in the average leaf B and Zn concentrations of the untreated controls (UTC's) occurred from the pre-application to the post-application sampling times. The reasons for this is not clear – it might be due to cross- contamination from neighbouring experimental plots (probably due to the buffer areas not being sufficiently large). For evaluation of the efficacy of the spray application treatments the increases in foliar nutrient concentration is therefore not compared to that of the UTC *after* application - the increase in concentration between the concentration prior to application and after application for each treatment was rather considered, as well as between the different treatments (the UTC results after application was therefore ignored).

If it is assumed that a part of the B and Zn in the spray mixture remained on the leaf surface within the cuticle and epidermal wall or bound to the surface of the plasmalemma (Swietlik and Fraust, 1984), it can be concluded that all the treatments showed a significant efficacy of deposition of the applied B and Zn. Given the difference in concentration of the B and Zn in the spray mixtures, as well as the method of application, it can be concluded that sufficiently effective application of these nutrients occurred with all the treatments, making all of them suitable methods of application. Addition of MasterLock® did not improve the efficacy of conventional methods of application, but it significantly improved the efficacy of aerial applications (Figures 5.3.2.3 & 5.3.2.4). The use of MasterLock® in aerial applications therefore increases spray efficacy, but not to the extent that it exceeds that of conventional methods of application.

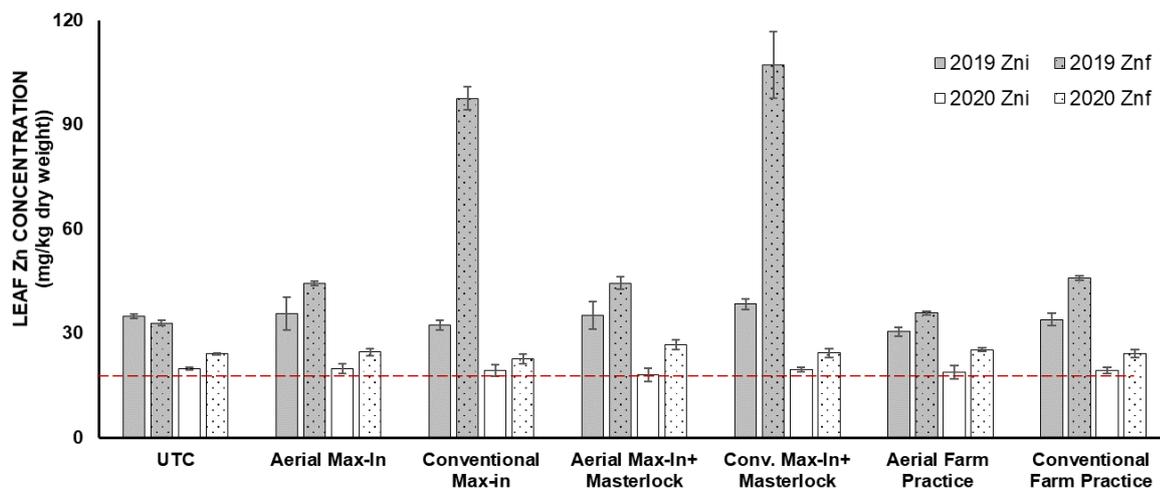
Furthermore, Figures 5.3.2.3 and 5.3.2.4 show that both conventionally and aerially applied B and Zn raised the leaf concentrations of these nutrients - there was no difference between the different aerial applications and the equivalent conventional method of application. It can therefore be concluded that aerial application of

foliar nutrition is just as effective to raise the total foliar B and Zn concentrations as the conventional methods of application.

Given the total application of B and Zn that were far higher for the Max-in treatments than the Farm Practice treatments, these results indicate that by raising the concentration of active nutrient above that which is effective will not further increase the efficacy of deposition or the total nutrient concentration of the leaves.



**Figure 5.3.2.3.** Comparison of pre-application (Bi) and post-application (Bf) boron concentrations of Valencia leaves as affected by foliar B nutrition and adjuvant application applied in September of 2019 and 2020 respectively – the horizontal line indicates the minimum acceptable B leaf norm.



**Figure 5.3.2.4.** Comparison of pre-application (Zni) and post-application (Znf) zinc concentrations of Valencia leaves as affected by foliar Zn nutrition and adjuvant application applied in September of 2019 and 2020 respectively - the horizontal line indicates the minimum acceptable Zn leaf norm.

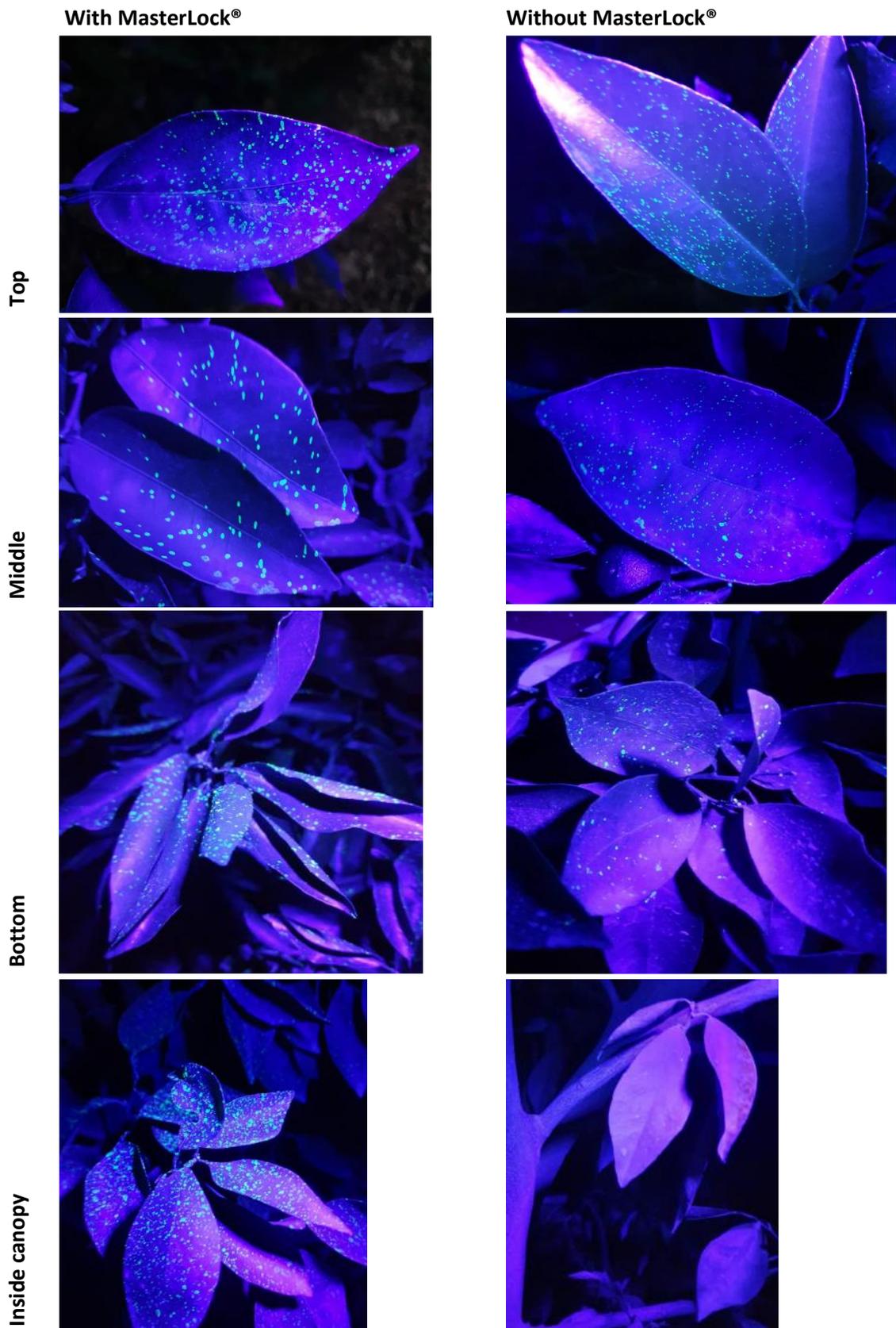
Due to the fact that much of the applied product can remain within the leaf's cuticle and is therefore not taken up to become metabolically active, as well as the fact that higher solute concentration can increase uptake of cations (Schreiber, 2005), analyses of cell layers of the leaves were done in cross sections (adaxial epidermis, mesophyll, abaxial epidermis) to quantify the B and Zn concentration. This was done by means of energy dispersive spectrometry and wavelength dispersive spectrometry using SEM (of the central analytical facility of Stellenbosch University (CAF)). The results were disappointing since it was only after analysis established that the detection limit of the method is 100 mg/kg, while **total dry mass** Zn and B concentrations typically is in the range of 25-100 mg/kg and 75-200 mg/kg respectively. The outcome was that no B was detected in the leaf tissues, while only in 5 of the 21 samples (replicates) Zn concentrations, ranging from 200 to 400 mg/kg (indicated as 0.2% -0.4%), were indicated. All three of the Aerial Farm Practice (T6) replications were included in the samples in which Zn was detected (data not shown) – whether this actually has any significant meaning,

is not clear. The variation in analysis results of the macronutrients, e.g. Na, K, Ca, Mg, is large and follows no meaningful pattern. For the purpose of this project, it is therefore inferred that either the method of analysis or the service provided by CAF of US is not utilisable.

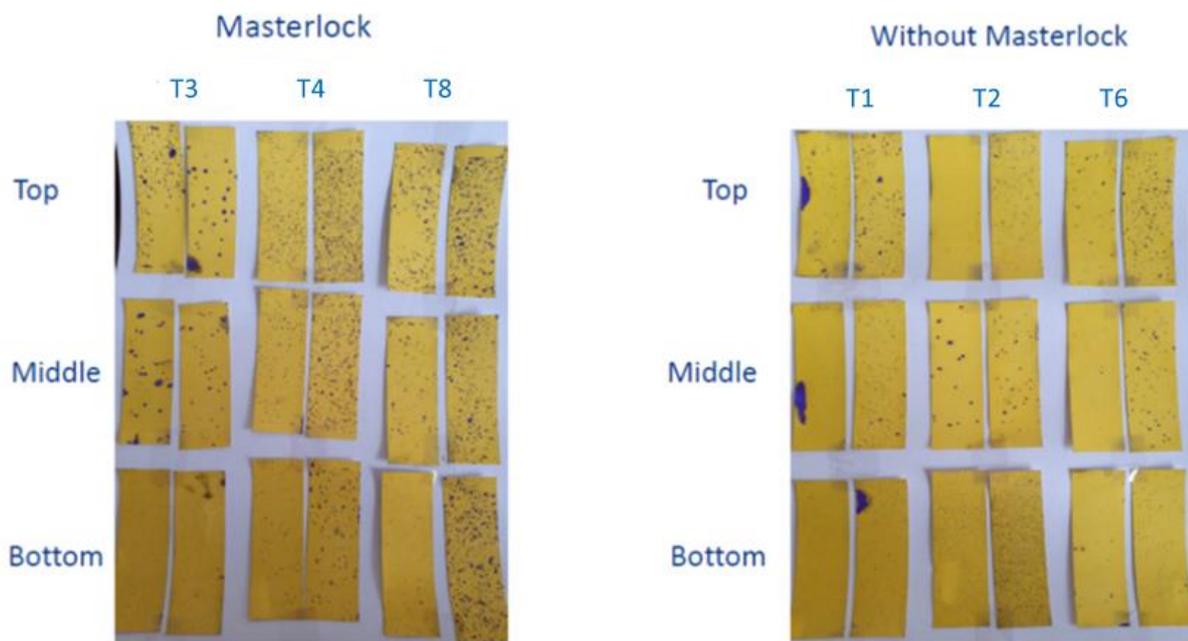
*Droplet size and wetting pattern:* Measurement of droplet sizes and the wetting pattern of the spray applications at different positions in the canopy, as determined using fluorescent dye (Figure 5.3.2.5) and water sensitive paper (Figures 5.3.2.6, 5.3.2.7 & 5.3.2.8) respectively, yielded the following results:

- A similar wetting pattern (distribution of droplets) were obtained for aerial applications on the top leaves, although the drop size was slightly larger where MasterLock® was used. Wetting in the middle and bottom sections of the trees was far better/higher with the use of MasterLock® (Figures 5.3.2.5 to 5.3.2.8).
- A dramatic increase in wetting of the leaf surface (percentage covered) was obtained inside the canopy with the use of MasterLock® (Figures 5.3.2.5 & 5.3.2.7).
- Drop size was also improved with the use of MasterLock®, especially in the middle and bottom sections of the tree canopy, as illustrated with the water sensitive paper in Figures 5.3.2.6 & 5.3.2.8).
- The total percentage of the leaf surface covered is increased dramatically, especially for the middle and bottom sections of the tree canopy (Figure 5.3.2.7).

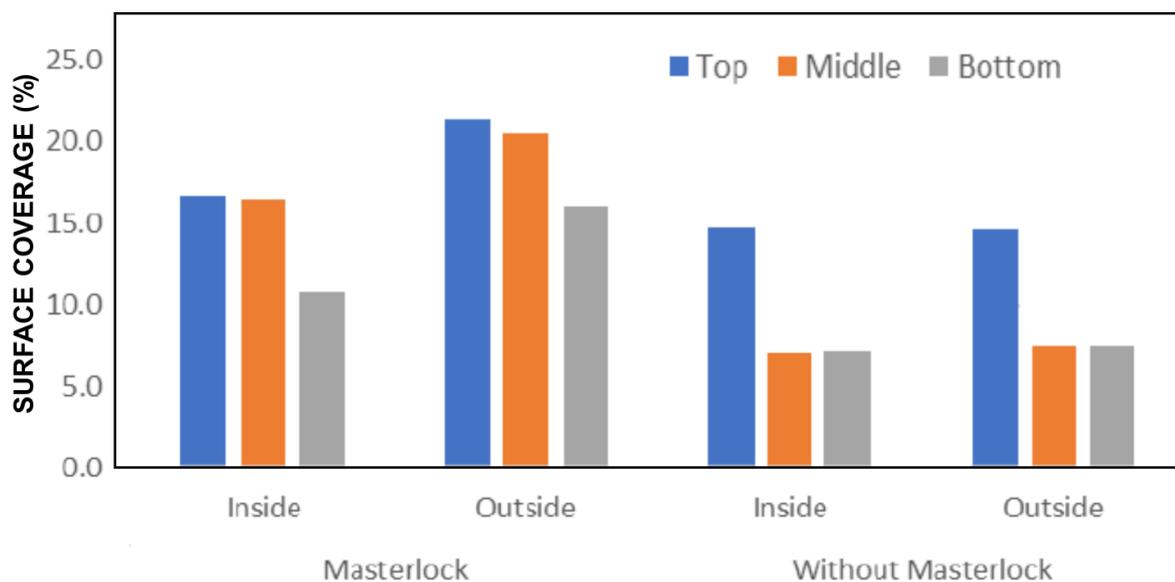
It can therefore be concluded that MasterLock® dramatically increased droplet distribution throughout the tree canopy. Drop size was also increased which explains the better distribution through the canopy, but it should also enhance nutrient uptake.



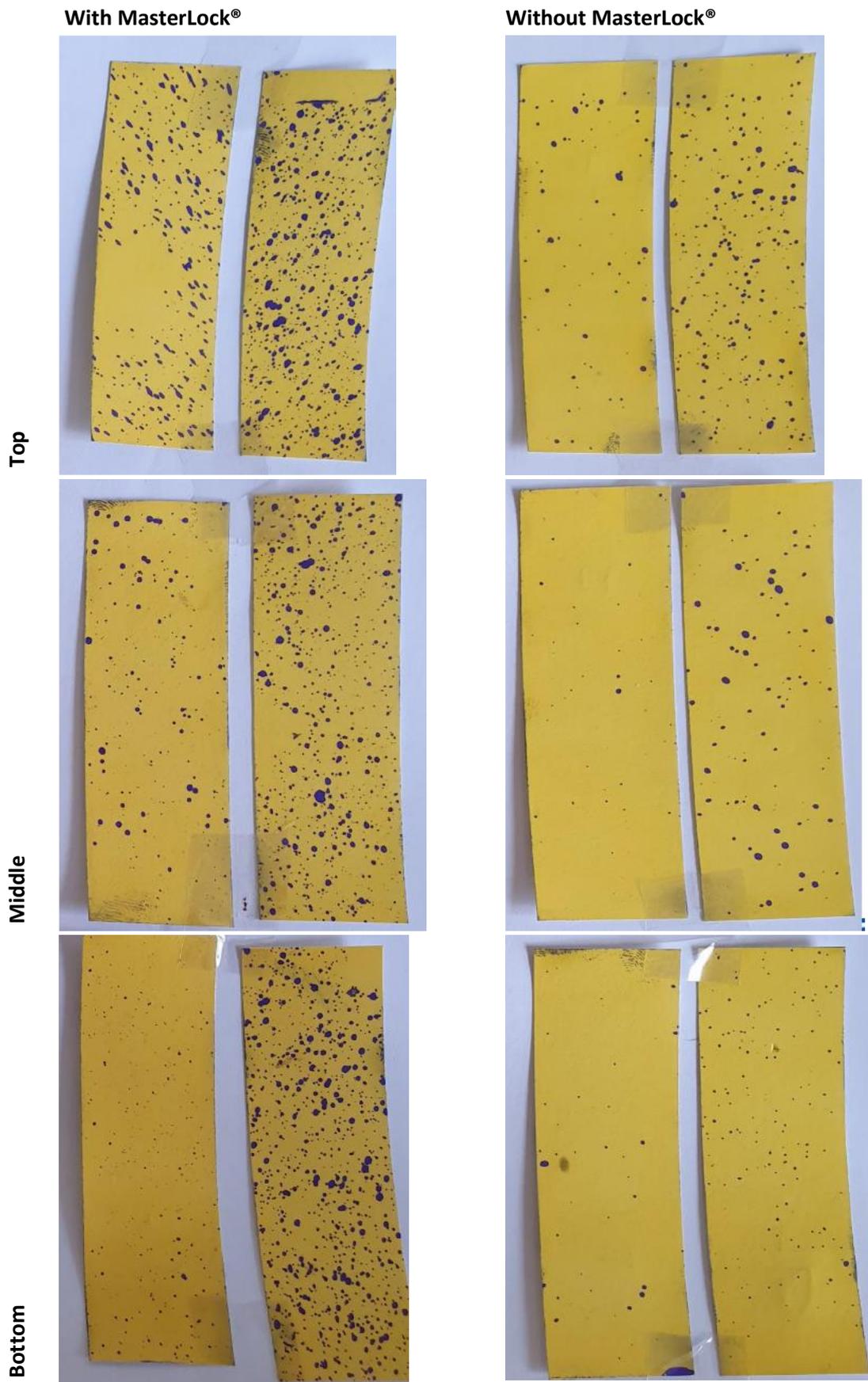
**Figure 5.3.2.5.** Difference in distribution of droplets and their sizes between aerial applications containing MasterLock® and without, at different positions in the tree canopy as shown with fluorescent dye.



**Figure 5.3.2.6.** Difference in distribution of droplets (wetting pattern) between treatments containing MasterLock® and without MasterLock®, at different positions in the tree canopy as shown with water sensitive paper.



**Figure 5.3.2.7.** Percentage surface coverage obtained for the two aerial application treatments that respectively contained MasterLock® (T8) and did not (T6).



**Figure 5.3.2.8.** Difference in distribution of droplets between treatments containing MasterLock® and without MasterLock®, at different positions in the tree canopy as shown with water sensitive paper.

## Conclusion

Aerial application of micronutrients increased the total foliar nutrient concentration and can be used to maintain the micronutrient nutrition status of trees. The total B and Zn was not increased significantly in the leaves with the use of Max-In products compared to the producer's products despite a far higher total amount per ha and concentration of B and Zn being applied with the use of Max-in products. Addition of MasterLock® did not improve the efficacy of conventional methods of application, but it significantly improved the efficacy of aerial applications (Figures 5.3.2.3 & 5.3.2.4). The use of MasterLock® in aerial applications therefore increases spray efficacy, but not to the extent, that it exceeds that of conventional methods of application.

The use of SEM technology, as employed in this report period, was unfruitful. It is, however, very important that the actual uptake of the nutrients be quantified since this will indicate the efficacy of the Max-in technology compared to other products.

MasterLock® dramatically increased droplet distribution and total wetted surface area throughout the tree canopy. Drop size was also increased which explains the better distribution through the canopy. It should therefore also enhance nutrient uptake.

In conclusion: aerial application of foliar nutrients is effective and can be used successfully – even at the conventionally prescribed rates. MasterLock® will significantly improve the distribution and uptake of the spray mixture of aerially applied nutrient mixtures.

## Future research

Method development for the proper quantification of the actual uptake of foliar applied nutrients will increase the value of future research in this field.

## Technology transfer

None so far - an article for the SAFJ is planned, as well as a presentation by the student at the next CRI research symposium.

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### 5.3.3 FINAL REPORT: Adaptive Nutrition Management Strategies for Improved Fruit Quality Project 1231/RCE-02-02 (April 2019 – March 2021) by PJ Raath (CRI)

## Summary

In this trial, the response of Midnight Valencia in Nelspruit and Orri mandarins in De Wet to excessive fertilisation with N, P, K and Mg was investigated. A specific focus was the changes in the trees' nutritional status as expressed in leaf and fruit analysis, as well as vegetative growth responses, fruit set and effects on fruit quality. The goal was to determine whether fertilisation requirements could be established from early season leaf and/or fruit mineral composition data so that for in-season adjustments of the fertilisation programme and/or manipulation of mandarin and Valencia quality could be achieved. Ultimately, development of a strategy for improved fertilisation management and fertiliser usage, possibly through better monitoring of tree nutrient (especially N, P and K) status, was the intention.

Significant insight regarding the lack of responsiveness of both Valencia and mandarin trees to excessive mineral nutrition was gained – with the exception of nitrogen (N). Consequently, it was concluded that it is not possible to do in-season regular short-term changes in fertilisation rates in orchards that are amply fertilised. Long-term tendencies should rather be used to establish the effect of over-fertilisation, and to adjust fertilisation programmes. The autumn sampling time ( $\pm 180$  days after full bloom) for foliar analysis was shown to sufficiently reveal the trees' overall nutritional response to a season's fertilisation and there is very limited value in using other sampling times. Furthermore, the limited usefulness of foliar analysis in conditions of ample to over-supply of nutrients was emphasised – the maximum norms were particularly found to be irrelevant. The sufficiency ranges should be regarded as secondary to observation of long-term trends in the foliar analysis, and foliar analysis should not be the only indication whether the fertilisation programme followed is appropriate, especially in conditions of ample to high rates of fertilisation. Finally, the lack of response in fruit quality in these situations of excessive fertilisation highlighted the senselessness of over-fertilisation - the ability to manipulate tree performance or fruit quality is completely lost.

## Opsomming

In hierdie projek is die reaksie van Midnight Valencia in Nelspruit en Orri mandaryne in De Wet op oormatige bemesting met N, P, K en Mg ondersoek. Daar is gefokus op veranderinge in die bome se voedingstatus, soos uitgedruk in blaar en vrugontledings, asook vegetatiewe groei, vrugset en die effek op vrugkwaliteit. Die doel was om vas te stel of bemestingsbehoefte bepaal kan word uit vroeë, in-seisoen, blaar- en/of vrugontledings, sodat aanpassings in die seisoen reeds gemaak kan word om vrugkwaliteit te manipuleer. Die uiteindelijke doelwit is om 'n strategie vir verbeterde bemestingsbestuur en verhoogde kunsmis gebruikseffektiwiteit met behulp van meer akkurate monitering daar te stel.

Met uitsondering van stikstof (N), was daar 'n afwesigheid van waargenome reaksie op oormatige bemesting by beide die Valencia and mandaryn bome. Die gevolgtrekking was dat dit nie moontlik is om in-seisoense, kort-termyn veranderinge in bemestingstoedienings te maak as die bome voldoende tot oorvoorsien aan voedingstowwe is nie. Lang-termyn neigings moet eerder gebruik word om die effek van oorbemesting te monitor en gebruik word om aanpassings in bemestingsprogramme te maak. Blaarontledings in die Herfs ( $\pm 180$  dae na volblom) is bevestig as geskik vir die evaluasie van bome se algemene voedingstatus en hul reaksie op die seisoen se bemestingsprogram – daar is 'n baie beperkte waarde in die neem van vroeëre monsters. Die beperkte waarde van blaarontledings in toestand van volop of oormatige bemesting is egter aangetoon, met die maksimum norme wat spesifiek as waardeloos bevind is. Die optimale grenswaardes moet as sekondêr tot die waarneming van lang-termyn neigings beskou word, end us nie as die enigste aanduiding van die geskiktheid van 'n bemestingsprogram beskou word nie – veral nie in toestand van volop of oorbemesting nie. Laastens, die tekort aan reaksie spver die vrugkwaliteit aangaan het die tekort aan logika van oorbemesting beklemtoon – die vermoë om vrugkwaliteit te manipuleer gaan verlore.

## Introduction

Fertilisation of citrus trees is regarded as an important factor that influences photosynthesis, flowering, fruit set, fruit growth and fruit quality. Ladaniya (2008) regards balanced nutrient management through chemical fertilisers and organic manures as the key for producing good quality citrus fruit with desired storage ability. Progress in citrus tree nutrition has led to the incorporation of various practices to better manipulate tree physiology and ensure improved set, higher production and superior fruit quality (Coetzee, 2007). Lately producers are confronted by concepts such as geoinformatics (to address spatial variation), open field hydroponics in a low-flow context using soil water extractions to establish fertilisation requirements (also to address spatial variation), site-specific nutrient management strategies, the use of biostimulants, exploiting nutrient-hormone synergies and the use of both organic matter and soil microbiology to improve fertilisation efficiency and improve the conventional methods of nutrient management (Srivastava, 2012).

Being bombarded by all these concepts, a critical need that citrus producers are experiencing concerning tree nutrition is the ability to practice effective decision making, *viz.*, to “nutrient management through soil and foliar applications, as the need arises”. Alternatively phrased, they need to have access to verify “adaptive

management strategies” to obtain a specific objective (e.g., improved set or fruit size, thicker skins, larger fruit, etc.). In addition to varying climatic conditions between seasons, individual producers have unique combinations of varieties, irrigation and fertilisation methodology, soil and climatic conditions and market-related demands. Improved decision-making aids, to assist individual producers make site and goal specific adaptations to their fertilisation, is therefore required. A research programme to meet this need and maintain fertilisation practices in the South African Citrus Industry at the forefront of global trends is being established. This research project was aimed at addressing the need for improved decision-making regarding N, P and K fertilisation through development and use of norms for early leaf sampling, fruit growth patterns and changes in mineral composition of fruit were elucidated. The ultimate goal being improved set and accurately managing fruit size, skin- and internal quality.

### **Stated objectives**

- To establish whether a fertilisation requirement model can be developed from early season leaf and/or fruit mineral composition data for *in-season* (i) adjustments of the fertilisation programme and (ii) manipulation of mandarin and Valencia quality.
- To establish to what extent *higher rates* of N, P, K and Mg fertilisation rates affect fruit set, fruit development, and fruit quality.
- Improve fertilisation management and optimise fertiliser usage (so called “best management practice”) by better monitoring tree nutrient (especially N, P and K) status.

### **Materials and methods**

#### Trial plot

The experiment was conducted in two locations, on two varieties, respectively:

Plot 1: ‘Orri’ mandarin trees budded onto ‘Carrizo’ citrange rootstock in a commercial four-year-old orchard in the De Wet area, Western Cape;

Plot 2: A mature, 10-year-old commercial ‘Midnight’ Valencia/C35 citrange orchard at Crocodile Valley, Nelspruit.

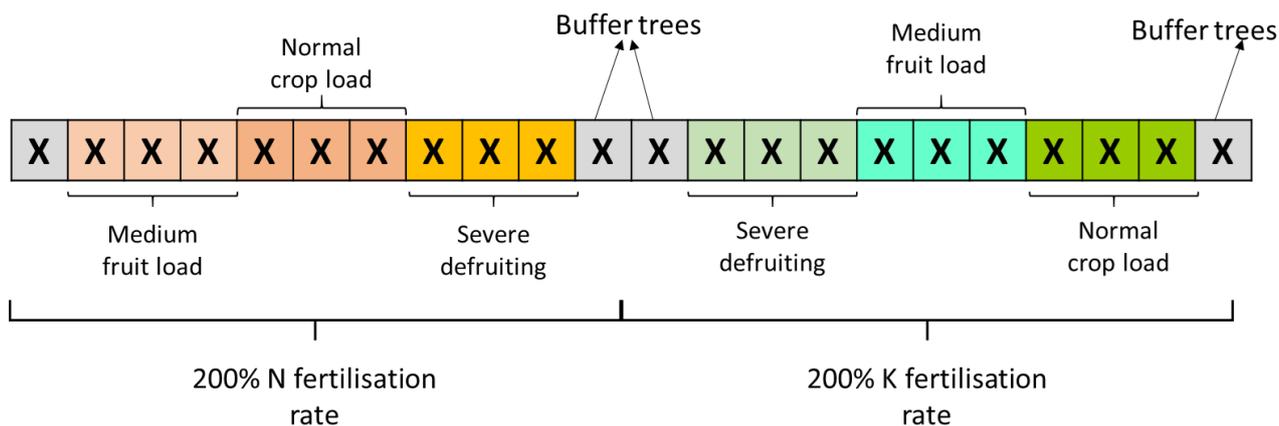
All trees received the standard farm practice fertiliser applications, with the rate of application based on OHS principles (“Orri” Mandarin block); and annual leaf mineral nutrient analysis together with an average target fruit yield (“Midnight” Valencia block).

#### Treatments and experimental design

The experiment consists of two phases:

Phase 1 (July 2018 to harvest 2019): In two locations, i.e., Stellenbosch and Nelspruit, two mandarin (Orri and Nadorcott) and Valencia (Midnight and Delta) cultivars were respectively selected to establish a baseline trend of changes in leaf and fruit mineral nutrient content throughout the season. In both locations, the respective cultivars were planted in blocks that were similar in age and on the same soil types. In each area, for both varieties, twenty trial trees that are uniform in size, fruit load and health were selected before the start of commercial harvest in 2018. They were divided into ten randomly located two-tree plots (n=10) from which mature leaves, flowers, fruitlets and fruit samples were collected at monthly intervals, starting in July (2018), and finishing in March (2019).

Phase 2 (July 2019 to harvest 2021): An experiment was set up in both locations in a randomised complete block design using plots of 11 trees, replicated four times. Five main treatments of fertiliser application were applied (Table 5.3.3.1). In each experimental plot, the middle nine trees were used for data collection (Figure 5.3.3.1). They were split in three groups of three trees, on which three de-fruiting treatments were respectively applied at random (Table 5.3.3.1) early December 2019. For the moderate de-fruiting treatment, all fruit smaller than 10 mm were removed by hand. For the severe de-fruiting treatment, all fruit smaller than 15 mm were removed.



**Figure 5.3.3.1.** Illustration of two neighbouring experimental plots indicating the randomised crop load treatments within each set of nine trees upon which the main fertilisation treatment is applied.

The fertilisation applied as main treatments are described in Tables 5.3.3.2 and 5.3.3.3, while the actual applied amounts of each nutrient are presented in Tables 5.3.3.4 and 5.3.3.5.

#### Data generation

*Quantify flowering, fruit set and vegetative shoot development:* The number of flowers and total number of new vegetative shoots after cessation of periods of vegetative shoot flushes in spring (November), summer (February) and autumn (April) were determined per tree. The phenological pattern in individual shoots were followed by randomly selecting ten shoots from each tree.

*Leaf, flower and fruit sampling for mineral nutrient analysis:* On a monthly basis, mature leaves were sampled from spring flushes (collected from vegetative shoots that developed during the previous season's vegetative shoot flushes), through summer (samples collected from vegetative shoots that developed during the current season's spring vegetative shoot flush), and autumn (samples collected from vegetative shoots that developed during the summer vegetative shoot flush). Selecting leaves on fully hardened, non-fruiting and purely vegetative shoots at a height of 1.5 m of the ground from the outer layer of the tree canopy, 20 leaves at the third to fifth position from the latest applicable flush, on at least four shoots per tree were sampled. An additional sample from fruit bearing shoots were also collected in autumn.

At anthesis, 80 full flowers were sampled at random around the tree from terminal positions at about 1.5 m from the ground.

Fifty fruitlets were sampled on a monthly basis from anthesis until autumn. They were taken at random around the tree from single-fruiting shoots on the terminal position, 1.5 m from the ground. In addition, fruit were also sampled at harvest, at random, i.e., different canopy positions.

*Fruit growth:* When the fruitlets reached a diameter of  $\pm 20$  mm, or after November fruit fall, ten were tagged per tree at 1.5 m from the ground per treatment/replicate. Fruit size (diameter) was then measured on a monthly basis until autumn (March/April), and then again at harvest, but then at random from different canopy positions. Fruit diameter were measured with an electronic fruit size measurer (EFM) and data logger [EFM & Data Logger; Güss Manufacturing (Pty) Ltd, Strand, South Africa].

*Fruit yield:* Total fruit yield was determined when harvest commenced (*viz.*, when the fruit quality indices complied with specifications established by fruit export markets) by stripping the trees, counting, and weighing each tree's fruit separately.

*Fruit quality:* One week before the orchards were commercially harvested, a sample of 12 randomly selected fruit per data tree were collected to determine the effects of treatments on fruit quality attributes. This was done

in the laboratory and the quality parameters evaluated were fruit size (diameter and weight), colour, rind thickness, total soluble solids (TSS), titratable acidity (TA) and juice percentage (%). Fruit size was measured using the Güss EFM and data logger, and then cut in half to measure rind thickness at opposite sides using an electronic calliper (CD-6" C; Mitutoyo Corp, Tokyo, Japan) and to calculate the average rind thickness. Fruit colour was evaluated using the standard colour charts. A fruit juicer (Sunkist®, Chicago, IL) was then used to extract the juice from the fruit to calculate the juice percentage, measure the TSS with a refractometer (PR-32 Palette, Atago Co., Tokyo, Japan) and determine the TA by titration with NaOH as base and phenolphthalein as indicator. The TSS:TA was calculated by dividing the TSS by the TA.

### Statistical analysis

SAS data analysis software (Version 9.4; SAS Institute Inc, Cary, USA) was used to analyse the data. Analysis of variance (ANOVA) or repeated-measures ANOVA was performed when responses were repeated on the same respondent. Mean separations were carried out using Fisher's least significant difference test where it was applicable, at a  $P \leq 0.05$ .

**Table 5.3.3.1.** Exposition of the various treatments that were applied.

Treatment number	Treatment name	Description of treatments
<b>Fertilisation trial</b>		
1.1	Control	Farm's prescribed standard fertilisation programme followed, with moderate defruiting in November/December to obtain medium fruit load
1.2	N	Application of 200% <b>N</b> of the standard recommended N fertilisation
1.3	P	Application of 200% <b>P</b> of the standard recommended P fertilisation
1.4	K	Application of 200% <b>K</b> of the standard recommended K fertilisation
1.5	Mg	Application of 200% <b>Mg</b> of the standard recommended Mg fertilisation
<b>Crop load trial</b>		
2.1	Control	Untreated trees, heavy fruit load, commercial practice without fruit thinning
2.2 (also 1.1)	Medium fruit load	Moderate defruiting in November/December to obtain medium fruit load
2.3	Severe defruiting	Severe defruiting in November/December to obtain low fruit load

**Table 5.3.3.2.** Fertilisation programme followed throughout the 2019/2020 and 2020/2021 seasons respectively, applied as standard farm practice on the De Wet/Orrri *Mandarin* trial plot – the indicated amounts were doubled (applied at 200% rate) for the respective treatments above.

Month 2019/20	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	TOTAL
N	12	17	35	52	69	75	75	75	69	35	35	29	577
P	0	0	1	1	1	1	1	1	1	1	1	1	10
K	4	6	13	19	26	28	28	28	26	13	13	11	215
Mg	2	3	5	8	11	12	12	12	11	5	5	5	90
Percentage of total annual N application	2%	3%	6%	9%	12%	13%	13%	13%	12%	6%	6%	5%	100%

**Table 5.3.3.3.** Fertilisation programme followed throughout the 2019/2020 and 2020/2021 seasons respectively, applied as standard farm practice on the Nelspruit/*Midknight Valencia* trial plot - the indicated amounts were doubled (applied at 200% rate) for the respective treatments above.

Month 2019/20		Jul	Aug	Sep	Oct	Nov	TOTAL
N	kg/ha	50	2.5	27	9	2	90
P		27	0	5	2	0.5	35
K		0	0	82	27	5	114
Mg*		0	0 (12)*	0 (12)*	0 (12)*	0	0 (36)*
Percentage of total annual N application		55%	3%	30%	10%	2%	100%

\*The total annual Mg that was applied in Treatment 1.5 was 36 kg/ha, split up in three instalments from August to October.

**Table 5.3.3.4.** The actual applied amounts of each nutrient to the *Orri Mandarin* orchard at DeWet during the two seasons.

<b>First season (2019/2020)</b>														
Supply	Nutrient (kg/ha)	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	Apr	May	Jun	TOTAL
Commercial nutrient supply*	N	11.5	17.3	34.6	51.9	69.2	75.0	75.0	75.0	69.2	34.6	34.6	28.9	<b>577</b>
	P	0.2	0.3	0.6	0.9	1.2	1.3	1.3	1.3	1.2	0.6	0.6	0.5	<b>10</b>
	K	4.3	6.5	12.9	19.4	25.8	28.0	28.0	28.0	25.8	12.9	12.9	10.8	<b>215</b>
	Mg	1.8	2.7	5.4	8.1	10.8	11.7	11.7	11.7	10.8	5.4	5.4	4.5	<b>90</b>
Additional treatment supply**	<b>N</b>	11.5	17.3	34.6	51.9	69.2	75.0	75.0	75.0	69.2	34.6	34.6	28.9	<b>577</b>
	<b>P</b>	0.1	0.15	0.3	0.45	0.6	0.65	0.65	0.65	0.6	0.3	0.3	0.25	<b>5</b>
	<b>K</b>	4.3	6.5	12.9	19.4	25.8	28.0	28.0	28.0	25.8	12.9	12.9	10.8	<b>215</b>
	<b>Mg</b>	1.8	2.7	5.4	8.1	10.8	11.7	11.7	11.7	10.8	5.4	5.4	4.5	<b>90</b>
Total Nutrient supply**	<b>N</b>	23.1	34.6	69.2	103.9	138.5	150.0	150.0	150.0	138.5	69.2	69.2	57.7	<b>1154</b>
	<b>P</b>	0.3	0.45	0.9	1.35	1.8	1.95	1.95	1.95	1.8	0.9	0.9	0.75	<b>15</b>
	<b>K</b>	8.6	12.9	25.8	38.7	51.6	55.9	55.9	55.9	51.6	25.8	25.8	21.5	<b>430</b>
	<b>Mg</b>	3.6	5.4	10.8	16.2	21.6	23.4	23.4	23.4	21.6	10.8	10.8	9.0	<b>180</b>
<b>Second season (2020/2021)</b>														
Supply	Nutrient (kg/ha)	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	Apr	May	Jun	TOTAL
Commercial nutrient supply*	N	11.5	17.3	34.6	51.9	69.2	75.0	75.0	75.0	69.2	34.6	34.6	28.9	<b>577</b>
	P	0.2	0.3	0.6	0.9	1.2	1.3	1.3	1.3	1.2	0.6	0.6	0.5	<b>10</b>
	K	4.3	6.5	12.9	19.4	25.8	28.0	28.0	28.0	25.8	12.9	12.9	10.8	<b>215</b>
	Mg	1.8	2.7	5.4	8.1	10.8	11.7	11.7	11.7	10.8	5.4	5.4	4.5	<b>90</b>
Additional treatment supply**	<b>N</b>	11.5	17.3	34.6	51.9	69.2	75.0	75.0	75.0	69.2	34.6	34.6	28.9	<b>577</b>
	<b>P</b>	0.2	0.3	0.6	0.9	1.2	1.3	1.3	1.3	1.2	0.6	0.6	0.5	<b>10</b>
	<b>K</b>	4.3	6.5	12.9	19.4	25.8	28.0	28.0	28.0	25.8	12.9	12.9	10.8	<b>215</b>
	<b>Mg</b>	1.8	2.7	5.4	8.1	10.8	11.7	11.7	11.7	10.8	5.4	5.4	4.5	<b>90</b>
Total Nutrient supply**	<b>N</b>	23.1	34.6	69.2	103.9	138.5	150.0	150.0	150.0	138.5	69.2	69.2	57.7	<b>1154</b>
	<b>P</b>	0.4	0.6	1.2	1.8	2.4	2.6	2.6	2.6	2.4	1.2	1.2	1.0	<b>20</b>
	<b>K</b>	8.6	12.9	25.8	38.7	51.6	55.9	55.9	55.9	51.6	25.8	25.8	21.5	<b>430</b>
	<b>Mg</b>	3.6	5.4	10.8	16.2	21.6	23.4	23.4	23.4	21.6	10.8	10.8	9.0	<b>180</b>

\*The control treatment received only the commercial nutrient supply, i.e., the producer's typical fertilisation rates.

\*\*N, P, K, Mg in this row represents the treatments.

**Table 5.3.3.5.** The actual applied amounts of each nutrient to the *Midknight Valencia* orchard at Nelspruit during the two seasons.

<b>First season (2019/2020)</b>														
Supply	Nutrient (kg/ha)	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	Apr	May	Jun	TOTAL
Commercial nutrient supply*	N	60.8	2.5	27.1	9.0	1.8	-	-	-	-	-	-	-	101
	P	24.3	-	5.4	1.8	0.4	-	-	-	-	-	-	-	32
	K	-	-	81.6	27.2	5.4	-	-	-	-	-	-	-	114
	Mg	-	-	-	-	-	-	-	-	-	-	-	-	0
Additional treatment supply**	N	-	64.7	28.8	9.0	-	-	-	-	-	-	-	-	103
	P	-	24.3	5.8	-	-	-	-	-	-	-	-	-	30
	K	-	-	80.9	27.2	-	-	-	-	-	-	-	-	108
	Mg	-	72.7	-	-	-	-	-	-	-	-	-	-	73
Total nutrient supply*	N	60.8	67.2	55.9	18.0	1.8	-	-	-	-	-	-	-	204
	P	24.3	24.3	11.2	1.8	0.4	-	-	-	-	-	-	-	62
	K	-	-	162.5	54.4	5.4	-	-	-	-	-	-	-	222
	Mg	-	72.7	-	-	-	-	-	-	-	-	-	-	73
<b>Second season (2020/2021)</b>														
Supply	Nutrient (kg/ha)	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	Apr	May	Jun	TOTAL
Commercial nutrient supply*	N	50.0	35.8	27.1	9.0	-	-	-	-	-	-	-	-	122
	P	-	24.3	5.4	1.8	-	-	-	-	-	-	-	-	32
	K	-	-	81.6	27.2	-	-	-	-	-	-	-	-	109
	Mg	-	-	-	-	-	-	-	-	-	-	-	-	0
Additional treatment supply**	<b>N</b>	-	85.9	27	9.7	-	-	-	-	-	-	-	-	123
	<b>P</b>	-	24.3	-	-	-	-	-	-	-	-	-	-	24
	<b>K</b>	-	-	82.8	27	-	-	-	-	-	-	-	-	110
	<b>Mg</b>	-	72.7	-	-	-	-	-	-	-	-	-	-	73
Total nutrient supply**	<b>N</b>	50.0	121.7	54.1	10.7	-	-	-	-	-	-	-	-	237
	<b>P</b>	-	44.6	5.4	1.8	-	-	-	-	-	-	-	-	52
	<b>K</b>	-	-	164.4	54.2	-	-	-	-	-	-	-	-	219
	<b>Mg</b>	-	72.7	-	-	-	-	-	-	-	-	-	-	73

\*The control treatment received only the commercial nutrient supply, i.e., the producer's typical fertilisation rates.

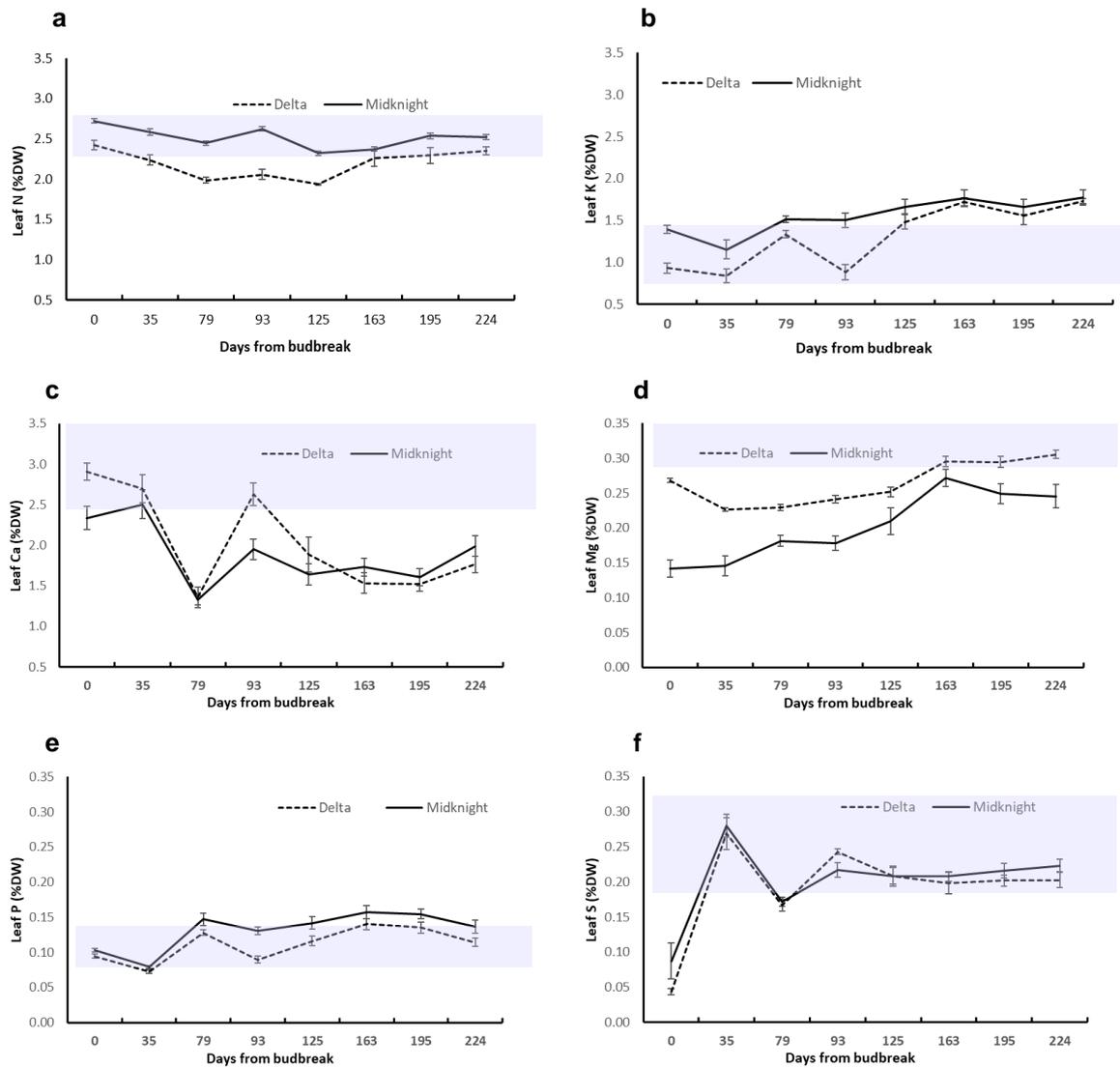
\*\*N, P, K, Mg in this row represents the treatments.

## Results and discussion

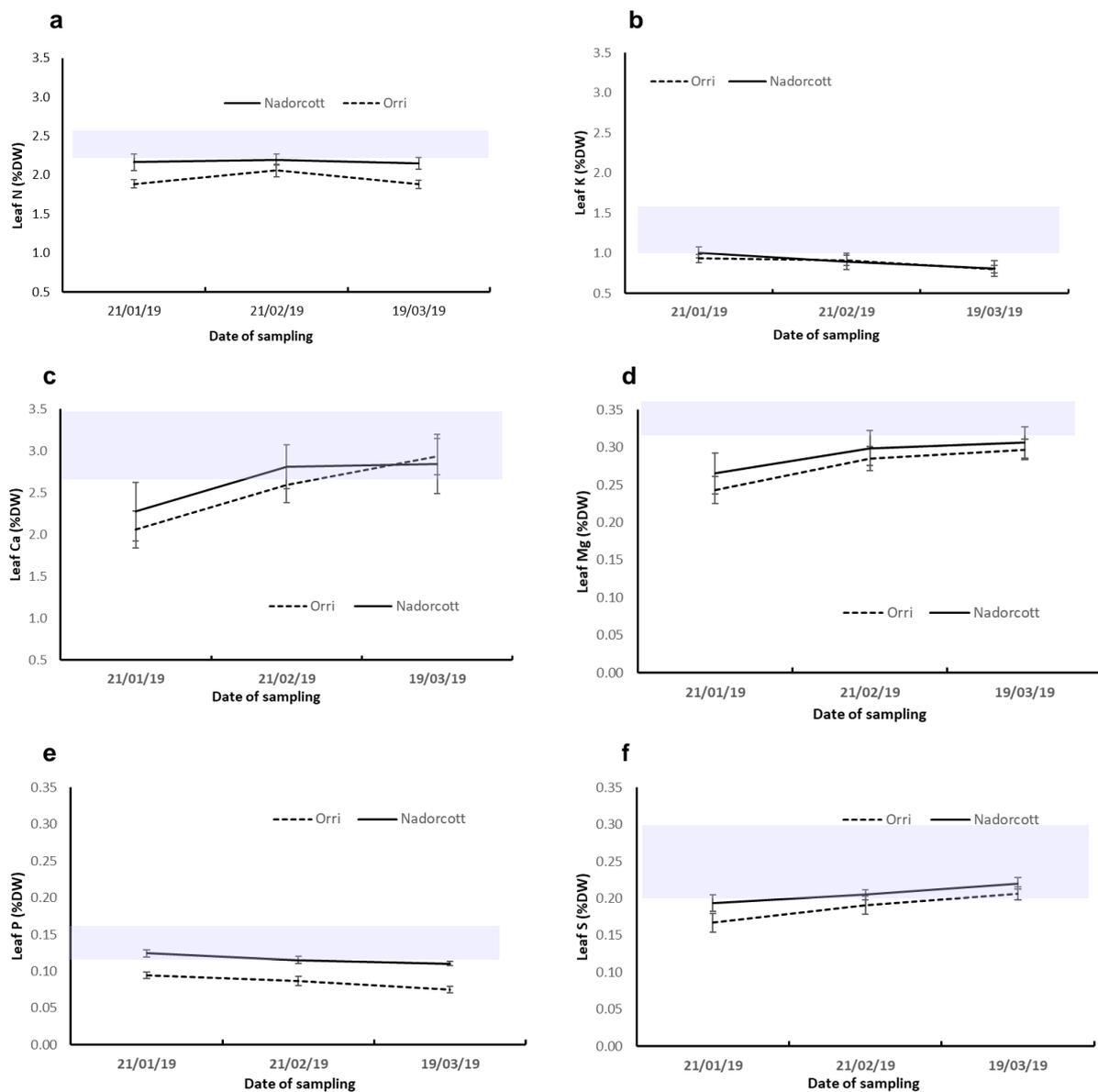
### Phase 1

Although significant differences were obtained between the Delta and Midnight Valencia leaf N, K, Ca, Mg, P and S concentrations, the seasonal trends in leaf nutrient concentrations were very similar (Figure 5.3.3.2). Likewise, for the Orri and Nadorcott mandarins (Figure 5.3.3.3). The differences in absolute concentrations between the respective cultivars can be due to various factors, e.g., differences in soil nutrient supply, levels of fertilisation, different rootstocks. Sheng *et al.* (2009) also found similar trends in foliar Ca and K nutrient concentrations of the two navel cultivars they studied, i.e., 'Newhall' and 'Skagg's Bonanza'. These patterns corresponded to those obtained in this trial and can be described as relatively constant throughout the whole season. The exception is Mg, where leaf Mg concentration in their studied cultivars showed a steady declining trend throughout the season. Except for a slight but steady decline in foliar K concentration of "Star Ruby" grapefruit, Singh *et al.* (2016) also found that the nutrient concentrations of both fruiting and non-fruiting terminals remained stable throughout the year.

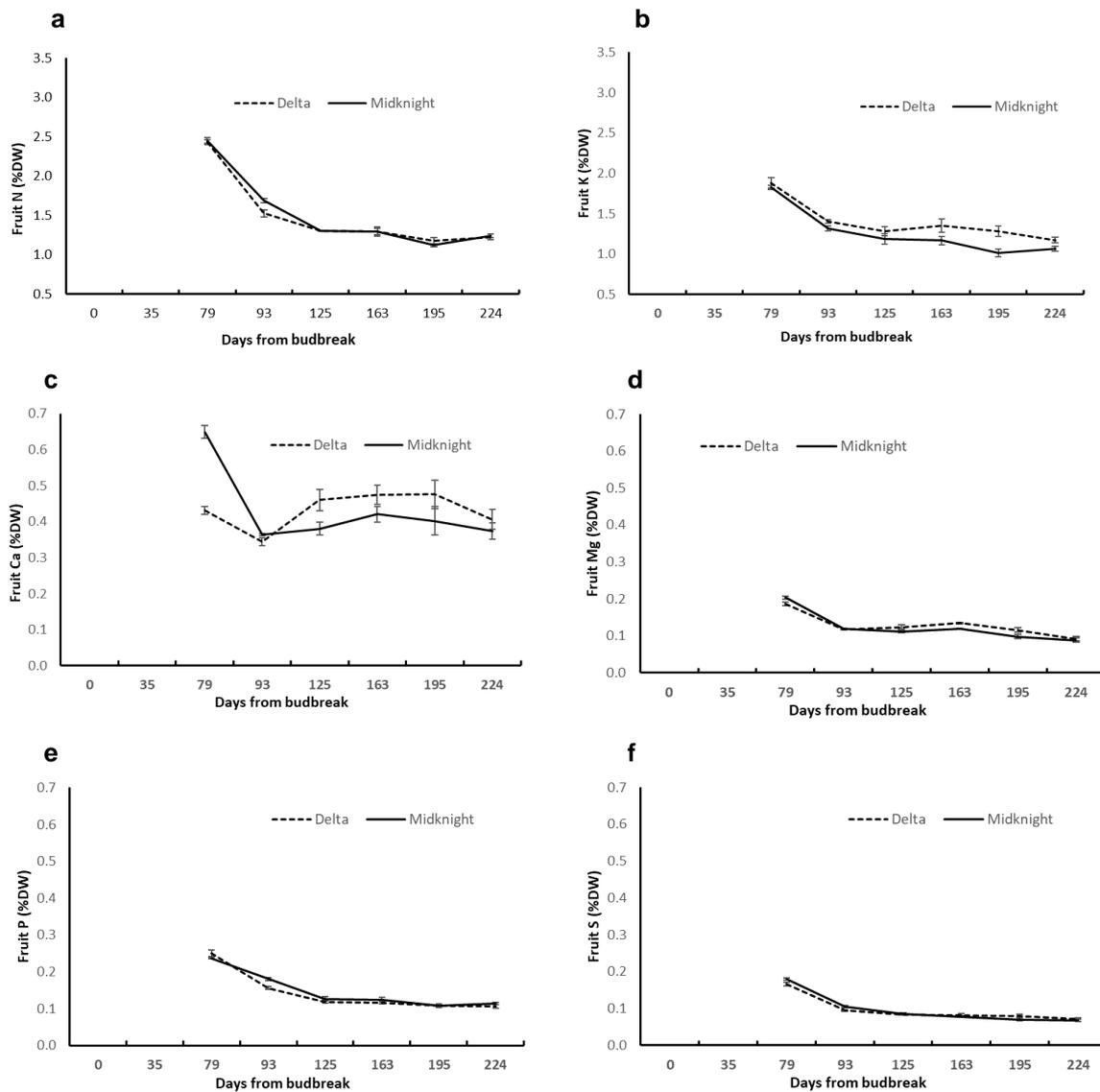
Fruit nutrient concentration differences between the respective pairs of cultivars were less pronounced, with slightly higher concentrations of only K and Ca in the Delta fruit compared to Midnight Valencia (Figure 5.3.3.4) and K in the Nadorcott compared to Orri Mandarins (Figure 5.3.3.5). The general trend of nutrient concentration during fruit development was to decrease rapidly during the first 14 days after fruit set (Figure 5.3.3.4). This corresponded to the trends obtained by Storey & Treeby (2000) for navel oranges, with the exception that they found an increase in the concentration of all elements during Stage I of fruit growth. Although different trends to that of Storey & Treeby (2000) were obtained regarding the concentration of nutrients in the fruit, these results confirmed their conclusion that Stage I is the period of fruit development in which there are the most rapid and extensive changes in nutrient concentration.



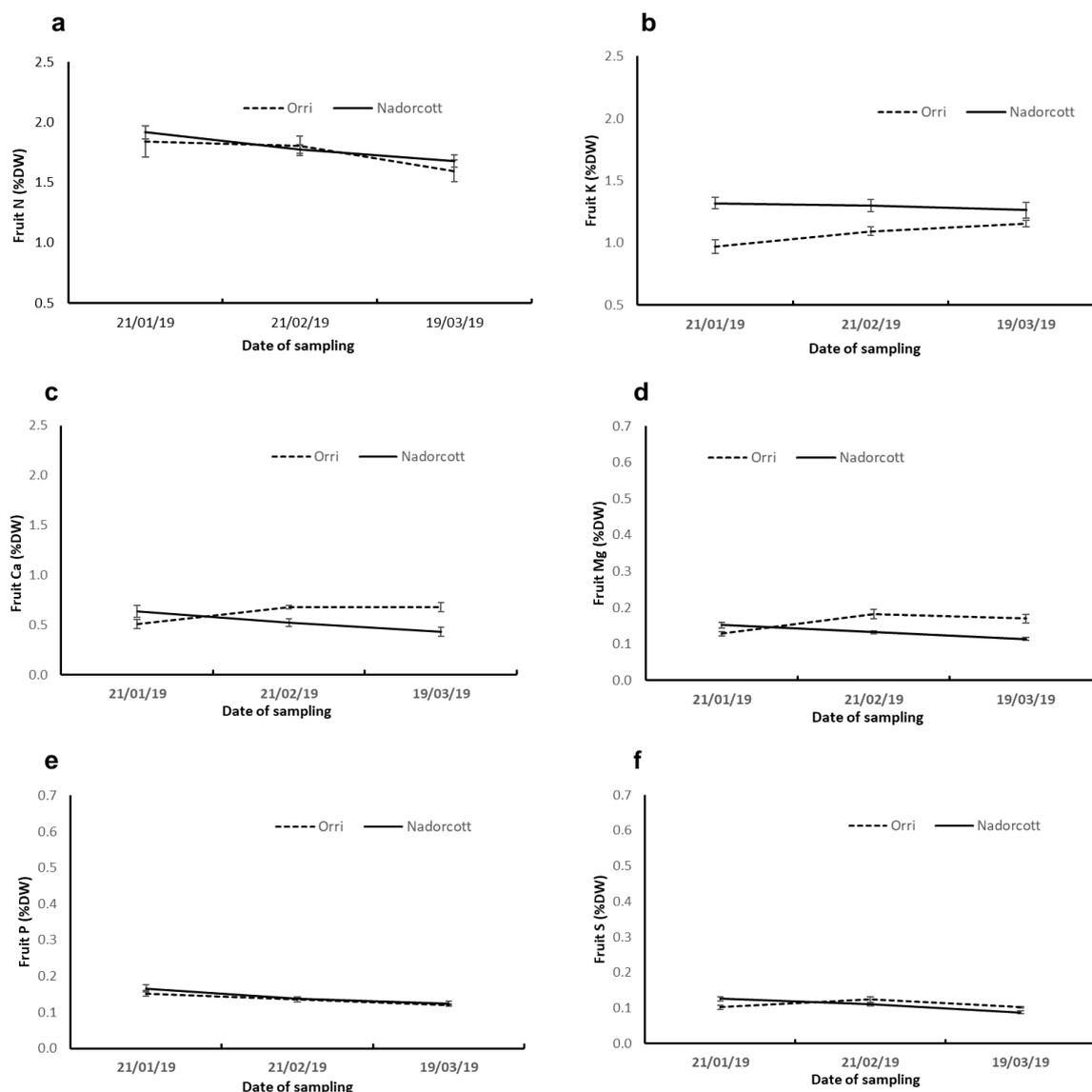
**Figure 5.3.3.2.** Concentration of nitrogen (a), potassium (b), calcium (c) magnesium (d), phosphorous (e) and sulphur (f) in leaves of mature *Delta* and *Midnight Valencia* trees during the 2018/2019 growing season in Nelspruit – mature leaves on the latest flush were respectively sampled. Purple shaded areas indicate the optimal range for fruiting terminals sampled in March-April.



**Figure 5.3.3.3.** Concentration of nitrogen (a), potassium (b), calcium (c) magnesium (d), phosphorous (e) and sulphur (f) in leaves of mature *Orri* and *Nadorcott* mandarin trees during the 2018/2019 growing season in Stellenbosch – mature leaves on the latest flush were respectively sampled. Purple shaded areas indicate the optimal range for fruiting terminals sampled in March-April.



**Figure 5.3.3.4.** Changes in the nitrogen (a), potassium (b), calcium (c) magnesium (d), phosphorous (e) and sulphur (f) concentrations of fruit from mature *Delta* and *Midnight Valencia* trees during the 2018/2019 growing season in Nelspruit - mature leaves on the latest flush were respectively sampled.



**Figure 5.3.3.5.** Changes in the nitrogen (a), potassium (b), calcium (c) magnesium (d), phosphorous (e) and sulphur (f) concentrations of fruit from mature *Orri* and *Nadorcott* mandarin trees during the 2018/2019 growing season in Stellenbosch.

## Phase 2

### Seasonal changes in leaf nutrient concentration and the effect of the fertilisation treatments on leaf nutrient concentration

*Midknight Valencia trees (Nelspruit):* A response in *only the leaf N* concentration of the trees that were fertilised at a 200% rate of the standard practices could be observed from 30 days after full bloom in the first season (Figure 5.3.3.6). This indicates to an ability of citrus trees to respond quickly to additional N fertilisation – Scholberg *et al.* (2002) even found that a residence time of 8 hours of increased N is sufficient to obtain a response. The same rapid response to the excessive fertilisation, however, did not occur for P, K or Mg (Figure 5.3.3.6). The trees seem to be especially non-responsive to the additionally applied P, with no shift in the leaf P concentrations even in the second season of the additionally applied P. This corresponds to Coetzee (2007) who maintains that well supplied citrus trees do not respond to additionally applied P. A delayed response to the excessive K applications were observed that only became significant in the second season, i.e., 2020/2021 (Figure 5.3.3.6). The same trend was observed for Mg, albeit not statistically significant even after two year's excessive Mg applications (Figure 5.3.3.6). Furthermore, N was the only nutrient that was raised through over-fertilisation to levels above the generally accepted limit associated with an excessive concentration – this

indicate that the maximum limit is might be too high, but definitely not helpful on its own to evaluate the accuracy of N fertilisation rates, or for that matter: for any of the nutrients.

*Orri mandarins (De Wet)*: No response in the leaf nutrient concentration of the trees that were fertilised at a 200% rate compared to the standard practices were obtained in the first season (Figure 5.3.3.7). During the second season, it is *only for K* that an increase in the leaf concentration was achieved - this became significant only from the middle of the 2020/21 season. Despite excessive levels of fertilisation (Table 5.3.3.4), the foliar nutrient concentration did not exceed any of the levels regarded as excessive for any of the nutrients (Figure 5.3.3.7). In addition, similar to the Midnight Valencia block, the trees also were especially non-responsive to the additionally applied P and Mg.

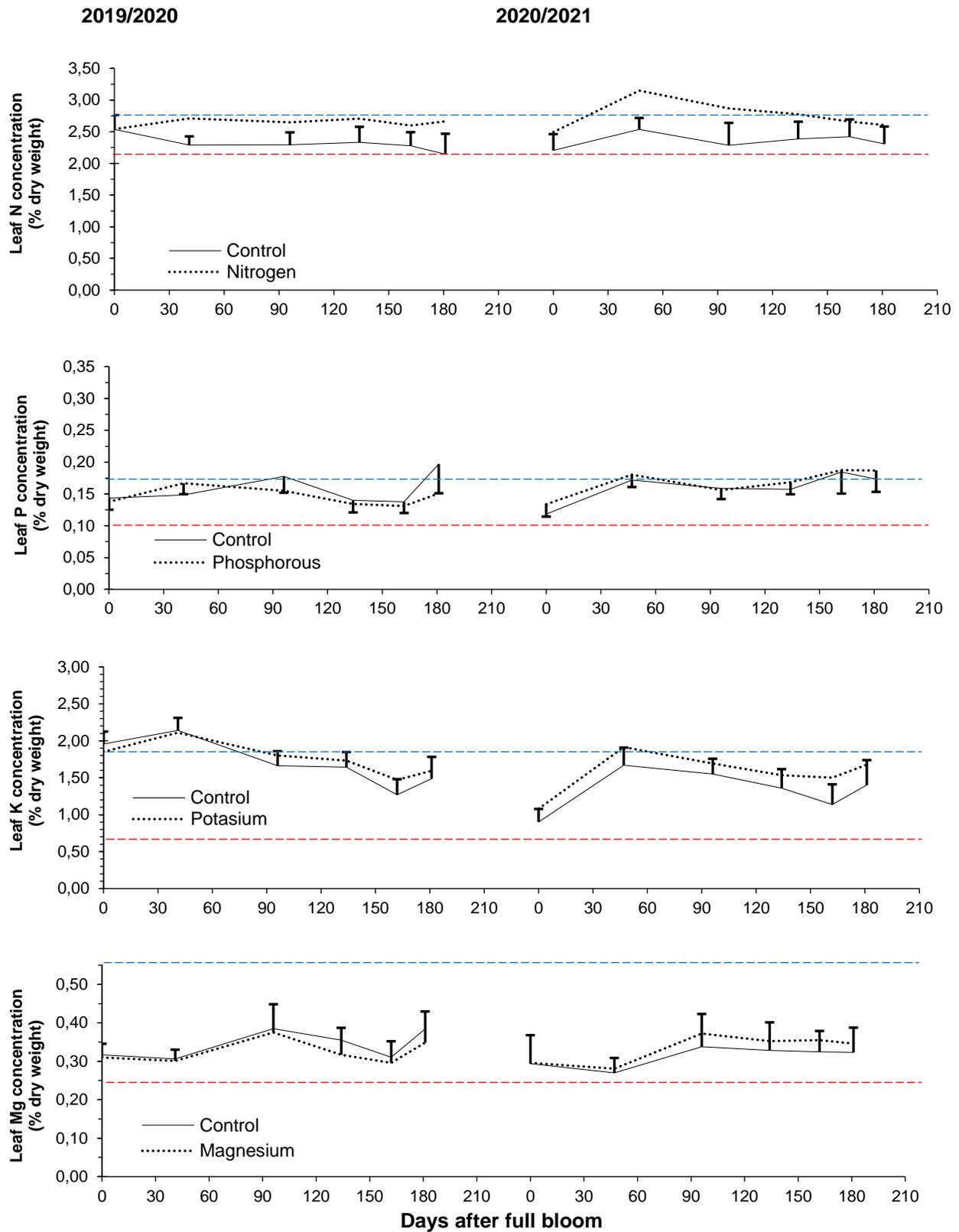
The results from the above two sites indicate that either:

- Orri mandarins are much less responsive to additional fertilisation than Midnight Valencia – which needs to be elucidated, or
- Additional fertilisation in a scenario of luxurious to excessive fertilisation will not result in a significant response from the trees.

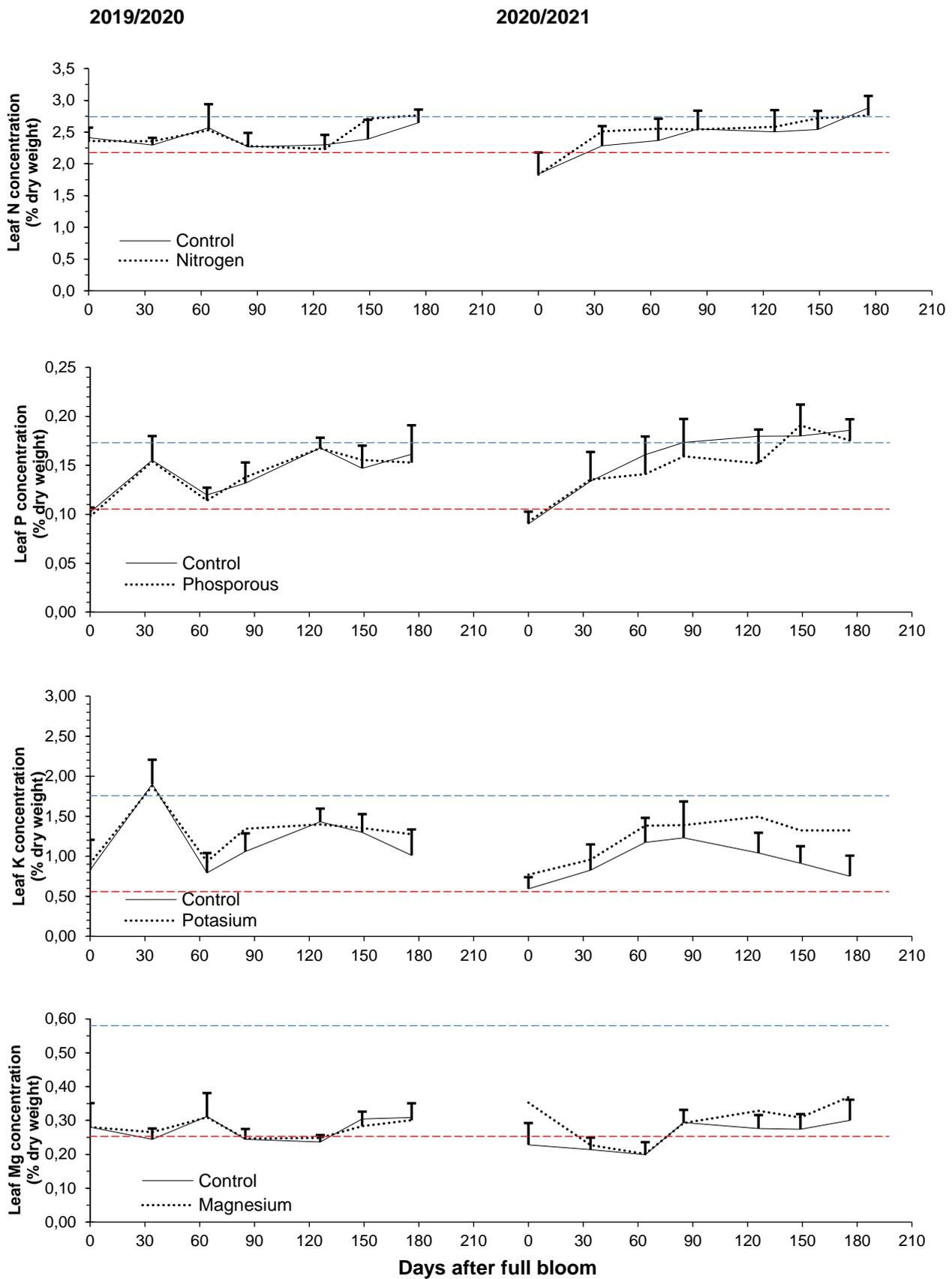
The implication for this concerning interpretation of foliar analysis is that:

- “In-season” leaf analysis has only application for possible adjustment to N-fertilisation requirements – and then only in conditions of conservative fertilisation;
- Once-off annual analysis of leaves for other nutrients are sufficient since the response of the trees to changes in P, K, Mg nutrition is so slow that one has to interpret trends over more than one season;
- Given the fact that the Midnight Valencia were conservatively fertilised (Table 5.3.3.5) compared to the Orri mandarins (Table 5.3.3.4), it seems that trees’ response to fertilisation is largely affected by the rate of fertilisation/level of nutrient availability – being more prone to respond at low levels of availability. This highlights again the validity of the “Law of diminishing returns” (\*see below);
- In scenarios of sufficient to excessive nutrient supply, not one particular sampling time necessarily best indicates the level of nutrient availability – but the autumn samples ( $\pm 180$  days after full bloom) seems to sufficiently reveal the trees’ overall nutritional response to a season’s fertilisation (discussed below);
- Furthermore, this data strongly supports the opinion of Menino (2012) that although sufficiency ranges are purported to improve flexibility in diagnosis, in reality, they decrease diagnostic precision because the limits are often too wide. It is therefore proposed that foliar analysis sufficiency ranges are regarded as secondary to observation of long-term trends in the foliar analysis - foliar analysis should never be used in isolation, i.e., it should not be used as the only indication whether the fertilisation programme followed are appropriate. Furthermore, one can conclude that where crop size remains fairly constant (discussed below), year-on-year changes in leaf nutrient status will remain constant in conditions of ample supply.

\* **The law of diminishing returns** states that in productive processes, increasing a factor of production by one unit, while holding all other production factors constant, will at some point return a lower unit of output per incremental unit of input. The law of diminishing returns does not cause a decrease in overall production capabilities, rather it defines a point on a production curve whereby producing an additional unit of output will result in a loss and is known as negative returns. Under diminishing returns, output remains positive, however, productivity and efficiency decrease (Shephard, 1970).



**Figure 5.3.3.6.** Seasonal changes in nutrient concentration of *Midknight Valencia* leaves as affected by fertilisation rates at 200% of the normally applied rate (control). The legend indicates the mineral that was applied – vertical bars indicate the LSD at  $p < 0.05$ . The blue horizontal line indicates the concentration that is regarded as an excessive level, while the red line indicates the minimum acceptable nutritional status.



**Figure 5.3.3.7.** Seasonal changes in nutrient concentration of *Orris mandarin* leaves as affected by fertilisation rates at 200% of the normally applied rate (control). The legend indicates the mineral that was applied – vertical bars indicate the LSD at  $p < 0.05$ . The blue horizontal line indicates the concentration that is regarded as an excessive level, while the red line indicates the minimum acceptable nutritional status.

### **Effect of excessive fertilisation on the mineral composition of leaves from both non-fruit bearing terminals and from fruit bearing terminals, sampled in autumn**

The effect of the excessive fertilisation on the mineral composition of leaves from both non-fruit bearing terminals and from fruit bearing terminals, sampled in autumn is demonstrated in Tables 5.3.3.6 & 5.3.3.7 for the Midnight Valencia and Orri mandarin trial sites respectively. A consistent, significant increase in the nutrient concentration in response to the fertilisation treatments was not obtained for any of the nutrients. Only N and K concentrations did show some response, but a definite distinction between the types of leaves cannot be observed. The suppression of N uptake in conditions of excessive K supply was noticeable in both leaf types and for both trial sites, albeit not consistently significant. As for the other nutrients (i.e., P & Mg), additional fertilisation rates did not affect the leaf concentration of the respective leaf types, and not the Ca-supply to the leaves.

### **Comparison between the mineral nutrient concentration of leaves from non-fruit bearing terminals and leaves from fruit bearing terminals sampled in autumn**

To elucidate whether the mineral nutrient concentration of leaves from non-fruit bearing terminals differ from those on fruit bearing terminals, samples from both positions were taken at the normal autumn sampling time ( $\pm 180$  days after full bloom), and the averages over all the treatments compared. The result of this is presented in Figure 5.3.3.8 for both varieties/sites. With the exception of S and Mg for the Midnight Valencia's in 2019/20, the differences between leaves from the different positions are consistent for both seasons and varieties. The following observations were made:

- the N, P and K concentration of leaves from non-fruit bearing terminals were higher than that of fruit bearing terminals – this was also found by Carranca *et al.* (1993) for Valencia late;
- the average Mg concentration, despite not showing a consistently significant trend, also was lower in fruit bearing terminals;
- the Ca concentration of leaves from fruit bearing terminals, however, are higher than that of non-fruit bearing terminals – this is expected due to the fruit-bearing terminal leaves being older than the non-fruit bearing terminals, and Ca that is also an immobile nutrient;
- analysis of leaves from non-fruit bearing terminals can also be used to assess tree nutritional status. For comparison to the norms, the adjustments provided below is, however, required for leaves from non-fruit bearing terminals compared to the reference norms used for leaves from fruit bearing terminals (i.e., the commonly used reference norms for South Africa as provided in Coetzee (2007)). For the purpose of this study, these adjustments is assumed to apply only to Valencia's and mandarins. Furthermore, care needs to be taken that, during sampling, leaves from only one position should be sampled (i.e., they should not be mixed).

N	P	K	Ca	Mg
+6.0% $\pm$ 0.8%	+35% $\pm$ 4.0%	+24% $\pm$ 6.0%	-15% $\pm$ 3.0%	+ 8.0% $\pm$ 1.0%

The higher concentration of N, P, K and Mg in the leaves from non-fruit bearing terminals is ascribed to the mobility of these nutrients, i.e., the crop is a strong sink for these nutrients (Mirsoleimani, 2014), and the younger leaves on the non-fruit bearing terminals are also more recent recipients of these nutrients. On the other hand, the higher Ca concentration in the fruit-bearing terminals is ascribed to the immobility of Ca, i.e., progressive accumulation, without transfer, of Ca has taken place in the leaves.

**Table 5.3.3.6.** Mineral nutrient concentration of the *Midknight Valencia* leaves sampled in autumn, as affected by fertilisation rates at 200% the normally applied rate (control) – numbers followed by different letters differ significantly at  $p < 0.05$ .

Type of leaf sample	Leaves on non-fruit bearing terminals										Leaves on fruit bearing terminals									
	2019/2020					2020/2021					2019/2020					2020/2021				
	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg
Control	2.1	0.2	1.49	2.8	0.38a	2.31	0.1	1.40	3.61	0.32	2.4	0.1	1.15	3.01	0.2	2.2	0.1	1.25	4.14	0.35
	5b	0a	ab	4		b	7b	ab	ab		1a	2b	a	b	7b	3b	4	a	ab	a
Nitrogen	<b>2.6</b>	0.1	1.29	2.8	0.33a	<b>2.61</b>	0.1	<u>1.17</u>	3.09	0.32	<b>2.3</b>	0.1	<u>0.92</u>	3.05	0.3	<b>2.5</b>	0.1	<u>0.82</u>	3.79	0.29
	<b>6a</b>	6ab	b	0	b	<b>a</b>	8ab	<u>b</u>	b		<b>1a</b>	4a	<u>b</u>	b	2a	<b>0a</b>	2	<u>b</u>	ab	b
Phosphorus	2.3	0.1	1.34	3.1	0.35a	2.28	0.1	1.27	3.66	0.31	2.0	0.1	1.20	3.13	0.3	2.2	0.1	1.06	3.99	0.27
	2b	5b	ab	1	b	b	9ab	b	ab		2b	3a	a	ab	3a	5b	4	ab	ab	b
Potassium	2.3	0.1	<b>1.59</b>	2.5	0.30b	2.34	0.2	<b>1.68</b>	3.69	0.33	2.0	0.1	1.18	3.29	0.3	2.1	0.1	1.22	3.77	0.24
	2b	7ab	<b>a</b>	3		ab	1a	<b>a</b>	ab		5b	3b	a	a	2a	7b	3	a	b	b
Magnesium	2.3	0.1	1.29	2.9	0.35a	2.46	0.1	1.44	3.90	0.35	2.3	0.1	1.11	3.05	0.3	2.2	0.1	1.25	4.51	0.36
	6ab	5b	b	5	b	ab	7b	ab	a		9a	2b	a	b	1a	7b	3	a	a	a
LSD	0.3	0.0	0.29	NS	0.081	0.27	0.0	0.34	0.75	NS	0.2	0.0	0.11	0.18	0.0	0.1	NS	0.26	0.73	0.05
( $p < 0.05$ )	2	46					34				4	06			4	7				

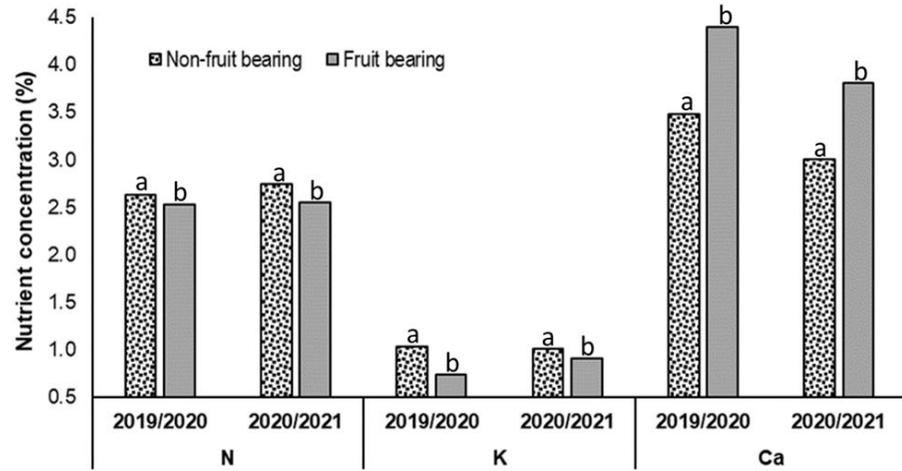
NS = not significant

**Table 5.3.3.7.** Mineral nutrient concentration of the *Orrri mandarin* leaves sampled in autumn, as affected by fertilisation rates at 200% the normally applied rate (control) – numbers followed by different letters differ significantly.

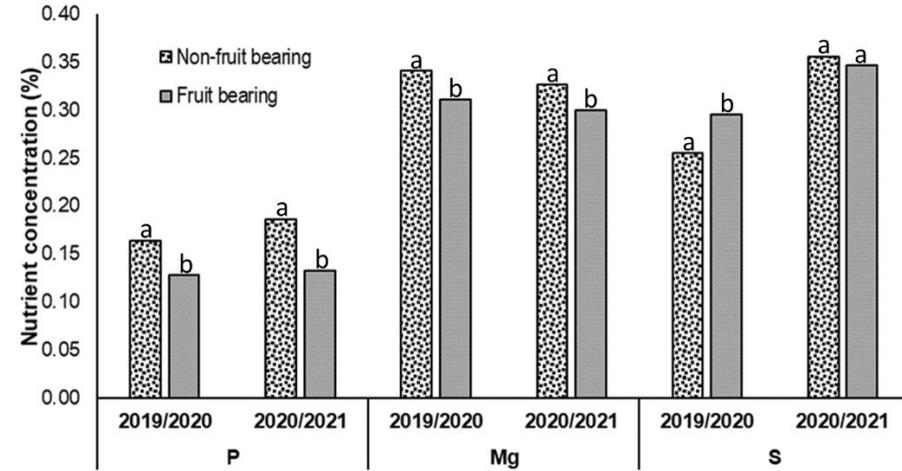
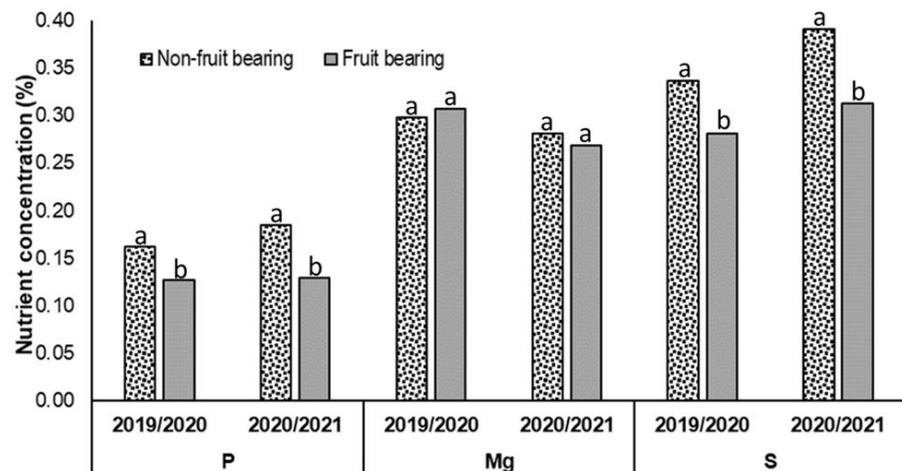
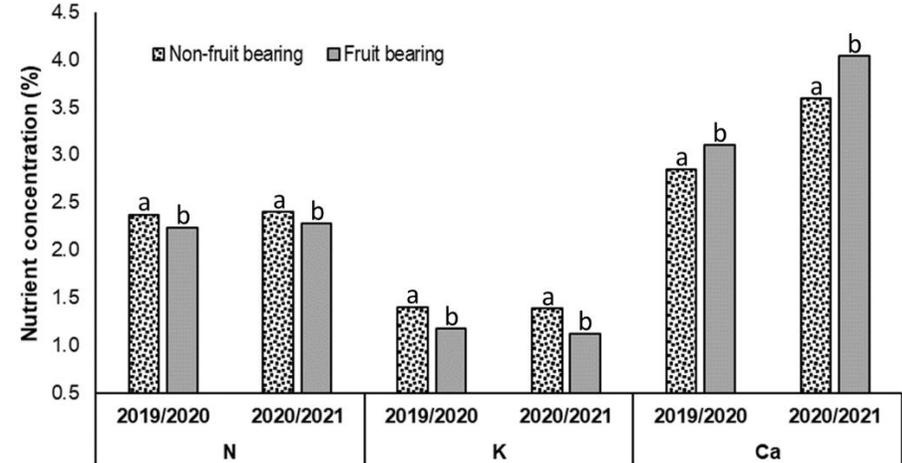
Type of leaf sample	Leaves on non-fruit bearing terminals										Leaves on fruit bearing terminals									
	2019/2020					2020/2021					2019/2020					2020/2021				
	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg
Control	2.6	0.16	1.01	3.75	0.31	2.54	0.18	0.91	2.93	0.27	2.52	0.13	0.69	4.97	0.32	2.50	0.12	0.70	4.00	0.28b
	5a		ab			b	ab	b		ab			b	ab				b		c
Nitrogen	<b>2.8</b>	0.16	0.93	3.14	0.28	2.72	0.18	<u>0.91</u>	3.07	0.28	2.63	0.13	<u>0.61</u>	4.28	0.31	2.62	0.13	<u>0.76</u>	3.72	0.25b
	<b>6a</b>		b			ab	ab	<u>b</u>		ab			<u>b</u>	ab				<u>b</u>		c
Phosphorus	2.4	0.15	0.93	3.62	0.32	2.85	0.19	0.86	3.15	0.29	2.38	0.12	0.74	4.34	0.33	2.61	0.13	0.88	4.03	0.29a
	0b		b			a	ab	b		ab			b	ab				ab		b
Potassium	2.7	0.17	<b>1.27</b>	3.37	0.27	2.77	0.20	<b>1.32</b>	2.83	0.25	2.44	0.13	<b>1.01</b>	4.29	<u>0.26</u>	2.51	0.13	<b>1.28</b>	3.36	<u>0.22c</u>
	8a		<b>a</b>			ab	a	<b>a</b>		b			<b>a</b>	<u>b</u>				<b>a</b>		
Magnesium	2.6	0.17	1.07	3.54	0.30	2.89	0.17	1.06	3.08	0.31	2.57	0.12	<u>0.66</u>	4.37	<b>0.35</b>	2.48	0.15	0.93	4.07	<b>0.34a</b>
	0ab		ab			a	b	b		a			<u>b</u>	<b>a</b>				ab		
LSD (p < 0.05)	0.2	NS	0.32	NS	NS	0.29	0.03	0.21	NS	0.04	NS	NS	0.21	NS	0.09	NS	NS	0.47	NS	0.06

NS = not significant

**Midnight Valencia's (Nelspruit)**



**Orri Mandarins (De Wet)**



**Figure 5.3.3.8.** Difference in nutrient concentrations of non-fruit bearing and fruit bearing terminals' leaves of Midnight Valencia and Orri mandarins, sampled in autumn (different symbols denote significant difference at  $p < 0.05$ ).

### **Correlation between nutrient concentrations of leaves at different times after full bloom and (i) that of the normal prescribed autumn sampling time; (ii) fruit at harvest**

The correlation between both Midnight Valencia and Orri mandarin leaf nutrient concentrations at various times after full bloom and that of the normal prescribed autumn sampling time is presented in Tables 5.3.3.8 and 5.3.3.9. In 2019/2020, the Midnight Valencia leaf analysis at most of the sampling dates showed a correlation with the autumn sampled non-fruit bearing terminal leaves only for N. However, in 2020/21 there was a significant correlation between all the sampling times for all the nutrients except P (Table 5.3.3.8).

With respect to nutrient concentration of autumn sampled leaves from the fruit bearing terminals, not one of the in-season sampling times' nutrient concentration correlated for any of the nutrients. However, in the 2020/2021 season there was significant correlations between the concentration of N and K of most of the sampling times with that of the fruit bearing terminal's leaves sampled in autumn at 180 days after full bloom (DAFB) (Table 5.3.3.8). Furthermore, N, K and Ca concentrations in 180 DAFB sampled leaves on non-fruit bearing terminals and fruit bearing terminals were significantly correlated (Table 5.3.3.8) – only in the 2020/2021 season.

The data presented in Table 5.3.3.9 shows that for the Orri mandarin trees in both seasons there was a significant correlation between leaf K and Mg concentration at almost all sampling times and that of the normal sampling time (176 DAFB) for both the leaf types. Furthermore there was a significant correlation between all nutrients' concentrations in the non-fruit bearing terminals and the fruit bearing terminals at 176 DAFB (i.e., the normal sampling time, autumn samples) in the 2019/2020 season, and for K, Ca and Mg in the 2020/2021 season.

This indicates a difference in the way foliar analysis can be used and interpreted between Midnight Valencia's (all Valencia's for that matter) and Orri mandarins (all mandarins), and also possible explanation for the rigidity with which sampling of leaves (i.e., only from fruit-bearing terminals) were prescribed by du Plessis & Smart (1970) and Coetzee (2007) since it is based on work done on Valencia's. The data in Tables 5.3.3.8 and 5.3.3.9 suggest that:

- in the case of Midnight Valencia's, autumn sampled leaves on both non-fruit bearing and fruit bearing terminals can be used to assess the tree nutritional status for N, K and Ca;
- in the case of Orri mandarins autumn sampled leaves on both non-fruit bearing and fruit bearing terminals can be used to assess the tree nutritional status for all nutrients;
- if concerns regarding tree N, K, Ca and Mg nutrition of Midnight Valencia's exist, the in-season leaf analysis can give a reliable indication of what the nutritional status of the trees will be later in the season – this applies to both leaves on non-fruit bearing terminals at the time of normal leaf analyses (autumn sampling) and with the exception of Mg also to leaves on fruit bearing terminals also.
- if concerns regarding tree K and Mg nutrition of Orri mandarins exist, the in-season leaf analysis can give a reliable indication of what the nutritional status of the trees will be later in the season, especially in relation to both types of leaves.

There were no meaningful correlations between leaf nutrient concentration and (i) fruit mineral concentration, (ii) fruit characteristics or (iii) yield found for the Midnight Valencia trial (Table 5.3.3.10). In the Orri Mandarin trial, non-fruit bearing terminal leaf N, P and K concentrations were significantly correlated with fruit yield (Table 5.3.3.11) by linear correlation analysis. Manivannan & Chadha (2011) found in their experiment that only N and Ca correlated with mandarin fruit yield.

**Table 5.3.3.8.** Correlations ( $r^2$ ) between mineral analysis results of mature *Midnight Valencia* leaves of the latest in-season flush, sampled at different times in both the 2019/2020 and 2020/2021 seasons, and the mineral nutrient content of leaves on both non-fruit bearing terminals and fruit bearing terminals sampled in March (normal practice) – the indicated  $r^2$  values are all significant at  $p < 0.05$ , while values in the blue blocks are significant at  $p < 0.07$ .

Nutrient	2019/2020 season										2020/2021 season										
	Sampling time (*DAFB)	Nutrient in leaves sampled in March (180 DAFB)					Nutrient in leaves sampled in March (180 DAFB)					Nutrient in leaves sampled in March (180 DAFB)					Nutrient in leaves sampled in March (180 DAFB)				
		From non-fruit bearing terminals					From fruit bearing terminals					From non-fruit bearing terminals					From fruit bearing terminals				
	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg	
N	0	NS				NS					NS					NS					
	**41/47	0.361				NS					0.228					0.388					
	96	0.257				NS					0.177					NS					
	134	NS				NS					0.231					0.328					
	162	0.243				NS					NS					NS					
	181	-				NS					-					0.446					
P	0		NS				NS					NS					NS				
	41/47		NS				NS					NS					NS				
	96		NS				NS					NS					NS				
	134		NS				NS					NS					NS				
	162		NS				NS					NS					NS				
	181		-				NS					-					NS				
K	0			0.218				NS					NS					NS			

	41/47			0.138					NS					0.251					0.331			
	96			0.371					NS					0.234					0.211			
	134			NS					NS					0.354					NS			
	162			NS					NS					0.581					NS			
	181			-					NS					-					0.391			
Ca	0				NS					NS				0.218						NS		
	41/47				NS					NS				NS						NS		
	96				NS					NS				0.292						NS		
	134				NS					NS				0.680						NS		
	162				NS					NS				0.309						0.216		
	181				-					NS				-						0.301		
Mg	0				NS					NS					NS						NS	
	41/47				NS					NS					0.261							NS
	96				0.402					NS					0.249							0.268
	134				NS					NS					0.148							NS
	162				NS					NS					0.278							NS
	181				-					NS					-							NS

\*DAFB = days after full bloom

\*\* Sampling was at 41 DAFB in 2019/2020 and 47 DAFB in 2020/2021

**Table 5.3.3.9.** Correlations ( $r^2$ ) between mineral analysis results of mature *Orris mandarin* leaves of the latest in-season flush, sampled at different times in both the 2019/2020 and 2020/2021 seasons, and the mineral nutrient content of leaves on both fruit-bearing terminals and fruit bearing terminals sampled in March (normal practice) – the indicated  $r^2$  values are all significant at  $p < 0.05$ , while values in the blue blocks are significant at  $p < 0.07$ .

Nutrient	Sampling time (*DAFB)	Nutrient in leaves sampled in March (176 DAFB)										Nutrient in leaves sampled in March (176 DAFB)									
		From non-fruit bearing terminals					From fruit bearing terminals					From non-fruit bearing terminals					From fruit bearing terminals				
		N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg
N	0	NS					NS					NS					NS				
	34	NS					NS					NS					NS				
	64	NS					NS					NS					NS				
	85	NS					0.233					NS					0.347				
	126	NS					NS					NS					NS				
	149	NS					NS					NS					NS				
	176	-					0.504					-					NS				
P	0		NS					NS					NS					NS			
	34		NS					0.423					0.267					NS			
	64		NS					0.279					NS					NS			
	85		NS					NS					0.378					NS			
	126		NS					NS					0.525					0.316			
	149		NS					NS					NS					NS			
	176		-					0.228					-					NS			
K	0			0.356					0.230					0.683					0.581		
	34			NS					NS					NS					0.192		

	64			0.52 4					0.32 7					0.74 8					0.66 7		
	85			0.30 8					0.21 0					0.62 8					0.54 6		
	126			0.31 9					NS					0.80 7					0.74 6		
	149			0.49 6					0.25 0					0.80 2					0.59 1		
	176			-					0.45 7					-					0.81 8		
Ca	0				NS				NS					NS					NS		
	34				NS				NS					NS					NS		
	64				NS				NS					NS					0.59 6		
	85				NS				NS					0.24 6					0.49 5		
	126				NS				NS					0.40 2					0.32 1		
	149				NS				NS					0.58 2					0.46 7		
	176				-				0.20 5					-					0.61 5		
Mg	0					0.46 6				0.43 4					0.75 2						NS
	34					0.62 1				0.36 1					0.52 4						0.24 8
	64					0.25 4				0.39 7					0.20 3						0.46 9
	85					0.57 9				0.45 4					0.50 0						0.65 6
	126					0.24 6				0.19 9					0.53 8						0.50 7
	149					0.18 2				0.33 6					0.35 6						

	176					-					0.50					-					0.43	
											7											6

\*DAFB = days after full bloom

**Table 5.3.3.10.** Correlations ( $r^2$ ) between mineral analysis results of mature *Midnight Valencia* leaves of the latest in-season flush, sampled at different times in the 2019/2020 season, and the mineral nutrient content of fruit at harvest, as well as fruit quality parameters – the indicated  $r^2$  values are all significant at  $p < 0.05$ , while values in the blue blocks are significant at  $p < 0.07$ .

Nutrient	Sampling time (*DAFB)	Nutrient in fruit sampled at harvest					Fruit quality parameter			
		N	P	K	Ca	Mg	Fruit size	Sugar (°Brix)	Acid	Yield
N	0	NS					NS	NS	NS	NS
	41	0.216					NS	NS	NS	NS
	96	NS					NS	NS	NS	NS
	134	0.297					NS	NS	NS	NS
	162	0.228					NS	NS	NS	NS
	181	NS					NS	NS	0.366	NS
	**FB 181	NS					NS	NS	NS	NS
P	0		NS				NS	NS	NS	NS
	41		NS				NS	NS	NS	NS
	96		NS				NS	NS	NS	NS
	134		NS				NS	NS	NS	NS
	162		NS				NS	NS	NS	NS
	181		NS				NS	NS	NS	NS
	FB 181		NS				-0.349	NS	0.262	NS
K	0			NS			NS	NS	NS	NS
	41			NS			0.280	NS	NS	NS
	96			NS			NS	NS	NS	NS
	134			NS			NS	NS	NS	NS
	162			NS			NS	NS	NS	NS
	181			NS			NS	NS	NS	NS
	FB 181			NS			NS	NS	NS	NS
Ca	0				NS		NS	NS	NS	NS
	41				NS		NS	NS	NS	NS
	96				NS		NS	NS	NS	NS
	134				NS		NS	NS	NS	NS
	162				NS		NS	NS	NS	NS
	181				NS		NS	NS	NS	NS
	FB 176				NS		NS	NS	NS	NS
Mg	0					NS	NS	NS	NS	NS
	41					NS	NS	NS	NS	NS
	96					0.321	NS	NS	NS	NS
	134					0.546	NS	NS	NS	NS
	162					0.266	NS	NS	NS	NS
	181					NS	NS	NS	NS	NS
	FB 181					NS	NS	NS	NS	NS

\*DAB = days after full bloom

FB = fruit bearing terminals

**Table 5.3.3.11.** Correlations ( $r^2$ ) between mineral analysis results of mature *Orrri mandarin* leaves of the latest in-season flush, sampled at different times in the 2019/2020 season, and the mineral nutrient content of fruit at harvest, as well as fruit quality parameters – the indicated  $r^2$  values are all significant at  $p < 0.05$ , while values in the blue blocks are significant at  $p < 0.07$ .

Nutrient	Sampling time (*DAFB)	Nutrient in fruit sampled at harvest					Fruit quality parameter			
		N	P	K	Ca	Mg	Fruit size	Sugar (°Brix)	Acid	Yield
N	0	NS					NS	NS	NS	NS
	34	NS					NS	NS	NS	NS
	64	NS					NS	NS	NS	NS
	85	NS					NS	NS	NS	NS
	126	NS					NS	NS	NS	0.480
	149	NS					NS	NS	NS	NS
	176	NS					NS	0.317	0.168	0.276
	**FB 176	NS					NS	0.361	0.446	NS
P	0		NS				NS	NS	NS	NS
	34		NS				NS	NS	NS	0.346
	64		NS				NS	NS	NS	0.539
	85		NS				NS	NS	NS	NS
	126		NS				NS	NS	NS	NS
	149		NS				NS	NS	NS	0.284
	176		NS				NS	NS	NS	0.316
	FB 176		NS				NS	0.307	NS	0.352
K	0			0.303			NS	NS	NS	0.477
	34			NS			NS	NS	NS	NS
	64			NS			NS	NS	NS	0.650
	85			NS			NS	0.421	NS	0.188
	126			NS			NS	NS	NS	0.282
	149			NS			NS	NS	NS	0.370
	176			NS			NS	NS	NS	0.560
	FB 176			NS			NS	NS	NS	NS
Ca	0				0.380		NS	NS	NS	NS
	34				NS		NS	NS	NS	NS
	64				NS		NS	NS	NS	NS
	85				NS		NS	NS	NS	NS
	126				NS		NS	NS	NS	NS
	149				NS		NS	NS	NS	NS
	176				NS		NS	NS	NS	NS
	FB 176				NS		NS	NS	NS	NS
Mg	0					NS	NS	NS	NS	NS
	34					0.398	NS	NS	NS	NS
	64					0.306	NS	NS	NS	NS
	85					NS	NS	NS	NS	NS
	126					NS	NS	NS	NS	NS
	149					NS	NS	NS	NS	NS
	176					0.231	NS	NS	NS	NS
	FB 176					0.306	NS	NS	NS	NS

\*DAB = days after full bloom

\*\*FB = fruit bearing terminals

## Seasonal changes in fruit nutrient concentration and the effect of fertilisation treatments on fruit nutrient concentration

Since the second season's fruit will only be harvested in July 2021, fruit analysis data could be generated only up to 180 DAFB for the 2020/2021 season.

*Midknight Valencia trees (Nelspruit)*: A similar response than for the leaf N concentration was obtained regarding fruit N concentration, i.e., the trees that were fertilised at a 200% rate to the standard practices showed significantly increased N concentrations, from fruit set (30 days after full bloom) onwards (Figure 5.3.3.9). Consistent trends of increased concentrations of the other elements were observed, but not at significant levels.

*Orri mandarins (De Wet)*: No response in the fruit nutrient concentration of the trees that were fertilised at a 200% rate to the standard practices were obtained – this is similar to the leaf nutrient concentration results (Figure 5.3.3.10). The difference in concentration of the fruit at onset of fruit development between the two seasons is probably related to a difference in early fruit set levels (Table 5.3.3.12), the higher total set in 2020/21 being a heavier sink for fruit, leading to dilution of available nutrients.

**Table 5.3.3.12.** Average number of *Orri mandarin* (De Wet) flowers and fruit per shoot counted in spring of the 2019/2020 and 2020/2021 seasons respectively.

Flowers per shoot		Fruit per shoot	
2019/2020	2020/2021	2019/2020	2020/2021
30.6	29.7	1.16	1.56

The decreasing trend in mineral nutrient concentration of the fruit of both cultivars (trial sites) in the earlier part of fruit development is directly related to fruit growth; while the flattening of the drop is probably related to accumulation of nutrients and sugar in the maturation stage while the fruit growth rate decreases (Alva *et al.*, 2001), as illustrated in Figures 5.3.3.11 & 5.3.3.12, respectively.

Fruit analysis at harvest reflects the additional N applied to trees of both varieties/sites as well as the differences in fertilisation rates between the two blocks (Figure 5.3.3.13). Furthermore, the possible suppressed Mg uptake by an excessive supply of K is also significantly reflected in the fruit analysis of both sites, and likewise for P and Ca in the *Orri mandarins*.

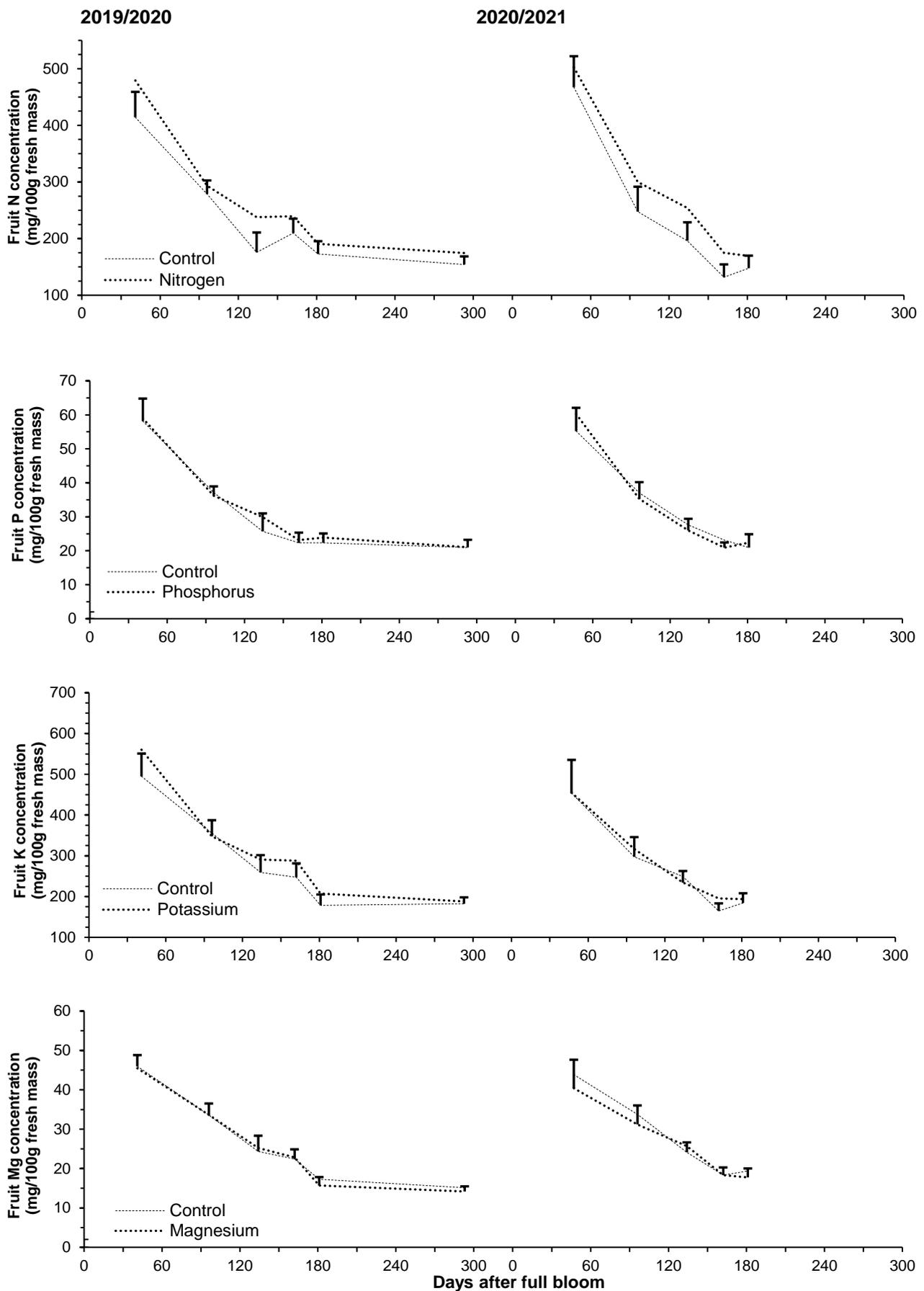
### Crop nutrient removal

Fruit analysis from the first season, i.e., 2019/2020, were used to calculate the nutrient removal per ton of fruit. It is shown in Table 5.3.3.13. For the *Midknight Valencia* this is lower than reported in Raath (2021), but in the case of the *Orri mandarins*, it is dramatically higher than that reported for mandarins in Raath (2021). For the *Midknight Valencia*, it is similar to that found by Alva *et al.* (2001) for *Valencia*. The very high removal rates found for the *Orri mandarins* could be induced by the high rate of nutrient supply.

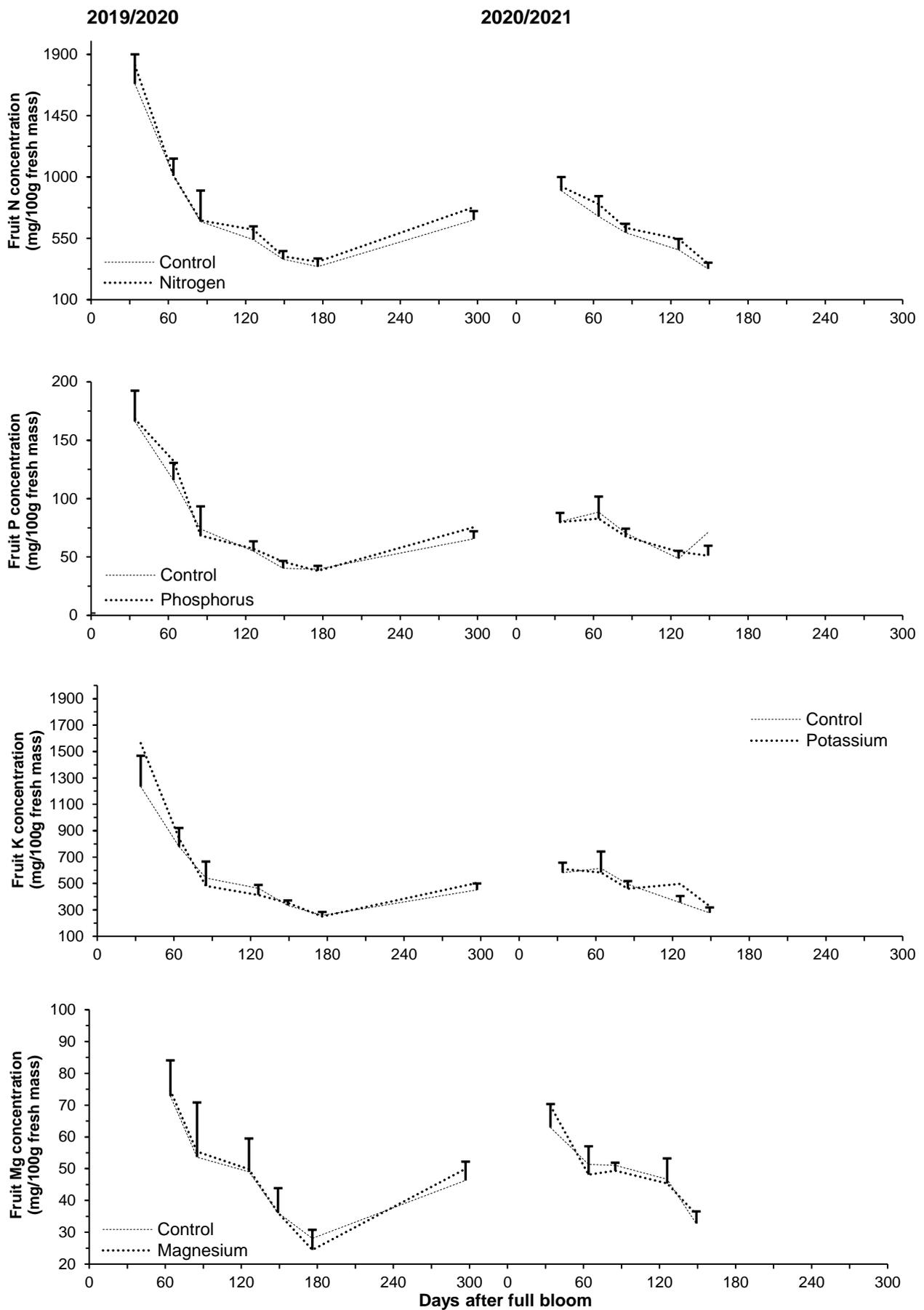
**Table 5.3.3.13.** Nutrient removal per ton of *Midknight Valencia* and *Orri mandarin* fruit produced.

Midknight Valencia					Orri mandarins				
N	P	K	Ca	Mg	N	P	K	Ca	Mg
kg/ton fruit					kg/ton fruit				
1.6 ± 0.21	± 0.03	1.81 ± 0.03	± 0.54	± 0.14	7.3 ± 0.71	± 0.02	4.9 ± 0.1	± 2.2	± 0.50 ± 0.01
*2.0-2.3	0.4	2.3	0.65	0.45	1.9-2.3	0.3	2.4	0.65	0.45

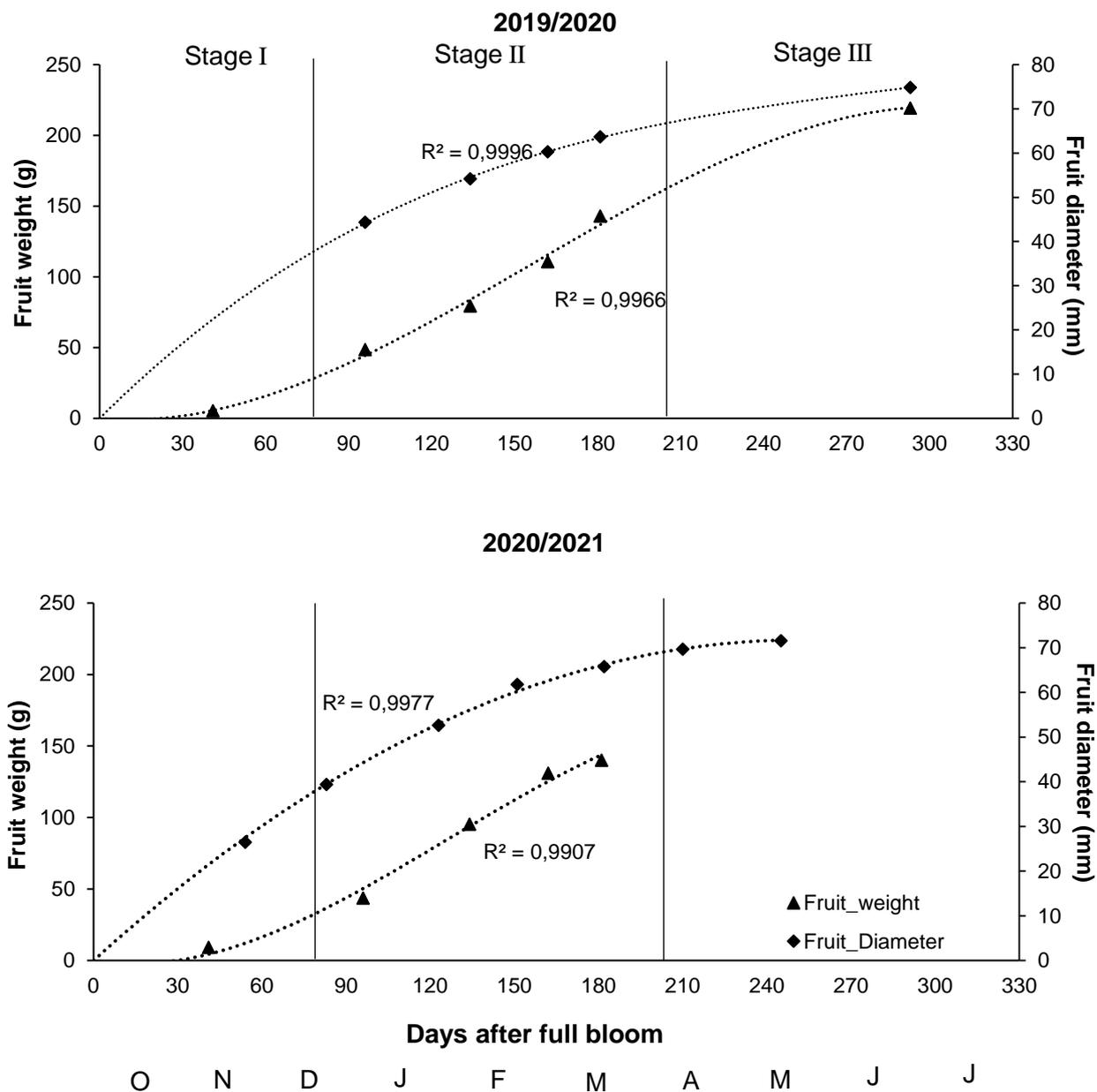
\*Removal of nutrients by the citrus crop as per Raath (2021)



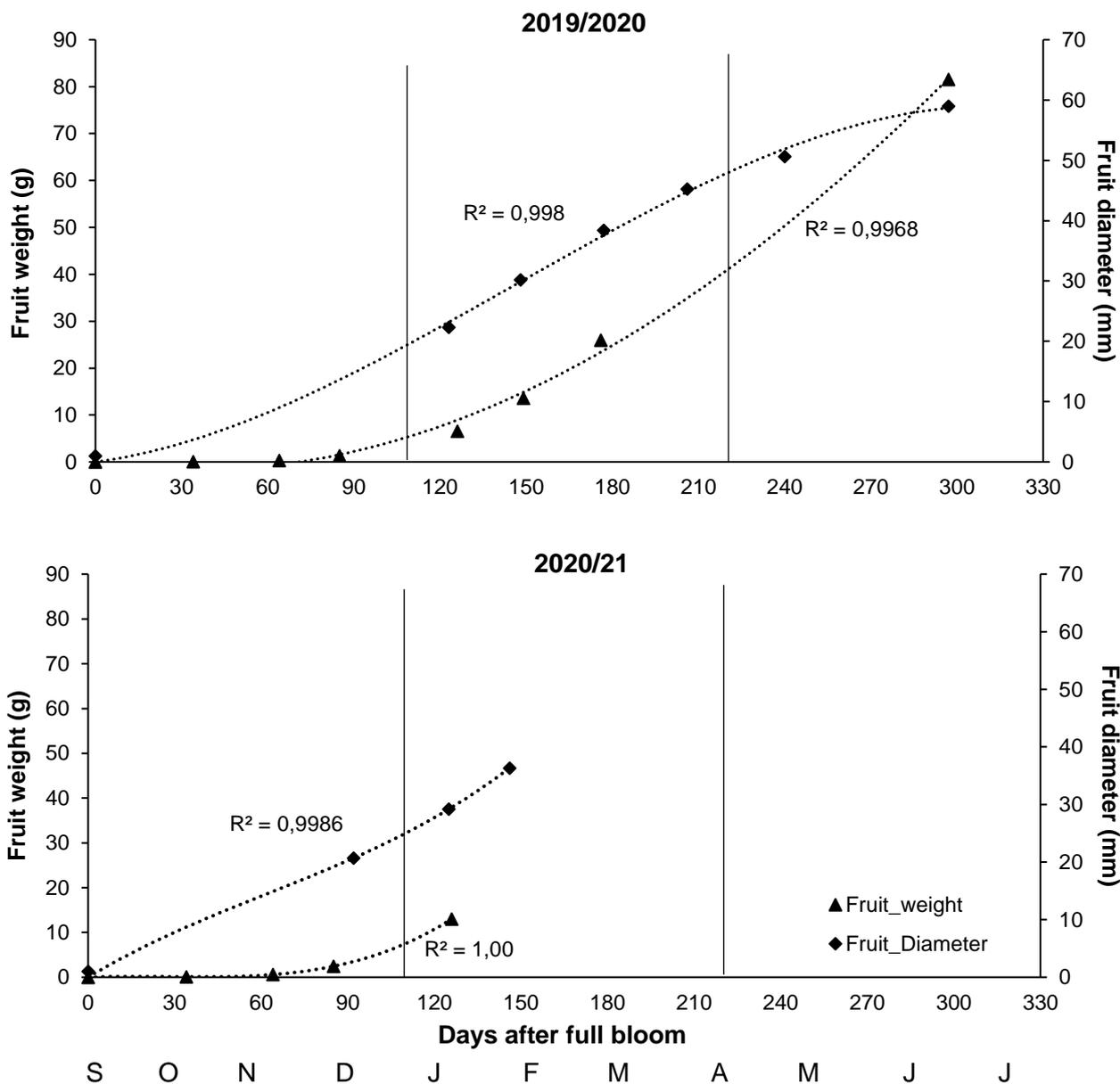
**Figure 5.3.3.9.** Seasonal changes in nutrient concentration of *Midnight Valencia* fruit as affected by fertilisation rates at 200% the normally applied rate (control) where the legend indicates the mineral that was applied – vertical bars indicate the LSD at  $p < 0.05$ .



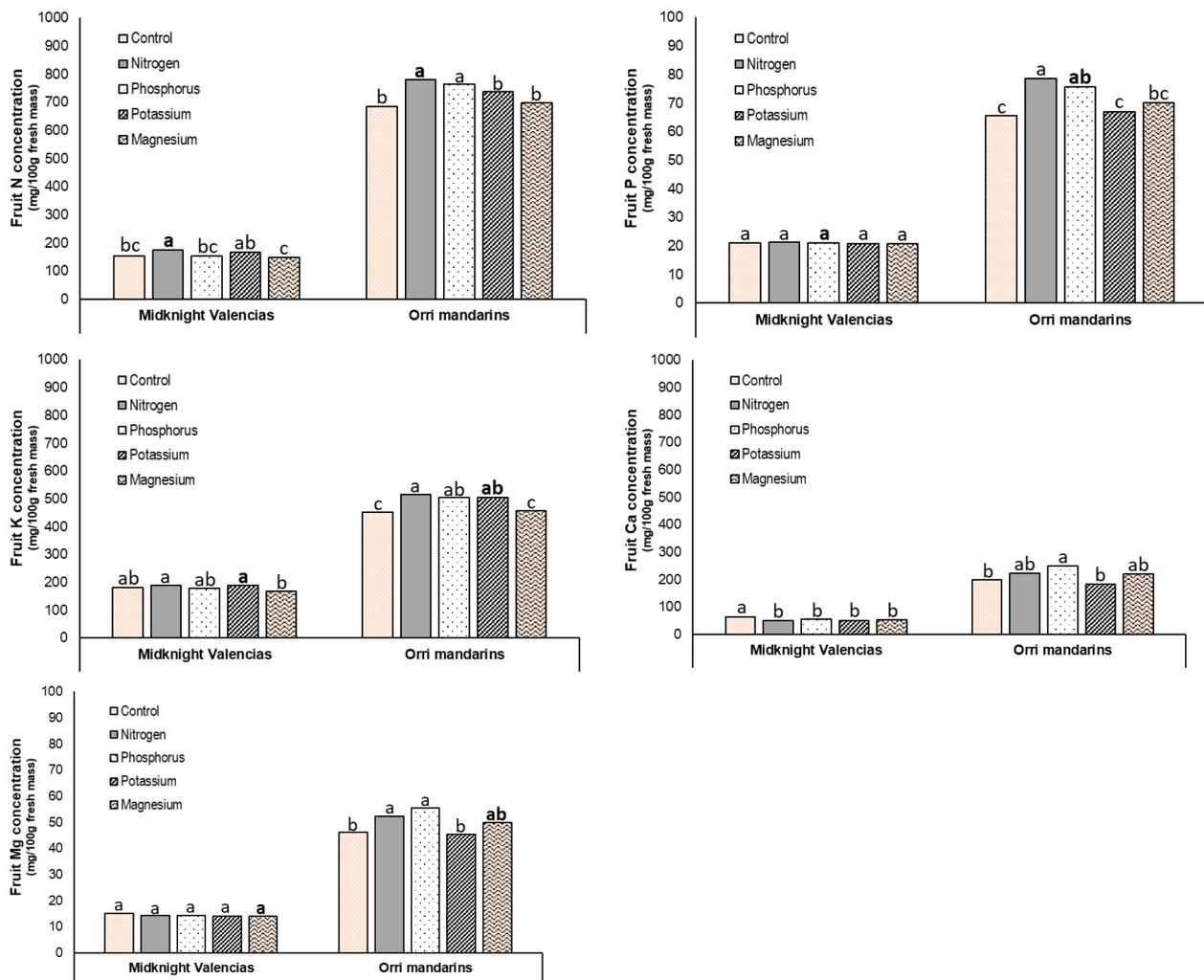
**Figure 5.3.3.10.** Seasonal changes in nutrient concentration of *Orri mandarin* fruit as affected by fertilisation rates at 200% the normally applied rate (control) where the legend indicates the mineral that was applied – vertical bars indicate the LSD at  $p < 0.05$ .



**Figure 5.3.3.11.** Seasonal changes in average fruit size of the *Midknight Valencia*, Nelspruit, during the 2019/2020 and 2020/2021 seasons.



**Figure 5.3.3.12.** Seasonal changes in average fruit size of the *Orri mandarins*, De Wet, during the 2019/2020 and 2020/2021 seasons.



**Figure 5.3.3.13.** Nutrient concentration of Midnight Valencia and Orri mandarin fruit at harvest, as affected by fertilisation rates at 200% the normally applied rate (control) where the legend indicates the mineral that was applied – vertical bars with different letters differ significantly at  $p < 0.05$ .

### Fruit characteristics at harvest

Only the first season's (2019/2020) final fruit characteristics and crop size could so far be evaluated. In Table 5.3.3.14 only the characteristics that was significantly affected by the fertilisation treatments are indicated. Parameters like sugar, acidity, colour, and juice % were not affected (data not shown). Various authors point to the fact that increasing rates of N fertilisation leads to decreasing citrus fruit size and mass (Reitz & Koo, 1959; Smith, 1966a; Chapman, 1968; Du Plessis & Koen, 1988; Koo, 1988; Quaggio *et al.*, 2006). In this trial the fruit weight of only the Midnight Valencia was affected (i.e., reduced) by excessive N applications. Likewise, Johnston (1950) found from observation in several orchards that there is no connection between N and fruit size.

Despite this treatment (excessively applied N) resulting in the smallest fruit (in terms of fruit weight), it was only in comparison with excessive K applications that a significant difference was obtained, and not compared to the control. The difference between the N and K treatments in terms of fruit weight might, to a large extent, be due to the increase in fruit size that high rates of K fertilisation causes (Du Plessis & Koen, 1988), as seen in Table 5.3.3.13. Ironically, the fruit N concentration of the Midnight Valencia were significantly increased by the N treatments, but not the K concentration (Figure 5.3.3.13).

The additionally applied N or K did not affect fruit weight of the Orri mandarins, although fruit N concentration was significantly increased by the N treatments (Figure 5.3.3.13). Furthermore, no significant effects on fruit

diameter were obtained for either cultivar/site, but the average diameter of the Midnight Valencia was the highest, to correspond to the significantly higher fruit weight obtained. The lack in response to excessive N and K applications, especially by the Orri mandarins, can be ascribed to the control and other treatments being excessively supplied by all nutrients. Corresponding with this lack of response in fruit characteristics, Castel & Ginestar (1996) found that no significant differences in any fruit quality parameters of 'Clementine' mandarin were obtained between N application rates of 120 and 210 kg N/ha per year. In another study, Montaña et al. (2004) also found no significant differences in fruit number, fruit size, rind colour, rind thickness, TDS or total acidity between annual N application rates of 178.5 kg N/ha and 297.5 kg N/ha respectively. And Alva *et al.* (2001) found no increase in fruit yield of Valencia for annual N application of 225 kg/ha and above.

The excessive K application treatment also increased the average rind thickness of the Midnight Valencia, but only significantly compared to the Mg treatment (Table 5.3.3.14). Although potassium is known for its positive effect on fruit size (Du Plessis and Koen, 1988) and rind thickness (Alva *et al.*, 2006; Koo, 1988; Smith, 1966b), it seems that a response is more prominent in situations of conservative or low supply of K.

The lack of treatment effects on fruit colour, particularly excessive N fertilisation, seems to suggest that high rates of N fertilisation does not necessarily affect fruit colour - if it is applied early enough on Midnight Valencia in Nelspruit, or even in February in Orri mandarins in the Western Cape.

**Table 5.3.3.14.** Midnight Valencia and Orri mandarin fruit characteristics at harvest, as affected by fertilisation rates at 200% the normally applied rate (control) – numbers followed by different letters differ significantly at  $p < 0.05$ .

Treatment	Fruit weight (g)		Fruit diameter (mm)		Rind thickness (mm)	
	Midnight Valencia	Orri mandarins	Midnight Valencia	Orri mandarins	Midnight Valencia	Orri mandarins
Control	225ab	95.4a	75.4	59.4	4.27ab	4.39
Nitrogen	<u>202b</u>	78.6b	74.3	59.0	4.62ab	4.33
Phosphorus	215ab	82.8b	73.7	59.1	4.29ab	4.55
Potassium	<b>233a</b>	75.1b	76.0	58.9	<b>4.76a</b>	4.62
Magnesium	222ab	75.8b	74.9	58.6	4.02b	4.43
LSD ( $p < 0.05$ )	25.4	10.2	NS	NS	0.70	0.35

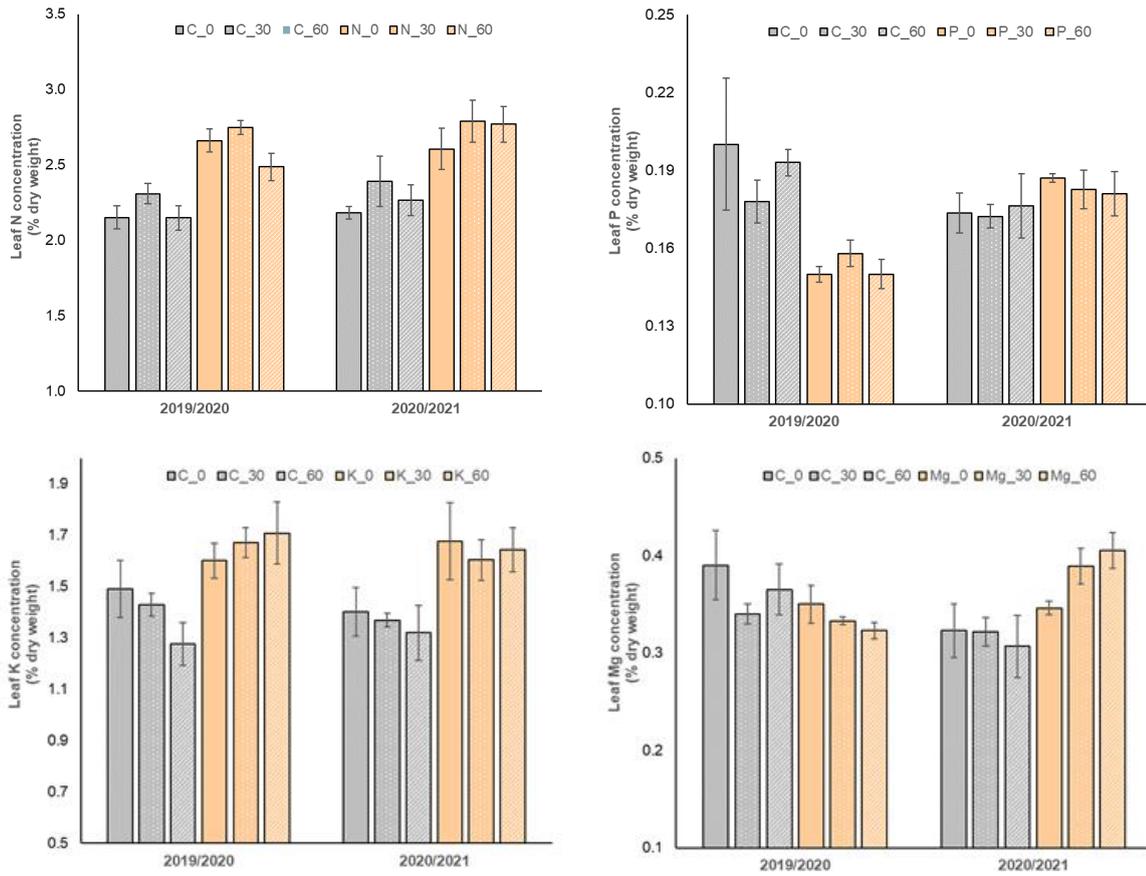
NS = not significant

#### The effect of fruit thinning in combination with excessively applied nutrients

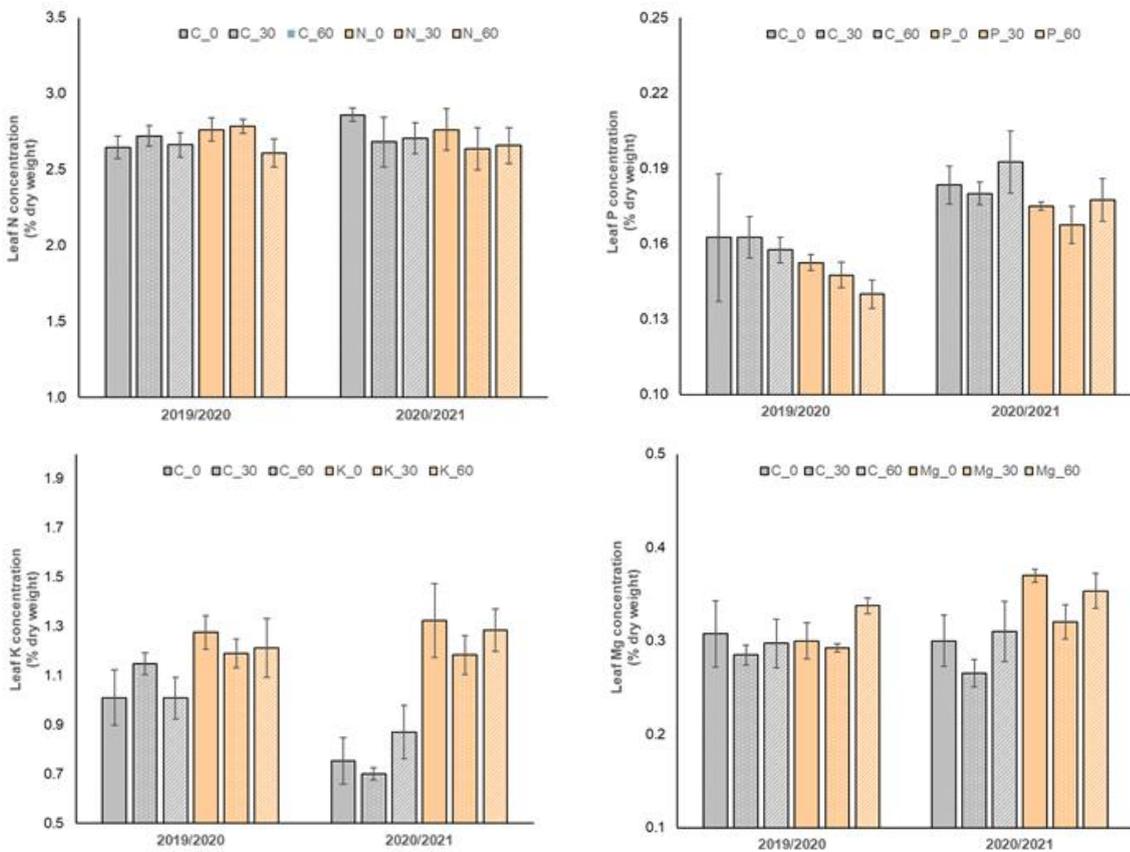
The level of fruit set was quantified only for the 2019/20 season, and will only be finalised for the 2020/2021 season at harvest in 2021. No significant effect on fruit set was obtained in the first season in response to the fertilisation or fruit thinning treatments. This is probably due to the slow, or lack, of response of the trees to the additional fertilisation applied, in terms of leaf nutrient concentrations and vegetative growth responses (data not shown).

When the control treatments are considered for both cultivars, fruit thinning did not have an effect on the nutrient concentration of leaves on non-fruit bearing terminals (Figure 5.3.3.14) or on fruit bearing terminals (data not shown). A response to fruit thinning was, however, obtained for leaves on non-fruit bearing terminals of the Midnight Valencia trees where excessive N (2020/2021), K (2019/2020) and Mg (2020/2021) were applied. No response, however, was found for the Orri Mandarins, or for leaves on the fruit bearing terminals (data not shown) of both cultivars.

### Midnight Valencia



### Orri Mandarins



**Figure 5.3.3.14.** The effect of fruit thinning and excessively applied nutrients on nutrient concentration in leaves on non-fruit bearing terminals of Midnight Valencia trees in Nelspruit and Orri mandarins in De Wet, leaves sampled in autumn (the vertical bars indicate standard errors).

## **Conclusion**

Significant insight was gained regarding the responsiveness of both Valencia and mandarin trees to mineral nutrition. Except for N, it can be concluded from this study that citrus trees have an ability to regulate their nutritional status in conditions of excessive fertilisation, making their leaf nutrient concentration fairly non-responsive to over-supplied levels of P, K, and Mg. This can explain the trend found in the Industry to over-fertilise, in an attempt to avoid deficiencies, without experiencing a negative effect on fruit quality. Long-term tendencies should rather be used to establish the effect of over-fertilisation, and be used to compile and manage fertilisation programmes to evaluate and avoid over-fertilisation, instead of in-season regular short-term changes in fertilisation rates.

The maximum limits used to evaluate foliar analysis are of little help to evaluate the accuracy of fertilisation rates of both Valencia and mandarins, since they were not exceeded despite excessively supplied nutritional conditions. The limited usefulness of foliar analysis in conditions of ample to over-supply of nutrients therefore needs to be emphasised.

The autumn sampling time ( $\pm 180$  days after full bloom) for foliar analysis was shown to sufficiently reveal the trees' overall nutritional response to a season's fertilisation and there is very limited value in using other sampling times. The sufficiency ranges also decrease diagnostic precision because they are too wide – they should therefore be regarded as secondary to observation of long-term trends in the foliar analysis, and foliar analysis should not be the only indication whether the fertilisation programme followed is appropriate, especially in conditions of ample to high rates of fertilisation.

When leaves were sampled for analysis, it showed that leaves from non-fruit bearing terminals can also be used successfully to assess tree nutritional status. Higher norms for N, P, K, Mg should however be used, while for Ca it must be slightly lower.

High rates of N fertilisation do not necessarily affect fruit colour - it must be applied early enough on Midnight Valencia in Nelspruit, but can even be applied as late as February on Orri mandarins in the Western Cape. The positive effect of K application on fruit size and rind thickness seems to apply mainly to situations of conservative or low supply of K.

This study has highlighted the senselessness of over-fertilisation, in that the ability to manipulate tree performance or fruit quality is completely lost. In addition, the anticipated increased cost-price squeeze as well as pressure on Industry to responsibly manage mineral fertilisation, will force producers to reduce their fertiliser inputs – and they will indeed find it to their advantage.

From the data generated in this project, two MSc theses are being prepared for graduation by the end of 2021. Furthermore, the role of nutrition to affect fruit set and quality of these two varieties will be elucidated using the final harvest data of the 2020/2021 season.

## **Future research**

Given that continuous excessive fertilisation has no positive, nor detrimental effect on tree nutritional status or fruit quality, the ability to manipulate fruit quality and tree performance in conditions of limited nutrient supply, with the goal of improving nutrient use efficiency, and reduced impact on the environment, needs to be elucidated.

## **Technology transfer**

Two presentations were given at a technical training event organised by Yara fertilisers (Paarl, June, 2021). The project's data will be incorporated in a CRI research symposium presentation in August 2021.

## **Acknowledgement**

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#### 5.3.4 **PROGRESS REPORT: Re-evaluating fruit set strategies for seedless 'Valencia' in the Letsitele region**

Project 1209 (RCE-2-01) (2018/19 - 2020/2) by Paul Cronje, Vivian White (CRI), Jakkie Stander (Philagro), Carel van Zyl and Karen Theron (SU)

##### **Summary**

The profitability of an orchard is determined by the quality and volume of the fruit it produces, but for seedless Valencia, oranges are influenced by variation in fruit set. Environmental factors and cultural practices on fruit set and quality under South African conditions are not well understood and contradictory results between climatic regions exist. This study researched possible factors that may influence the amount and quality of seedless Valencia produced in South Africa. A review of climatic conditions and production trends in two climatically diverse seedless Valencia production regions of South African (Kirkwood and Letsitele) revealed that tracking weather extremes should help explain the yields produced within orchards during growing seasons. However, the study highlighted the lack of targeted climatic studies in the various citrus producing regions to identify possible climatic factors that contribute to inconsistent fruit set in these regions. Improved understanding should help manage their financial risk in high-value orchards by allowing them to take preventative measures actively, plan their irrigation – and nutritional schedule, and to help them select the soundest horticultural practices. Secondly, the study included research into the cost of low set and - yield of seedless Valencia cultivars in the Letsitele areas, using the orchard level budget model. The model highlighted that high fruit prices would decrease the possible financial loss an orchard incurs, but to reach these high fruit prices for high-quality fruit, the producers must use innovative horticultural practices in the orchard. This reduces the risk of the orchard not sustainably producing economically viable levels of high-quality seedless Valencia oranges. The effects of Nitrogen, GA<sub>3</sub> and uniconazole on 'Midnight' and 'Delta' Valencia to improve fruit set, was tested and delivered inconsistent results, but it showed that standard levels of GA<sub>3</sub> should be applied to seedless Valencia. However, uniconazole applications can increase the yield, and if high N applications are made, uniconazole should be applied to counteract the possible excessive vegetative growth that it may cause. The influence of higher levels of N consistently led to the production of seedless Valencia oranges with a decreased internal quality. Finally, the study aimed to measure the effect of the soil potential and root development on fruit set of seedless Valencia oranges and provide an accurate classification system specifically for citrus, which should help the producer's decision making, especially in making pre-establishment corrections, the optimisation of fertilisation and irrigation. The study also showed that higher potential soils improved the fruit size distribution produced, improving the orchards' profitability. The improvement is possible because of improved nutrients, oxygen and water allocation to the root zone, and the tree and fruit itself during fruit development.

##### **Opsomming**

Die winsgewendheid van 'n sitrusboord word hoofsaaklik bepaal deur die kwaliteit en volume van die vrugte wat dit produseer en in saadlose Valencia-lemoene beïnvloed vrugset hierdie aspek. Die invloed van omgewingsfaktore en kulturele praktyke op die totale produksie (vrugset) en produksie kwaliteit van sitrusbome, word nie goed begryp nie en teenstrydige resultate tussen klimaatstreke is gevind. Die doel van die studie was om faktore te identifiseer wat die hoeveelheid en kwaliteit van saadlose Valencia, wat in Suid-Afrika geproduseer word, kan beïnvloed. Die studie het 'n oorsig van klimaatstoestand en produksietendense in twee saadlose Valencia-produksiestreke van Suid-Afrika (Kirkwood en Letsitele) gedoen. Die klimaat-onderzoek het gewys dat weersomstandighede die variasie in opbrengs tot 'n mate kan verklaar. Die studie het egter die gebrek aan detail klimaatstudies, om klimaatsfaktore te identifiseer wat bydra tot 'n inkonsekwente vrugset, uitgelig. Soortgelyke studies kan dit moontlik maak om aktief voorsorg maatreëls te tref, besproeiings- en voedingskodes te beplan, en om op tyd die beste praktyke te kies. 'n Boordvlak-begrotingsmodel, het die feit beklemtoon dat hoë vrugpryse die moontlike finansiële verlies wat 'n boord kan ly, sal verminder. Maar om dit te bereik moet die produsente innoverende oesmanipulasie praktyke gebruik. Die invloed van stikstof, GA<sub>3</sub> en uniconazole op 'Midnight' en 'Delta' Valencia om vrugset te verbeter, is getoets, maar inkonsekwente

resultate is gelewer. Die studie het getoon dat ten minste standaardvlakke van GA<sub>3</sub> op saadlose Valencia toegedien moet word. 'n Uniconazole toediening kan egter die opbrengs van 'n boom verhoog, en indien hoë N toedienings gedoen word, moet uniconazole toegedien word om die moontlike oormatige vegetatiewe groei wat kan plaasvind te voorkom. Laastens het die studie probeer om die invloed van die grond - en wortelontwikkeling op vrugset van saadlose Valencia-lemoene te meet, en 'n akkurate klassifikasiestelsel spesifiek vir sitrus te ontwikkel, wat die produsent se besluitneming kan verbeter in terme van tydige regstellings voor vestiging en om bemesting en besproeiing te optimaliseer. Die studie het ook getoon dat hoër potensiaal grond die vruggrootheid verspreiding verbeter, wat die winsgewendheid van boorde verbeter. Die verbetering is moontlik as gevolg van verbeterde toediening van voedingstowwe, suurstof en water aan die wortelsone en dus 'n beter boom en vrugte tydens vrugontwikkeling.

### 5.3.5 **PROGRESS REPORT: Handbook for Citrus Nutrition in Southern Africa**

Project RCE-2-03 (1232) (2019/20 – 2020/1) by Pieter Raath (CRI)

#### **Summary**

In line with Citrus Research International's (CRI) mandate to maximise the long-term global competitiveness of the Southern African Citrus Growers, an initiative was taken to provide the Industry with renewed fertilisation guidelines. A Handbook that supplements the Fertilisation Guidelines, as published in the CRI Production Guidelines, was therefore compiled. The objective of the original Fertilisation Guidelines was to provide detailed background information and recommendations to develop a rigorous citrus fertilisation programme that optimises financial returns, sustains yields and maintains soil and water quality. This Handbook updates these invaluable guidelines, combining age-old scientific principles with new practical implementations, to reflect the dynamic nature of the South African Citrus Industry. The goal with this Handbook remains central to the CRI mandate to empower both citrus growers and advisors to obtain a thorough understanding of the theoretical and practical elements that need to be considered for optimal citrus nutrition management.

#### **Opsomming**

In ooreenstemming met CRI se mandaat om Suider-Afrika se sitrusprodusente oor die langtermyn op die voorpunt te hou is besluit om opgedateerde bemestingsriglyne aan die Bedryf te verskaf. 'n Handboek wat die CRI Bemestingsriglyne aanvul, is dus saamgestel. Die doel van die oorspronklike riglyne was om detail agtergrondinligting en aanbevelings te verskaf sodat deeglike sitrusbemestingsprogramme wat maksimale finansiële wins, volhoubare oeste versker, tesame met handhawing van grond en watergehalte. Die Handboek is 'n opdatering hiervan, waar bewese wetenskaplike gekombineer is met nuwe praktyke sodat die dinamiese aard van die Suid-Afrikaanse Sitrus Bedryf gehandhaaf kan word. Hierdie Handboek het ten doel om, in lyn met CRI se mandaat, beide produsente en konsultante te bemagtig deurdat hulle 'n deeglike begrip van beide die teoretiese en praktiese aspekte wat benodig word vir optimale sitrus bemestingsbestuur kan bekom.

### 5.4 **PROGRAMME: CULTIVAR EVALUATION**

Programme coordinator: Johan Joubert (CRI)

#### 5.4.1 **Programme summary**

Eureka, followed by Lisbon, Limoneira and Genoa (single crop production per season) plantings, remain high in all the citrus-producing areas, as lemon establishment is possible in any citrus climatic region. One of the most important fruit characteristics remains oblong (longer) fruit shape, followed by prolonged picking window (best prices early in the season) and low seed numbers. Another important aspect on the tree's side is low thorn numbers or smaller thorns on the bearing branches to facilitate the harvesting process. Eureka remains the number one lemon selection preferred by citrus growers and consumers for several reasons: good quality fruit with high juice content; fairly long fruit shape; ability to bear a good crop on the trees with two to three main crops; and limited thorns on the bearing branches for optimal picking. Lisbon and Limoneira will be the next option for commercial plantings, due to compatibility on citrumelo and citrange rootstocks (Carizzo citrange, Swingle citrumelo and C35) and good production and fruit quality. There are several new lemon

selections included in the trial sites to challenge Eureka, with a specific goal towards completely seedless fruit along with optimum yield on the trees.

The mandarin trials in the hot citrus production areas (5.4.4, 5.4.7) remain a priority with the reality of good quality fruit with good colour development early in the season and optimum Brix:acid ratios being critical, options are improving with new selections becoming available, but require good management practices. The cool and intermediate production areas remain the best mandarin producing options (5.4.6, 5.4.8, 5.4.10, 5.4.14, 5.4.15, 5.4.16, 5.4.17) due to specific climatic requirements (better early colour and acids). The focus remains to plant earlier or later maturing cultivars outside the Tango, ARCCIT9 (Nadorcott LS), and Nadorcott picking windows since very high numbers of these trees have been planted. The best quality fruit produced in this picking window will be in high demand, but marginal fruit quality areas will be in trouble. The consumer demands fruit with low seed numbers, or seedless fruit, that peels easily, has good colour development and excellent flavour. Numerous new experimental options went into trial sites in the main citrus production areas, on different rootstocks, to determine the commercial value of these cultivars, including in the hotter production regions (early- and late maturing possibilities).

To address the satsuma and Clementine requirements, there are numerous trials representing the cooler citrus production areas of South-Africa, focusing on the early mandarin demand (5.4.13, 5.4.26, 5.4.23, 5.4.24, 5.4.25)

Star Ruby remains the number one grapefruit planted in South Africa due to excellent internal colour development and very good internal quality with high Brix content. The Star Ruby early and late were included at trial sites to allow the possibility of a longer picking window for red grapefruit. There are numerous new red grapefruit selections included in the trial sites with lower naringin (bitter taste) levels to improve flavour and eating experience (5.4.12).

We need early maturing Valencia options to replace or supplement Turkey because of the rind disorders and shelf life (adding experimental options in trials). The focus on late-maturing Valencia selections increased (5.4.2, 5.4.3, 5.4.5, 5.4.11, 5.4.21, 5.4.22) in the suitable citrus production areas (Letsitele) where demand for low seeded or seedless Valencia with good crop production increased. The problem with high chimera numbers on some of the late Valencia selections stimulated the need for alternative options to replace problem orchards or to establish new plantings (Jassie, Kobus du Toit Late, McClean SL etc).

Navel prices remained fairly low due to a combination of shelf life problems (low acid levels), postharvest performance and open navel ends, all contributing to the decrease in new navel plantings (5.4.9, 5.4.18, 5.4.19, 5.4.20). There is a demand for red pigmented navel fruit in the market and specifically new Cara Cara plantings increased in the navel production areas. The focus will remain on lemon, mandarin and Valencia options to fill the requirements in the production cycle of the packhouse programme.

Rootstock evaluations (including semi-commercial trials) expand the range of new rootstock trials, including the mainstream (commercial), semi-commercial range, as well as several Florida options (with the possibility of HLB tolerance) to evaluate production performance and suitability in different soil types (pH, clay content, salinity etc). There is also a range of new Argentinian rootstocks in the new trials (experimental and semi-commercial) to address the need for lemon scion compatibility and specific conditions and smaller tree volumes (5.4.5, 5.4.11).

## **Programopsomming**

Eureka, gevolg deur Lisbon, Limoneira en Genoa (enkel oes produksie per seisoen) aanplantings, bly hoog in al die sitrus produserende areas; deurdat suurlemoen vestiging moontlik is in enige sitrus klimaatsones. Een van die belangrikste vrug eienskappe is steeds langwerpige (silindriese) vrug vorm, gevolg deur verlengde plukvenster (beste pryse vroeg in die seisoen) en lae saad getalle. Ander belangrike aspekte is lae en of kleiner dorings op die draetakke om die oes proses te vergemaklik. Eureka bly steeds die nommer een suurlemoenseleksie wat deur Sitrusprodusente en verbruikers verkies word om verskillende redes: vrugte van goeie gehalte met 'n hoë sapinhoud; redelike lang vrugvorm; potensiaal vir 'n goeie drag met twee tot drie

vrugsette asook min dorings. Lisbon en Limoneira is die volgende kommersiele opsie vir aanplantings, a.g.v. verenigbaarheid op citrumelo en citrange onderstamme (carizzo citange, swingle citrumelo en citrange 35) asook goeie produksie en vrugkwaliteit. Lemongold (2 PH SL Eureka) aanplantings neem toe as 'n kommersiele saadlose opsie. Daar is 'n aantal nuwe suurlemoen seleksies wat by die proefpersele ingesluit word om met Eureka te vergelyk, met die spesifieke doel om totaal saadlose langwerpige vrugte met optimale opbrengs te produseer.

Die mandaryn proewe in die warm sitrus produksie areas (5.4.4, 5.4.7) bly 'n prioriteit van goeie vrugkwaliteit (optimale Brix:suur) met goeie kleurontwikkeling vroeg in die seisoen. Die nuwe seleksie brei die opsies uit vir hierdie doelwit, maar met goeie bestuurs praktyke. Sitrus wat in die koel en intermediêre produksieareas verbou word, bly die beste mandaryn produserende opsies (5.4.6, 5.4.8, 5.4.10, 5.4.14, 5.4.15, 5.4.16, 5.4.17) as gevolg van spesifieke klimaatvereistes (beter vroeë kleur en sure). Die fokus bly op die aanplant van vroer of later rypwordende kultivars buiten die Tango, ARCCIT9 (Nadorcott LS) en Nadorcott plukvensters, aangesien groot volumes van hierdie kultivars geplant is. Die beste kwaliteit vrugte wat in hierdie plukvenster geproduseer word, sal in groot aanvraag wees, maar die gebiede met marginale vrugkwaliteit kan problematies wees. Die verbruikers voorkeur bly vrugte met 'n lae saadinhoud, of totaal saadlose vrugte, wat maklik skil met 'n goeie kleurontwikkeling en uitstekende smaak. Verskeie nuwe eksperimentele opsies word ingesluit in proefpersele in die belangrikste sitrus produkserende areas, op verskillende onderstam opsies, om die kommersiële waarde van hierdie kultivars te bepaal, insluitend die warmer produksie areas (vroeë en laat rypwordende opsies).

Die satsuma en Clementine behoeftes word aangepreek in verskeie proewe wat die koeler sitrus produserende areas van Suid-Afrika insluit en die vroeë mandaryn aanvraag bedien (5.4.13, 5.4.26, 5.4.23, 5.4.24, 5.4.25)

Star Ruby is die nommer een pomelo wat in Suid-Afrika aangeplant word a.g.v. uitstekende interne kleurontwikkeling en baie goeie interne gehalte met 'n hoë Brix-inhoud. Die eksperimentele vroeë- en laat rypwordende Star Ruby seleksie word in proef persele ingesluit met die moontlikheid om die plukvenster te verleng van die rooi pomelos. Daar is verskeie nuwe rooi pomelo seleksies wat by die proefpersele ingesluit word met laer naringien vlakke (bitter smaak) om die smaak en eet-ervaring te verbeter (5.4.12).

Vroeë Valencia opsies word benodig om die Turkey aan te vul of te vervang, a.g.v. problematiese skildefekte en hou vermoë (nuwe opsies word ingesluit in eksperimente). Die fokus op laat rypwordende Valencia-seleksies bly hoog (5.4.2, 5.4.3, 5.4.5, 5.4.11, 5.4.21, 5.4.22) in die geskikte sitrusproduksie areas (Letsitele), waar die vraag na Valencia met 'n lae saadinhoud of totaal saadlose vrugte met goeie produksie toegeneem het. Die probleem van 'n hoë chimera voorkoms by sommige van die laat Valencia seleksies, het die behoefte laat ontstaan om alternatiewe opsies vir die vervanging van probleem boorde of nuwe aanplantings te gebruik (Jassie, Kobus du Toit Late, McClean SL ens.).

Die nawel pryse het redelik laag gebly as gevolg van die kombinasie van rakkelyd probleme (lae suurvlakke), na-oes prestasie en oop nawel-ente, wat alles bygedra het tot die afname in nuwe nawel aanplantings (5.4.9, 5.4.18, 5.4.19, 5.4.20). Daar is wel 'n aanvraag vir rooi gepigmenteerde vrugte in die mark en spesifiek het nuwe Cara Cara aanplantings toegeneem in die nawel produksie areas. Die fokus bly egter op suurlemoen, mandaryn en Valencia opsies om die vereistes in die produksie siklus van die pakhuisprogram aan te spreek.

Onderstam evaluasies word uitgebrei (insluitend semi-kommersiele proewe) met 'n nuwe reeks onderstam proewe, ingesluit die hoofstroom reeks (kommersiele opsies), semi-kommersiele reeks asook verskeie Florida opsies (met moontlike HLB weerstandbiedendheid) om produksie potensiaal te evalueer en geskiktheid vir verskillende grond tipes (pH, klei inhoud, sout vlakke) te bepaal. Daar is 'n reek Argentynse onderstamme in nuwe proewe ingesluit (eksperimenteel en semi-kommersieel) om die suurlemoen-bostam verenigbaarheids kwessie aan te spreek asook kleiner boom volumes te bevorder (5.4.5, 5.4.11).

## 5.4.2 PROGRESS REPORT: Evaluation of Valencia selections in hot humid inland areas (Onderberg) Project 75A by J. Joubert (CRI)

### Summary

Selections that performed well in this season, in this hot, humid production area, according to optimal maturity from early to late, were as follows. Val early, one of the new early maturing (internal quality) cultivars that matures before Turkey. There was still a delayed colour development on the fruit by the time of optimum maturity with Valearly, but deeper orange colour compared to the more yellow of Turkey when fully coloured. Turkey will follow, but bear in mind that this selection has a sensitive rind. Do not allow the fruit to hang for too long because the optimal picking period is no longer than 4-6 weeks.

Delta would follow, with good internal quality, production and fruit size, followed by McClean SL and Gusocora representing the middle of the Valencia season for this area. Alpha will be next in line, maturing later in this area than other areas, but keep the young tree age in mind. The later selections can broaden the list of choices to extend the season, commencing with Skilderkrans, Kobus du Toit Late and Jassie (optimum fruit size distribution) and followed by Lavalley with higher acid levels an ultra-late possibility.

### Opsomming

Seleksies wat hierdie seisoen, volgens optimum rypheid van vroeg tot laat goed presteer het vir hierdie vottige warm produksie area, is soos volg. Valearly is een van die nuwe vroeë Valencia opsies (vroeg intern ryp) wat voor Turkey inpas. Daar was steeds vertraagde kleurontwikkeling op die vrugte gewees met optimum rypheid by Valearly, maar wel dieper orange kleur wanneer opgekleur in vergelyking met meer geel by Turkey. Turkey sal dan volg, wees net versigtig om nie die seleksie te lank te hang nie. Baie skil probleme kan ontwikkel, want die optimum oes tydperk strek oor gemiddeld vier weke, ses weke maksimum dan moet die vrugte af wees.

Delta kan dan volg wat goeie interne kwaliteit, produksie en vruggrootte lewer, gevolg deur McClean SL en Gusocora wat dan die middel van die Valencia seisoen vir hierdie area verteenwoordig. Alpha pas dan in, wat later in hierdie area rypword in vergelyking met ander areas, maar hou die jong boom ouderdom in gedagte. Die later seleksies wat kan bydra tot die keuse om die seisoen te verleng, kan bestaan uit Skilderkrans, Kobus du Toit Laat en Jassie (optimum vruggrootte verspreiding) gevolg deur Lavalley met hoër suurvlakke as die ultra-laat opsie.

### Objective

- To find suitable Valencia selections with superior characteristics for the hot inland citrus production areas.

### Materials and methods

Field evaluations and laboratory analyses were conducted on Alpha, Delta (control), Gusocora, Jassie, Kobus du Toit Late, Lavalley, McClean SL, Skilderkrans, Turkey (control) and Valearly at Riverside in Malelane, Mpumalanga.

**Table 5.4.2.1.** Internal fruit quality minimum export requirements for Valencia types.

Variety	Juice %	Brix °	Min Acids	Max Acids	Ratio	Colour
Valencia EU	48	8.5	0.6	1.8%	7.5:1	Colour plate 3 of set no. 34
Midnight	52	9.5	0.85	1.8%	7.5:1	Colour plate 3 of set no. 34
*Turkey	50	10.0	0.85	1.5%	7.5:1	Colour plate 3 of set no. 34

\*Interim internal fruit quality standards.

**Table 5.4.2.2.** List of Valencia selections evaluated at Riverside (Malelane) during 2020.

Selection	Rootstock	Year Planted	Topworked	No. of trees
Alpha	CC	NA	2015	4
Delta	C35/CC/SC	2012	NA	5/5/5
Gusocora	CC	NA	2015	4
Jassie	C35/CC/SC	2012	NA	5/5/5
Kobus du Toit Late	C35/CC/SC	2012	NA	5/5/5
Lavalle	CC	NA	2015	4
McClearn SL	C35/CC/SC	2012	NA	5/5/5
Skilderkrans	C35/CC	2012	NA	5/5
Turkey	C35/CC/SC	2012	NA	5/5/5
Valearly	C35/CC/SC	2012	NA	5/5/5

## Results and discussion

This project is ongoing – all evaluations and tasks have been completed to date. Trees were visually evaluated at Riverside (Malelane) during the 2020 season.

### Delta (control)

Delta produced a fair to good crop, bearing fruit for the fifth set of evaluations this season. Fruit size range was slightly smaller this season and ranged from medium to large/extra-large (count 88 to 48) with good internal quality values, juice levels above 58, Brix of up to 12.4 and acids above 1.0%. Colour development at peak maturity on all three rootstocks were very similar (between T1 and 3). Delta remained completely seedless and complied with the export requirements. Based on the internal quality results in Table 5.4.2.3, maturity will be from the beginning to the middle of July.

### Jassie

The trees were bearing another good crop this year on all three rootstock combinations, averaging between 80 and 90 kg per tree. The fruit size was smaller and varied from medium to large, count 72 to 56 (average). The rind texture improved this season, becoming smoother with time. Seed count per fruit was decreased even more this season and varied from 1.5 to 7 seeds per fruit. Internal quality improved with tree age and produced better juice levels (above 57%), good Brix (above 11 at maturity) and higher acids (above 1.0). External colour development improved and peaked between T1 and 3 with the final evaluations. Maturity seems to be the middle of July to the beginning of August based on the results in Table 5.4.2.3.

### Kobus du Toit Late

Kobus du Toit Late was evaluated at the Riverside trial site on three rootstocks (C35, CC, SC) and produced medium to large fruit size (count 88 to 56/48) on the trees due to a lighter crop, with 2.4 seeds average. The colour development was very similar on all three rootstocks at peak maturity. The internal quality was good, juice levels above 60%, Brix up to 11.9, and lower acids earlier in the season (below 1.0 on CC) for the later maturing selection. External colour peaked from T1 to 4. Maturity seems to be middle to end of July, according to Table 5.4.2.3.

### McClearn SL

The standard McClearn will be included in future trials as a control to compare the SL selection's performance, although McClearn developed high chimera incidences on the fruit (up to 40%) in commercial plantings. McClearn SL produced fairly round fruit with soft fiber strength that peeled easily, containing low rind oil levels. All the fruit evaluated remained seedless. Many totally seedless selections have fruit set problems and bear less fruit, but this does not appear to be the case with this cultivar (GA<sub>3</sub> sprays are recommended). The fruit size increased and peaked at medium-large to large/extra-large (count 72-56/48). The internal quality improved from good (2017) to very good with high juice levels for the trial site up to 65%, Brix up to 13 and acceptable

acid levels (above 0.9%). There was a slight delay in external colour ranging from T1-3. Based on the internal quality results in Table 5.4.2.3, maturity will be mid to end of July.

#### Skilderkrans

Skilderkrans bore fruit on C35, Carrizo and Swingle at the Riverside trial site. Fruit size increased due a lighter crop and varied from medium to large/extra-large (count 72-48/40). Internally the Brix content improved (up to 12.5) and the acid level of 1.2 to 1.5% indicated a later maturing Valencia selection. Juice level increased to an average 62%; above the minimum required export figure. There was no delay in external colour (T1-3) on any of the rootstocks evaluated. The fruit developed a smooth rind, fibre strength was fairly soft and the fruit shape was round. Ratios were lower this season due to the low Brix and higher acid levels, delaying peak maturity to the end of July and mid-August on all three rootstocks (Table 5.4.2.3).

#### Turkey (Control)

Fruit size improved this season, ranging from count 72/64 to 48/40 average, with medium to large/extra-large fruit size. Fruit characteristics for Turkey were round fruit shape, smooth rind texture, very good flavour, soft rag, fairly thin rind, easy fruit peeling, and higher seed count per fruit ranging from 1.0 to 4.2 seeds per fruit (higher than 2019). The internal colour was light yellow, and externally the fruit remained yellow up to over-matured fruit. It should be borne in mind that this selection is not a true Valencia and has the qualities of a mid-season orange; for instance, the exceptionally soft rag of the fruit, and the soft rind result in rind problems if managed incorrectly. Turkey should not be harvested over more than four weeks as extending the harvesting season can lead to rind disorders. Based on the internal quality results in Table 5.4.2.3, the estimated maturity will be middle to end of May.

#### Valearly

Valearly, bearing a good crop (70 kg/tree) on the trees, developed low seed numbers (0.0 to 0.2 seeds per fruit) this season. The internal quality of the fruit was good early in the season with medium-high juice (above 52%, highest for Carrizo – 57%), Brix above 11 and acid above 0.8 on C35 and Carrizo. Acids on Swingle remained fairly high till later in the season (1.3%). Compared to the other early maturing selections, Valearly seems to be at least two weeks earlier, with good internal quality but delayed external colour ranging from T2-4. Estimated maturity according to Table 5.4.2.3, seems to be the end of April.

#### Additional selection

Alpha, Gusocora and Lavallo bore a decent crop for the second time this season and evaluations were possible, all topworked onto Carrizo rootstocks. All three selections were completely seedless. Alpha matured first; middle to end of July with good acid levels and very good Brix, ranging from 10.3 to 12.8. The highest acid level between the three cultivars was on Lavallo; also developing very good Brix and juice content. Colour development remained very similar between T1 and T3 up to peak maturity. Fruit size peaked on two of the three selections, except for Lavallo with bigger fruit, between count 72 and 56/48; very good for Valencia production and export.

### **Conclusions**

The internal quality for this season for all the selections evaluated, complied with the export standards at peak maturity of the fruit. These acid levels will decrease towards the end of the season, indicating extended shelf-life of the selection for example, Jassie. Jassie also indicated very low chimera fruit numbers on the trees, providing another good late maturing Valencia option to be included in future plantings. There was no Brix: acid ratio below 7.5:1 at peak maturity this season, which is often associated with later maturing selections having higher acid levels, but rather the opposite scenario with lower acids in general on most of the fruit samples being evaluated. When the acid levels decrease, the ratio increases. There was a better colour development with most of the selections towards peak maturity time, except in the case of Valearly, more specifically on Swingle rootstock, where the colour development was delayed even after peak quality. The average seed count for this season remained fairly low, including Kobus du Toit Late and Jassie (average 2.4 and 3.3 seeds per fruit), indicating lower cross pollination in the mixed trial block. McClean SL remained completely seedless. Jassie and Kobus du Toit Late will be future possibilities to include in new Valencia plantings (optimum Valencia fruit size distribution, high juice levels, low seed counts and late maturing). Fruit

size improved on the trees as they matured, between count 88/72 and up to count 56/48 on selections with lighter yields.

**Table 5.4.2.3.** Internal fruit quality data for Valencia and late orange selections at Riverside (Malelane) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Alpha	CC	06/05/2020	78 - 85	64 - 48	59,5	11,9	1,60	7,4	0,0	T4 - 6
Alpha	CC	20/05/2020	72 - 83	88 - 56	60,5	10,3	1,40	7,4	0,0	T3 - 5
Alpha	CC	10/06/2020	79 - 86	64 - 48	61,7	10,9	1,45	7,5	0,0	T1 - 4
Alpha	CC	02/07/2020	78 - 89	64 - 48	57,6	11,6	1,35	8,6	0,0	T1 - 3
Alpha	CC	16/07/2020	80 - 87	64 - 48	62,1	12,2	1,20	10,2	0,0	T1 - 3
Alpha	CC	05/08/2020	73 - 86	72 - 48	62,4	12,7	1,30	9,8	0,0	T1 - 3
Delta	C35	10/06/2020	72 - 81	88 - 64	64,3	11,3	1,30	8,7	0,0	T 1 - 4
Delta	C35	02/07/2020	76 - 87	72 - 48	60,1	11,4	1,10	10,4	0,0	T 1 - 3
Delta	C35	16/07/2020	75 - 86	72 - 48	58,1	12,1	1,00	12,1	0,0	T 1 - 3
Delta	CC	10/06/2020	78 - 89	64 - 48	59,6	11,5	1,25	9,2	0,0	T 1 - 4
Delta	CC	02/07/2020	79 - 89	64 - 48	58,8	11,4	1,10	10,4	0,0	T 1 - 3
Delta	CC	16/07/2020	78 - 84	64 - 56	63,7	12,1	1,20	10,1	0,0	T 1 - 3
Delta	CC	05/08/2020	78 - 83	64 - 56	60,7	12,4	1,05	11,8	0,0	T 1 - 3
Delta	SC	10/06/2020	78 - 89	64 - 48	58,2	10,9	1,20	9,1	0,0	T 1 - 4
Delta	SC	02/07/2020	73 - 84	72 - 56	60,2	11,5	1,05	11,0	0,0	T 1 - 3
Delta	SC	16/07/2020	73 - 83	72 - 56	60,8	11,7	1,15	10,2	0,0	T 1 - 3
Delta	SC	05/08/2020	69 - 95	88 - 40	62,1	11,8	1,00	11,8	0,0	T 1 - 3
Gusocora	CC	06/05/2020	74 - 80	72 - 64	57,0	11,0	1,35	8,1	0,0	T5 - 6
Gusocora	CC	20/05/2020	76 - 82	72 - 56	58,1	10,8	1,20	9,0	0,0	T4 - 5
Gusocora	CC	10/06/2020	77 - 89	72 - 48	58,9	10,9	1,30	8,4	0,0	T 1 - 5
Gusocora	CC	02/07/2020	76 - 83	72 - 56	56,6	10,9	1,15	9,5	0,0	T 1 - 3
Gusocora	CC	16/07/2020	74 - 89	72 - 48	66,9	12,8	1,15	11,1	0,0	T 1 - 3
Gusocora	CC	05/08/2020	75 - 89	72 - 48	67,0	12,6	1,15	11,0	0,0	T 1 - 3
Gusocora	CC	14/08/2020	75 - 86	72 - 56	66,8	12,5	1,10	11,4	0,0	T 1 - 3
Jassie	C35	10/06/2020	79 - 86	64 - 56	57,2	11,3	1,70	6,6	2,0	T 1 - 3
Jassie	C35	02/07/2020	75 - 81	72 - 64	62,5	12,8	1,45	8,8	4,3	T 1 - 3
Jassie	C35	16/07/2020	79 - 82	64 - 56	61,9	11,7	1,55	7,5	3,7	T 1 - 3
Jassie	C35	05/08/2020	75 - 82	72 - 56	65,4	10,7	1,65	6,5	3,7	T 1 - 3
Jassie	CC	20/05/2020	75 - 80	72 - 64	58,9	11,0	1,75	6,3	1,9	T2 - 4
Jassie	CC	10/06/2020	81 - 86	64 - 56	61,3	10,3	1,35	7,6	2,1	T 1 - 4
Jassie	CC	02/07/2020	74 - 81	72 - 64	59,8	11,5	1,65	7,0	7,0	T 1 - 3
Jassie	CC	16/07/2020	76 - 86	72 - 56	62,6	11,5	1,45	7,9	3,0	T 1 - 3
Jassie	CC	05/08/2020	74 - 84	72 - 56	62,2	12,0	1,15	10,4	3,3	T 1 - 3
Jassie	SC	20/05/2020	75 - 85	72 - 56	60,0	12,6	1,50	8,4	4,4	T2 - 4
Jassie	SC	10/06/2020	78 - 85	64 - 56	58,5	11,0	1,45	7,6	1,5	T 1 - 4
Jassie	SC	02/07/2020	72 - 82	88 - 56	58,1	11,4	1,70	6,7	7,0	T 1 - 3
Jassie	SC	16/07/2020	77 - 88	72 - 48	61,8	11,6	1,05	11,0	2,2	T 1 - 3
Jassie	SC	05/08/2020	77 - 85	72 - 56	59,7	12,6	1,00	12,6	3,6	T 1 - 3
K du Toit Late	C35	10/06/2020	70 - 80	88 - 64	62,4	10,9	1,35	8,1	1,3	T 2 - 4
K du Toit Late	CC	06/05/2020	77 - 85	72 - 56	64,6	10,9	1,20	9,1	1,3	T4 - 6
K du Toit Late	CC	10/06/2020	77 - 85	72 - 56	60,3	10,7	1,20	8,9	2,0	T 1 - 4
K du Toit Late	CC	02/07/2020	73 - 82	72 - 56	61,2	11,1	1,25	8,9	5,3	T 1 - 3
K du Toit Late	CC	16/07/2020	77 - 84	72 - 56	61,5	11,9	1,75	6,8	2,0	T 1 - 3
K du Toit Late	CC	05/08/2020	73 - 84	72 - 56	62,3	11,1	1,25	8,9	3,2	T 1 - 4

K du Toit Late	CC	14/08/2020	72 - 89	88 - 48	61,3	11,6	0,90	12,9	2,7	T1 - 3
K du Toit Late	SC	01/06/2020	75 - 85	72 - 56	61,8	10,7	1,15	9,3	3,0	T3 - 5
K du Toit Late	SC	02/07/2020	75 - 81	72 - 64	61,8	11,6	1,00	11,6	2,5	T1 - 3
K du Toit Late	SC	16/07/2020	74 - 85	72 - 56	64,0	11,6	1,05	11,0	1,8	T1 - 3
K du Toit Late	SC	05/08/2020	78 - 85	88 - 58	62,6	11,4	0,95	12,0	2,3	T1 - 3
K du Toit Late	SC	14/08/2020	76 - 83	88 - 48	60,7	11,6	0,80	14,5	3,3	T1 - 3
Lavalle	CC	10/06/2020	83 - 90	72 - 40	61,7	14,4	2,00	7,2	0,0	T1 - 4
Lavalle	CC	02/07/2020	78 - 90	64 - 40	62,2	11,5	1,60	7,2	0,0	T1 - 3
Lavalle	CC	16/07/2020	80 - 92	64 - 40	62,7	12,4	1,40	8,9	0,0	T1 - 3
McClearn SL	C35	06/05/2020	74 - 80	72 - 64	63,3	11,3	1,30	8,7	0,0	T3 - 5
McClearn SL	C35	20/05/2020	73 - 85	72 - 56	58,2	11,1	1,40	7,9	0,0	T3 - 4
McClearn SL	C35	02/07/2020	75 - 84	72 - 56	58,6	11,2	1,35	8,3	0,0	T1 - 3
McClearn SL	C35	16/07/2020	74 - 85	72 - 56	65,0	12,6	1,25	10,1	0,0	T1 - 3
McClearn SL	C35	05/08/2020	74 - 86	72 - 48	60,0	12,4	1,40	8,9	0,0	T1 - 3
McClearn SL	C35	14/08/2020	73 - 82	72 - 56	61,5	13,1	0,90	14,6	0,0	T1 - 3
McClearn SL	CC	06/05/2020	73 - 82	72 - 56	63,4	11,4	1,30	8,8	0,0	T3 - 5
McClearn SL	CC	20/05/2020	74 - 83	72 - 56	62,9	11,0	1,10	10,0	0,0	T4 - 5
McClearn SL	CC	10/06/2020	79 - 85	64 - 56	63,7	10,9	1,45	7,5	0,0	T1 - 4
McClearn SL	CC	02/07/2020	72 - 88	88 - 56	51,6	11,6	1,05	11,0	0,0	T1 - 3
McClearn SL	CC	16/07/2020	74 - 87	72 - 48	64,7	12,4	1,10	11,3	0,0	T1 - 3
McClearn SL	CC	05/08/2020	72 - 81	88 - 64	63,0	11,9	1,15	10,3	0,0	T1 - 3
McClearn SL	CC	14/08/2020	73 - 84	72 - 56	65,8	13,0	1,05	12,4	0,0	T1 - 3
McClearn SL	SC	10/06/2020	77 - 85	72 - 56	58,0	10,7	1,05	10,2	1,5	T1 - 3
McClearn SL	SC	02/07/2020	71 - 84	88 - 56	61,1	12,5	1,05	11,9	0,0	T1 - 3
McClearn SL	SC	16/07/2020	74 - 87	72 - 48	65,3	11,8	0,95	12,4	0,0	T1 - 3
McClearn SL	SC	05/08/2020	76 - 86	72 - 56	64,7	12,0	1,05	11,4	0,0	T1 - 3
Skilderkrans	C35	02/07/2020	77 - 85	72 - 56	58,5	12,0	1,30	9,2	0,0	T1 - 3
Skilderkrans	C35	16/07/2020	78 - 89	64 - 48	64,7	11,7	1,70	6,9	0,0	T1 - 3
Skilderkrans	C35	05/08/2020	79 - 90	64 - 40	60,5	12,2	1,30	9,4	0,0	T1 - 3
Skilderkrans	C35	14/08/2020	75 - 85	88 - 48	64,0	12,3	1,10	11,2	0,0	T1 - 3
Skilderkrans	CC	02/07/2020	75 - 86	72 - 48	57,0	11,3	1,45	7,8	0,0	T1 - 3
Skilderkrans	CC	16/07/2020	75 - 85	72 - 48	62,0	12,5	1,15	10,9	0,0	T1 - 3
Skilderkrans	CC	05/08/2020	79 - 90	64 - 40	62,2	11,5	1,25	9,2	0,0	T1 - 3
Skilderkrans	CC	14/08/2020	75 - 91	72 - 40	58,0	12,6	1,25	10,1	0,0	T1 - 3
Skilderkrans	SC	02/07/2020	77 - 81	72 - 64	61,4	11,5	1,40	8,2	0,0	T1 - 4
Skilderkrans	SC	16/07/2020	75 - 92	72 - 40	60,9	12,0	1,45	8,3	1,5	T1 - 3
Skilderkrans	SC	05/08/2020	77 - 91	72 - 40	65,4	11,6	1,35	8,6	0,0	T1 - 3
Skilderkrans	SC	14/08/2020	79 - 91	64 - 40	66,7	11,7	1,45	8,1	0,0	T1 - 3
Turkey	C35	06/05/2020	82 - 88	56 - 48	64,0	11,4	0,90	12,7	4,2	T3 - 5
Turkey	C35	20/05/2020	72 - 91	88 - 40	60,5	11,8	0,95	12,4	1,0	T1 - 3
Turkey	C35	10/06/2020	73 - 87	72 - 40	64,2	12,2	0,85	14,4	1,3	T1 - 3
Turkey	CC	06/05/2020	77 - 83	72 - 56	59,9	11,9	1,05	11,3	3,8	T3 - 5
Turkey	CC	20/05/2020	78 - 92	64 - 40	58,5	11,7	1,05	11,1	1,5	T1 - 4
Turkey	CC	10/06/2020	77 - 87	72 - 48	62,6	11,7	1,10	10,6	1,1	T1 - 3
Turkey	SC	06/05/2020	79 - 86	64 - 48	58,5	11,5	1,05	11,0	3,2	T3 - 5
Turkey	SC	20/05/2020	79 - 91	64 - 40	56,4	11,0	0,95	11,6	0,8	T1 - 4
Turkey	SC	10/06/2020	80 - 90	64 - 40	69,8	10,8	1,20	9,0	1,0	T1 - 3
Valearly	C35	06/05/2020	77 - 88	72 - 48	54,0	11,1	1,00	11,1	0,0	T3 - 5
Valearly	C35	20/05/2020	75 - 81	72 - 64	56,9	11,9	0,80	14,9	0,0	T1 - 4
Valearly	C35	10/06/2020	77 - 87	72 - 48	52,0	12,2	0,95	12,8	0,0	T1 - 3
Valearly	CC	06/05/2020	75 - 80	72 - 64	57,1	11,9	0,80	14,9	0,0	T3 - 5
Valearly	CC	20/05/2020	72 - 89	88 - 48	55,3	11,4	0,90	12,7	0,0	T2 - 4

Valearly	CC	10/06/2020	72 - 81	88 - 64	56,2	11,7	0,90	13,0	0,2	T1 - 3
Valearly	SC	06/05/2020	75 - 79	72 - 64	53,8	11,9	1,30	9,2	0,0	T4 - 6
Valearly	SC	10/06/2020	75 - 80	72 - 64	56,2	11,5	1,20	9,6	0,0	T1 - 4

#### 5.4.3 PROGRESS REPORT: Evaluation of Valencia selections in the hot, dry inland areas (Letsitele and Hoedspruit)

Project 75B by J. Joubert (CRI)

##### Summary

The season starts with early selections and proceeds to the late maturing selections suitable for these hot-dry production areas. Recommendations have therefore been made accordingly. Valearly will start the season as an early maturing Valencia. Turkey will follow, producing large fruit size with good internal quality and soft fibre. The optimal picking window will be within the first four weeks of peak maturity. Bennie 1 and 2 can follow after Turkey with good production and medium to large fruit size, but we recommend harvesting the cultivar after the middle of the Valencia season to prevent rind pitting problems (in problem areas). Delta, as a control fits in before Gusocora. Gusocora and McClean SL follow next with completely seedless fruit and very good Brix: acid ratios. Midnight 1 and 2 cover the middle of the Valencia season with good internal quality fruit, large fruit size, smooth rind and low seed counts per fruit. Du Roi follows with an excellent crop on the trees and medium to medium-large fruit size (count 72 to 56). Valencia Late, followed by Lavallo are currently the latest maturing Valencia selections that are being planted commercially, developing large to extra-large fruit size, pebbly rind texture, delayed colour development and good yield.

A series of experimental/semi-commercial selections have also been included in the hot production areas. The selection range follows from mid-, to late-maturing options. The mid-season starts with Skilderkrans. Jassie with optimum fruit size as well as good internal quality and Kobus Du Toit late mature more towards the end of the Valencia season with medium to large fruit size.

##### Opsomming

Die seisoen begin met vroeg rypwordende seleksies en duur voort met die laat rypwordende seleksies in die warm droë produksie areas en aanbevelings is daarvolgens gebaseer. Valearly kan die seisoen begin as 'n vroeg rypwordende Valencia. Turkey kan nou volg, wat groot vrugte produseer met goeie interne kwaliteit en sagte vesel. Optimum plukvenster is binne die eerste vier weke van piek rypheid. Bennie 1 en 2 kan na Turkey volg met goeie produksie en medium tot groot vuggroote, maar ons beveel aan om die kultivar na die middel van die Valencia seisoen te oes om gepokte skil te voorkom (in probleem areas). Delta as kontrole pas in voor Gusocora. Gusocora en McClean SL volg dan met totaal saadlose vrugte en goeie Brix: suur verhoudings. Midnight 1 en 2 vul die middel van die Valencia seisoen met goeie interne kwaliteit vrugte, groot vuggroote, gladde skille en lae saadtellings per vrug. Du Roi is volgende met uitstekende oeste op die bome en medium tot medium/groot vrugte (telling 72 tot 56). Valencia late, gevolg deur Lavallo, wat huidiglik die laatste rypwordende Valencia seleksie wat kommersieel aangeplant word is, met groot tot ekstra-groot vuggroote, growwe tekstuur, vertraagde kleurontwikkeling en goeie produksie.

Daar is 'n reeks eksperimentele/semi-kommersiele seleksies wat ook vir die warm produksie areas ingesluit is. Hier volg die seleksies van middel, tot laat rypwordende kultivars. Die mid-seisoen kan begin word met Skilderkrans. Jassie met optimum vuggroote asook goeie interne kwaliteit en Kobus Du Toit Laat word meer aan die einde van die Valencia seisoen ryp met medium tot groot vuggroote.

##### Objective

- To find suitable Valencia selections with superior characteristics for the hot inland citrus production areas.

## Materials and methods

Field evaluations and laboratory analyses were conducted on Alpha, Beli, Bennie 1 & 2, Delta, Du Roi, Kobus Du Toit Late, Gusocora, Jassie, Lavalley 1, McClean, McClean SL, Midnight 1 & 2, Skilderkrans, Turkey, Valearly and Val Late at Bosveld Citrus (Letsitele) and Groep 91 (Letsitele).

**Table 5.4.3.1.** List of Valencia selections evaluated at Bosveld Citrus (Letsitele) during the 2020 season.

Selection	Rootstock	Planted
Alpha	SC	2009
Bennie 1	SC	2009
Delta (control)	SC	2009
Du Roi	SC	2009
Gusocora	SC	2009
Kobus Du Toit Late	C35/CC	2011
Lavalley 1	SC	2009
McClean SL	SC	2009
Midnight 1	SC	2009
Midnight 2	SC	2009
Turkey	C35/SC	2011
Valearly	CC	2011
Val Late	SC	2009

**Table 5.4.3.2.** List of Valencia selections evaluated at Groep 91 (Letsitele) during the 2020 season.

Selection	Rootstock	Planted
Bennie 1	CC/SC	2006
Bennie 2	CC/SC	2006
Kobus du Toit Late	CC/SC/Sunki 812/X639	2013
Jassie	CC/SC	2013
McClean	CC/SC/Sunki 812/X639	2013
McClean SL	CC/SC/Sunki 812/X639	2013
Midnight 1 & 2	CC/SC	2006
Skilderkrans	CC/SC	2006
Turkey	C35/CC/SC	2006
Valearly	SC/Sunki 812/X639	2013

## Results and discussion

### Alpha

Fruit production on the Alpha trees was good this season in the Letsitele area, despite the terrible drought conditions with limited water supply. Alpha was planted on C35 and Swingle at the Bosveld trial site to compare tree development (vigour) and yield production (trees on C35 almost a third smaller). The internal quality was still very good compared to 2019, juice levels peaked at 59%, Brix was above 11 and acids were fairly high (1.7%). Fruit size increased slightly and varied from count 72 to 56, excellent for Valencia production and export. External colour was delayed on Swingle (typical characteristic on this rootstock) and peaked from T1 to T3. Maturity seems to be the end of June to the middle of July (Table 5.4.3.3).

### Bennie 1 and 2

Bennie was evaluated at two trial sites this season: Bosveld and Groep 91. There was a good crop on the Bennie trees at both sites despite the remaining drought conditions and severe high temperatures. Fruit size peaked between count 72 and 48 (Bosveld) and 88 up to 48 (Groep 91); fruit size was smaller at Groep 91 compared to Bosveld. The internal colour of the fruit was deep yellow, fruit shape round, rind texture fairly

smooth, medium rag content and medium rind thickness. Bennie 1 and 2 internally produced similar juice levels (average 60.1%), Brix (average 11.5), acid (1.2%) and seed counts (average 2.9 seeds per fruit). External colour on both selections by the time of harvest varied between T1 and T4 (better colour on SC this season). Based on ratios, Bennie 1 and 2 mature end of June to the beginning of July (Tables 5.4.3.3 & 5.4.3.4).

#### Delta (control)

Delta on Swingle rootstock, as control cultivar, produced completely seedless fruit and a good yield. Fruit size peaked between count 72 and 56 (good crop) with good internal quality, reaching juice levels of 56%, Brix of 12 and acid content of 1.3%. The external colour of the fruit was between T1 and T4. Maturity is middle to the end of June (Table 5.4.3.3).

#### Du Roi

Du Roi was planted on two rootstocks, C35 and Swingle at the Bosveld trial, and for this season the Swingle combination was evaluated as a control selection due to C35's severe susceptibility to Blight in the Letsitele production area. There was a good yield and fruit size peaked between count 72 and 56 (better crop load on the trees). The external colour peaked between T1 and T3 (maturity) and the average seed count was 2.3 seeds per fruit (slightly higher). Swingle developed a juice content of 61%, Brix of 11.4 and acids of 1.5%. Maturity is end of July to middle August (Table 5.4.3.3).

#### Gusocora

Gusocora was evaluated at Bosveld Citrus this year on Swingle rootstock. The fruit was completely seedless and developed a good internal quality where juice (56%), Brix (11.8) and acid (1.3) complied with export requirements. The external colour varied from T1 to T4, correlating with the internal quality and Brix:acid ratio of 12; although delayed. Fruit size was bigger and peaked between counts 72 and 56/48 (lighter crop), optimal fruit size for export Valencia (medium to large). There was a good crop on the trees, bearing in mind that Swingle induces good yields and internal quality. It is apparent that Gusocora maturity is middle to end of July (Table 5.4.3.3).

#### Jassie

Fruit size at Groep 91 on Carrizo and Swingle was slightly smaller (peaked between count 88 and 56) smaller this season. Production was good on all the rootstock combinations. Internal quality was good with juice levels of 65%, Brix up to 12 (C35) at Groep 91 and average acid levels of 1.3. Seed count increased this year and varied from 2.7 to 4.0 (avg. 3.2) seeds per fruit. Fruit shape was round, with a smooth rind texture, internal colour was light yellow, and juice flavour was good. Fibre strength was fairly soft, rind thickness was medium and the fruit peeled easily. Jassie bore high numbers of fruit inside the tree (good quality and colour). Maturity is the middle of July to the beginning of August in this area (Tables 5.4.3.3. & 5.4.3.4).

#### Kobus Du Toit Late

Kobus Du Toit Late was evaluated at the Bosveld trial site on two rootstocks (C35, CC) and Groep 91 on four rootstock (CC, SC, Sunki 812, X639) and produced medium and large fruit size (count 88 to 56) on the trees, with 3.4 seeds average (higher compared to 2019). The colour development was very similar on all the rootstock combinations this season (T1 to T3/4). The internal quality was good, juice levels above 56%, Brix up to 13 (lower levels at Groep 91 – average 11) and good acids for the later maturing selection. Maturity seems to be middle to end of July to middle August according to Tables 5.4.3.3. & 5.4.3.4.

#### Lavalle 1

The seed production this season decreased and Lavalle still produced no seeds per fruit at Bosveld. The internal quality complied with export requirements and acid level was above 1.0% at the final evaluation in the middle of August. Keep in mind that Lavalle is a late Valencia selection with good shelf life and the optimal harvest time will be in August. The navel end on some fruit seems to develop a button and there was split fruit on some of the trees evaluated, but this varies from season to season (seen mainly in 2013). From the ratio on this date, it is apparent that Lavalle 1 maturity is the end of August to middle of September (Table 5.4.3.3).

#### McClellan and McClellan SL

Both selections were planted and evaluated this season on Carrizo, Swingle, X639 and Sunki 812 at Groep 91 to compare the performance of McClean SL with the old clone Mclean selection. McClean SL was planted on C35, Carrizo and Swingle at the Bosveld trial site with good crop production and remained completely seedless (Bosveld & Groep 91); similar to all the other trial sites where the selection was included. Fruit quality improved at the Groep 91 site due to trees bearing their fourth crop now; the Sunki 812 combination followed by Swingle were the best for 2020 (Carrizo was best 2019). Fruit size peaked from count 88 to 56 (excellent for Valencia production). External colour varied from T1-3/4 at Bosveld and Groep 91; improved from 2019 where SC had a delayed colour. At both sites juice was 57% and above (as high as 64% on Sunki 812 and SC), Brix improved to as high as 13.1 and acids were above 1.0% (peak maturity) towards the end of the season, resulting in good Brix:acid ratios (above 12:1). Maturity seems to be the end of June to the middle of July (Tables 5.4.3.3. & 5.4.3.4).

#### Midnight 1 & 2

Midnight 1 and 2 bore an average to good yield on the two rootstocks Carrizo and Swingle. The fruit size this year was very uniform and ranged between count 64 and 56/48, juice content was around 60% at Groep 91 and 57% at Bosveld, Brix levels lower around 10.5 (peak maturity) and acids around 1.1%. The two Midnight selections performed very similarly this season. Midnight 1 and 2 developed low seed numbers in the fruit, ranging from seedless up to 1.8 seeds per fruit. The characteristic Midnight die-back was more visible on Midnight 1 compared to Midnight 2. Fruit shape was round, rind texture was fairly smooth, and fruit was raggy with a medium rind thickness which peeled moderately. Maturity seems to be the middle of July to the end of July (Table 5.4.3.3. & 5.4.3.4).

#### Skilderkrans

Skilderkrans at Group 91 is the back-up site (was evaluated) and the trial block at Bosveld bore no fruit on C35, Carrizo and Swingle due to the water shortages. Fruit size was smaller this season and more favourable for good quality fruit; varied from medium to large (count 88-56). Internally the Brix content was good (up to 13) and high acid level of 1.4% (CC) to 1.5% (SC) indicated a later maturing Valencia selection. Juice level increased to an average 59% later in the season; above the minimum required export figure. There was a good external colour at Groep 91 (T1-3). The fruit developed a smooth rind, fibre strength was fairly soft and the fruit shape was round. Ratios were lower at Groep 91 this season due to the higher acid levels on both Carrizo and Swingle, delaying peak maturity to end of July and mid-August. Peak maturity by the end of July (Tables 5.4.3.3. & 5.4.3.4).

#### Turkey

Turkey was planted on three rootstocks at both trial sites: Carrizo, Swingle and C35, to determine the compatibility status. All three combinations performed well; yield was the best in combination with Carrizo relative to tree size. Fruit size distribution ranged from medium to large (count 88-56/48), good Brix content with highest levels at Groep 91 (average 11.6), similar acid levels at Bosveld compared to Groep 91 and Brix:acid ratio. The average seed count per fruit decreased to 1.6 and peaked at 3.7 seeds. The external colour (between T1 and T3/4) in the middle of June was similar for all three rootstocks and both trial sites. Yield production and tree size showed Carrizo to be the best rootstock combination for Turkey. C35 developed the smallest tree size (2.5 m) in combination with Turkey. The Swingle trees were declining and die-back was visible. Based on the ratios, maturity will be end of May to middle June (Tables 5.4.3.3. & 5.4.3.4).

#### Valearly

Valearly, bearing an average to good crop, remained low-seeded this season (0.5 seeds per fruit). The internal quality of the fruit was fair to good with juice levels above 50% at Groep 91 (above 50% at Bosveld) early in the season, higher Brix on C35 (up to 12.1) compared to Carrizo (11.9) at Groep 91 and acid of 0.9. Compared with the other early maturing selections (Turkey), Valearly seems to be at least two weeks earlier, with delayed external colour development. Estimated maturity, according to Table 5.4.3.5 seems to be second week to the end of May.

#### Valencia Late (control)

The Valencia Late was included as one of the control selections in this trial at Bosveld Citrus. Yield production on the trees improved this season and fruit size peaked at large (count 64/56), optimal Valencia export quality.

Acid levels were above 1.3% (average) when the evaluation was completed, indicating the late maturity qualities of the selection. The juice content was similar to 2018 & 2019 this season at 56% and Brix 11.6 with the last evaluation. Seed count went up from 1.5 seeds per fruit to 1.8. Maturity will be late in the season and according to Table 5.4.3.5, peak in the middle to end of August.

## Conclusion

Alpha performed average to well compared to the 2019 evaluation, developing a good crop on the trees. The internal quality was good (juice levels lower – 59%) and fruit size peaked bigger between counts 72 to 56 (picked up one count size on smaller side).

Bennie 1 and 2 produced similar fruit quality this season, as well as yield production and medium to large/Extra fruit size (peaked from count 88 to 48 between the two trial sites). Delta was the control cultivar for the trial; fruit size peaked between count 72 and 56 (slightly smaller compared to 2019 due to a better crop) with good internal quality, reaching juice levels of 56%, Brix of 12 and acid content of 1.3%.

Du Roi was evaluated on Swingle this season with a bigger fruit size ranging from count 72 to 56 (up by one count size compared to 2019 season). Kobus du Toit Late performed well with good fruit size and promising juice and Brix levels. Gusocora performed well on Swingle (delayed colour development), meeting the export standards (acid levels improved).

Jassie produced an excellent internal quality (high Brix and acid) on Carrizo and Swingle, with medium to large fruit size (count 88-56) with a good crop load on the trees. Lavalley 1 was ultra-late, peak maturity middle of August on Swingle rootstock; colour peaked from T1 to 4.

McClellan SL remained completely seedless at Bosveld and Groep 91 with good internal quality and optimum fruit size (count 88-56).

Fruit quality on Midnight 1 was better, with higher Brix than Midnight 2 (Bosveld trial site). The external colour was similar on both Midnight selections this season (better compared to 2019); range between T1 and T3.

Turkey performed best in combination with Carrizo when Brix:acid ratio and yield production were considered. Valearly produced a better crop on the trees, better juice levels and improved colour development compared to Turkey this season. Future evaluations will determine the value of this cultivar for the citrus industry.

**Table 5.4.3.3.** Internal fruit quality data for Valencia orange selections at Bosveld Citrus (Letsitele) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Alpha	SC	08/07/2020	74 - 82	72 - 56	58,9	11,8	1,70	6,9	3,0	T1 - 3
Beli	CC	18/06/2020	76 - 85	72 - 56	55,1	10,1	0,75	13,5	0,0	T3 - 5
Beli	CC	08/07/2020	74 - 87	72 - 48	53,7	10,9	0,80	13,6	0,0	T1 - 4
Bennie	SC	08/07/2020	75 - 88	72 - 48	55,1	10,6	1,15	9,2	3,5	T1 - 3
Delta	SC	08/07/2020	75 - 83	72 - 56	56,3	12,1	1,25	9,7	0,0	T1 - 4
Du Roi	SC	08/07/2020	73 - 82	72 - 56	60,6	11,4	1,45	7,9	2,3	T1 - 3
Gusocora	SC	08/07/2020	74 - 88	72 - 48	55,8	11,8	1,30	9,1	0,0	T1 - 4
K du Toit Late	CC	28/05/2020	70 - 79	88 - 64	63,0	11,5	1,10	10,5	0,0	T1 - 4
K du Toit Late	C35	18/06/2020	70 - 83	88 - 56	61,6	12,6	1,45	8,7	1,5	T3 - 5
Lavalley	SC	08/07/2020	80 - 90	64 - 40	58,6	11,3	1,40	8,1	0,0	T1 - 4
McClellan SL	SC	08/07/2020	74 - 87	72 - 48	56,0	10,8	1,15	9,4	0,0	T1 - 3

Midnight 1	SC	08/07/2020	79 - 89	64 - 48	57,2	11,0	1,15	9,6	0,0	T1 - 3
Midnight 2	SC	08/07/2020	79 - 86	64 - 56	56,9	9,1	0,95	9,6	0,0	T1 - 3
Turkey	C35	14/05/2020	68 - 80	88 - 64	51,0	11,3	1,20	9,4	1,3	T3 - 5
Turkey	C35	28/05/2020	77 - 84	72 - 56	48,1	11,5	1,00	11,5	0,0	T1 - 4
Turkey	SC	28/05/2020	72 - 82	88 - 56	61,3	10,9	1,25	8,7	2,8	T1 - 3
Val Early	CC	14/05/2020	77 - 85	72 - 56	56,0	10,0	0,75	13,3	0,0	T2 - 5
Val Early	CC	28/05/2020	75 - 92	72 - 40	58,9	10,1	0,80	12,6	0,0	T1 - 5
Val Late	SC	08/07/2020	79 - 82	64 - 56	55,5	11,6	1,30	8,9	1,8	T1 - 3

**Table 5.4.3.4.** Internal fruit quality data for Valencia orange selections at Groep 91 (Letsitele) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Bennie1	CC	14/05/2020	73 - 79	72 - 64	59,0	10,9	1,35	8,1	0,0	T3 - 5
Bennie1	CC	18/06/2020	75 - 84	72 - 56	54,1	13,3	1,45	9,2	1,0	T1 - 3
Bennie1	CC	08/07/2020	72 - 83	88 - 56	60,2	12,0	1,20	10,0	4,9	T1 - 3
Bennie1	CC	28/07/2020	70 - 83	88 - 56	61,9	11,5	1,05	11,0	3,2	T1 - 3
Bennie1	CC	17/08/2020	76 - 86	72 - 48	64,9	12,7	0,90	14,1	2,8	T1 - 3
Bennie1	SC	18/06/2020	75 - 82	72 - 56	60,8	11,6	1,40	8,3	1,8	T1 - 3
Bennie1	SC	08/07/2020	77 - 90	72 - 36	57,7	12,6	1,35	9,3	5,3	T1 - 3
Bennie1	SC	28/07/2020	75 - 90	72 - 40	64,8	12,7	1,25	10,2	2,0	T1 - 3
Bennie1	SC	17/08/2020	76 - 84	72 - 56	64,4	3,0	0,85	3,5	3,4	T1 - 3
Bennie 2	CC	18/06/2020	76 - 88	72 - 48	56,3	11,3	1,25	9,0	4,0	T1 - 3
Bennie 2	CC	08/07/2020	71 - 87	88 - 48	60,3	11,1	1,05	10,6	2,0	T1 - 3
Bennie 2	CC	28/07/2020	72 - 85	88 - 56	66,0	12,2	1,20	10,2	1,7	T1 - 3
Bennie 2	SC	14/05/2020	77 - 79	72 - 64	53,8	10,9	1,20	9,1	2,5	T5 - 7
Bennie 2	SC	18/06/2020	77 - 81	72 - 64	57,0	12,2	1,10	11,1	4,8	T1 - 3
Bennie 2	SC	08/07/2020	73 - 84	72 - 56	58,4	12,4	1,20	10,3	3,7	T1 - 3
Bennie 2	SC	28/07/2020	75 - 81	72 - 64	59,1	12,7	1,10	11,5	2,5	T1 - 3
Bennie 2	SC	17/08/2020	77 - 87	72 - 48	63,6	12,7	1,30	9,8	3,6	T1 - 3
Du Toit Late	CC	07/07/2020	74 - 79	72 - 64	62,3	11,3	1,10	10,3	3,2	T1 - 3
Du Toit Late	CC	08/07/2020	76 - 82	72 - 56	57,7	11,2	1,20	9,3	2,3	T1 - 3
Du Toit Late	CC	28/07/2020	73 - 84	72 - 56	58,2	11,7	1,05	11,1	2,7	T1 - 3
Du Toit Late	CC	17/08/2020	78 - 83	64 - 56	56,4	11,2	0,95	11,8	4,3	T1 - 3
Du Toit Late	SC	18/06/2020	77 - 79	72 - 64	56,7	11,0	1,05	10,5	1,8	T2 - 4
Du Toit Late	SC	08/07/2020	71 - 80	88 - 64	60,0	11,2	1,05	10,7	2,3	T1 - 3
Du Toit Late	SC	28/07/2020	70 - 80	88 - 64	59,6	11,4	1,10	10,4	2,7	T1 - 3
Du Toit Late	SC	17/08/2020	75 - 85	72 - 56	60,5	11,5	0,90	12,8	4,0	T1 - 3
Du Toit Late	X639	18/06/2020	70 - 73	88 - 72	59,3	11,5	1,35	8,5	4,0	T2 - 4
Du Toit Late	X639	08/07/2020	73 - 84	72 - 56	61,6	11,4	1,15	9,9	5,2	T1 - 4
Du Toit Late	X639	28/07/2020	73 - 79	72 - 64	61,8	11,8	1,10	10,7	2,0	T1 - 3
Du Toit Late	X639	17/08/2020	72 - 83	88 - 56	60,8	11,6	1,00	11,6	3,5	T1 - 3
Du Toit Late	US812	18/06/2020	74 - 83	72 - 56	56,2	11,2	1,35	8,3	5,7	T2 - 4
Du Toit Late	US812	28/07/2020	71 - 76	88 - 72	61,1	1,3	1,10	1,2	3,5	T1 - 3
Du Toit Late	US812	17/08/2020	75 - 85	72 - 56	60,0	12,5	1,05	11,9	3,8	T1 - 3
Jassie	CC	18/06/2020	75 - 85	72 - 56	54,1	11,6	1,20	9,7	4,0	T2 - 4
Jassie	CC	28/07/2020	72 - 81	88 - 64	63,2	12,2	1,10	11,1	2,7	T1 - 3
Jassie	SC	18/06/2020	77 - 82	72 - 56	54,6	11,5	1,35	8,5	2,5	T2 - 4

Jassie	SC	28/07/2020	70 - 84	88 - 56	65,2	12,4	1,30	9,5	3,5	T1 - 3
McClean	CC	14/05/2020	74 - 81	72 - 64	58,8	10,7	1,45	7,4	1,7	T4 - 5
McClean	CC	28/05/2020	74 - 80	72 - 64	60,1	11,0	1,40	7,9	0,0	T3 - 5
McClean	CC	18/06/2020	74 - 87	72 - 48	58,8	10,7	0,85	12,6	1,5	T1 - 4
McClean	CC	08/07/2020	73 - 79	72 - 64	56,1	11,7	1,40	8,4	2,0	T1 - 3
McClean	CC	28/07/2020	76 - 91	72 - 40	65,5	12,3	1,25	9,8	2,7	T1 - 3
McClean	CC	17/08/2020	74 - 86	72 - 48	58,8	12,7	0,95	13,4	3,7	T1 - 3
McClean	SC	14/05/2020	75 - 78	72 - 64	54,0	10,6	1,40	7,6	3,8	T4 - 5
McClean	SC	28/05/2020	75 - 84	72 - 56	57,0	10,7	1,50	7,1	1,7	T3 - 5
McClean	SC	18/06/2020	72 - 82	88 - 56	62,5	10,8	1,60	6,8	4,0	T2 - 4
McClean	SC	08/07/2020	73 - 82	72 - 56	58,6	12,1	1,15	10,5	2,5	T1 - 4
McClean	SC	28/07/2020	72 - 82	88 - 56	58,8	12,3	1,25	9,8	2,8	T1 - 3
McClean	SC	17/08/2020	73 - 83	72 - 56	64,1	13,9	1,15	12,1	2,8	T1 - 3
McClean	X639	14/05/2020	75 - 79	72 - 64	58,6	10,1	1,65	6,1	2,0	T5 - 7
McClean	X639	28/05/2020	77 - 80	72 - 64	58,6	10,5	1,35	7,8	0,0	T2 - 5
McClean	X639	18/06/2020	70 - 79	88 - 64	60,3	11,4	1,30	8,8	0,0	T2 - 4
McClean	X639	08/07/2020	75 - 84	72 - 56	57,6	11,3	1,45	7,8	2,2	T1 - 3
McClean	X639	28/07/2020	74 - 81	72 - 64	59,6	12,7	1,05	12,1	2,0	T1 - 3
McClean	X639	17/08/2020	77 - 87	72 - 48	60,0	11,9	1,10	10,8	2,7	T1 - 3
McClean	US812	14/05/2020	68 - 72	88 - 64	57,0	10,2	1,45	7,0	1,0	T4 - 6
McClean	US812	18/06/2020	72 - 84	88 - 56	56,3	11,0	1,25	8,8	2,3	T2 - 4
McClean	US812	08/07/2020	73 - 78	72 - 64	59,5	11,5	1,30	8,8	2,7	T1 - 3
McClean	US812	28/07/2020	72 - 82	88 - 56	62,2	12,5	1,30	9,6	2,3	T1 - 3
McClean	US812	17/08/2020	74 - 83	72 - 56	55,4	12,7	1,10	11,5	2,8	T1 - 3
McClean SL	CC	18/06/2020	72 - 81	88 - 64	59,4	13,1	1,05	12,5	0,0	T1 - 3
McClean SL	CC	08/07/2020	75 - 82	72 - 56	60,7	11,7	1,05	11,1	0,0	T1 - 3
McClean SL	CC	28/07/2020	74 - 78	72 - 64	61,2	11,7	1,00	11,7	0,0	T1 - 3
McClean SL	CC	17/08/2020	77 - 87	72 - 56	62,8	12,4	0,85	14,6	0,0	T1 - 3
McClean SL	SC	18/06/2020	74 - 79	72 - 64	60,6	10,9	1,10	9,9	0,0	T2 - 4
McClean SL	SC	08/07/2020	76 - 86	72 - 56	60,5	10,9	1,25	8,7	0,0	T1 - 3
McClean SL	SC	28/07/2020	75 - 84	72 - 56	61,4	11,1	1,15	9,7	0,0	T1 - 3
McClean SL	SC	17/08/2020	76 - 87	72 - 56	64,9	11,7	0,95	12,3	0,0	T1 - 3
McClean SL	X636	18/06/2020	72 - 82	88 - 56	58,6	10,6	1,00	10,6	0,0	T2 - 4
McClean SL	X636	08/07/2020	75 - 84	72 - 56	62,3	11,1	1,00	11,1	0,0	T1 - 3
McClean SL	X636	28/07/2020	75 - 84	72 - 56	60,4	11,6	0,90	12,9	0,0	T1 - 4
McClean SL	X636	17/08/2020	75 - 85	72 - 56	62,3	12,4	1,00	12,4	0,0	T1 - 3
McClean SL	US812	18/06/2020	78 - 88	64 - 48	57,4	10,7	1,00	10,7	0,0	T2 - 4
McClean SL	US812	08/07/2020	78 - 83	64 - 56	58,0	10,4	0,90	11,6	0,0	T1 - 3
McClean SL	US812	27/07/2020	70 - 86	88 - 56	57,4	11,9	0,95	12,5	0,0	T1 - 3
McClean SL	US812	17/08/2020	76 - 87	72 - 56	64,0	12,1	0,85	14,2	0,0	T1 - 3
Midnight 1	CC	14/05/2020	72 - 80	88 - 64	60,9	10,8	1,05	10,3	0,3	T3 - 5
Midnight 1	CC	28/05/2020	75 - 86	72 - 56	68,8	10,5	1,20	8,8	0,0	T2 - 4
Midnight 1	CC	18/06/2020	78 - 89	64 - 48	57,1	11,4	1,20	9,5	0,0	T1 - 3
Midnight 1	CC	08/07/2020	76 - 86	72 - 48	58,2	13,1	1,25	10,5	0,0	T1 - 3
Midnight 1	CC	28/07/2020	81 - 83	64 - 56	60,8	12,8	1,05	12,2	0,0	T1 - 3
Midnight 1	SC	14/05/2020	70 - 86	88 - 48	54,5	9,9	1,20	8,3	1,8	T3 - 5
Midnight 1	SC	28/05/2020	79 - 85	64 - 56	60,5	9,9	1,25	7,9	0,0	T2 - 4
Midnight 1	SC	18/06/2020	74 - 89	72 - 48	58,4	11,3	0,95	11,9	0,0	T1 - 3
Midnight 1	SC	08/07/2020	79 - 90	64 - 48	58,6	12,1	1,05	11,5	0,0	T1 - 3
Midnight 1	SC	28/07/2020	77 - 82	72 - 56	60,1	12,3	1,10	11,2	0,0	T1 - 3
Skilderkrans	CC	08/07/2020	75 - 78	72 - 64	54,9	13,1	1,40	9,4	0,0	T1 - 4
Skilderkrans	CC	17/08/2020	70 - 82	88 - 56	64,5	13,0	1,35	9,6	3,0	T1 - 3

Skilderkrans	SC	08/07/2020	72 - 83	88 - 56	56,2	11,3	1,85	6,1	0,0	T1 - 3
Skilderkrans	SC	17/08/2020	77 - 83	72 - 56	61,2	11,3	1,45	7,8	1,2	T1 - 3
Turkey	C35	21/04/2020	69 - 76	88 - 72	59,2	11,8	1,25	9,4	0,0	T3 - 5
Turkey	C35	14/05/2020	72 - 81	72 - 64	60,0	12,1	1,20	10,1	0,8	T2 - 3
Turkey	C35	28/05/2020	72 - 84	72 - 56	60,6	11,7	1,20	9,8	3,7	T1 - 3
Turkey	C35	18/06/2020	70 - 85	88 - 56	61,9	12,9	1,35	9,6	0,5	T1 - 3
Turkey	CC	21/04/2020	72 - 76	88 - 72	58,5	11,5	1,25	9,2	0,0	T3 - 5
Turkey	CC	14/05/2020	72 - 84	88 - 56	62,0	11,9	1,15	10,3	2,5	T2 - 4
Turkey	CC	28/05/2020	74 - 80	72 - 64	60,6	11,5	1,05	11,0	0,0	T1 - 3
Turkey	CC	18/06/2020	69 - 73	88 - 72	60,5	11,7	1,00	11,7	2,4	T1 - 3
Turkey	SC	21/04/2020	72 - 77	88 - 72	57,1	10,9	1,30	8,4	0,0	T3 - 5
Turkey	SC	14/05/2020	75 - 86	72 - 48	51,0	11,5	1,10	10,5	0,0	T3 - 4
Turkey	SC	28/05/2020	75 - 83	72 - 56	63,1	10,8	1,20	9,0	1,0	T1 - 4
Turkey	SC	18/06/2020	73 - 81	72 - 64	58,8	10,7	1,10	9,7	0,0	T1 - 3
Val Early	CC	14/05/2020	71 - 81	88 - 64	54,3	10,7	0,80	13,4	0,8	T3 - 5
Val Early	CC	28/05/2020	77 - 85	72 - 56	51,6	11,3	0,75	15,1	0,0	T1 - 5
Val Early	CC	18/06/2020	74 - 85	72 - 56	49,8	11,0	0,75	14,7	0,0	T1 - 3
Val Early	SC	14/05/2020	71 - 84	88 - 56	51,9	10,7	0,80	13,4	0,0	T3 - 5
Val Early	SC	28/05/2020	73 - 77	72	49,2	10,7	0,85	12,6	0,0	T2 - 4
Val Early	SC	18/06/2020	76 - 85	72 - 56	50,9	11,0	1,35	8,1	0,0	T1 - 3
Val Early	X639	14/05/2020	70 - 83	88 - 56	53,2	11,0	0,75	14,7	1,2	T2 - 5
Val Early	X639	28/05/2020	80 - 85	64 - 56	60,6	10,3	1,00	10,3	2,0	T2 - 4
Val Early	X639	18/06/2020	75 - 77	72 -	61,2	11,3	0,75	15,1	0,5	T1 - 3
Val Early	US812	14/05/2020	74 - 79	72 - 64	57,4	10,3	0,75	13,7	0,0	T3 - 5
Val Early	US812	28/05/2020	74 - 80	72 - 64	57,0	11,3	0,80	14,1	0,7	T2 - 4
Val Early	US812	18/06/2020	74 - 84	72 - 56	42,7	11,8	0,70	16,9	0,8	T1 - 3

**5.4.4 PROGRESS REPORT: Evaluation of Mandarin hybrid selections in the hot inland areas (Letsitele and Hoedspruit)**  
Project 75C by J. Joubert (CRI)

**Summary**

RHM, Etna, Sirio and Tango mature first according to the results of the 2020 season for the warm production areas, and Tango developed the smallest fruit size and good internal quality. Samba and Leanri with high juice levels, fit in before Furr that followed with the highest seed count per fruit for this trial. Next will be Orah, also developing fairly high seed counts per fruit. The mid-maturing mandarins are represented by Mor 26, which developed high Brix levels compared to the other selections (up to Brix of 13). Tambor, followed by Tanor Late were the last selections to mature at these trial sites, ending the Mandarin Hybrid season. Picking periods should not be longer than 3 - 4 weeks to maintain good internal quality and avoid rind disorders.

**Opsomming**

RHM, Etna, Sirio en Tango word die vroegste ryp volgens resultate van die 2020 seisoen vir hierdie warm produksie area, met Tango die kleinste vruggrootte en goeie interne kwaliteit. Samba en Leanri, met hoë sap vlakke, pas in voor Furr, wat daarna volg met die hoogste saadtelling per vrug vir hierdie proef. Volgende is Orah, met redelike hoë saad tellings per vrug. Die middel van die mandaryne word verteenwoordig deur Mor 26 met die hoë Brix vlakke in vergelyking met die ander seleksies (tot Brix van 13). Tambor, gevolg deur Tanor Late was die laaste seleksie om ryp te word op hierdie proef persele, wat ook die Mandaryn Hibried seisoen afsluit vir hierdie proef. Daar word aanbeveel om nie die oesperiode langer as 3 tot 4 weke te verleng nie om goeie interne kwaliteit te verseker met minimum skil probleme.

**Objectives**

- To select Mandarin Hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new Mandarin Hybrid cultivars and to determine the climatic suitability of these cultivars in hot production regions.

### Materials and methods

Field evaluations and laboratory analyses were conducted on Mandarin Hybrid selections from Bosveld Citrus (Letsitele), Overbrug (Hoedspruit), Mahela Citrus (Letsitele) and Moriah Citrus (Hoedspruit) from the Limpopo region.

The following cultivars were evaluated:

**Table 5.4.4.1.** List of Mandarin Hybrid selections evaluated at Bosveld Citrus (Letsitele) during the 2020 season.

Selection	Rootstock	Topwork
Saint Andre	CC	2015
Samba	CC	2015

**Table 5.4.4.2.** List of Mandarin Hybrid selections evaluated at Mahela (Letsitele) during the 2020 season.

Selection	Rootstock	Planted
Etna	CC	2014
Furr	CC	2014
Saint André	CC	2014
Samba	CC	2014
Sirio	CC	2014
Tango	CC	2013
Tanor Late	CC	2014
Tasty 1	CC	2014

**Table 5.4.4.3.** List of Mandarin Hybrid selections evaluated at Moriah (Hoedspruit) during the 2020 season.

Selection	Rootstock	Topwork
ARCCIT 9 LS	C35	2015
Furr (Clemcott)	MxT	2011
IRM 1	C35	2015
Leanri	C35	2015
Mor 26	MxT	2011
Nova SL ARC	C35	2014
Orah	MxT	2011
Orri	C35	2015
RHM	C35	2015
Saint Andre	C35	2014
Samba	C35	2014
Tambor	MxT	2011
Tanor Late	C35	2015

**Table 5.4.4.4.** List of Mandarin Hybrid selections evaluated at Overbrug (Hoedspruit) during the 2020 season.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
ARCCIT 9 LS	CC	2011
Ma'ayana (Dina)	CC	2011
IRM 2	CC	2011

## **Results and discussion**

For Mandarin Hybrid selections, a ratio of 11:1 is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over-mature. This process from the start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a higher instance of quality and rind issues.

### Etna

Etna bore a good crop with large to extra-large fruit (count 1X to 1XX) and good internal quality for a hot production area, but low acids. External colour was delayed from the start (varied from T2 to 4). The fruit was completely seedless this season, even with cross-pollination in the trial block (lower than in 2019). Maturity seems to be the middle of April for the hot production areas, according to the information in Table 5.4.4.5.

### Furr (Clemcott)

Furr developed large to extra-large fruit size (count 1XX – 1XXX) on the trees at Mahela and Moriah Estate, one of the characteristics of the cultivar, as well as an excellent crop on the trees. The external colour development on the fruit was good for the Hoedspruit area (T1-3), and delayed in the Letsitele area (T3-5). Internally the fruit quality was very good, developing high juice (up to 63%) and Brix (up to 13) levels with average to good acids (better at Bosveld). Another quality of the fruit is the high seed count (high self-and cross pollination). Maturity seems to be middle to the end of May to the middle of June for the hot production areas, according to the information in Table 5.4.4.5.

### Mor 26

Mor 26 produced a light (Moriah) crop on the trees for the 2020 season. The fruit size increased due to the light crop this season compared to 2019 and peaked between count 1XX and 1XXX, large to extra-large fruit. The external colour development was yellow and peaked at T1-3. The internal quality was good with high juice levels of up to 60%, Brix up to 13 and acceptable acid levels (1.0% - peak maturity). There were on average no seed in the fruit. Based on the internal quality results in Table 5.4.4.5, estimated maturity will be the end of May to the beginning of June.

### ARCCIT 9 LS

The crop was average to good this season to evaluate (management improvement). The fruit shape was very similar to the Nadorcott selection. Rind texture was very smooth with a natural shine (similar to packhouse waxing). The fruit size was bigger this season, ranging from count 1 and peaked at count 1XX/XXX even with the crop load on the trees. ARCCIT 9 LS produced low seed numbers in the fruit (up to 1.4 seeds per fruit) this season. Maturity seems to be two weeks earlier on the ARCCIT 9 LS selection, according to Table 5.4.4.5, but the information was limited due to only two evaluations (beginning to middle of June).

### Orah

Orah, producing a good crop with medium to large/extra-large (avg. count 2-1XX) fruit size. The average seed count in the fruit went up from 2019 to 13.1 seeds per fruit, one of the selection characteristics (high seed numbers). Internal quality was good, the Brix levels were above 11.0 by time of harvest, good juice levels (above 57%) and acceptable acids. Early external colour development ranged from T1 to T3 (only two evaluations). Based on the internal quality results in Table 5.4.4.5, estimated maturity will be end of May (degreening) to the middle of June.

### Samba

Samba on Carrizo rootstock produced a very good crop with good internal quality on the large fast growing thornless trees at Mahela and Bosveld (Letsitele). The trees at Moriah planted on C35 bore their third crop this season, producing large fruit size due to the young tree age (from count 1 up to count 1 XX). Colour development was early in the season (deep orange) with a smooth rind texture on the fruit. Fruit were completely seedless this season in the combined trial blocks, except one evaluation at Moriah with 0.8 seeds per fruit (future evaluations will confirm low seed numbers) and peaked from medium to large/extra-large fruit size at Bosveld and Mahela (average count 2 to 1XX/1XXX). Internal quality was good with high juice and Brix levels, and lower acids (average 0.8%). Based on the internal quality results in Table 5.4.4.5, the estimated maturity will be middle to the end of April.

#### Tambor

Tambor is an addition to the late maturing mandarin selections for the hot production areas, producing seedless fruit this season (seedless in 2019 too), fairly low compared to the Furr and Orah selections. The external colour was on the yellow side at peak maturity, but with good internal quality, developing juice levels above 61%, Brix above 11 and acids above 1.0 at the last evaluation. Fruit size peaked from count 1XX to count 1XXX, very large for mandarin cultivars. Based on the internal quality results in Table 5.4.4.5, estimated maturity will be the end of July to the middle of August.

#### Tango

There was a good crop on the trees at all the trial sites this season compared to the average last year. Tango was completely seedless at all sites (in 2016 there were 0.2 seeds at Mahela). The fruit shape was similar to the Nadorcott selection. Rind texture was very smooth with a natural shine (similar to packhouse waxing). The Tango trees were thornless with an upright growth pattern and tree shape. The fruit was firm and the rind thin, fibre was soft and peeled very easily. Internally the fruit was high in juice content (above 60%), Brix levels were higher (average 10.6) and peaked at 11.5 for the Moriah site, acid levels were on the lower side early in the season (indicating a shorter shelf life), and deep orange coloured fibre. Fruit size peaked at count 2 to 1XX (medium to large). Based on the internal quality results in Table 5.4.4.5, estimated maturity will be end of April to the middle of May with delayed external colour development.

#### Additional selections

The internal quality of Tasty (Bruce) remained below average this season with low juice levels (46.2%), higher Brix (above 11) and acids were low (0.70%), questioning the potential of the cultivar in the hot production areas. The fruit size peaked from count 1XX to count 1XXX with low seed numbers (3.0 seeds/fruit).

Sirio produced large to extra-large fruit (count 1XX to 1XXX) on the trees due to a fair crop (large fruit size in hot areas and young trees) with average internal quality (low juice levels) in the hot production areas. The fruit was seedless with three evaluations completed.

Ma'yana (Dina) bore fruit on the Overbrug (Hoedspruit) trial trees this year. The tree shape is very upright with a dark bark colour. Fruit size varied from count 1 to 1XX/XXX (large fruit size similar to 2019), and seedless fruit in the crosspollination environment. The internal quality was good on the young trees; juice was above 56%, Brix levels average 11.7 and better acid levels (remained on the lower but stable side).

Leanri cropped the second time this season at Moriah, the fruit size varied from large to extra-large (1X-1XXX) with good internal quality; high juice levels and good flavoured fruit, good Brix (average 12.9) and fairly low acids (average 0.8%). Seed numbers added up to 1.6 seeds per fruit; not completely seedless.

One of the new ultra-late selections to include at the Bosveld trial site will be Sugar Belle, bearing no fruit this season due to the water problems in the area.

Nova SL was included as a control for Saint André in the trial, the fruit is fairly difficult to peel and low numbers of seed were discovered in the fruit with the last evaluation. External colour was late and the fruit size varied between count 1X and count 1XX/XXX (large to extra-large fruit). Nova SL (ARC) produced a coarse rind texture on the fruit with large fruit size (count 1 to count 1XX). The acid levels in the fruit were similar compared to Saint André (slightly lower) and the external colour development better (T1 to 3).

Tanor Late cropped fruit at Mahela in Letsitele and Moriah in Hoedspruit; the peak maturity was by the middle of July (late maturing selection). Fruit size was extra-large (count 1XX to 1XXX) and completely seedless.

IRM 1, ARCCIT 9 LS, Orri and RHM bore their second crop at Moriah Estate this season. IRM 1, ARCCIT 9 LS and Orri cropped large to extra-large fruit on the trees with acceptable internal quality and low seed numbers in the crosspollination trial block. RHM performed well on C35 and developed good internal qualities with lower acid levels. The fruit size varied from count 1 to 1XXX.

## Conclusion

There was an improvement in the external colour delay in the hot areas that was a problem in the past; future evaluations will clarify the situation. Degreening may be an option for Nadorcott. Leanri, Orri, IRM 1, Furr, Tambor and Orah (fruit colour development was yellow with degreening), but ethylene reacted slowly with Tango (W. Murcott selection). In the hot areas, it will be crucial to cover the mandarin orchards with shade net, to minimise sunburn and improve the packout percentage of the fruit.

Furr and Mor 26 had the larger fruit size, followed by Tambor, Samba and then Orah. The smaller fruit size was produced on Tango. Furr and Orah developed the highest number of seeds, followed by RHM, IRM 1 and Saint Andre.

Ma'yana (Dina), Tanor Late and Leanri were evaluated for the third time; and Etna, Sirio and Tasty 1 for the fourth time this season; future evaluations will continue to determine suitability for this production area.

**Table 5.4.4.5.** Internal fruit quality data for Mandarin hybrid selections at Bosveld (Letsitele), Mahela (Letsitele), Moriah (Hoedspruit) and Overbrug (Hoedspruit) during the 2020 season.

<b>Bosveld</b>										
<b>Cultivar</b>	<b>Root-stock</b>	<b>Date harvested</b>	<b>Fruit size (mm)</b>	<b>Count</b>	<b>Juice (%)</b>	<b>Brix °</b>	<b>Acid (%)</b>	<b>Ratio</b>	<b>Avg seed</b>	<b>Fruit external colour</b>
Saint Andre	CC	17/03/2020	68 - 75	1X - 1XX	56,0	10,4	0,80	13,0	0,0	T4 - 6
Samba	CC	17/03/2020	60 - 70	2 - 1X	59,5	11,3	0,95	11,9	0,0	T3 - 5
<b>Mahela</b>										
<b>Cultivar</b>	<b>Root-stock</b>	<b>Date harvested</b>	<b>Fruit size (mm)</b>	<b>Count</b>	<b>Juice (%)</b>	<b>Brix °</b>	<b>Acid (%)</b>	<b>Ratio</b>	<b>Avg seed</b>	<b>Fruit external colour</b>
Etna	CC	18/03/2020	69 - 74	1X - 1XX	35,6	9,6	0,65	14,8	0,0	T4 - 6
Etna	CC	21/04/2020	70 - 78	1X - 1XXX	95,4	9,8	0,60	16,3	0,0	T2 - 4
Etna	CC	14/05/2020	75 - 90	1XX - 1XXX	52,2	9,2	0,50	18,4	0,0	T1 - 3
Furr	CC	21/04/2020	72 - 82	1XX - 1XXX	59,5	11,4	1,05	10,9	4,1	T3 - 5
Tanor Late	CC	14/05/2020	80 - 90	1XXX	54,9	10,7	1,25	8,6	0,0	T2 - 4
Tanor Late	CC	28/05/2020	63 - 78	2 - 1XXX	56,6	11,2	1,60	7,0	0,0	T1 - 3
Tanor Late	CC	08/07/2020	73 - 86	1XX - 1XXX	54,8	13,5	1,65	8,2	0,0	T1 - 3

Saint Andre	CC	18/03/2020	73 - 84	1XX - 1XXX	37,0	10,2	0,75	13,6	15,3	T5 - 7
Saint Andre	CC	21/04/2020	72 - 80	1XX - 1XXX	54,8	12,6	0,70	18,0	0,9	T1 - 3
Samba	CC	18/03/2020	68 - 75	1X - 1XX	60,6	9,6	0,80	12,0	0,0	T3 - 5
Samba	CC	14/05/2020	69 - 83	1X - 1XXX	62,4	12,0	0,70	17,1	0,0	T1 - 3
Sirio	CC	21/04/2020	72 - 83	1XX - 1XXX	52,2	11,8	0,85	13,9	0,0	T1 - 3
Sirio	CC	14/05/2020	80 - 94	1XXX	81,4	10,1	0,65	15,5	0,0	T1 - 4
Tango	CC	21/04/2020	69 - 73	1X - 1XX	61,6	9,0	0,85	10,6	0,0	T4 - 6
Tango	CC	14/05/2020	65 - 70	1 - 1XX	59,6	10,1	0,70	14,4	0,0	T2 - 3
Tango	CC	17/06/2020	68 - 74	1X - 1XX	60,2	10,6	0,60	17,7	0,0	T1 - 3
Tasty	CC	14/05/2020	72 - 86	1XX - 1XXX	44,8	11,4	0,75	15,2	6,0	T2 - 4
Tasty	CC	28/05/2020	76 - 89	1XX - 1XXX	47,6	10,9	0,55	19,8	0,0	T1 - 3
<b>Moriah</b>										
Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
ARCCIT 9 LS	C35	12/03/2020	64 - 70	1 - 1X	64,2	9,4	1,00	9,4	1,3	T6 - 7
ARCCIT 9 LS	C35	22/04/2020	65 - 77	1 - 1XX	47,2	9,8	1,00	9,8	1,4	T3 - 5
ARCCIT 9 LS	C35	15/05/2020	67 - 81	1 - 1XXX	66,7	10,6	0,55	19,3	0,8	T1 - 3
ARCCIT 9 LS	C35	29/05/2020	74 - 83	1XX - 1XXX	59,6	9,9	0,60	16,5	1,3	T2 - 5
Furr	MxT/C35	14/05/2020	70 - 82	1XX - 1XXX	63,2	12,6	0,65	19,4	6,7	T1 - 3
IRM1	C35	29/05/2020	74 - 90	1XX - 1XXX	62,9	11,3	0,85	13,3	0,0	T1 - 3
Leanri	C36	12/03/2020	69 - 77	1X - 1XX	63,6	11,1	0,85	13,1	1,1	T4 - 6
Leanri	C35	22/04/2020	79 - 86	1XXX	57,9	12,3	0,60	20,5	0,0	T1 - 3
Leanri	C35	14/05/2020	75 - 85	1XX - 1XXX	64,0	13,2	0,65	20,3	0,0	T1 - 2
Leanri	C35	20/06/2020	69 - 79	1X - 1XXX	62,8	15,1	1,00	15,1	0,5	T1 - 3
Mor 26	MxT/C35	15/05/2020	85 - 100	1XXX	60,1	11,1	0,75	14,8	0,0	T1 - 3
Mor 26	MxT/C35	29/06/2020	74 - 83	1XX - 1XXX	57,5	12,6	1,00	12,6	0,0	T1 - 3
Nova ARC	C35	12/03/2020	65 - 69	1 - 1X	62,3	11,6	0,75	15,5	0,0	T3 - 5

Nova ARC	C35	22/04/2020	65 73	-	1 - 1XX	54,6	11,9	0,70	17,0	0,0	T1 - 3
Orri	C35	12/03/2020	63 70	-	2 - 1X	56,9	10,6	1,40	7,6	1,7	T5 - 7
Orri	C35	14/05/2020	72 82	-	1XX 1XXX	62,7	11,7	0,80	14,6	0,0	T1 - 2
Orah	C35	12/03/2020	63 70	-	2 - 1X	56,3	10,9	1,30	8,4	12,0	T5 - 7
Orah	C35	15/05/2020	62 73	-	2 - 1XX	57,8	11,5	0,65	17,7	4,2	T1 - 3
RHM	C35	22/04/2020	72 80	-	1XX 1XXX	59,4	11,5	0,50	23,0	2,8	T1 - 3
RHM	C35	20/06/2020	67 74	-	1 - 1XX	60,2	13,2	0,75	17,6	0,0	T1 - 3
Saint Andre	C35	12/03/2020	68 74	-	1X - 1XX	63,2	11,1	0,75	14,8	3,1	T4 - 6
Saint Andre	C35	22/04/2020	71 80	-	1X - 1XXX	53,5	11,9	0,60	19,8	0,0	T1 - 3
Samba	C35	12/03/2020	65 69	-	1 - 1X	64,0	11,1	0,70	15,9	0,8	T3 - 5
Samba	C35	22/04/2020	67 73	-	1 - 1XX	57,6	12,6	0,55	22,9	0,0	T1 - 3
Tambor	C35	29/05/2020	76 85	-	1XX 1XXX	61,3	11,2	1,15	9,7	0,0	T1 - 4
Tango	C35	22/04/2020	67 78	-	1 - 1XXX	62,8	11,1	1,00	11,1	0,0	T3 - 5
Tango	C35	15/05/2020	62 75	-	2 - 1XX	60,5	11,5	0,70	16,4	0,0	T1 - 3
Tango	C35	29/05/2020	63 70	-	2 - 1X	60,0	11,3	0,75	15,1	0,0	T2 - 4
Tanor Late	C35	29/05/2020	81 100	-	1XXX	48,6	10,7	1,25	8,6	0,0	T1 - 4
<b>Overbrug</b>											
Cultivar	Root-stock	Date harvested	Fruit size (mm)		Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Ma'ayana	CC	29/05/2020	69 79	-	1X - 1XXX	57,7	10,0	0,90	11,1	0,0	T1 - 3
Ma'ayana	CC	19/06/2020	69 92	-	1 - 1XXX	56,8	13,4	1,00	13,4	0,0	T1 - 3
IRM 2	CC	29/05/2020	70 78	-	1X - 1XXX	61,2	11,8	0,55	21,5	0,0	T1 - 3
IRM 2	CC	19/06/2020	73 83	-	1XX 1XXX	62,3	13,4	1,10	12,2	1,3	T1 - 3
ARCCIT 9 LS	CC	29/05/2020	68 80	-	1X - 1XXX	64,3	12,6	0,95	13,3	0,0	T1 - 3
ARCCIT 9 LS	CC	19/06/2020	60 78	-	2 - 1XXX	55,3	12,5	0,80	15,6	0,0	T1 - 3

#### 5.4.5 PROGRESS REPORT: Evaluation of Valencia selections in the hot, dry production areas (Tshipise)

Project 899A by J. Joubert (CRI)

#### Summary

This was the fifth season for the Alicedale site due to fruit numbers on the trees, and meaningful data was collected. Turkey will start the season as the earliest maturing Valencia with a colour delay on the over mature fruit. Delta will be next in line, followed by Alpha, Skilderkrans and McClean SL with improved colour and completely seedless fruit. Kobus du Toit Late follows as part of the mid-maturing Valencia section. Gusocora with seedless fruit, as well as Henrietta and Bennie will be next, followed by Louisa, and Jassie, towards the late Valencia section, with excellent internal quality and optimal colour development (120 kg per tree). Lavalle will end of the Valencia season in the warm dry production areas.

### Opsomming

Hierdie was die vyfde seisoen vir Alicedale as gevolg van voldoende vrugte aan die bome, betekenisvolle data kon versamel word. Turkey begin die seisoen as die vroegste Valencia met 'n vertraagde vrugkleur op oorryp vrugte. Delta sal volgende in lyn wees, gevolg deur Alpha, Skilderkrans en McClean SL met later vrugkleur en totaal saadlose vrugte. Kobus du Toit Laat volg as deel van die mid-rypwordende Valencia gedeelte. Gusocora met saadlose vrugte, asook Henrietta en Bennie is volgende, gevolg deur Louisa en Jassie, nader aan die laat Valencia periode met uitstekende interne kwaliteit en optimum kleur ontwikkeling (120 kg per boom). Lavalle sal die Valencia seisoen afsluit in die warm droë produksie areas.

### Objective

- To find suitable Valencia selections with superior characteristics for the hot dry inland citrus production areas.

### Materials and methods

Field evaluations and laboratory analyses were conducted on the list below at Alicedale (Tshipise).

**Table 5.4.5.1.** List of Valencia selections evaluated at Alicedale (Tshipise) during 2020.

Selection	Rootstock	Planted
Alpha	C35/Sunki 812/RL/X639	2013
Bennie 2	C35/Sunki 812/RL/X639	2013
Delta	C35/Sunki 812/RL/X639	2013
Gusocora	C35/Sunki 812/RL/X639	2013
Henrietta	C35/Sunki 812/RL/X639	2013
Jassie	C35/Sunki 812/RL/X639	2013
Kobus du Toit Late	C35/Sunki 812/RL/X639	2013
Lavalle	C35/Sunki 812/RL/X639	2013
Louisa	C35/Sunki 812/RL/X639	2013
McClean SL	C35/Sunki 812/RL/X639	2013
Skilderkrans	C35/Sunki 812/RL/X639	2013
Turkey	C35/Sunki 812/RL/X639	2013

### Results and discussion

The Alicedale trial site at Tshipise bore fruit on all the cultivars on different rootstocks and evaluations were done accordingly. There was a good fruit set on the trees for 2020 (determine yield production) and all cultivar combinations will be evaluated in the next season.

#### Alpha

The fruit was completely seedless on all four rootstock combinations with medium to large fruit size (count 72 to 48). X639 matured first, Brix:acid ratio above 9.0 and high juice content (above 55%). It is apparent that Alpha's maturity is middle to end of July (Table 5.4.5.3).

#### Bennie

There was a good crop on Bennie and the fruit size peaked between count 72 and 48/40 (very good for Valencia production). The internal colour of the fruit was deep yellow, fruit shape round, rind texture fairly smooth, medium rag content and medium rind thickness. Bennie produced good juice levels (average 62%), Brix (average 10.6) and acid (1.2%) and fairly low seed counts (average 2.3 seeds per fruit). External colour by the time of harvest varied between T1 and T3. Based on ratios, maturity end of June to the beginning of July (Table 5.4.5.3).

#### Delta

The Delta (control) trees were planted at Alicedale on four rootstocks. Fruit size distribution was uniform and ranged from count 88/72 to 48, medium to large fruit and optimum Valencia requirements. Internal quality was good with high juice (up to 62%), higher Brix (11) and good acid levels through the season (average 1.0%). Based on the internal quality results in Table 5.4.5.3, the estimated maturity will be mid-June to mid-July.

#### Gusocora

Gusocora was evaluated at Alicedale this year on C35, RL, Sunki 812 and X639 rootstocks. The fruit was completely seedless and developed a good internal quality where juice (up to 64%), Brix (up to 11.3) and acid above 0.85 complied with export requirements. The external colour (improved) varied from T1 to T3, correlating with the internal quality and Brix:acid ratio (10:1 for maturity). Fruit size was bigger and peaked between counts 72 and 48, optimal fruit size for export Valencia (medium to large). There was a good crop on the trees. It is apparent that Gusocora maturity is middle to end of July (Table 5.4.5.3).

#### Henrietta

Henrietta was evaluated on all four rootstock combinations at Alicedale, Tshipise this season. Juice levels peaked above 59% average with higher Brix (up to 11.1) and acids 1.30 (Sunki 812). The external colour development improved and peaked between T1 and T4 for the season. Average seeds per fruit increased to 0.9 seeds per fruit (0.5 seeds for 2019). Based on the internal quality results in Table 5.4.5.3, estimated maturity will be mid-July to mid-August.

#### Jassie

Jassie seems to be one of the most promising new Valencia selections being tested and evaluated in the different citrus production and climatic areas. Fruit size distribution was excellent even with the high yield on the trees; the counts were from 88 to 56. Fruit quality was good with high juice (average 59%), Brix of up to 12.3 (X639) and fairly high acid levels (above 0.9%) at the final evaluation, indicating the late characteristics of the cultivar. The seed counts varied from 1.0 up to 5.3 seeds per fruit (average 2.2 seeds per fruit - lower). Based on the internal quality results in Table 5.4.5.3, the estimated maturity will be mid-July to mid-August.

#### Kobus du Toit Late

There was an external colour delay that improved on the fruit during the season, ranging from T1 to T3/4 up until the last evaluation. Fruit average size varied from medium to large, count 88 to 56/48. Internal quality was good depending on the age of the trees and the rootstock combinations. Kobus du Toit Late performed the best on X639 and Sunki 812 at Alicedale, Tshipise. Seed production was slightly higher this season for a seeded selection (average 2.8 seeds per fruit). Acid levels were above 0.90% the entire season. Maturity, based on the internal quality results in Table 5.4.5.3, is estimated to be the end of June to the middle of July for these hot production areas.

#### Lavalle

Lavalle was evaluated on all four rootstocks this season. There was one combination with seed production in the fruit (on Sunki 812) this season and Lavalle produced completely seedless fruit for the rest of the other evaluations. The internal quality complied with export requirements and acid level was above 1.1% at the final evaluation at the middle of July (Alicedale harvested fruit to determine final crop). Keep in mind that Lavalle is

a late Valencia selection with good shelf life and the optimal harvest time will be in August. The navel end on some fruit seems to develop a button and there were split fruit on some of the trees evaluated, but this varies from season to season. From the ratio on this date it is apparent that Lavelle's maturity is end of August to middle of September (Table 5.4.5.3).

#### Louisa

There was a good crop on all four rootstock combinations at Alicedale with RL cropping 140 kg per tree. The fruit was completely seedless and internal quality was average to good with improved juice (63%) and good Brix levels (up to 11). The fruit colour was fairly yellow by the time of peak maturity between T1 and T3/4. Fruit size peaked from medium to large, count 88/72 to count 48/40. Based on the internal quality results in Table 5.4.5.3, estimated maturity will be middle to the end of July.

#### McClellan SL

Compared to all the other Valencia trial sites, McClellan SL remained completely seedless. This year all the combinations with McClellan SL were bearing fruit (110 to 120 kg per tree average) at Alicedale to evaluate, indicating the potential for the future (crop improvement due to Gibb spray). The fruit size peaked between count 88 and 48 (medium to large/very large) with good internal quality (juice up to 63%, Brix 13, acid 0.9). Maturity (Table 5.4.5.3) is estimated to be the end of June to middle of July.

#### Skilderkrans

Skilderkrans was evaluated at Alicedale in the hot production areas. Fruit size varied from medium to large/extra-large (count 88-48/40). Internally the Brix content was good (up to 11.4) and the acid level of 0.90 to 1.5% (peak maturity) indicated a later maturing Valencia selection. Juice level increased to an average 59% later in the season; above the minimum required export figure. There was an improvement in external colour at Alicedale on all four of the rootstocks evaluated, including on RL (T1-4). The fruit developed a smooth rind, fibre strength was fairly soft and the fruit shape was round. Ratios were lower this season due to the higher acid levels, delaying peak maturity to the end of July and mid-August on Swingle and C35 (Table 5.4.5.3).

### **Conclusions**

Bennie matures well on the trees and reduces rind pitting problems. The recommendation will be to harvest the fruit from middle July onwards (stronger rind). Gusocora seems to have delayed colour development at peak maturity and degreening might be an option on swingle rootstock and heavier soil types.

Skilderkrans developed high numbers of Chimeras on the fruit this season, questioning the selections' stability.

All the selections evaluated developed seeds in their fruit, except for Alpha, Delta, Gusocora, Louisa, Lavelle, McClellan seedless and Skilderkrans. All the selections comply with the minimum export standards. The ideal fruit size distribution for Valencia exports was achieved and peaked from count 88 to count 56 (excellent).

**Table 5.4.5.3.** Internal fruit quality data for Valencia orange selections at Alicedale (Tshipise) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Alpha	C35	04/06/2020	76 - 84	72 - 56	60,2	9,8	1,35	7,3	0,0	T3 - 5
Alpha	C35	07/07/2020	75 - 85	72 - 48	56,9	10,8	1,20	9,0	0,0	T1 - 3
Alpha	X639	27/05/2020	78 - 85	64 - 56	55,4	9,4	1,15	8,2	0,0	T3 - 5
Alpha	X639	17/06/2020	75 - 87	72 - 48	54,2	9,8	1,10	8,9	0,0	T2 - 5
Alpha	X639	07/07/2020	79 - 89	64 - 48	59,4	10,0	1,05	9,5	0,0	T1 - 3
Alpha	X639	27/07/2020	79 - 88	64 - 48	57,7	10,4	1,15	9,0	0,0	T1 - 3
Alpha	RL	27/05/2020	79 - 85	64 - 48	54,7	9,9	1,45	6,8	0,0	T3 - 5

Alpha	RL	17/06/2020	75 - 86	72 - 48	58,2	10,8	1,35	8,0	0,0	T2 - 4
Alpha	RL	07/07/2020	79 - 85	64 - 56	61,0	10,4	1,15	9,0	0,0	T1 - 3
Alpha	RL	27/07/2020	75 - 89	72 - 48	60,0	11,4	1,15	9,9	0,0	T1 - 3
Alpha	Sunki 812	17/06/2020	74 - 87	72 - 48	61,0	10,3	1,40	7,4	0,0	T2 - 5
Alpha	Sunki 812	07/07/2020	78 - 89	64 - 48	63,1	10,7	1,40	7,6	0,0	T1 - 3
Alpha	Sunki 812	27/07/2020	82 - 86	56 - 48	62,5	11,4	1,25	9,1	0,0	T1 - 3
Bennie 2	C35	13/05/2020	80 - 85	64 - 56	61,7	10,2	1,25	8,2	2,3	T2 - 5
Bennie 2	C35	27/05/2020	82 - 86	56 - 48	63,1	11,2	1,45	7,7	3,7	T2 - 4
Bennie 2	C35	17/06/2020	75 - 95	72 - 36	55,6	11,2	1,05	10,7	0,8	T1 - 3
Bennie 2	C35	07/07/2020	79 - 91	64 - 40	60,7	10,9	1,25	8,7	1,5	T1 - 3
Bennie 2	C35	27/07/2020	80 - 89	64 - 48	63,2	11,3	1,05	10,8	3,5	T1 - 3
Bennie 2	X639	13/05/2020	76 - 83	72 - 56	59,7	9,6	1,35	7,1	5,5	T2 - 4
Bennie 2	X639	17/06/2020	79 - 85	64 - 48	60,7	10,5	1,35	7,8	3,0	T1 - 3
Bennie 2	X639	07/07/2020	83 - 88	56 - 48	61,9	10,3	1,30	7,9	1,3	T1 - 3
Bennie 2	X639	27/07/2020	82 - 91	56 - 40	63,5	10,7	1,05	10,2	1,7	T1 - 3
Bennie 2	RL	13/05/2020	80 - 87	64 - 48	58,2	9,3	1,30	7,2	2,3	T3 - 5
Bennie 2	RL	17/06/2020	76 - 91	72 - 40	60,2	9,4	1,10	8,5	1,5	T1 - 3
Bennie 2	RL	07/07/2020	78 - 82	64 - 56	67,1	10,3	1,10	9,4	2,8	T1 - 3
Bennie 2	RL	27/07/2020	72 - 85	88 - 56	63,0	10,4	1,00	10,4	1,8	T1 - 3
Bennie 2	Sunki 812	27/05/2020	76 - 82	72 - 56	59,7	10,6	1,35	7,9	0,0	T2 - 4
Bennie 2	Sunki 812	17/06/2020	76 - 83	72 - 56	65,4	10,8	1,30	8,3	2,8	T1 - 3
Bennie 2	Sunki 812	07/07/2020	80 - 90	64 - 40	64,3	11,2	1,30	8,6	3,0	T1 - 3
Bennie 2	Sunki 812	27/07/2020	77 - 86	72 - 48	68,8	13,1	1,05	12,5	2,0	T1 - 3
Delta	C35	17/06/2020	80 - 82	64 - 56	53,5	10,2	0,90	11,3	0,0	T3 - 5
Delta	C35	07/07/2020	74 - 84	72 - 56	58,1	9,8	1,00	9,8	0,0	T1 - 4
Delta	C35	27/07/2020	77 - 87	72 - 48	58,8	10,5	1,00	10,5	0,0	T1 - 3
Delta	X639	17/06/2020	76 - 83	72 - 56	58,6	9,3	1,00	9,3	0,0	T2 - 5
Delta	X639	07/07/2020	76 - 84	72 - 56	59,3	10,0	1,00	10,0	0,0	T1 - 3
Delta	X639	27/07/2020	76 - 81	72 - 64	61,9	10,3	0,90	11,4	0,0	T1 - 3
Delta	RL	27/05/2020	81 - 89	64 - 48	62,1	9,9	1,30	7,6	0,0	T2 - 4
Delta	RL	17/06/2020	72 - 89	88 - 48	53,7	9,2	0,95	9,7	0,0	T1 - 4
Delta	RL	07/07/2020	74 - 81	72 - 64	54,4	10,7	1,10	9,7	0,0	T1 - 4
Delta	RL	27/07/2020	75 - 88	72 - 48	58,7	11,2	0,95	11,8	0,0	T1 - 3
Delta	Sunki 812	17/06/2020	76 - 83	72 - 56	55,5	10,4	1,15	9,0	0,0	T2 - 5
Delta	Sunki 812	07/07/2020	78 - -89	64 - 48	57,8	10,6	1,25	8,5	0,0	T1 - 4
Delta	Sunki 812	27/07/2020	69 - 82	88 - 56	54,4	11,1	0,95	11,7	0,0	T1 - 3
Gusocora	C35	17/06/2020	77 - 83	72 - 56	57,5	10,8	1,10	9,8	0,0	T1 - 3
Gusocora	C35	07/07/2020	78 - 85	64 - 56	59,1	10,9	1,05	10,4	0,0	T1 - 3
Gusocora	C35	27/07/2020	74 - 85	72 - 56	60,1	10,7	1,05	10,2	0,0	T1 - 3

Gusocora	X639	17/06/2020	75 - 82	72 - 56	57,9	10,0	1,05	9,5	0,0	T2 - 5
Gusocora	X639	07/07/2020	80 - 89	64 - 48	57,1	10,6	1,00	10,6	0,0	T1 - 4
Gusocora	X639	27/07/2020	77 - 83	72 - 56	63,8	10,2	0,95	10,7	0,0	T1 - 3
Gusocora	RL	17/06/2020	76 - 85	72 - 56	57,9	8,9	0,95	9,4	0,0	T2 - 5
Gusocora	RL	07/07/2020	77 - 92	72 - 40	56,7	9,5	0,85	11,2	0,0	T1 - 4
Gusocora	RL	27/07/2020	78 - 85	64 - 56	54,2	9,6	0,90	10,7	0,0	T1 - 3
Gusocora	Sunki 812	17/06/2020	78 - 83	64 - 56	55,4	10,9	1,10	9,9	0,0	T1 - 4
Gusocora	Sunki 812	07/07/2020	77 - 84	72 - 56	57,7	10,8	0,95	11,4	0,0	T1 - 3
Gusocora	Sunki 812	27/07/2020	70 - 82	88 - 56	57,7	11,3	0,90	12,6	0,0	T1 - 3
Henrietta	C35	17/06/2020	79 - 83	64 - 56	58,7	10,2	1,30	7,8	1,7	T2 - 5
Henrietta	C35	07/07/2020	77 - 87	72 - 48	59,9	10,1	1,20	8,4	2,1	T1 - 3
Henrietta	C35	27/07/2020	73 - 91	72 - 40	64,2	10,8	1,05	10,3	2,3	T1 - 3
Henrietta	X639	17/06/2020	79 - 82	64 - 56	59,1	9,2	1,25	7,4	0,0	T2 - 4
Henrietta	X639	07/07/2020	78 - 82	64 - 56	59,9	9,9	1,25	7,9	1,0	T1 - 3
Henrietta	X639	27/07/2020	76 - 85	72 - 56	62,3	10,6	1,20	8,8	0,0	T1 - 3
Henrietta	RL	17/06/2020	75 - 81	72 - 64	59,0	8,9	1,15	7,7	0,0	T3 - 5
Henrietta	RL	07/07/2020	75 - 87	72 - 48	56,7	9,6	1,00	9,6	0,0	T1 - 4
Henrietta	RL	27/07/2020	80 - 97	64 - 36	58,8	8,4	0,90	9,3	0,0	T1 - 4
Henrietta	Sunki 812	17/06/2020	73 - 83	72 - 56	58,4	10,6	1,15	9,2	0,0	T2 - 5
Henrietta	Sunki 812	07/07/2020	77 - 86	72 - 48	62,3	11,1	1,30	8,5	1,8	T1 - 4
Henrietta	Sunki 812	27/07/2020	77 - 89	72 - 48	53,3	11,4	1,15	9,9	2,3	T1 - 3
Jassie	C35	17/07/2020	77 - 84	72 - 56	59,0	10,1	1,10	9,2	1,0	T3 - 5
Jassie	C35	07/07/2020	76 - 86	72 - 48	59,4	12,2	1,15	10,6	1,8	T1 - 3
Jassie	C35	27/07/2020	70 - 81	72 - 64	58,0	12,3	1,20	10,3	2,0	T1 - 3
Jassie	X639	17/06/2020	78 - 85	64 - 56	58,0	10,3	1,25	8,2	1,0	T2 - 5
Jassie	X639	07/07/2020	72 - 83	88 - 56	60,3	10,5	0,90	11,7	5,3	T1 - 5
Jassie	X639	27/07/2020	77 - 84	72 - 56	64,3	10,3	1,00	10,3	1,8	T1 - 4
Jassie	RL	17/06/2020	79 - 84	64 - 56	54,9	9,3	1,10	8,5	1,0	T2 - 5
Jassie	RL	07/07/2020	72 - 87	88 - 48	57,9	9,7	1,15	8,4	4,1	T1 - 4
Jassie	RL	27/07/2020	77 - 83	72 - 56	55,8	8,9	0,90	9,9	1,7	T1 - 3
Jassie	Sunki 812	17/06/2020	72 - 81	88 - 64	67,3	11,0	1,25	8,8	3,3	T2 - 5
Jassie	Sunki 812	07/07/2020	73 - 85	72 - 56	52,0	11,3	1,30	8,7	1,5	T1 - 4
Jassie	Sunki 812	27/07/2020	73 - 84	72 - 64	56,8	11,1	1,10	10,1	1,5	T1 - 4
K du Toit Late	C35	17/06/2020	76 - 84	72 - 56	61,1	10,6	1,05	10,1	5,3	T2 - 5
K du Toit Late	C35	07/07/2020	72 - 88	76 - 48	57,5	10,5	1,05	10,0	3,6	T1 - 3
K du Toit Late	C35	27/07/2020	78 - 84	64 - 56	65,7	11,0	1,14	9,6	1,8	T1 - 3
K du Toit Late	X639	17/06/2020	72 - 80	88 - 64	52,9	10,5	1,15	9,1	4,2	T2 - 4

K du Toit Late	X639	07/07/2020	73 - 80	72 - 64	60,0	9,9	1,05	9,4	1,9	T1 - 3
K du Toit Late	X639	27/07/2020	75 - 83	72 - 56	62,9	10,8	1,10	9,8	2,7	T1 - 3
K du Toit Late	RL	20/06/2020	74 - 77	72	58,7	9,0	0,95	9,5	3,3	T3 - 5
K du Toit Late	RL	07/07/2020	75 - 85	75 - 56	60,1	10,0	1,00	10,0	3,8	T1 - 4
K du Toit Late	RL	20/07/2020	71 - 79	88 - 64	61,5	10,1	0,90	11,2	2,7	T1 - 3
K du Toit Late	Sunki 812	17/06/2020	72 - 79	88 - 64	19,4	10,9	1,30	8,4	1,7	T2 - 4
K du Toit Late	Sunki 812	07/07/2020	74 - 83	72 - 64	56,4	10,1	1,35	7,5	0,0	T2 - 4
K du Toit Late	Sunki 812	27/07/2020	69 - 80	88 - 64	62,4	11,8	1,15	10,3	3,2	T1 - 4
Lavalle 2	C35	17/06/2020	85 - 92	56 - 36	55,3	10,8	1,55	7,0	0,0	T3 - 6
Lavalle 2	C35	07/07/2020	86 - 90	48 - 40	55,4	10,5	1,45	7,2	0,0	T2 - 5
Lavalle 2	C35	27/07/2020	77 - 91	72 - 40	58,2	11,8	1,40	8,4	0,0	T1 - 4
Lavalle 2	X639	17/06/2020	75 - 82	72 - 56	60,5	10,5	1,40	7,5	0,0	T2 - 5
Lavalle 2	X639	07/07/2020	77 - 88	72 - 48	58,9	10,7	1,50	7,1	0,0	T2 - 5
Lavalle 2	X639	27/07/2020	76 - 86	72 - 48	61,0	12,4	1,35	9,2	0,0	T1 - 4
Lavalle 2	RL	17/06/2020	76 - 80	72 - 64	57,5	9,5	1,45	6,6	0,0	T3 - 5
Lavalle 2	RL	07/07/2020	77 - 85	72 - 56	46,5	9,1	0,85	10,7	0,0	T1 - 4
Lavalle 2	RL	27/07/2020	69 - 80	88 - 64	60,1	10,0	1,10	9,1	0,0	T1 - 4
Lavalle 2	Sunki 812	17/06/2020	79 - 84	64 - 56	57,8	11,4	1,80	6,3	1,3	T3 - 6
Lavalle 2	Sunki 812	07/07/2020	85 - 93	56 - 40	56,4	12,6	1,60	7,9	0,0	T2 - 5
Lavalle 2	Sunki 812	27/07/2020	81 - 93	64 - 40	60,9	12,6	1,65	7,6	0,0	T1 - 4
Louisa	C35	17/06/2020	75 - 83	72 - 56	57,1	10,8	1,10	9,8	0,0	T2 - 4
Louisa	C35	07/07/2020	72 - 87	88 - 48	55,9	10,2	1,15	8,9	0,0	T1 - 4
Louisa	C35	27/07/2020	75 - 90	72 - 40	60,1	10,9	1,05	10,4	0,0	T1 - 3
Louisa	X639	17/06/2020	72 - 77	88 - 72	56,9	8,9	1,15	7,7	0,0	T3 - 5
Louisa	X639	07/07/2020	80 - 89	64 - 48	56,1	9,5	1,00	9,5	0,0	T1 - 3
Louisa	X639	27/07/2020	78 - 91	64 - 40	57,0	9,4	0,95	9,9	0,0	T1 - 4
Louisa	RL	17/06/2020	77 - 84	72 - 56	56,5	8,6	1,15	7,5	0,0	T3 - 6
Louisa	RL	07/07/2020	74 - 85	72 - 56	57,1	8,1	1,00	8,1	0,0	T2 - 4
Louisa	RL	27/07/2020	74 - 84	72 - 56	53,7	8,9	0,80	11,1	0,0	T1 - 3
Louisa	Sunki 812	17/06/2020	77 - 84	72 - 56	56,6	9,6	1,30	7,4	0,0	T3 - 5
Louisa	Sunki 812	07/07/2020	81 - 90	64 - 48	62,8	10,1	1,40	7,2	0,0	T1 - 4
Louisa	Sunki 812	27/07/2020	77 - 91	72 - 40	68,8	11,0	1,35	8,1	0,0	T1 - 3
McClellan SL	C35	17/06/2020	80 - 89	64 - 48	52,7	10,4	1,05	9,9	0,0	T2 - 4
McClellan SL	C35	07/07/2020	75 - 86	72 - 48	58,0	13,8	1,05	13,1	0,0	T1 - 3
McClellan SL	C35	27/07/2020	75 - 83	72 - 56	62,9	10,4	1,00	10,4	0,0	T1 - 3
McClellan SL	X639	17/06/2020	76 - 85	72 - 56	56,9	10,0	1,10	9,1	0,0	T2 - 5

McClean SL	X639	07/07/2020	74 - 84	72 - 56	58,7	10,3	1,00	10,3	0,0	T2 - 5
McClean SL	X639	27/07/2020	75 - 85	72 - 56	63,0	10,6	0,95	11,2	0,0	T1 - 3
McClean SL	RL	17/06/2020	74 - 82	72 - 56	62,3	10,6	0,90	11,8	0,0	T2 - 5
McClean SL	RL	07/07/2020	72 - 88	88 - 48	57,4	9,7	0,95	10,2	0,0	T1 - 4
McClean SL	RL	27/07/2020	75 - 82	72 - 56	62,5	10,7	1,00	10,7	0,0	T1 - 4
McClean SL	Sunki 812	17/06/2020	76 - 86	72 - 48	56,9	10,9	1,15	9,5	0,0	T1 - 4
McClean SL	Sunki 812	07/07/2020	78 - 90	64 - 40	57,6	10,7	1,10	9,7	0,0	T1 - 3
McClean SL	Sunki 812	27/07/2020	71 - 83	88 - 56	63,9	11,4	1,25	9,1	0,0	T1 - 3
Skilderkrans	C35	07/07/2020	78 - 92	64 - 40	60,0	9,6	1,20	8,0	0,0	T2 - 4
Skilderkrans	C35	27/07/2020	77 - 90	72 - 40	62,9	10,5	1,15	9,1	0,0	T1 - 4
Skilderkrans	X639	07/07/2020	77 - 93	72 - 40	54,9	9,1	0,90	10,1	0,0	T2 - 4
Skilderkrans	X639	27/07/2020	79 - 84	64 - 56	57,2	9,6	1,00	9,6	0,0	T1 - 4
Skilderkrans	RL	07/07/2020	75 - 89	72 - 48	57,0	8,8	1,05	8,4	0,0	T1 - 5
Skilderkrans	RL	27/07/2020	70 - 84	88 - 56	57,7	10,7	1,10	9,7	1,0	T1 - 4
Skilderkrans	Sunki 812	07/07/2020	79 - 89	64 - 48	58,2	10,3	1,40	7,4	0,0	T1 - 4
Skilderkrans	Sunki 812	27/07/2020	82 - 89	56 - 48	60,4	10,8	1,20	9,0	0,0	T1 - 4
Turkey	C35	13/05/2020	78 - 80	64	57,7	11,5	1,15	10,0	0,5	T2 - 5
Turkey	C35	27/05/2020	79 - 84	64 - 56	58,9	12,6	1,45	8,7	1,8	T2 - 4
Turkey	C35	17/06/2020	76 - 84	72 - 56	59,6	11,3	1,10	10,3	4,0	T1 - 3
Turkey	X639	13/05/2020	74 - 82	72 - 56	59,4	10,4	1,00	10,4	2,1	T3 - 5
Turkey	X639	27/05/2020	79 - 85	64 - 56	60,9	10,7	1,05	10,2	0,3	T1 - 4
Turkey	X639	17/06/2020	75 - 79	72 - 64	59,1	10,1	0,95	10,6	0,2	T1 - 4
Turkey	RL	13/05/2020	79 - 81	64	56,4	9,5	1,10	8,6	1,2	T3 - 5
Turkey	RL	27/05/2020	75 - 86	72 - 48	58,2	9,7	0,95	10,2	1,3	T1 - 4
Turkey	RL	17/06/2020	72 - 84	88 - 56	53,5	9,6	0,95	10,1	1,3	T1 - 4
Turkey	Sunki 812	13/05/2020	73 - 80	72 - 64	58,3	11,5	1,05	11,0	1,0	T2 - 4
Turkey	Sunki 812	27/05/2020	72 - 83	88 - 56	59,1	12,1	1,10	11,0	0,3	T1 - 3
Turkey	Sunki 812	17/06/2020	77 - 85	72 - 56	61,4	12,5	1,10	11,4	4,0	T1 - 4

#### 5.4.6 PROGRESS REPORT: Evaluation of Mandarin hybrid selections in the intermediate production areas (Marble Hall)

Project 941C by J. Joubert (CRI)

##### Summary

The quality of the Mandarin Hybrid fruit improved in this climatic region (intermediate area). The results indicated that RHM with higher seed counts and low acid levels, followed by Leanri mature first. Tango was next in line with an increase in fruit size and fair to good internal quality. Tango and Ma'ayan were completely seedless this season. Ma'ayana, Meirav 63 & 119 and Michal 6/47 & 89/64 seem to fit in with the mid-maturing selections with deep orange rind colour. Picking periods should not be longer than 3 - 4 weeks to maintain good internal quality and to avoid rind disorders.

##### Opsomming

Die kwaliteit van die Mandaryn Hibried vrugte het verbeter in die klimaatzone (intermediere areas). Die resultate het aangedui dat RHM met hoër saadtellings en lae suurvlakke, gevolg deur Leanri die vroegste ryp geword het. Tango is volgende, met 'n toename in vruggrootte en gemiddelde tot goeie interne kwaliteit. Tango en Ma'ayana was total saadloos gewees hierdie seisoen. Ma'ayana, Meirav 63 & 119 en Michal 6/47 & 89/64 pas hier in saam met die mid seleksies met diep oranje skilkleur Daar word aanbeveel om nie die oesperiode langer as 3 tot 4 weke te verleng nie om goeie interne kwaliteit te verseker met minimum skil probleme.

## Objectives

- To select Mandarin Hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new Mandarin Hybrid cultivars and to determine the climatic suitability of these cultivars in intermediate, inland production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on Mandarin Hybrid selections from Moosrivier Estate (Marble Hall).

**Table 5.4.6.1.** List of Mandarin Hybrid selections evaluated at Moosrivier Estate (Marble Hall) during the 2020 season.

Selection	Rootstock	Planted
Ma'ayana (Dina)	Sunki 812	2013
Leanri	Sunki 812	2013
Meirav 63	Sunki 812	2013
Meirav 119	Sunki 812	2013
Michal 6/47	Sunki 812	2013
Michal 89/64	Sunki 812	2013
RHM	Sunki 812	2013
Tango	CC/C35	2013

## Results and discussion

The trial site at Moosrivier was relocated to a new site and trees were established for future evaluations due to cold damage and soil quality at the old site. All the trees at Moosrivier bore their fifth crop for this season with improved fruit numbers and more mature tree internal quality and fruit size characteristics.

For Mandarin Hybrid selections, a ratio of 11:1 is considered to be the build-up towards peak maturity, with a ratio of 12:1. After reaching the peak, the ratio increases to 13:1, after which the fruit is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

### Tango

Tango remained completely seedless at the trial site and there was a good to very good crop on the trees at Moosrivier and the fruit shape was similar to the Nadorcott selection. Rind texture was very smooth with a natural wax shine on the fruit. The Tango trees were thornless with a V-tree shape. The fruit was firm and the rind thin, fibre was soft and peeled very easily. Internally the fruit was high in juice content (up to 57%), Brix was average (up to 10) and the acid levels below 1.0% during the season (CC outperformed C35 this season). Fruit size varied on the trees and peaked at count 2 to 1XXX (medium/large to extra-large). Based on the internal quality results in Table 5.4.6.2, estimated maturity will be end of April to the middle of May (slightly delayed external colour).

### Additional selections

The internal quality of Ma'ayana (Dina) was very good this season with high juice levels (average 62.1%) and no granulation problems in the fruit compared to Nova. Brix (average 12.3) and similar acids (1.1%), indicating the early-mid maturing characteristics of the selection in the intermediate production areas, with seedless fruit this season. The fruit size peaked from count 2 to count 1XXX.

Leanri developed a fairly large fruit size between count 1 and 1XXX, slightly smaller than the hot production areas. 2020 was the fourth crop on the trees and internal quality was very good on Sunki 812 (average juice 55%, Brix 11.9, acid 1.1%). Seed numbers were fairly low; from 0.8 up to 3.8 seeds per fruit.

Meirav 63 (experimental) developed a deep orange rind colour (T1 with peak maturity), as well as Meirav 119. Internal quality improved; juice content was average to good (average above 55%), Brix of 11.3 and acids above 0.9%. The fruit evaluated was seedless to low seeded (up to 2.5) at the Moosrivier trial site.

Michal 6/47 and 89/64 bore a fair crop with medium/large to extra-large fruit size (count 2 to count 1XX) and deep orange rind colour (T1-T3). Internal quality was good early in the season with good juice (up to 58%), Brix (10.8) and low acids (average 0.7%) levels. The fruit average seed count was 2.1 seeds per fruit this season.

RHM cropped fruit for the fourth time this season with average seed numbers during the evaluation (2.4 seeds per fruit) and are prone to cross-pollination. There was a delayed colour development (T2 to 4) from the first evaluation with low acids (average 0.70 to 0.75), indicating peak maturity Brix: acid ratio over 12. Future evaluations will determine the optimum quality of the fruit evaluated.

### **Conclusion**

The delay in external colour development improved this season due to the age of the trees (more mature); future evaluation will confirm this.

This was the fourth evaluation of RHM, Leanri, Meirav 63 & 119; the fifth evaluation of Ma'ayana (Dina) at Moosrivier; so, information is becoming available and future evaluations will improve recommendations on these cultivars (management improvement). The highest seed numbers were on RHM and the Michal, Meirav selections this season, followed by Leanri. All the other selections developed very low seed numbers in the fruit. Meirav 63 and 119 performed well with deep orange colour development and Ma'ayana's (Dina) internal quality improved substantially. RHM continued with the delayed external colour development on the fruit and lower acids.

**Table 5.4.6.2.** Internal fruit quality data for Mandarin hybrid selections at Moosrivier (Marble Hall) during the 2020 season.

Cultivar	Rootstock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Ma'yana	Sunki 812	12/05/2020	62 - 82	2 - 1XXX	60,1	11,9	1,20	9,9	0,0	T1 - 2
Ma'yana	Sunki 812	03/06/2020	70 - 90	1 - 1XXX	64,2	12,7	0,95	13,4	0,0	T1 - 2
Leanri	Sunki 812	24/03/2020	68 - 77	1X - 1XX	53,2	10,9	1,05	10,4	3,8	T3 - 5
Leanri	Sunki 812	03/06/2020	75 - 86	1XX - 1XXX	56,9	13,0	0,95	13,7	1,2	T1 - 3
Leanri	Sunki 812	12/05/2020	76 - 85	1XX - 1XXX	54,3	11,7	1,20	9,8	0,8	T1 - 2
Meirav 63	Sunki 812	24/03/2020	63 - 70	2 - 1X	48,6	11,0	1,55	7,1	2,5	T4 - 7
Meirav 63	Sunki 812	03/06/2020	68 - 79	1X - 1XXX	55,1	11,9	0,90	13,2	0,0	T1 - 3

Meirav 119	Sunki 812	24/03/2020	64 - 70	1 - 1X	63,9	10,9	0,25	43,6	2,2	T3 - 5
Michal 6/47	Sunki 812	24/03/2020	60 - 64	2 - 1	48,4	10,3	0,70	14,7	3,0	T4 - 6
Michal 6/47	Sunki 812	12/05/2020	60 - 74	2 - 1XX	58,9	10,8	0,60	18,0	3,5	T1 - 2
Michal 6/47	Sunki 812	03/06/2020	67 - 77	1 - 1XX	46,0	12,6	0,96	13,1	0,4	T1 - 3
Michal 89/64	Sunki 812	24/03/2020	57 - 64	3 - 1	50,0	9,0	0,65	13,8	2,3	T3 - 6
Michal 89/64	Sunki 812	03/06/2020	63 - 75	2 - 1XX	48,1	11,4	0,65	17,5	1,0	T1 - 3
RHM	C35	12/05/2020	70 - 76	1X - 1XX	55,8	9,2	0,75	12,3	0,0	T2 - 4
RHM	C35	03/06/2020	72 - 79	1XX - 1XXX	56,6	11,2	0,80	14,0	0,0	T1 - 2
RHM	CC	12/05/2020	67 - 79	1 - 1XXX	67,9	11,1	0,75	14,8	7,0	T2 - 4
RHM	CC	03/06/2020	67 - 90	1 - 1XXX	64,9	11,7	0,85	13,8	0,0	T1 - 4
RHM	Sunki 812	12/05/2020	71 - 81	1X - 1XXX	60,3	10,9	0,70	15,6	6,0	T1 - 3
RHM	Sunki 812	03/06/2020	65 - 80	1 - 1XXX	60,2	11,9	0,70	17,0	1,7	T1 - 3
Tango	CC	24/03/2020	60 - 69	2 - 1X	48,4	7,0	1,55	4,5	0,0	T5 - 7
Tango	CC	03/06/2020	70 - 80	1X - 1XXX	57,4	10,1	0,90	11,2	0,0	T1 - 3
Tango	C35	24/03/2020	62 - 67	2-1	48,4	8,3	1,45	5,7	0,0	T5 - 7
Tango	C35	03/06/2020	68 - 79	1X - 1XXX	55,1	10,0	0,90	11,1	0,0	T1 - 4

#### 5.4.7 PROGRESS REPORT: Evaluation of Mandarin hybrid selections in the hot, dry inland areas (Tshipise)

Project 899B by J. Joubert (CRI)

##### Summary

The quality of the Mandarin Hybrid fruit between the different production areas was very different, indicating how important it is to decide what cultivar to plant where, as well as the suitable rootstock for that area. The results of the 2020 season still indicated that for the warm production areas Tango matures first with smaller fruit size and good internal quality (acid levels drop early in the season).

Etna was first to mature from the new additional selections, followed by Sirio with low-seeded fruit this season. Saint André, Nova and Nova SL followed with good external colour development and seedless fruit. Next to mature was Samba, followed by Furr with high seed numbers in the fruit. Tambor 1, 2 and Tanor Late mature last, ending off the mandarin season for the hot areas.

Evaluations on the latest additions indicated that Goldup would be very early maturing in the hot areas, followed by RHM with lower acids. Ma'ayana, Meirav and Leanri will be next in line, cropping large fruit on the trees with good internal quality. Taylor Lee follows; developing low seed number and good internal quality as well as colour on the fruit. The Nadorcott selections will be next, cropping a good crop on the trees; ARCCIT 9 LS had no seeds in the fruit.

Picking periods should not be longer than 3 - 4 weeks to maintain good internal quality and avoid rind disorders.

##### Opsomming

Die kwaliteit van die Mandaryn Hibried vrugte het aansienlik verskil tussen die verskillende produksie areas, wat 'n baie belangrike punt uitlig wanneer dit by die keuse van kultivars vir aanplantings kom, sowel as die onderstam wat gebruik word. Die resultate van die 2020 seisoen vir hierdie warm produksie areas het steeds

aangedui dat Tango die vroegste ryp geword het met kleiner vruggrootte en goeie interne kwaliteit (suurvlakke daal vinnig in begin van seisoen).

Etna het eerste ryp geword van die nuwe addisionele seleksies, gevolg deur Sirio met lae-saad vrugte vir die seisoen. Saint André, Nova en Nova SL het gevolg met goeie kleur ontwikkeling en saadlose vrugte. Volgende om ryp te word sal Samba wees, gevolg deur Furr met hoë saadtellings in die vrugte. Mor 26 volg nou, met 'n ligte oes op die bome (tussenstam opsie) en goeie interne kwaliteit. Tambor 1, 2 en Tanor Late word laaste ryp en eindig die mandaryn seisoen vir die warm produksie area.

Evaluasies op die nuutste toevoegings het aangedui dat Goldup die vroegste ryp word in die warm area, gevolg deur RHM met lae suur vlakke. Ma'ayana, Meirav en Leanri is volgende in lyn, wat groot vrugte op die bome dra en goeie interne kwaliteit. Taylor Lee pas nou in; lae saad tellings, goeie interne kwaliteit en eksterne kleur ontwikkeling. Die Nadorcott seleksies pas nou in met 'n goeie oes op die bome; ARCCIT 9 se vrugte was totaal saadloos gewees.

Daar word aanbeveel om nie die oesperiode langer as 3 tot 4 weke te verleng nie om goeie interne kwaliteit te verseker met minimum skil probleme.

### Objectives

- To select Mandarin Hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new Mandarin Hybrid cultivars and to determine the climatic suitability of these cultivars in hot, dry production regions.

### Materials and methods

Field evaluations and laboratory analyses were conducted on Mandarin Hybrid selections from Alicedale (Tshipise) in the Limpopo region.

**Table 5.4.7.1.** List of Mandarin Hybrid selections evaluated at Alicedale (Tshipise) during the 2020 season.

Selection	Rootstock	Topworked
Ma'ayana (Dina)	X639	2015
Etna	X639	2013/2014
Furr (Clemcott)	X639	2013/2014
IRM 1&2	X639	2015
Leanri	X639	2015
Meirav 119	X639	2015
Nadorcott ARCCIT 9	X639	2015
Nova	X639	2013/2014
Nova SL (ARC)	X639	2013/2014
Sirio	X639	2013/2014
Tango	X639	2010
Taylor Lee	X639	2015

### Results and discussion

More information was available at Alicedale (new and existing trial site) due to older trees with better crops. Tanor Late fruit was picked earlier in the season before late maturing evaluations started and there was no fruit to sample. Evaluations were completed on trees bearing fruit.

For Mandarin Hybrids, a ratio of 11:1 is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which the fruit is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

Tango remained completely seedless at Alicedale this season. The fruit shape was similar to the Nadorcott selection. Rind texture was very smooth with a natural wax shine on the fruit. The Tango trees were thornless and an upright V-shape. The fruit was firm and the rind thin, fibre was soft and peeled very easily. Internally the fruit was high in juice content (above 58%), Brix improved for this selection (average 10.8), acid levels (below 1.0) decreased early in the season (indicating a short shelf life in the hot production areas) and deep orange coloured fibre. Fruit size increased and peaked at count 1 to 1XX (medium/large to large). Based on the internal quality results in Table 5.4.7.3, estimated maturity will be the middle of April.

Etna bore a good crop with medium to very large fruit (count 1 to 1XX) and average/good internal quality (high juice and Brix, and average acids of 0.90). The external colour was delayed at peak maturity (T3 to T5) and all the fruit evaluated were seedless. Furr (Clemcott) was included as a control for the hot production areas and to compare with Leanri. Fruit size was large to very large (count 1XXX), good internal quality with acceptable acids (average 0.80%) early in the season and high number of seeds in the fruit (14.7).

Nova was included as a control for Nova SL and Saint André in the trial, the fruit is fairly difficult to peel and low numbers of seed developed in the fruit. The external colour was late and the fruit size increased between count 1 and 1XX (medium to large). Nova SL (ARC) produced a coarse rind texture on the fruit with similar fruit size, medium to large (count 2 to count 1XX). The acid levels in the fruit were similar compared to Nova (control) and the external colour development was delayed this season at peak maturity.

Orri trees were very aggressively growing, producing a very light crop on the trees this season and no evaluation was possible.

Samba produced an average third (X639 rootstock) crop on the large fast-growing thorn-less trees. Colour development was early in the season (deep orange) with a smooth rind texture on the fruit. Seed counts were very low, completely seedless this season in the mixed block and the fruit size peaked from count 2 to count 1XX. Sirio produced erratic fruit sizes last season from medium to large coarse fruit (count 2 to 1XXX), this season the fruit size peaked at count 1XXX (very large fruit) with average to good internal quality; juice of 52%, Brix 11 and acids below 1.0% and better colour development on the trees (T1 to T3).

The Tambor fruit matured late this season after the last evaluation was completed. We will make provision to evaluate the fruit next season.

Nadorcott (control) and Nadorcott ARCCIT 9 cropped the best yield on the young trees, followed by Leanri with large fruit size and good colour development. Meirav 119 developed very good colour on the young trees (from T1 to T3); acids were also fairly low (0.6%) early in the season for this hot dry production area. The yield on Ma'ayana (Dina) improved and three evaluations were possible with good juice and Brix levels; acids improve on the higher side, but with the early colour development Ma'ayana can be harvested as one of the earlier mandarin selections. Ma'ayana developed trees that were very upright in shape and pruning will be crucial to develop proper bearing branches. IRM 1 & 2 cropped minimal fruit this season and evaluations will continue next year.

Taylor Lee bore a better crop this season to evaluate with high juice (up to 63%), Brix (11) and acceptable acids. Seed numbers were on the low side, between 2.3 and 3.7 seeds per fruit compared to the Furr control (14.7 seeds per fruit).

## **Conclusion**

The external colour delay (internal quality improved with more mature trees) in the hotter areas remained a problem; future evaluations will confirm this. Degreening may be an option for the early selections included in the trial, but ethylene reacted slowly or not at all for Tango and Nadorcott (W. Murcott selections).

This was the fifth evaluation of Etna, Furr (control), Nova (Control), Nova SL, Page, Saint André, Samba, Sirio, Tambor 1 (control) and 2, Tasty 1 and 2, information is progressing with trees maturing and better fruit quality; so future evaluations will improve recommendations on these varieties. The promising selections at this early stage were Page (good colour development and low seeded fruit), Saint André (bigger fruit size and later maturing) and Samba with good internal quality fruit (early maturing), good colour development and crop on the trees in combination with X639. Seed numbers on these selections were very low to completely seedless in the combination trial block with cross pollinating cultivars included. Furr (control for Leanri) developed the highest seed numbers per fruit, a typical characteristic of the selection with good colour development and internal quality.

This was the third evaluation (trees five years old – topworked end of 2015) of Nadorcott, Nadorcott ARCCIT 9, Ma'ayana (Dina), IRM 1&2, Leanri, Meirav 119 and RHM. The Nadorcott selections performed well with large fruit and good internal quality. Ma'ayana (Dina) and Leanri matures early in the season with improved low acid levels. IRM 1&2 cropped a very light yield possibly due to alternate bearing patterns, future evaluations will confirm and fairly ribbed fruit.

Goldup and Taylor Lee were evaluated for the second time this year. The Goldup fruit seems fairly soft and not this suitable for this hot area. Taylor Lee performed well in terms of internal quality, colour development and low seed numbers, but large to extra-large fruit size.

**Table 5.4.7.2.** Internal fruit quality data for Mandarin hybrid selections at Alicedale (Tshipise) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
ARCCIT 9 LS	X639	13/05/2020	72 - 81	1XX - 1XXX	53,0	8,9	1,20	7,4	0,0	T2 - 4
ARCCIT 9 LS	X639	27/05/2020	75 - 80	1XX - 1XXX	58,6	9,4	1,05	9,0	0,0	T3 - 5
Ma'ayana	X639	17/03/2020	60 - 62	2	57,9	11,4	1,00	11,4	0,0	T4 - 6
Ma'ayana	X639	13/05/2020	60 - 73	2 - 1XX	63,6	12,6	0,75	16,8	0,0	T1 - 3
Ma'ayana	X639	27/05/2020	71 - 79	1X - 1XXX	57,8	10,5	0,95	11,1	0,0	T1 - 3
Etna	X639	17/03/2020	64 - 72	1 - 1XX	60,8	10,8	0,90	12,0	0,0	T3 - 5
Furr	X639	27/05/2020	80 - 90	1XXX	60,0	10,0	0,80	12,5	14,7	T2 - 4
IRM 1	X639	27/05/2020	72 - 83	1XX - 1XXX	55,8	11,7	0,75	15,6	1,7	T2 - 4
IRM 2	X639	13/05/2020	70 - 82	1X - 1XXX	51,7	10,6	0,60	17,7	1,7	T2 - 4
IRM 2	X639	27/05/2020	67 - 78	1 - 1XXX	60,9	11,1	0,55	20,2	0,0	T2 - 3
Leanri	X639	17/03/2020	73 - 77	1XX	64,0	10,3	0,80	12,9	1,2	T5 - 7
Leanri	X639	13/05/2020	75 - 88	1XX - 1XXX	64,0	11,2	1,05	10,7	0,0	T 2 - 4
Leanri	X639	27/05/2020	75 - 86	1XX - 1XXX	59,6	10,6	1,05	10,1	0,0	T1 - 4
Meirav 119	X639	13/05/2020	68 - 80	1X - 1XXX	62,0	11,2	0,55	20,4	0,0	T1 - 3
Meirav 119	X639	27/05/2020	70 - 80	1X - 1XXX	60,8	10,6	0,55	19,3	0,0	T1 - 3
Nadorcott	X639	13/05/2020	68 - 83	1X - 1XXX	52,7	9,4	0,50	18,8	1,5	T2 -- 4
Nadorcott	X639	27/05/2020	72 - 81	1XX - 1XXX	59,6	9,4	0,65	14,5	0,0	T1 - 3
Nova	X639	17/03/2020	67 - 76	1 - 1XX	60,0	10,8	0,80	13,5	0,0	T3 - 5
Sirio	X639	17/03/2020	71 - 76	1X - 1XX	50,0	10,5	0,85	12,4	5,3	T3 - 5
Sirio	X639	13/05/2020	63 - 81	2 - 1XXX	54,1	11,2	0,90	12,4	0,5	T1 - 3
Tango	X639	13/05/2020	68 - 75	1X - 1XX	58,8	10,9	0,90	12,1	0,0	T2 - 4
Tango	X639	27/05/2020	66 - 75	1 - 1XX	61,7	10,6	0,80	13,3	0,0	T1 - 4
Tanor Late	X639	27/05/2020	70 - 91	1X - 1XXX	52,2	10,6	1,10	9,6	0,3	T1 - 4

Taylor Lee	X639	13/05/2020	75 - 84	1XX - 1XXX	62,7	10,9	0,70	15,6	2,3	T2 - 4
Taylor Lee	X639	27/05/2020	80 - 86	1XXX	58,5	11,1	0,80	13,9	3,7	T1 - 4

#### 5.4.8 PROGRESS REPORT: Evaluation of Mandarin hybrid selections in the intermediate production areas (Karino and Ngonini)

Project 963C by J. Joubert (CRI)

##### Summary

The quality of the Mandarin Hybrid fruit was good in the Nelspruit production area, due to the similar climatic region (intermediate areas). The results indicated that in the Nelspruit production area, Nadorcott ARCCIT9 LS matures first (two weeks before ARC selection), followed by Nadorcott ARC with medium to large fruit size for this season and excellent colour development. Ma'ayana and Meirav 63 also indicated to be fairly early maturing selections, followed by Samba with good colour development. IRM 2 mature next towards the middle of the mandarin season with less ribbing on the fruit compared to IRM 1 at the other trial sites. All the fruit evaluated this season had very low seed numbers due to cross pollination impact from the seeded cultivars close by, except for Sugar Belle, maturing ultra-late in the mandarin season.

##### Opsomming

Die kwaliteit van die Mandaryn Hibried vrugte was goed in die Nelspruit produksie area, a.g.v. die klimaatsone (intermediêre areas). Die resultate vir die Nelspruit produksie area het aangedui dat Nadorcott ARCCIT9 LS eerste gereed was vir die oesproses (twee weke voor die ARC seleksie), gevolg deur Nadorcott ARC met medium tot groot vrugte vir hierdie seisoen en baie goeie kleur ontwikkeling. Ma'ayana en Meirav 63 het ook onder die vroeë seleksies ingepas gevolg deur Samba met goeie kleur ontwikkeling. IRM 2 was volgende gereed vir oes gewees meer na die middel van die mandaryn seisoen; met minder ribbing op die vrugte in vergelyking met IRM 1 by die ander proef persele. Al die vrugte geëvalueer het baie lae saadinhoud aangedui a.g.v. die kruisbestuivings impak van aangrensende varieteite met saad, behalwe vir Sugar Belle, wat ultra-laas ryp word in die mandaryn seisoen.

##### Objectives

- To select Mandarin Hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new Mandarin Hybrid cultivars and to determine the climatic suitability of these cultivars in intermediate, inland production regions.

##### Materials and methods

Field evaluations and laboratory analyses were conducted on Mandarin Hybrid selections from Karino-koöp (Nelspruit) in the Mpumalanga region.

**Table 5.4.8.1.** List of Mandarin Hybrid selections evaluated at Karino-koöp (Nelspruit) during the 2020 season.

Selection	Rootstock	Planted
Ma'ayana (Dina)	CC	2011
IRM 1	CC	2014
IRM 2	CC	2011
Meirav 63	CC	2011
Meirav 119	CC	2014
Michal 6/47	CC	2014
Michal 89/64	CC	2014
Mor 2/15/26	CC	2014

Nadorcott ARC	CC	2011
Nadorcott ARCCIT9 LS	CC	2011
Shani SL	CC	2011
Sugar Belle	CC	2016
Tango	CC	2014
Winola	CC	2014

## Results and discussion

The mature trees at Karino-koöp's new trial site were evaluated this season; this is having an impact on the quality and quantity of the fruit. More information was available and future evaluations will be conducted.

For Mandarin Hybrids, a ratio of 11:1 is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which the fruit is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

### IRM 1 & IRM 2

The tree shape of the IRM selections was very upright (V-shaped) with no thorns and aggressive growth. IRM 1 and 2 produced an alternative crop on the trees, with an off-year this season, and the fruit size peaked on IRM 2 from medium to large (count 1 to 1XX). The seed numbers on IRM 1 increased this season and averaged from seedless to 1.7 seeds per fruit, peels easily with some ribbing on the fruit (typical Murcott characteristic). Juice levels on IRM 1&2 increased compared to 2019 and averaged above 62%, Brix was very good (up to 14.) and acids were above 1.2%. The external colour was deep orange and peaked between T1 and 3. Based on the internal quality results in Table 5.4.8.3, estimated maturity will be the middle to end of June

### Nadorcott ARC & ARCCIT9 LS

The fruit shape was similar to the Nadorcott selection. Rind texture was very smooth with a natural shine (similar to packhouse waxing). Both selections developed good internal quality with high juice levels (up to 63.9%), Brix averaging 10.2 and higher acids (avg. 0.9%). Nadorcott ARCCIT9 LS produced a better crop on the trees compared to the ARC selection and the fruit size was bigger this season in spite of a good crop load; varied from count 1-1XX/1XXX. ARCCIT9 LS were completely seedless at the Karino trial site. Maturity seems to be two weeks earlier on the ARCCIT9 LS selection, but the information was limited due to the fourth year of evaluation (end of April to the end of May), according to Table 5.4.8.3.

### Additional selections

The internal quality of Ma'ayana (Dina) was good with high juice levels above 60%, no granulation problems in the fruit compared to Nova. Brix (above 11 – second and third evaluation) and acceptable acids (0.8%), indicating the early to mid-maturing characteristics of the selection in the intermediate production areas, with low seeded fruit. The fruit size peaked from count 1 to count 1XXX.

Meirav 63 and 119 developed a deep orange rind colour (T1 with peak maturity). Internal quality was good with high juice content (above 59% at peak), Brix of 12.2 and good acids (average 0.8%). The seed content decreased back from 1.3 seeds to 0.8 seeds per fruit evaluated at the Karino trial site (cross pollination).

Michal 6/47 & 89/64, Tango and Winola were included in the Karino trial site in 2014 and bore their third crop on the trees this season. Fruit size peaked from medium to large on Michal 89/64 and 6/47 (count 2 to 1XX) with seedless fruit on all four selections. Tango cropped completely seedless fruit and Winola developed juice levels of 64%.

Sugar Belle was planted at the Karino trial site in 2016 and bore its second crop this season. The fruit size peaked from count 2 to count 1XX and colour development was favourable between T1 and T3 early in the season. Internal quality was very good with high juice and Brix levels, but exceptionally high acids, 1.35% with the end of May evaluation.

## Conclusion

This was the fifth evaluation of Ma'ayana (Dina) and Meirav 63; Ma'ayana cropped large fruit size on the trees with good colour development (precocious bearing pattern). IRM 1&2, Nadorcott ARC and Nadorcott ARCCIT9 LS was evaluated in the new trial block; where the two Nadorcott selections performed similarly, except for a better crop on ARCCIT 9.

Meirav 119, Michal 6/47 & 89/64, Tango and Winola at the Karino site were evaluated for the third time on the young tree, so information becomes available and future evaluations will improve recommendations on these varieties. All the selections developed no seeds in the fruit compared to the previous season, where they were also completely seedless for this trial. There was a good external colour development on the Nadorcott, Meirav 63&119 and Michal 6/47 & 89/64 selections (deep orange).

Sugar Belle bore fruit to evaluate; the cultivar had a good colour development early in the season, but Sugar Bell will be an ultra-late maturing option due to very high acids.

**Table 5.4.8.2.** Internal fruit quality data for Mandarin hybrid selections at Karino- koöp (Nelspruit) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
ARCCIT 9 LS	CC	21/05/2020	70 - 82	1X 1XXX	50,3	9,4	0,60	15,7	0,8	T2 - 3
ARCCIT 9 LS	CC	08/06/2020	70 - 80	1X 1XXX	57,5	11,0	0,75	14,7	0,0	T2 - 4
ARCCIT 9 LS	CC	01/07/2020	70 - 85	1X 1XXX	53,2	10,9	0,80	13,6	0,0	T1 - 3
Etna	CC	29/04/2020	67 - 78	1 - 1XXX	62,3	8,0	0,60	13,3	0,0	T2 - 4
Etna	CC	21/05/2020	68 - 90	1X 1XXX	59,5	7,7	0,55	14,0	0,0	T1 - 3
Furr	CC	21/05/2020	78 - 88	1XXX	55,1	11,7	0,80	14,6	2,1	T1 - 3
Furr	CC	08/06/2020	74 - 85	1XX 1XXX	60,3	12,2	0,60	20,3	3,7	T1 - 3
Furr	CC	15/07/2020	75 - 88	1XX 1XXX	64,3	14,0	1,05	13,3	2,0	T1 - 3
Gold UP	CC	04/05/2020	63 - 77	2 - 1XX	50,6	7,4	0,50	14,8	0,0	T2 - 3
IRM 1	CC	21/05/2020	71 - 95	1X 1XXX	68,1	11,3	0,70	16,1	1,5	T2 - 4
IRM 1	CC	01/07/2020	66 - 81	1 - 1XXX	62,9	11,2	0,85	13,2	0,3	T1 - 3
IRM 1	CC	04/08/2020	71 - 87	1X 1XXX	65,7	14,2	1,00	14,2	1,7	T1 - 3
IRM 1	CC	21/05/2020	63 - 76	2 - 1XX	56,6	10,1	0,85	11,9	0,0	T2 - 4
IRM 2	CC	08/06/2020	65 - 78	1 - 1XXX	66,1	11,2	0,90	12,4	0,0	T1 - 4
IRM 2	CC	01/07/2020	72 - 83	1XX 1XXX	62,1	13,6	1,25	10,9	0,0	T1 - 3
IRM 2	CC	04/08/2020	73 - 81	1XX 1XXX	56,5	13,3	0,80	16,6	1,0	T1 - 3
Leanri	CC	04/05/2020	72 - 79	1XX 1XXX	58,6	11,8	0,80	14,8	0,0	T2 - 3
Leanri	CC	21/05/2020	74 - 90	1XX 1XXX	54,1	9,8	0,75	13,1	0,0	T1 - 4
Ma'ayana (Dina)	CC	04/05/2020	73 - 82	1XX 1XXX	59,7	9,9	0,80	12,4	0,0	T2 - 3

Ma'ayana (Dina)	CC	21/05/2020	72 - 85	1XX 1XXX	-	53,1	11,7	0,85	13,8	2,1	T1 - 3
Ma'ayana (Dina)	CC	29/04/2020	64 - 78	1 - 1XXX		60,2	12,3	0,75	16,4	0,0	T2 - 3
Meirav 63	CC	29/04/2020	59 - 67	2 - 1,		61,8	10,8	0,90	12,0	0,0	T3 - 5
Meirav 63	CC	08/06/2020	70 - 78	1X 1XXX	-	66,4	12,50	0,85	14,7	0,8	T1 - 3
Meirav 63	CC	01/07/2020	67 - 79	1 - 1XXX		55,8	12,3	0,70	17,6	0,0	T1 - 3
Meirav 63	CC	15/07/2020	72 - 85	1XX 1XXX	-	62,8	13	0,95	13,7	0,0	T1 - 3
Meirav 119	CC	21/05/2020	65 - 75	1 - 1XX		59,2	11,8	0,65	18,2	0,0	T1 - 3
Meirav 119	CC	08/06/2020	65 - 77	1 - 1XX		63,2	12,8	0,70	18,3	0,0	T1 - 3
Michal 6/47	CC	08/06/2020	64 - 72	1 - 1XX		56,3	11,50	0,60	19,2	0,0	T1 - 4
Michal 89/64	CC	29/04/2020	63 - 75	2 - 1XX		58,9	10,4	0,50	20,8	0,0	T1 - 3
Michal 89/64	CC	21/05/2020	62 - 72	2 - 1XX		60,8	10,7	0,50	21,4	0,0	T1 - 3
Michal 89/64	CC	08/06/2020	60 - 72	2 - 1XX		55,4	10,9	0,55	19,8	0,0	T1 - 2
Michal 89/64	CC	15/07/2020	67 - 76	1 - 1XX		51,1	12,8	0,50	25,6	0,0	T1 - 3
Nadorcott	CC	21/05/2020	70 - 83	1X 1XXX	-	53,2	9,7	5,50	1,8	0,0	T2 - 3
Nadorcott	CC	08/06/2020	68 - 82	1X 1XXX	-	63,3	11,9	0,95	12,5	0,0	T1 - 3
Nadorcott	CC	01/07/2020	64 - 76	1 - 1XX		51,1	11,5	0,85	13,5	0,6	T1 - 3
Nadorcott	CC	15/07/2020	67 - 85	1 - 1XXX		63,9	10,5	0,70	15,0	1,8	T1 - 3
Nova ARC	CC	29/04/2020	68 - 76	1X - 1XX		63,6	11,1	0,80	13,9	0,0	T2 - 4
Nova ARC	CC	21/05/2020	73 - 78	1XX 1XXX	-	53,3	11,5	0,70	16,4	0,0	T1 - 3
Nova (Control)	CC	29/04/2020	68 - 79	1X 1XXX	-	12,8	10,2	0,70	14,6	2,3	T2 - 4
Nova (Control)	CC	21/05/2020	73 - 85	1XX 1XXX	-	54,4	9,9	0,70	14,1	0,8	T1 - 3
Or 1	CC	21/05/2020	60 - 75	2 - 1XX		50,0	10,7	0,75	14,3	0,0	T2 - 4
Or 1	CC	01/07/2020	56 - 79	3 - 1XXX		50,0	12,5	0,65	19,2	0,0	T1 - 3
Or 1	CC	15/07/2020	65 - 74	1 - 1XX		68,3	14,5	0,65	22,3	0,0	T1 - 3
Or 1	CC	04/08/2020	57 - 88	3 - 1XXX		63,1	15,1	0,85	17,8	0,0	T1 - 3
Or 4	CC	21/05/2020	72 - 84	1XX 1XXX	-	51,2	10,3	0,55	18,7	0,0	T1 - 3
Or 4	CC	01/07/2020	56 - 80	3 - 1XXX		71,6	11,8	0,65	18,2	0,0	T1 - 3
Or 4	CC	15/07/2020	53 - 79	4 - 1XXX		61,2	14,4	0,75	19,2	0,0	T1 - 3
Or 4	CC	04/08/2020	60 - 76	2 - 1XX		59,0	14,7	0,90	16,3	0,0	T1 - 3
RHM	CC	04/05/2020	68 - 76	1X - 1XX		62,3	10,7	0,45	23,8	0,0	T1 - 3
RHM	CC	21/05/2020	66 - 85	1 - 1XXX		54,9	10,3	0,45	22,9	0,0	T2 - 3
RHM	CC	01/07/2020	63 - 81	2 - 1XXX		58,6	12,4	1,00	12,4	1,5	T1 - 3
RHM	CC	15/07/2020	65 - 82	1 - 1XXX		67,2	15,0	1,10	13,6	1,5	T1 - 3
Saint Andre	CC	04/05/2020	68 - 79	1X 1XXX	-	50,5	10,3	0,65	15,8	0,0	T1 - 3
Saint andre	CC	21/05/2020	69 - 80	1X 1XXX	-	52,5	9,8	0,65	15,1	0,0	T1 - 3
Samba	CC	04/05/2020	71 - 81	1X 1XXX	-	56,1	9,2	0,60	15,3	0,0	T1 - 2
Sirio	CC	29/04/2020	73 - 85	1XX 1XXX	-	53,5	9,4	0,75	12,5	0,0	T2 - 4
Sirio	CC	21/05/2020	70 - 88	1X 1XXX	-	48,3	10,1	0,80	12,6	1,7	T1 - 3

Sugar Belle	CC	29/04/2020	68 - 75	1X - 1XX	64,0	11,6	1,75	6,6	7,0	T2 - 4
Sugar Belle	CC	21/05/2020	60 - 75	2 - 1XX	63,2	12,5	1,35	9,3	6,0	T1 - 3
Tambor 2	CC	01/07/2020	69 - 74	1x - 1xx	66,5	10,1	0,90	11,2	0,0	T1 - 3
Tambor 2	CC	15/07/2020	68 - 92	1X 1XXX	62,3	11,6	0,85	13,6	5,7	T1 - 3
Tambor 2	CC	04/08/2020	75 - 83	1XX 1XXX	57,7	11,4	1,00	11,4	4,8	T1 - 3
Tambor 1	CC	01/07/2020	79 - 89	1XXX	57,2	10,9	0,85	12,8	3,8	T1 - 3
Tambor 1	CC	15/07/2020	78 - 89	1XXX	58,6	11,2	0,85	13,2	5,2	T1 - 3
Tambor 1	CC	04/08/2020	79 - 93	1XXX	56,5	11,4	0,85	13,4	8,2	T1 - 3
Tango	CC	29/04/2020	67 - 78	1 - 1XXX	60,0	10,5	0,70	15,0	0,0	T3 - 5
Tango	CC	21/05/2020	65 - 83	1 - 1XXX	51,8	10,5	0,65	16,2	0,0	T2 - 3
Tango	CC	08/06/2020	69 - 82	1X 1XXX	55,7	10,3	0,60	17,2	0,0	T1 - 3
Tango	CC	15/07/2020	70 - 84	1X 1XXX	50,8	12,0	0,55	21,8	0,0	T1 - 3
Tango	CC	04/08/2020	67 - 88	1 - 1XXX	47,2	12,2	0,65	18,8	0,0	T1 - 3
Tanor Late	CC	01/07/2020	70 - 88	1X 1XXX	46,1	10,7	1,05	10,2	0,0	T1 - 3
Tasty 1	CC	08/06/2020	65 - 80	1 - 1XXX	60,9	11,1	0,60	18,5	0,0	T1 - 4
Tasty 2	CC	21/05/2020	73 - 84	1XX 1XXX	54,9	10,4	0,60	17,3	0,0	T1 - 3
Tasty 2	CC	08/06/2020	73 - 93	1XX- 1XXX	61,0	11,2	0,70	16,0	0,0	T1 - 2
Winola	CC	21/05/2020	59 - 78	2 - 1XXX	64,3	12,2	1,25	9,8	0,0	T1 - 3

#### 5.4.9 PROGRESS REPORT: Evaluation of Navel selections in the intermediate production areas (Karino)

Project 963B by J. Joubert (CRI)

##### Summary

In the Nelspruit production area, M7 matures first with good internal quality (high Brix) for the trial, followed by Fukumoto 1 and 2 with medium to large fruit size for this season and low acid levels. Bahianinha matures next with good juice levels and earlier external colour on the fruit. Clarke, Fischer and Dream matured next, towards the middle of the navel orange range, with medium to large fruit size and good Brix levels (up to 12.8). De Wet 1 and Hutton grouped well with Dream and Clarke this season, with good Brix and juice content. Kirkwood Red, the only red pigmented navel included in the trial, fits in well with the mid-maturing options. The late selections will include Glenora Late and Gloudi with good acids, followed by Lane Late and Suitangi. The ultra-late bracket will be filled with Carninka ending of the navel season.

##### Opsomming

In die Nelspruit produksie area word M7 eerste ryp met goeie interne kwaliteit (hoë Brix) vir hierdie proef, gevolg deur Fukumoto 1 and 2 met medium tot groot vrugsgrootte vir hierdie seisoen en lae suur vlakke. Bahianinha word volgende ryp met goeie sap vlakke en vroeër eksterne kleur op die vrugte. Clarke, Fischer en Dream volg, om die middel van die nawel soetleemoen reeks te vul, met medium tot groot vrugsgrootte en goeie Brix vlakke (tot 12.8). De Wet 1 en Hutton se rypwordings inligting plaas die kultivar saam met Dream en Clarke met goeie Brix en sap inhoud. Kirkwood Red, die enigste rooi gepigmenteerde nawel wat in die proef ingesluit is, pas goed in by die mid-rypwordende opsies. Die laat seleksies sluit in Glenora Late en Gloudi met goeie sure, gevolg deur Lane Late en Suitangi. Die ultra-laat gaping word gevul deur Carninka wat die nawel seisoen afsluit.

##### Objectives

- To select navel cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (juice, Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new navel cultivars and to determine the climatic suitability of these cultivars in intermediate production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on navel selections from Karino-koöp (Nelspruit) in the Mpumalanga region.

When the ratio between sugar and acid is 10:1, the navel fruit is considered to be at peak maturity. A ratio of 9:1 is considered to be the build-up towards peak maturity of 10:1. After reaching the peak, the ratio increases to 11:1, after which the fruit are considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in greater instances of quality and rind issues.

**Table 5.4.9.1.** List of navel selections evaluated at Karino-koöp (Nelspruit) during the 2020 season.

Selection	Rootstock	Planted
Bahianinha	CC	2017
Carninka	CC	2017
Clarke	CC	2017
De Wet 1	CC	2017
Dream	CC	2017
Fischer	CC	2017
Fukumoto 1&2	C35/CC/SC	2017
Glenora Late	CC	2017
Gloudi	CC	2017
Hutton	CC	2017
Kirkwood Red	CC	2017
Lane Late	CC	2017
Lazy Boy 1	CC	2017
M7	CC	2017
Suitangi	CC	2017
Witkrans	CC	2017

## Results and discussion

The new trial block trees at Karino-koöp were evaluated for the first time this season; this having an impact on the quality and quantity of the fruit. More information was available and future evaluations will be conducted.

The internal quality varied from fair to good on the first crop and due to high rainfall figures later in the summer season, acids on all the selections (early and late), was on the low side (peaked from 0.45 up to 0.80%). The exception on internal quality was Gloudi and Glenora Late, two of the late nawels included in the trial with acids above 1.0%. Juice (average 52.2%) and Brix levels (up to 12.6 – Glenora Late) were good to very good this season; external colour peaked from T 1 to T3 /4 at peak maturity early in the season. Fruit size distribution peaked from count 88 to 48/40 and will be in demand for export purposes.

## Conclusion

This was the first evaluation of all the navel selections at this new trial site in Karino, so information becomes available and future evaluations will improve these cultivars recommendations. The juice levels on most of the combinations improved from low to average/good; above the minimum export requirement of 48% with the exception of Fisher and Fukumoto. Acids remained low from the beginning of the season up to peak maturity with the exception of Gloudi and Glenora Late. The external colour development improved on all the selections the season, creating a more ideal situation with better internal quality and more specifically high Brix levels.

Future evaluations will be crucial to determine the performance of these early to mid/late-navel selections for the Karino area.

**Table 5.4.9.2.** Internal fruit quality data for Navel selections at Karino- koöp (Nelspruit) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Babianinha	CC	21/05/2020	80 - 94	64 - 40	54,3	11,0	0,70	15,7	0,0	T1 - 5
Babianinha	CC	08/06/2020	79 - 96	64 - 40	52,5	10,6	0,75	14,1	0,0	T1 - 4
Cambria	CC	01/07/2020	66 - 81	105 - 64	52,8	10,6	0,65	16,3	0,0	T1 - 5
Carninka	CC	21/05/2020	79 - 92	64 - 40	51,4	10,3	0,65	15,8	0,0	T3 - 5
Carninka	CC	01/07/2020	75 - 86	72 - 56	51,6	10,6	0,65	16,3	0,0	T1 - 3
Carninka	CC	15/07/2020	69 - 96	88 - 40	52,3	11,7	0,75	15,6	0,0	T1 - 3
Clarke	CC	08/06/2020	72 - 90	88 - 40	53,6	12,8	0,90	14,2	0,0	T1 - 4
Clarke	CC	01/07/2020	68 - 89	88 - 48	50,0	10,7	0,80	13,4	0,0	T1 - 4
De Wet 1	CC	21/05/2020	79 - 89	64 - 48	50,0	9,9	0,60	16,5	0,0	T1 - 5
De Wet 1	CC	08/06/2020	75 - 82	72 - 56	57,7	11,2	0,75	14,9	0,0	T1 - 5
Dream	CC	21/05/2020	82 - 93	56 - 40	50,3	8,8	0,55	16,0	0,0	T2 - 5
Dream	CC	08/06/2020	67 - 85	67 - 85	62,4	10,9	0,65	16,8	0,0	T1 - 3
Fisher	CC	21/05/2020	79 - 93	64 - 40	44,4	9,7	0,55	17,6	0,0	T2 - 5
Fukumoto 1	C35	04/05/2020	83 - 89	56 - 48	50,5	9,7	0,50	19,4	0,0	T2 - 4
Fukumoto 1	C35	21/05/2020	78 - 92	64 - 40	47,6	9,7	0,50	19,4	0,0	T2 - 4
Fukumoto 1	CC	04/05/2020	75 - 88	72 - 48	47,4	10,5	0,50	21,0	0,0	T1 - 3
Fukumoto 1	CC	21/05/2020	79 - 89	64 - 48	54,1	11,0	0,65	16,9	0,0	T1 - 4
Fukumoto 1	SC	04/05/2020	79 - 88	64 - 48	47,7	9,3	0,50	18,6	0,0	T2 - 4
Fukumoto 1	SC	21/05/2020	75 - 93	72 - 40	45,3	9,4	0,50	18,8	0,0	T3 - 5
Fukumoto 2	C35	04/05/2020	80 - 93	64 - 40	50,5	9,7	0,45	21,6	0,0	T2 - 4
Fukumoto 2	C35	21/05/2020	77 - 93	72 - 40	46,1	10,5	0,40	26,3	0,0	T1 - 4
Fukumoto 2	CC	04/05/2020	80 - 91	64 - 40	49,9	9,4	0,50	18,8	0,0	T2 - 4
Fukumoto 2	SC	04/05/2020	79 - 88	64 - 48	51,1	9,0	0,50	18,0	0,0	T2 - 4
Fukumoto 2	SC	21/05/2020	83 - 94	56 - 40	46,2	9,8	0,50	19,6	0,0	T1 - 4
Glenora Late	CC	01/07/2020	71 - 81	88 - 64	52,4	12,6	0,85	14,8	0,0	T1 - 4
Glenora Late	CC	15/07/2020	82 - 91	56 - 40	51,1	11,2	1,05	10,7	0,0	T1-3
Glenora Late	CC	04/08/2020	73 - 89	72 - 48	50,0	11,4	0,70	16,3	0,0	T1 - 4
Gloudi	CC	01/07/2020	67 - 79	105 - 64	52,9	10,8	1,10	9,8	0,0	T1 - 3
Gloudi	CC	15/07/2020	62 - 83	125 - 56	53,7	11,6	1,05	11,1	0,0	T1 - 4
Gloudi	CC	04/08/2020	79 - 90	64 - 40	52,4	11,3	1,05	10,8	0,0	T1 - 3
Hutton	CC	08/06/2020	85 - 90	56 - 40	55,4	11,6	0,70	16,6	0,0	T1 - 3
Hutton	CC	01/07/2020	72 - 85	88 - 56	50,5	10,9	0,75	14,5	0,0	T1 - 3

Kirkwood Red	CC	08/06/2020	82 - 90	56 - 48	53,2	10,3	0,85	12,1	0,0	T1 - 5
Kirkwood Red	CC	01/07/2020	72 - 89	88 - 48	57,5	11,0	0,75	14,7	0,0	T1 - 3
Kirkwood Red	CC	15/07/2020	72 - 93	88 - 40	59,3	10,9	0,75	14,5	0,0	T1 - 3
Lane Late	CC	21/05/2020	71 - 85	88 - 56	54,4	9,8	0,85	11,5	0,0	T2 - 5
Lane Late	CC	01/07/2020	78 - 92	64 - 40	52,3	11,4	0,75	15,2	0,0	T1 - 3
Lane Late	CC	15/07/2020	72 - 93	88 - 40	61,4	12,7	0,70	18,1	0,0	T1 - 3
Lazy boy 1	CC	21/05/2020	76 - 83	72 - 56	53,5	10,1	0,65	15,5	0,0	T4 - 5
Lazy boy 1	CC	01/07/2020	75 - 86	72 - 56	51,7	10,0	0,55	18,2	0,0	T1 - 4
M7	CC	04/05/2020	79 - 93	64 - 40	51,2	11,5	0,70	16,4	0,0	T1 - 3
M7	CC	21/05/2020	80 - 94	64 - 40	51,2	12,4	0,65	19,1	0,0	T1 - 3
Suitangi	CC	01/07/2020	73 - 84	72 - 56	53,8	10,4	0,65	16,0	0,0	T1 - 4
Suitangi	CC	15/07/2020	90 - 92	48 - 40	54,8	11,9	0,65	18,3	0,0	T1 - 4
Witkrans	CC	21/05/2020	73 - 81	72 - 64	56,1	10,2	0,75	13,6	0,0	T3 - 6
Witkrans	CC	01/07/2020	72 - 81	88 - 64	53,6	11,1	0,80	13,9	0,0	T1 - 4
Witkrans	CC	15/07/2020	68 - 89	88 - 48	53,5	11,1	0,75	14,8	0,0	T1 - -3

#### 5.4.10 PROGRESS REPORT: Evaluation of Mandarin hybrid selections in the cool-inland production areas (Orighstad and Burgersfort)

Project 990A by J. Joubert (CRI)

#### Summary

Nova, Nova SL and Saint André mature first according to the results of the 2020 season for the cool inland production areas, and all three selections developed large to extra-large fruit calibre and good internal quality. Ma`ayana, Samba and RHM with high juice levels and Meirav 119, fit in before Leanri with large fruit size, which follows. Furr developed the highest seed count per fruit for this trial under net and in the open blocks followed by RHM, developing the second-highest seed count per fruit. The mid-maturing mandarins are represented by ARCCIT 9 LS Nadorcott and ARC Nadorcott, which developed good Brix levels and higher acids than the other selections (up to acid of 0.9%) this season. Picking periods should not be longer than 3 - 4 weeks to maintain good internal quality and to avoid rind disorders.

#### Opsomming

Nova, Nova SL and Saint André word die vroegste ryp volgens resultate van die 2020 seisoen vir hierdie koel binnelandse produksie area, met al drie seleksies groot tot ekstra-groot vrugt kaliber en goeie interne kwaliteit. Ma`ayana, Samba en RHM, met hoë sap vlakke en Meirav 119, pas in voor Leanri, wat daarna volg met die groot vruggrootte. Furr het die hoogste saadtellings per vrug ontwikkel vir hierdie proef onder net en in die oop blokke. Volgende is RHM, met die tweede hoogste saad telling per vrug. Die middel van die mandaryne word verteenwoordig deur ARCCIT 9 LS Nadorcott en ARC Nadorcott, met goeie Brix vlakke en hoër sure in vergelyking met die ander seleksies (tot suur van 0.9%). Daar word aanbeveel om nie die oesperiode langer as 3 tot 4 weke te verleng nie om goeie interne kwaliteit te verseker met minimum skil probleme.

#### Objectives

- To select Mandarin Hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new Mandarin Hybrid cultivars and to determine the climatic suitability of these cultivars in cool, inland production regions.

#### Materials and methods

Field evaluations and laboratory analyses were conducted on Mandarin Hybrid selections from Mountain Haven (Orighstad), Waterval (Burgersfort), Futsela Iglobhu Investment (Ryton/Ngodwana) and Buffelsfontein (Mooinooi).

**Table 5.4.10.1.** List of Mandarin Hybrid selections evaluated at Mountain Haven (Orighstad) during the 2020 season.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
ARCCIT 9 LS	CC	2017
Ma`ayana (Dina)	CC	2017
Etna	CC	2017
Furr	CC	2017
Gloudup	CC	2017
IRM 1&2	CC	2017
Leanri	CC	2017
Meirav 119	CC	2017
Mor 26	CC	2017
Nadorcott	CC	2017
Nova ARC	CC	2017
Nova	CC	2017
Or 1&4	CC	2017
Page	CC	2017
RHM	CC	2017
Saint André	CC	2017
Samba	CC	2017
Shani SL	CC	2017
Sirio	CC	2017
Tambor 1&2	CC	2017
Taylor Lee	CC	2017
Tasty 1	CC	2017

**Table 5.4.10.2.** List of Mandarin Hybrid selections evaluated at Waterval (Burgersfort) during the 2020 season.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
ARCCIT 9 LS	CC	2017
Etna		
Furr	CC	2017
Leanri	CC	2017
Ma`ayana (Dina)	CC	2017
Meirav 119	CC	2017
Nova	CC	2017
Page	CC	2017
RHM	CC	2017
Saint André	CC	2017
Samba	CC	2017

**Table 5.4.10.3.** List of Mandarin Hybrid selections evaluated at Futsela Iglobhu Investment (Ryton/Ngodwana) during the 2020 season.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
ARCCIT 9 LS	CC	2017

Etna	CC	2017
Furr	CC	2017
Goldup	CC	2017
IRM 1&2	CC	2017
Leanri	CC	2017
Ma`ayana (Dina)	CC	2017
Meirav 119	CC	2017
Mor 26	CC	2017
Nadorcott (Control)	CC	2017
Nova	CC	2017
Nova ARC	CC	2017
Page	CC	2017
RHM	CC	2017
Saint André	CC	2017
Samba	CC	2017
Shani SL	CC	2017
Sirio	CC	2017
Tambor 1&2	CC	2017
Tanor Late	CC	2017
Tasty 1	CC	2017
Taylor Lee	CC	2017

**Table 5.4.10.4.** List of Mandarin Hybrid selections evaluated at Buffelsfontein (Mooinooi) during the 2020 season.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
ARCCIT 9 LS	CC	2017
Bruce	CC	2017
Etna	CC	2017
Furr	CC	2017
Goldup	CC	2017
IRM 1&2	CC	2017
Ma`ayana (Dina)	CC	2017
Meirav 119	CC	2017
Mor 26	CC	2017
Nadorcott (Control)	CC	2017
Nova	CC	2017
Nova ARC	CC	2017
Or 1	CC	2017
Or 4	CC	2017
Page	CC	2017
RHM	CC	2017
Saint André	CC	2017
Samba	CC	2017
Shani SL	CC	2017
Sirio	CC	2017

Tambor 1&2	CC	2017
Tasty 1	CC	2017
Taylor Lee	CC	2017

## Results and discussion

The trees at Mountain Haven and Waterval bore their second crop for this season with improved fruit numbers and more mature tree internal quality and fruit size characteristics. Mountain Haven was planted under net completely; Waterval consists of two adjacent trial blocks, one side under net and the other side open, duplicating the same selections.

The new additional trial blocks at Futsela Iglobhu Investment (Ryton) and Mooinooi bore their first crop on the trees; additional information to add for future evaluations.

For Mandarin Hybrid selections, a ratio of 11:1 is considered to be the build-up towards peak maturity, with a ratio of 12:1. After reaching the peak, the ratio increases to 13:1, after which the fruit is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

### Ma`ayana (Dina)

The internal quality of Ma`ayana was good this season at Mountain Haven, with high juice levels (average 55%) and no granulation problems in the fruit compared to Nova. Juice level at Waterval under the net was (52.3%) compared to higher juices (average 54.6%) in the open plantings. Brix (average 10.5) and similar acids (0.9%), indicating the early-mid maturing characteristics of the selection in the cool inland production areas, with seedless fruit this season. The fruit size peaked from large to extra-large, count 1 to count 1XX/1XXX.

### Furr (Clemcott)

Furr developed large to extra-large fruit size (count 1X – 1XXX) on the trees at three trial sites (average bigger size counts at Waterval, smallest at Buffelsfontein), one of the cultivar's characteristics as well as a good crop on the trees. The external colour development on the fruit was good for the cool areas, as expected (T1-3/4); slight delay at the Mooinooi site. Internally the fruit quality was very good, developing high juice (up to 62%) and Brix (up to 14.0) levels with good acids. Another quality of the fruit is the high seed count (high self-and cross pollination, up to 18 seeds per fruit). Maturity seems to be middle to the end of May to the middle of June for the cool production areas, according to the information in Table 5.4.10.5.

### Leanri

Leanri developed a fairly large to extra-large fruit size between count 2/1 and 1XXX, smaller compared to the hot production areas with the smallest fruit produced this season at Ryton. 2020 was the first (Ryton, Mooinooi) and third (Mountain Haven, Waterval) crop on the trees and internal quality was good (Mountain Haven) to very good (Waterval) on the Carrizo rootstocks (average juice 53%, Brix 11, acid 0.9%). Seed numbers were fairly low; completely seedless up to 3.3 seeds per fruit.

### Meirav 119

Meirav 119 developed a deep orange rind colour (T1 to 3 with peak maturity). Internal quality improved; juice content was better at Waterval compared to Mountain Haven, average to good (average above 55.4% up to 55.7%), Brix of 12 and acids above 0.8%. The fruit evaluated was seedless at Mountain Haven and low seeded at Waterval (0.5 seeds per fruit).

### Nadorcott & ARCCIT 9 LS

The crop on both selections was good this season to evaluate. The fruit shape was very similar to the Nadorcott selection. Rind texture was very smooth with a natural shine (similar to packhouse waxing). Both selections produced a fruit size that ranged from count 1 and peaked at count 1XX/1XXX even with the better crop load on the trees. Nadorcott ARC and ARCCIT 9 LS produced low seed numbers in the fruit (up to 1.6 seeds per

fruit only with one evaluation) this season. Maturity seems to be two weeks earlier on the ARCCIT 9 LS selection, according to Table 5.4.10.3, but the information was limited due to the first crop on the trees being evaluated (beginning to middle of June).

#### Nova, Nova SL, Saint André

Nova was included at Waterval as an early maturing control, and as a control for Nova SL and Saint André in the trial at Mountain Haven. The fruit is fairly difficult to peel and low numbers of seed were discovered in the fruit with some of the evaluations. External colour was later on Nova and Saint André, and the fruit size varied between count 2/1 and count 1XX/XXX (medium/large to extra-large fruit); smaller fruit size at Mooinooi and Rytton (cooler areas in summer). Nova SL (ARC) produced a coarse rind texture on the fruit compared to the other two selections. The acid levels at Mountain Haven in the fruit was similar compared to higher acids under the net structures at Waterval than the open block, and the external colour development better (T1 to 2).

#### RHM

RHM cropped fruit for the third time this season with low seed numbers at Waterval under net and open blocks (from 0.8 up to 6.3 seeds per fruit), and even lower numbers in the net block at Mountain Haven (avg. 1.3 seeds per fruit) with the crosspollination. There was a delayed colour development (T2 to 4/5) from the first evaluation with low acids (avg. 0.70%), indicating peak maturity Brix: acid ratio over 12. Future evaluations will determine optimum quality of the fruit evaluated.

#### Samba

Samba on Carrizo rootstock produced a good second crop with good internal quality on the larger fast growing thornless trees at both trial sites. Colour development was better early in the season (deep orange) at Mountain Haven with higher acids (0.80%) compared to Waterval, with a smooth rind texture on the fruit. Fruit was completely seedless this season, except for two evaluations in the open block at Waterval, in the combined trial blocks (future evaluations will confirm low seed numbers) and peaked from medium to large fruit size at Mountain Haven (average count 2 to 1XX, cooler area) and count 1X to 1XXX at Waterval (large to extra-large fruit size). Internal quality was good with similar higher juice's at Orighstad and Burgersfort (avg. 55 to 57%), high Brix levels (from 10 to 12), and lower acids (avg. 0.7 to 0.8%). Based on the internal quality results in Table 5.4.10.5, the estimated maturity will be middle to the end of April.

#### Additional selections

Page matured before Samba; juice and Brix was higher at Waterval and the opposite at Mahela with delayed colour development, but similar acids on the lower side for the cooler regions, except for Mountain Haven where Samba developed good acids throughout the season.

There are new selections in the four trial sites bearing their first crop and future evaluations will include performance.

#### **Conclusion**

This was the second evaluation of all the sections at the two trial sites, with Mountain Haven (Orighstad) cooler than Burgersfort. Trees are still young and fruit quality will improve with future evaluations.

The highest seed numbers were on Page, RHM, Saint André, followed then by Leanri with lower numbers in the open trial block at Waterval (cross-pollination). All the other selections developed very low seed numbers in the fruit. Ma`ayana, Meirav 119, Leanri, Samba and the Nadorcott selections performed well with deep orange colour development, as well as the Nova selections and Saint André. RHM continued with delayed external colour development on the fruit and lower acids and large fruit size at Waterval.

**Table 5.4.10.5.** Internal fruit quality data for Mandarin hybrid selections at Mountain Haven (Orighstad), Waterval (Burgersfort), Futsela Iglobhu Investment (Ryton) and Mooinooi during the 2020 season.

Mountain Haven										
Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
ARCCIT 9 LS	CC	15/04/2020	71 - 80	1X - 1XXX	52,4	9,9	0,70	14,1	0,0	T2 - 4
ARCCIT 9 LS	CC	02/06/2020	70 - 84	1X - 1XXX	53,2	10,0	0,85	11,8	0,0	T2 - 4
ARCCIT 9 LS	CC	22/06/2020	70 - 88	1X - 1XXX	49,5	10,1	0,75	13,5	0,0	T1 - 3
Ma'ayana (Dina)	CC	22/04/2020	69 - 76	1X - 1XX	56,7	11,2	0,90	12,4	0,0	T3 - 5
Ma'ayana (Dina)	CC	15/05/2020	77 - 85	1XX - 1XXX	53,5	11,6	1,00	11,6	0,0	T1 - 3
Ma'ayana (Dina)	CC	02/06/2020	74 - 83	1XX - 1XXX	54,3	12,2	1,10	11,1	0,0	T1 - 3
Etna	CC	25/03/2020	70 - 74	1X - 1XXX	58,5	9,2	0,80	11,5	0,0	T6 - 7
Etna	CC	22/04/2020	74 - 80	1XX - 1XXX	54,4	9,2	0,70	13,1	0,0	T2 - 3
Etna	CC	15/05/2020	77 - 85	1XX - 1XXX	50,9	9,9	0,65	15,2	0,0	T1 - 3
Goldup	CC	25/03/2020	63 - 74	2 - 1XX	46,1	11,1	0,70	15,9	0,0	T6 - 7
Goldup	CC	22/04/2020	71 - 77	1X - 1XX	36,0	9,0	0,60	15,0	0,0	T1 - 3
IRM 1	CC	22/06/2020	66 - 73	1 - 1XX	61,2	13,4	1,30	10,3	0,0	T1 - 3
IRM 1	CC	20/07/2020	70 - 76	1X - 1XX	62,0	15,4	1,40	11,0	2,0	T1 - 3
IRM 2	CC	22/04/2020	62 - 73	2 - 1XX	59,0	10,3	1,30	7,9	0,0	T3 - 5
IRM 2	CC	15/05/2020	67 - 77	1 - 1XX	59,1	10,9	1,20	9,1	0,8	T2 - 4
IRM 2	CC	02/06/2020	65 - 72	1 - 1XX	57,3	11,4	1,05	10,9	0,3	T1 - 4
IRM 2	CC	22/06/2020	64 - 79	1 - 1XXX	63,5	12,4	1,25	9,9	1,3	T1 - 3
IRM 2	CC	20/07/2020	65 - 72	1 - 1XX	62,0	14,5	1,20	12,1	1,2	T1 - 3
Leanri	CC	22/04/2020	71 - 82	1X - 1XXX	52,9	10,7	0,56	19,1	0,0	T3 - 5
Leanri	CC	15/05/2020	69 - 81	1X - 1XXX	52,2	10,3	0,90	11,4	0,0	T2 - 4
Leanri	CC	02/06/2020	73 - 80	1XX - 1XXX	56,2	11,1	0,90	12,3	0,0	T1 - 3
Meirav 119	CC	22/04/2020	68 - 77	1X - 1XX	56,8	10,9	0,85	12,8	1,3	T3 - 5
Meirav 119	CC	02/06/2020	69 - 83	1X - 1XXX	54,3	11,7	0,75	15,6	0,0	T1 - 3
Meirav 119	CC	22/06/2020	75 - 92	1XX - 1XXX	55,2	11,7	0,80	14,6	0,0	T1 - 3
Mor 26	CC	15/05/2020	67 - 78	1 - 1XXX	56,8	11,8	1,15	10,3	0,0	T2 - 5
Mor 26	CC	02/06/2020	65 - 77	1 - 1XX	62,0	12,6	1,10	11,5	0,0	T1 - 4
Mor 26	CC	22/06/2020	60 - 75	2 - 1XX	62,2	13,5	1,10	12,3	0,0	T1 - 3
Mor 26	CC	20/07/2020	63 - 79	2 - 1XXX	61,3	15,6	0,95	16,4	0,0	T1 - 3
Nadorcott	CC	22/04/2020	65 - 76	1 - 1XX	52,5	9,7	1,00	9,7	0,0	T2 - 4
Nadorcott	CC	15/05/2020	65 - 74	1 - 1XX	47,9	10,3	0,85	12,1	0,0	T1 - 3
Nadorcott	CC	02/06/2020	70 - 82	1X - 1XXX	44,8	10,9	0,85	12,8	3,0	T1 - 3
Nadorcott	CC	22/06/2020	67 - 86	1 - 1XXX	59,4	11,4	0,90	12,7	0,0	T1 - 3
Nadorcott	CC	20/07/2020	69 - 78	1X - 1XXX	46,3	12,5	0,80	15,6	0,8	T1 - 3
Nova ARC	CC	25/03/2020	65 - 76	1 - 1XX	52,7	10,5	0,95	11,1	0,0	T3 - 4
Nova ARC	CC	22/04/2020	76 - 87	1XX - 1XXX	60,0	10,0	0,80	12,5	0,0	T3 - 5
Nova ARC	CC	15/05/2020	81 - 90	1XXX	45,2	9,5	0,85	11,2	0,0	T1 - 4
OR 1	CC	22/06/2020	68 - 79	1X - 1XXX	49,4	10,6	0,90	11,8	0,7	T1 - 3
OR 4	CC	20/07/2020	63 - 69	2 - 1X	56,8	14,2	0,80	17,8	0,0	T1 - 3
Page	CC	25/03/2020	65 - 69	1 - 1X	55,8	9,7	0,85	11,4	0,0	T6 - 7
Page	CC	22/04/2020	65 - 74	1 - 1XX	52,8	10,3	0,70	14,7	0,0	T2 - 3
RHM	CC	15/05/2020	63 - 80	2 - 1XXX	61,2	10,8	0,65	16,6	1,3	T1 - 4
Saint Andre	CC	22/04/2020	65 - 71	1 - 1X	55,2	11,0	0,80	13,8	0,0	T1 - 3
Saint Andre	CC	15/05/2020	73 - 88	1XX - 1XXX	50,6	10,0	0,70	14,3	1,2	T1 - 3
Samba	CC	25/03/2020	60 - 68	2 - 1X	51,3	10,5	0,80	13,1	0,0	T3 - 5
Samba	CC	22/04/2020	64 - 73	1 - 1XX	55,7	11,1	0,75	14,8	0,0	T2 - 4
Samba	CC	15/05/2020	69 - 80	1X - 1XXX	55,6	11,3	0,80	14,1	0,0	T1 - 3
Samba	CC	02/06/2020	67 - 85	1 - 1XXX	57,0	11,7	0,90	13,0	0,0	T1 - 2
Shani SL	CC	22/04/2020	64 - 69	1 - 1X	54,4	10,8	1,25	8,6	0,0	T3 - 5

Shani SL	CC	15/05/2020	57 - 66	3 - 1	54,5	12,3	1,25	9,8	0,0	T2 - 4
Shani SL	CC	02/06/2020	64 - 80	1 - 1XXX	53,4	12,2	1,25	9,8	0,0	T1 - 4
Shani SL	CC	22/06/2020	65 - 76	1 - 1XX	62,9	14,2	1,40	10,1	0,0	T1 - 3
Sirio	CC	25/03/2020	72 - 78	1XX - 1XXX	46,5	6,8	0,85	8,0	0,0	T6 - 7
Sirio	CC	22/04/2020	73 - 88	1XX - 1XXX	41,6	10,4	0,85	12,2	0,0	T1 - 3
Tambor 1	CC	02/06/2020	79 - 95	1XXX	59,3	9,9	1,00	9,9	0,3	T1 - 3
Tambor 1	CC	22/06/2020	81 - 95	1XXX	54,3	9,2	1,05	8,8	0,8	T1 - 3
Tambor 1	CC	20/07/2020	79 - 94	1XXX	52,8	11,0	1,10	10,0	1,3	T1 - 3
Tambor 2	CC	15/05/2020	76 - 90	1XX - 1XXX	59,5	9,4	1,00	9,4	0,0	T2 - 4
Tambor 2	CC	02/06/2020	74 - 92	1XX - 1XXX	59,4	12,8	1,10	11,6	0,3	T2 - 4
Tambor 2	CC	22/06/2020	75 - 83	1XX - 1XXX	58,5	11,4	1,20	9,5	0,0	T1 - 3
Tambor 2	CC	20/07/2020	76 - 94	1XX - 1XXX	51,8	14,0	1,10	12,7	0,0	T1 - 3
Taylor Lee	CC	22/04/2020	68 - 73	1X - 1XX	63,3	10,6	1,00	10,6	0,0	T3 - 5
Taylor Lee	CC	15/05/2020	69 - 78	1X - 1XXX	60,9	11,7	1,05	11,1	0,7	T1 - 4
Taylor Lee	CC	22/06/2020	72 - 79	1XX - 1XXX	55,0	13,1	1,05	12,5	0,5	T1 - 3
Taylor Lee	CC	20/07/2020	65 - 73	1 - 1XX	59,2	15,9	1,15	13,8	1,3	T1 - 3
Taylor Lee	CC	11/08/2020	67 - 75	1 - 1XX	59,9	13,3	0,95	14,0	5,7	T1 - 3
Tanor Late	CC	22/06/2020	92 - 101	1XXX	42,6	11,0	0,70	15,7	0,0	T1 - 3
Tasty 1	CC	22/04/2020	75 - 83	1XX - 1XXX	49,6	9,4	0,70	13,4	0,0	T2 - 4
Tasty 1	CC	15/05/2020	76 - 85	1XX - 1XXX	42,7	10,9	0,70	15,6	1,0	T1 - 4

Waterval										
Cultivar	Root-stock	Date harvested	Fruit size (mm)	Net/Out of Net	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed
ARCCIT 9 SL	CC	22/04/2020	66 - 77	Net	1 - 1XX	53,9	11,6	1,40	8,3	0,0
ARCCIT 9 SL	CC	15/05/2020	56- 80	Net	3 - 1XXX	56,6	11,1	1,05	10,6	0,3
ARCCIT 9 SL	CC	02/06/2020	65 - 85	Net	1 - 1XXX	60,9	12,8	1,15	11,1	0,0
ARCCIT 9 SL	CC	20/07/2020	72 - 90	Net	1XX - 1XXX	46,8	14,0	1,25	11,2	0,0
ARCCIT 9 SL	CC	11/08/2020	69 - 81	Net	1X - 1XXX	56,5	14,9	1,40	10,6	0,0
ARCCIT 9 SL	CC	22/04/2020	70 - 75	Out of Net	1X - 1XX	53,8	11,2	1,15	9,7	0,0
ARCCIT 9 SL	CC	15/05/2020	65 - 76	Out of Net	1 - 1XX	55,0	12,0	1,05	11,4	0,0
ARCCIT 9 SL	CC	02/06/2020	70 - 79	Out of Net	1X - 1XXX	55,2	13,1	1,30	10,1	0,8
ARCCIT 9 SL	CC	22/06/2020	66 - 80	Out of Net	1 - 1XXX	55,6	13,3	1,20	11,1	0,0
ARCCIT 9 SL	CC	20/07/2020	73 - 84	Out of Net	1XX - 1XXX	52,4	15,3	1,45	10,6	1,6
ARCCIT 9 SL	CC	11/08/2020	68 - 84	Out of Net	1X - 1XXX	54,7	15,7	1,45	10,8	1,3
Etna	CC	22/04/2020	74 - 80	Net	1XX - 1XXX	58,4	9,5	0,70	13,6	0,0
Etna	CC	15/05/2020	80 - 95	Net	1XXX	48,7	10,7	0,65	16,5	1,8
Etna	CC	24/03/2020	75 - 84	Out of Net	1XX - 1XXX	57,5	9,2	0,80	11,5	2,7
Etna	CC	22/04//2020	74 - 87	Out of Net	1XX - 1XXX	55,3	9,6	0,70	13,7	0,0
Etna	CC	15/05/2020	77 - 88	Out of Net	1XX - 1XXX	60,8	10,7	0,65	16,5	1,4
Furr	CC	22/04/2020	81 - 86	Net	1XXX	59,9	11,4	0,85	13,4	10,8
Furr	CC	15/05/2020	79 - 90	Net	1XXX	57,2	11,1	0,95	11,7	17,0
Furr	CC	02/06/2020	78 - 94	Net	1XXX	59,7	12,9	1,05	12,3	9,4

Furr	CC	22/04/2020	82 - 94	Out of Net	1XXX	56,9	12,4	0,90	13,8	0,0
Furr	CC	02/06/2020	84 - 95	Out of Net	1XXX	61,6	13,0	1,10	11,8	7,0
Furr	CC	20/07/2020	84 - 93	Out of Net	1XXX	54,9	14,4	1,10	13,1	11,8
IRM 1	CC	22/04/2020	65 - 76	Net	1 1XX	56,2	12,2	1,20	10,2	7,7
IRM 1	CC	02/06/2020	75 - 84	Net	1XX - 1XXX	59,3	12,9	1,20	10,8	3,2
IRM 1	CC	22/06/2020	72 - 85	Net	1XX - 1XXX	59,7	12,4	1,00	12,4	2,7
IRM 1	CC	20/07/2020	75 - 84	Net	1XX - 1XXX	61,2	13,5	1,20	11,3	2,2
IRM 1	CC	02/06/2020	65 - 78	Out of Net	1 - 1XXX	60,3	14,5	1,45	10,0	5,4
IRM 1	CC	22/06/2020	73 - 82	Out of Net	1XX	61,3	14,1	0,95	14,8	2,1
IRM 1	CC	20/07/2020	69 - 78	Out of Net	1X - 1XXX	62,9	16,5	1,30	12,7	5,5
IRM 2	CC	15/05/2020	70 - 86	Net	1X - 1XXX	55,6	11,9	0,75	15,9	1,7
IRM 2	CC	02/06/2020	75 - 80	Net	1XX - 1XXX	62,2	10,9	0,90	12,1	1,7
IRM 2	CC	22/06/2020	69 - 80	Net	1X - 1XXX	62,4	11,8	0,95	12,4	0,0
IRM 2	CC	20/07/2020	67 - 82	Net	1 - 1XXX	60,6	11,9	0,95	12,5	2,0
IRM 2	CC	02/06/2020	65 - 75	Out of Net	1 - 1XX	63,2	13,1	1,10	11,9	0,8
IRM 2	CC	22/06/2020	72 - 85	Out of Net	1XX - 1XXX	64,5	10,9	0,85	12,8	1,5
IRM 2	CC	20/07/2020	64 - 81	Out of Net	1 - 1XXX	61,6	12,0	0,80	15,0	2,7
Leanri	CC	22/04/2020	77 - 83	Net	1XX - 1XXX	60,2	10,9	1,00	10,9	0,0
Leanri	CC	15/05/2020	76 - 95	Net	1XX - 1XXX	56,0	10,6	0,90	11,8	0,0
Leanri	CC	02/06/2020	77 - 95	Net	1XX - 1XXX	52,3	10,5	1,00	10,5	0,0
Leanri	CC	22/04/2020	75 - 84	Out of Net	1XX - 1XXX	56,1	12,7	0,80	15,9	2,3
Leanri	CC	15/05/2020	67 - 80	Out of Net	1 - 1XXX	59,2	12,5	0,85	14,7	3,2
Leanri	CC	02/06/2020	68 - 85	Out of Net	1X - 1XXX	48,4	13,3	0,95	14,0	3,3
Ma'ayana (Dina)	CC	15/05/2020	80 - 87	Net	1XXX	52,9	11,5	0,90	12,8	0,0
Ma'ayana (Dina)	CC	02/06/2020	78 - 90	Net	1XXX	51,8	12,4	1,15	10,8	0,0
Ma'ayana (Dina)	CC	22/04/2020	68 - 77	Out of Net	1X - 1XX	55,7	11,1	0,80	13,9	0,0
Ma'ayana (Dina)	CC	15/05/2020	73 - 84	Out of Net	1XX - 1XXX	53,6	10,8	0,90	12,0	0,0
Meirav 119	CC	22/04/2020	69 - 80	Net	1X - 1XXX	55,8	12,4	0,95	13,1	0,0
Meirav 119	CC	15/05/2020	65 - 85	Net	1 - 1XXX	58,2	12,2	0,89	13,7	1,5
Meirav 119	CC	20/07/2020	69 - 82	Net	1X - 1XXX	53,2	16,8	1,20	14,0	1,5
Meirav 119	CC	22/04/2020	66 - 74	Out of Net	1 - 1XX	55,7	11,5	1,05	11,0	2,3
Meirav 119	CC	15/05/2020	64 - 72	Out of Net	1 - 1XX	56,8	11,3	0,85	13,3	0,8
Meirav 119	CC	02/06/2020	79 - 85	Out of Net	1XXX	57,1	13,6	0,95	14,3	1,4
Meirav 119	CC	20/07/2020	69 - 82	Out of Net	1X - 1XXX	53,2	16,5	1,20	13,8	1,5
Mor 26	CC	02/06/2020	71 - 77	Net	1X - 1XX	61,9	12,5	1,05	11,9	0,0
Mor 26	CC	22/06/2020	69 - 78	Net	1X - 1XXX	62,7	13,1	0,84	15,6	0,0

Mor 26	CC	20/07/2020	72 - 82	Net	1XX - 1XXX	64,6	14,8	1,00	14,8	0,0
Mor 26	CC	22/06/2020	71 - 81	Out of Net	1X - 1XXX	64,0	11,4	0,90	12,7	1,3
Mor 26	CC	20/07/2020	71 - 79	Out of Net	1X - 1XXX	63,8	11,5	0,90	12,8	3,0
Nova (Control)	CC	22/04/2020	78 - 86	Net	1XXX	53,4	10,2	0,70	14,6	0,0
Nova (Control)	CC	15/05/2020	78 - 94	Net	1XXX	52,6	10,4	0,80	13,0	0,0
Nova (Control)	CC	24/03/2020	74 - 77	Out of Net	1XX	58,7	10,6	0,95	11,2	0,0
Nova (Control)	CC	22/04/2020	76 - 85	Out of Net	1XX - 1XXX	63,4	9,9	0,70	14,1	2,8
OR4	CC	02/06/2020	67 - 73	Net	1 - 1XX	51,4	12,7	2,05	6,2	1,3
OR4	CC	22/06/2020	64 - 80	Net	1 - 1XXX	47,6	11,7	1,75	6,7	0,0
OR4	CC	02/06/2020	63 - 75	Out of Net	2 - 1XX	56,1	11,8	1,50	7,9	3,7
RHM	CC	22/04/2020	71 - 77	Net	1X - 1XX	62,0	10,8	0,65	16,6	4,8
Page	CC	24/03/2020	64 - 70	Net	1 - 1X	59,0	11,5	1,00	11,5	2,7
Page	CC	22/04/2020	65 - 77	Net	1 - 1XX	52,9	11,7	0,80	14,6	0,0
Page	CC	24/03/2020	69 - 79	Out of Net	1X - 1XXX	56,6	10,8	0,95	11,4	4,7
Page	CC	22/04/2020	66 - 78	Out of Net	1 - 1XXX	50,7	11,8	0,75	15,7	0,0
Page	CC	15/05/2020	65 - 75	Out of Net	1 - 1XX	60,9	10,2	0,65	15,7	0,0
Page	CC	02/06/2020	75 - 85	Out of Net	1XX - 1XXX	54,6	11,8	0,85	13,9	3,4
RHM	CC	22/04/2020	77 - 83	Net	1XX - 1XXX	57,6	10,4	0,65	16,0	6,3
RHM	CC	15/05/2020	70 - 85	Net	1X - 1XXX	54,2	10,4	0,60	17,3	1,2
RHM	CC	02/06/2020	79 - 90	Net	1XXX	59,8	12,0	0,75	16,0	1,3
RHM	CC	22/04/2020	73 - 77	Out of Net	1XX	59,6	11,6	0,65	17,8	4,5
RHM	CC	15/05/2020	70 - 82	Out of Net	1X - 1XXX	62,8	13,0	0,70	18,6	1,5
RHM	CC	02/06/2020	68 - 80	Out of Net	1X - 1XXX	58,8	13,7	0,75	18,3	0,8
Saint Andre	CC	15/05/2020	69 - 84	Net	1X - 1XXX	54,2	11,8	0,70	16,9	0,0
Saint Andre	CC	24/03/2020	79 - 88	Out of Net	1XXX	49,6	9,5	0,80	11,9	0,0
Saint Andre	CC	15/05/2020	79 - 90	Out of Net	1XXX	50,2	9,2	0,60	15,3	9,1
Samba	CC	24/03/2020	74 - 82	Net	1XX - 1XXX	56,7	11,0	0,75	14,7	0,0
Samba	CC	22/04/2020	70 - 82	Net	1X - 1XXX	55,8	11,4	0,75	15,2	0,0
Samba	CC	22/05/2020	69 - 73	Out of Net	1X - 1XX	57,7	11,3	0,70	16,1	3,5
Samba	CC	15/05/2020	70 - 80	Out of Net	1X - 1XXX	58,8	9,8	0,70	14,0	1,3
Shani SL	CC	15/05/2020	55 - 72	Net	3 - 1XX	60,4	11,0	1,65	6,7	0,5
Shani SL	CC	02/06/2020	65 - 73	Net	1 - 1XX	61,1	14,8	1,70	8,7	0,0
Shani SL	CC	22/06/2020	62 - 74	Net	2 - 1XX	63,4	14,5	1,65	8,8	0,0
Shani SL	CC	20/07/2020	64 - 75	Net	1 - 1XX	62,1	15,0	1,50	10,0	0,0
Shani SL	CC	15/05/2020	61 - 65	Out of Net	2 - 1	61,2	10,2	2,00	5,1	1,8
Shani SL	CC	02/06/2020	65 - 72	Out of Net	1 - 1XX	58,2	12,6	1,45	8,7	1,4
Shani SL	CC	22/6/2020	67 - 82	Out of Net	1 - 1XXX	61,1	13,5	1,35	10,0	0,4

Shani SL	CC	20/07/2020	65 - 79	Out of Net	1 - 1XXX	42,3	15,3	1,70	9,0	2,3
Sirio	CC	22/04/2020	79 - 85	Net	1XXX	70,5	11,7	1,05	11,1	0,0
Sirio	CC	15/02/2020	80 - 93	Net	1XXX	52,5	11,9	0,95	12,5	0,0
Sirio	CC	24/03/2020	75 - 85	Out of Net	1XX - 1XXX	55,0	9,9	0,55	18,0	3,5
Sirio	CC	22/04/2020	74 - 85	Out of Net	1XX - 1XXX	57,6	10,9	0,90	12,1	2,7
Sugar Belle	CC	15/05/2020	72 - 83	Net	1XX - 1XXX	54,5	11,8	1,25	9,4	1,7
Sugar Belle	CC	02/06/2020	70 - 84	Net	1X - 1XXX	56,2	12,2	1,85	6,6	3,7
Tambor 1	CC	22/06/2020	80 - 85	Out of Net	1XXX	57,7	12,4	1,60	7,8	0,8
Tambor 1	CC	20/07/2020	77 - 96	Out of Net	1XX - 1XXX	54,3	12,4	1,45	8,6	8,5
Tambor 1	CC	11/08/2020	79 - 91	Out of Net	1XXX	55,2	13,1	1,50	8,7	8,5
Tambor 2	CC	02/06/2020	81 - 85	Net	1XXX	60,8	10,9	1,45	7,5	15,2
Tambor 2	CC	22/06/2020	85 - 93	Net	1XXX	57,9	11,7	1,20	9,8	6,3
Tambor 2	CC	20/07/2020	82 - 101	Net	1XXX	49,5	10,8	1,30	8,3	12,3
Tambor 2	CC	11/08/2020	90 - 106	Net	1XXX	46,8	11,2	1,10	10,2	12,0
Tambor 2	CC	22/06/2020	72 - 83	Out of Net	1XX - 1XXX	59,5	11,3	1,40	8,1	7,5
Tambor 2	CC	20/07/2020	78 - 83	Out of Net	1XXX	59,4	11,1	1,55	7,2	12,9
Tambor 2	CC	11/08/2020	79 - 91	Out of Net	1XXX	55,8	12,9	1,50	8,6	13,5
Tanor late	CC	02/06/2020	85 - 99	Net	1XXX	47,7	12,4	1,35	9,2	0,0
Tanor late	CC	22/06/2020	96 - 102	Net	1XXX	47,7	11,4	1,60	7,1	0,7
Tanor late	CC	20/07/2020	99 - 107	Net	1XXX	48,0	12,9	1,15	11,2	0,0
Tanor late	CC	11/08/2020	85 - 106	Net	1XXX	49,5	14,7	1,40	10,5	0,0
Tasty 1	CC	15/05/2020	75 - 92	Net	1XX - 1XXX	43,2	10,9	0,85	12,8	1,8
Tasty 1	CC	02/06/2020	81 - 94	Net	1XXX	38,1	11,6	0,90	12,9	0,0
Tasty 1	CC	15/05/2020	97 - 75	Out of Net	1XXX	41,4	12,6	0,95	13,3	3,2
Tasty 2	CC	15/05/2020	77 - 86	Net	1XX - 1XXX	52,9	11,4	0,85	13,4	8,0
Tasty 2	CC	15/05/2020	74 - 89	Out of Net	1XX - 1XXX	38,7	11,1	0,85	13,1	17,5

Futsela Iglobhu Investment (Ryton)										
Cultivar	Roots tock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
ARCCIT 9 LS	CC	29/04/2020	64 - 75	1 - 1XX	62,8	9,2	0,75	12,3	0,0	T3 - 5
ARCCIT 9 LS	CC	21/05/2020	71 - 78	1X - 1XXX	55,7	8,6	0,65	13,2	1,9	T2 - 4
ARCCIT 9 LS	CC	08/06/2020	65 - 75	1 - 1XX	48,8	10,7	0,75	14,3	2,6	T1 - 3
ARCCIT 9 LS	CC	17/07/2020	65 - 89	1 - 1XXX	43,8	12,1	0,70	17,3	4,0	T1 - 3
Etna	CC	19/03/2020	62 - 67	2 - 1XX	57,1	8,4	0,70	12,0	5,5	T5 - 7
Etna	CC	29/04/2020	71 - 80	1X - XXX	57,3	8,9	0,60	14,8	4,3	T1 - 3
Furr	CC	29/04/2020	75 - 83	1XX - 1XXX	50,0	11,2	0,85	13,2	15,0	T3 - 5
Furr	CC	21/05/2020	84 - 90	1XXX	57,1	11,9	0,85	14,0	12,7	T1 - 3
Furr	CC	08/06/2020	77 - 86	1XX - 1XXX	59,0	11,7	1,05	11,1	7,4	T1 - 3
Furr	CC	01/07/2020	75 - 86	1XX - 1XXX	57,1	12,7	0,95	13,4	8,2	T1 - 3
Furr	CC	17/07/2020	74 - 90	1XX - 1XXX	58,5	13,9	0,90	15,4	13,8	T1 - 3
Goldup	CC	05/03/2020	55 - 60	3 - 2	56,5	9,7	0,80	12,1	4,4	T6 - 4
IRM 1	CC	21/05/2020	65 - 72	1 - 1XX	62,0	11,0	1,05	10,5	4,0	T2 - 4
IRM 1	CC	08/06/2020	64 - 77	1 - 1XX	62,6	11,2	1,00	11,2	1,7	T1 - 4
IRM 1	CC	01/07/2020	61 - 75	2 - 1XX	64,6	12,0	0,95	12,6	1,3	T1 - 3
IRM 1	CC	17/07/2020	63 - 85	2 - 1XXX	63,5	14,3	1,00	14,3	2,3	T1 - 3
IRM 2	CC	29/04/2020	63 - 70	2 - 1X	58,1	9,4	0,90	10,4	2,8	T4 - 6
IRM 2	CC	21/05/2020	69 - 78	1 - 1XXX	55,0	10,6	0,90	11,8	0,8	T2 - 4

IRM 2	CC	08/06/2020	63 - 74	2 - 1XX	61,6	11,9	0,95	12,5	1,0	T1 - 3
IRM 2	CC	02/07/2020	64 - 77	1 - 1XX	62,9	13,5	0,90	15,0	3,5	T1 - 3
IRM 2	CC	17/07/2020	58 - 67	3 - 1	61,1	15,0	1,05	14,3	1,8	T1 - 3
Leanri	CC	19/03/2020	62 - 72	2 - 1XX	50,0	10,5	0,95	11,1	3,7	T4 - 6
Ma'ayana (Dina)	CC	29/04/2020	65 - 73	1 - 1XX	58,2	12,0	0,80	15,0	7,8	T2 - 4
Ma'ayana (Dina)	CC	21/02/2020	71 - 76	1X - 1XX	55,9	10,9	0,95	11,5	2,3	T2 - 4
Meirav 119	CC	19/03/2020	55 - 63	3 - 2	41,7	9,5	0,65	14,6	2,1	T2 - 4
Meirav 119	CC	29/04/2020	69 - -80	1X - 1XXX	54,3	10,6	0,70	15,1	3,2	T2 - 4
Meirav 119	CC	21/05/2020	69 - 76	1X - 1XX	58,5	10,8	0,65	16,6	0,8	T1 - 3
Mor 26	CC	29/04/2020	62 - 66	2 - 1	59,1	10,7	0,90	11,9	2,5	T2 - 4
Mor 26	CC	21/05/2020	61 - 68	2 - 1X	58,6	11,0	0,90	12,2	3,5	T2 - 4
Mor 26	CC	08/06/2020	61 - 70	2 - 1X	64,4	12,8	1,00	12,8	1,6	T1 - 3
Mor 26	CC	01/07/2020	60 - 72	2 - 1XX	59,5	12,6	0,70	18,0	1,1	T1 - 3
Mor 26	CC	17/07/2020	63 - 68	2 - 1X	61,0	15,6	0,95	16,4	2,4	T1 - 3
Nadorcott	CC	08/06/2020	65 - 72	1 - 1XX	58,0	11,8	0,85	13,9	1,7	T1 - 4
Nadorcott	CC	01/07/2020	66 - 70	1 - 1X	54,9	12,1	0,80	15,1	0,8	T1 - 3
Nadorcott	CC	17/07/2020	66 - 72	1 - 1XX	54,6	13,7	0,80	17,1	2,1	T1 - 3
Nova	CC	19/03/2020	64 - 73	1 - 1XX	50,6	8,5	0,80	10,6	2,8	T6 - 7
Nova	CC	29/04/2020	72 - 80	1XX - 1XXX	54,5	8,3	0,65	12,8	0,0	T3 - 5
Nova	CC	21/50/2020	71 - 83	1X - 1XXX	50,9	9,6	0,70	13,7	0,0	T2 - 3
Nova ARC	CC	19/03/2020	63 - 72	2 - 1XX	52,4	9,5	0,85	11,2	0,8	T5 - 7
Nova ARC	CC	29/04/2020	73 - 84	1XX - 1XXX	48,7	9,7	0,75	12,9	9,2	T3 - 4
Nova ARC	CC	21/05/2020	72 - 85	1XX - 1XXX	47,3	10,2	0,75	13,6	5,0	T1 - 3
Page	CC	29/04/2020	69 - 80	1X - 1XXX	56,3	10,2	0,75	13,6	2,8	T2 - 4
Page	CC	21/05/2020	67 - 79	1 - 1XXX	58,5	10,8	0,65	16,6	3,7	T1 - 3
RHM	CC	19/03/2020	60 - 67	2 - 1	61,9	9,3	0,60	15,5	9,8	T6 - 7
RHM	CC	29/04/2020	62 - 72	2 - 1XX	55,6	10,7	0,60	17,8	4,1	T2 - 4
RHM	CC	21/05/2020	60 - 72	2 - 1XX	66,5	9,9	0,50	19,8	4,3	T1 - 3
RHM	CC	08/06/2020	65 - 72	1 - 1XX	53,5	12,8	0,60	21,3	5,3	T1 - 3
Saint Andre	CC	19/03/2020	72 - 75	1XX - 1XXX	56,3	9,4	0,75	12,5	3,1	T6 - 7
Saint Andre	CC	29/04/2020	76 - 84	1XX - 1XXX	48,6	9,2	0,65	14,2	4,1	T3 - 5
Saint Andre	CC	21/05/2020	75 - 88	1XX - 1XXX	45,5	9,4	0,60	15,7	2,5	T1 - 3
Samba	CC	19/03/2020	56 - 61	3 - 2	53,3	10,3	0,85	12,1	3,8	T3 - 5
Samba	CC	29/04/2020	59 - 68	2 - 1X	62,0	9,8	0,65	15,1	2,6	T2 - 3
Shani SL	CC	29/04/2020	64 - 72	1 - 1XX	59,2	9,9	0,90	11,0	1,8	T3 - 5
Shani SL	CC	21/05/2020	63 - 72	2 - 1XX	59,7	11,6	0,90	12,9	0,8	T2 - 4
Sirio	CC	19/03/2020	66 - 70	1 - 1X	51,7	8,8	0,85	10,4	5,7	T6 - 7
Sirio	CC	29/04/2020	72 - 83	1XX - 1XXX	49,1	10,4	0,75	13,9	5,8	T2 - 3
Sirio	CC	21/05/2020	75 - 87	1XX - 1XXX	55,8	8,9	0,60	14,8	1,6	T1 - 3
Tambor 1	CC	08/06/2020	77 - 84	1XX - 1XXX	59,7	10,1	0,90	11,2	7,0	T1 - 4
Tambor 1	CC	17/07/2020	80 - 93	1XXX	55,9	11,5	1,00	11,5	8,0	T1 - 3
Tambor 1	CC	04/08/2020	70 - 89	1X - 1XXX	53,6	11,2	1,00	11,2	8,7	T1 - 3
Tambor 2	CC	08/06/2020	74 - 85	1XX - 1XXX	56,7	9,2	0,75	12,3	14,0	T2 - 4
Tambor 2	CC	17/07/2020	70 - 98	1X - 1XXX	54,6	10,5	1,00	10,5	5,3	T1 - 3
Tambor 2	CC	04/08/2020	69 - 85	1X - 1XXX	55,7	11,1	1,00	11,1	7,0	T1 - 3
Tanor Late	CC	08/06/2020	93 - 102	1XXX	52,1	10,9	0,85	12,8	0,3	T1 - 3
Tanor Late	CC	01/07/2020	72 - 84	1XX - 1XXX	56,3	9,7	0,90	10,8	10,7	T1 - 4
Tasty 1	CC	29/04/2020	72 - 84	1XX - 1XXX	55,4	10,5	0,85	12,4	13,7	T2 - 4
Tasty 1	CC	21/05/2020	70 - 80	1X - 1XXX	47,9	9,8	0,55	17,8	1,2	T2 - 4
Tasty 1	CC	08/06/2020	72 - 88	1XX - 1XXX	47,8	10,5	0,55	19,1	4,0	T1 - 3
Taylor Lee	CC	29/04/2020	73 - 78	1XX - 1XXX	61,5	11,1	0,80	13,9	5,7	T3 - 5
Taylor Lee	CC	08/06/2020	75 - 86	1XX - 1XXX	64,5	12,7	0,90	14,1	2,2	T1 - 3

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Cultivar	Roots tock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
ARCCIT 9 LS	CC	19/05/2020	58 - 67	3 - 1	63,9	11,4	1,30	8,8	2,2	T1 - 3
ARCCIT 9 LS	CC	01/07/2020	54 - 65	4 - 1	58,8	11,1	1,25	8,9	2,2	T1 - 3
Bruce (Tasty 1)	CC	19/05/2020	83 - 92	1XXX	47,4	11,3	0,80	14,1	8,2	T1 - 3
Bruce (Tasty 1)	CC	01/07/2020	80 - 96	1XXX	43,3	13,1	0,70	18,7	22,5	T1 - 3
Ma'ayana (Dina)	CC	19/05/2020	70 - 76	1X - 1XX	61,8	9,1	1,10	8,3	4,0	T1 - 2
Ma'ayana (Dina)	CC	01/07/2020	64 - 75	1 - 1XX	62,2	15,7	1,20	13,1	5,3	T1 - 3
Etna	CC	19/05/2020	76 - 96	1XX - 1XXX	56,0	9,1	0,70	13,0	8,0	T1 - 2
Etna	CC	01/07/2020	77 - 100	1XXX	44,9	10,3	0,55	18,7	13,7	T1 - 3
Furr	CC	19/05/2020	70 - 89	1X - 1XXX	61,9	12,5	1,10	11,4	5,5	T1 - 4
Furr	CC	01/07/2020	71 - 82	1X - 1XXX	62,6	13,5	1,15	11,7	18,3	T1 - 4
Goldup	CC	19/05/2020	62 - 71	2 - 1X	56,0	11,2	0,80	14,0	2,0	T1 - 2
Goldup	CC	01/07/2020	64 - 70	1 - 1X	44,8	13,5	0,90	15,0	2,5	T1 - 2
IRM 1	CC	19/05/2020	57 - 74	3 - 1XX	57,7	11,1	1,25	8,9	1,3	T2 - 4
IRM 1	CC	01/07/2020	62 - 71	2 - 1X	57,3	14,2	1,30	10,9	4,2	T1 - 3
IRM 2	CC	19/07/2020	63 - 66	2 - 1	64,9	12,4	1,15	10,8	1,0	T2 - 4
IRM 2	CC	01/07/2020	57 - 69	3 - 1X	62,1	14,9	1,30	11,5	5,2	T1 - 3
Sugar Belle	CC	19/05/2020	63 - 74	2 - 1XX	56,8	11,3	1,70	6,6	3,3	T1 - 3
Sugar Belle	CC	01/07/2020	65 - 71	1 - 1X	61,1	13,4	1,75	7,7	26,8	T1 - 3
Meirav 119	CC	19/05/2020	60 - 77	2 - 1XX	56,7	13,5	1,30	10,4	1,0	T2 - 4
Meirav 119	CC	19/05/2020	64 - 74	1 - 1XX	59,8	12,2	1,00	12,2	0,0	T1 - 3
Mor 26	CC	01/07/2020	62 - 69	2 - 1X	57,4	15,0	1,30	11,5	4,3	T1 - 3
Nadorcott	CC	19/05/2020	70 - 75	1X - 1XX	58,1	11,1	1,10	10,1	4,2	T1 - 3
Nadorcott	CC	01/07/2020	69 - 76	1X - 1XX	57,6	13,7	1,25	11,0	10,0	T1 - 3
Nova (control)	CC	01/07/2020	67 - 71	1 - 1X	60,0	13,0	0,90	14,4	4,3	T1 - 3
Nova ARC	CC	19/05/2020	73 - 81	1XX - 1XXX	55,6	12,1	1,00	12,1	0,0	T1 - 2
Nova ARC	CC	01/07/2020	67 - 80	1 - 1XXX	48,4	12,9	1,30	9,9	3,3	T1 - 3
OR 1	CC	19/05/2020	66 - 74	1 - 1XX	57,0	12,1	1,10	11,0	3,0	T1 - 3
OR 1	CC	01/07/2020	72 - 76	1XX	54,2	12,9	0,90	14,3	7,5	T1 - 3
Or 4	CC	01/07/2020	63 - 76	2 - 1XX	54,8	13,3	1,10	12,1	2,2	T1 - 3
Page	CC	19/05/2020	62 - 68	2 - 1X	62,7	13,6	0,85	16,0	1,7	T1 - 3
Page	CC	01/07/2020	59 - 66	2 - 1	61,8	14,9	1,10	13,5	4,2	T1 - 3
RHM	CC	19/05/2020	69 - 72	1X - 1XX	61,5	11,6	0,65	17,8	7,3	T1 - 2
RHM	CC	01/07/2020	66 - 73	1 - 1XX	59,6	13,5	0,65	20,8	16,3	T1 - 2
Saint Andre	CC	19/05/2020	76 - 85	1XX - 1XXX	57,1	11,6	0,85	13,6	13,8	T1 - 2
Saint Andre	CC	01/07/2020	75 - 84	1XX - 1XXX	53,7	12,9	0,85	15,2	7,5	T1 - 2
Samba	CC	19/05/2020	63 - 74	2 - 1XX	61,3	11,9	0,90	13,2	2,0	T1 - 2
Samba	CC	01/07/2020	55 - 71	3 - 1X	56,3	12,2	1,30	9,4	2,5	T1 - 2
Shani SL	CC	01/07/2020	59 - 68	2 - 1X	53,8	12,9	1,60	8,1	0,5	T1 - 3
Sirio	CC	19/05/2020	80 - 90	1XXX	52,4	11,0	0,85	12,9	7,4	T1 - 2
Sirio	CC	01/07/2020	75 - 90	1XX - 1XXX	54,5	12,5	0,90	13,9	10,7	T1 - 2
Tambor 1	CC	01/07/2020	72 - 89	1XX - 1XXX	54,8	10,9	1,05	10,4	22,2	T1 - 3
Tambor 2	CC	01/07/2020	60 - 78	2 - 1XXX	54,8	11,1	1,50	7,4	8,0	T1 - 3
Tasty 2	CC	19/05/2020	72 - 78	1XX - 1XXX	56,8	11,4	0,80	14,3	2,5	T1 - 2
Taylor lee	CC	19/05/2020	65 - 80	1 - 1XXX	61,3	12,7	1,15	11,0	1,6	T1 - 3
Taylor lee	CC	01/07/2020	68 - 77	1X - 1XX	61,3	13,5	1,05	12,9	8,0	T1 - 3

#### 5.4.11 PROGRESS REPORT: Evaluation of Valencia selections in the intermediate production areas (Nelspruit)

Project 963A by J. Joubert (CRI)

#### Summary

The Valencia season starts with mid-maturing selections and proceeds to the late maturing selections suitable for this intermediate production area. Recommendations have therefore, been made accordingly. Midnight was first to mature in the middle of the Valencia season as a control for the trial; fruit size peaked at count 48. McClean SL followed, producing the second best crop on the trees (201 kg per tree) with good internal quality and soft fibre. Jassie followed after McClean SL with good production (166 kg per tree avg) and compact tree canopy. Kobus du Toit Late followed slightly after Jassie and fits in before Skilderkrans. Skilderkrans follows next with completely seedless fruit and very good juice levels on Sunki 812 (62%). Valencia Late is currently one of the latest maturing Valencia selections that are being planted commercially, developing small to medium fruit size, smooth rind texture and good yield.

## Opsomming

Die Valencia seisoen begin met mid-rypwordende seleksies en duur voort met die laat rypwordende seleksies in die intermediere produksie areas en aanbevelings is daarvolgens gebaseer. Midnight sal eerste ryp word in die middel van die Valencia seisoen as kontrole vir die proef; vruggrootte piek by telling 48. McClean SL kan nou volg, wat die tweede beste oes op die bome produseer het (201 kg per boom) met goeie interne kwaliteit en sagte vesel. Jassie volg na McClean SL met goeie produksie (166 kg per boom gem) en kompakte boom volume. Kobus du Toit Laat volg net na Jassie en pas in voor Skilderkrans. Skilderkrans volg dan met totaal saadlose vrugte en baie goeie sap vlakke op Sunki 812 (62%). Val Late is huidiglik een van die laatste rypwordende Valencia seleksie wat kommersieel aangeplant word, met klein tot medium vruggrootte, gladde skil tekstuurvertraagde en goeie produksie.

## Objectives

Determine the suitability of seven Valencia selections on Swingle Citrumelo (SC) and Sunki 812 citrandarin (Sunki) rootstocks, in an intermediate citrus production area, by evaluating internal fruit quality, crop production and export fruit counts.

## Materials and methods

Trees were planted at Crocodile Valley, Nelspruit in February 2012 and harvested for the first time in August 2019 to determine crop production, internal quality and fruit size distribution. Scion cultivars included Jassie, McClean SL, Kobus du Toit Late, Late Valencia (control), Skilderkrans, and Midnight (control) for this intermediate production area. SC and Sunki rootstocks were used to compare the performance of the different combinations. Trees were planted in a commercial Midnight Valencia orchard under optimal commercial production management and practices.

**Table 5.4.11.1.** List of Valencia selections evaluated at Crocodile Valley (Nelspruit) during the 2020 season.

Selection	Rootstock	Planted
Jassie	SC, Sunki 812	2012
Kobus du Toit Late	SC, Sunki 812	2012
McClean SL	SC, Sunki 812	2012
Midnight	SC, Sunki 812	2012
Skilderkrans	SC, Sunki 812	2012
Val Late	SC, Sunki 812	2012

## Results and discussion

Brix:acid ratio indicates harvest maturity and 'Midnight' on Sunki and SC were first to mature, followed by 'McClean SL' on both rootstocks, Late Valencia on Sunki and then 'Kobus du Toit Late'. 'Skilderkrans' with ratios between 7.3 (SC) and 8.8 (Sunki) was next in line. Jassie confirmed its status as a late maturing Valencia selection, reaching optimum just before 'Late Valencia' on SC which was the last cultivar to mature.

The highest juice content was obtained with 'McClean SL' on Sunki (63%) and Midnight on Swingle (12.7) had the highest Brix levels. Kobus du Toit Late' had the highest seed counts, from 0.8 up to 2 seeds per fruit, followed by Val Late with 1.7 seeds per fruit and Jassie 1.5 seeds. 'Kobus du Toit Late' on SC cropped the best average yield (207 kg/tree), followed by 'McClean SL' on SC with 201 kg/tree and 'Skilderkrans' on SC producing 195 kg/tree. The lowest yield was 'Midnight' on Sunki (140.5 kg/tree), bearing in mind it is a low seeded variety and requires fruit set manipulation. All the combinations peaked at count 88 due to a heavy fruit set, except for 'Midnight' on Sunki 812 and SC, which peaked at count 48.

## Conclusion

The 2020 season was the second harvest and evaluation of all the sections at Crocodile Valley's trial site. Trees are still young; we need to generate production data with future evaluations over numerous seasons to ensure good comparisons between the different scion:rootstock combinations.

Documentations of all aspects relevant to sustainable Valencia production need to be part of scion, rootstock combination evaluation. The second season's results in this trial indicate clear interactions between rootstock and scion i.t.o. yield, fruit size, internal quality as well as canopy volume. Over the next few seasons, data from this project will give direction in identifying the optimum tree canopy size (reduce harvest time, pruning, spray volume) without compromising on yield or fruit quality.

**Table 5.4.11.2.** Internal fruit quality data for Valencia selections at Crocodile Valley (Nelspruit) during the 2020 season.

Cultivar	Root-stock	Date harvested	Fruit size (mm)	Count	Juice (%)	Brix °	Acid (%)	Ratio	Avg seed	Fruit external colour
Jassie	SC	22/07/2020	76 - 85	72 - 56	62,9	11,9	1,40	8,5	0,0	T1 - 3
Jassie	US 812	22/07/2020	71 - 81	88 - 64	59,7	12,4	1,35	9,2	1,5	T1 - 3
K duToit Late	SC	22/07/2020	73 - 79	72 - 64	61,8	11,9	1,35	8,8	2,0	T1 - 3
K duToit Late	US 812	22/07/2020	70 - 80	88 - 64	62,7	11,5	1,65	7,0	0,8	T1 - 3
McClean SL	SC	22/07/2020	72 - 80	88 - 64	59,7	11,9	1,30	9,2	0,0	T1 - 3
McClean SL	US 812	22/07/2020	70 - 81	88 - 64	63,0	12,1	1,45	8,3	0,0	T1 - 3
Midnight	SC	22/07/2020	76 - 84	72 - 56	61,3	12,7	1,35	9,4	0,0	T1 - 3
Midnight	US 812	22/07/2020	75 - 81	72 - 64	60,9	11,9	1,15	10,3	0,0	T1 - 3
Skilderkrans	SC	22/07/2020	71 - 78	88 - 64	61,9	11,0	1,50	7,3	0,0	T1 - 3
Skilderkrans	US 812	22/07/2020	74 - 82	72 - 56	62,4	12,3	1,40	8,8	0,0	T1 - 3
Val Late	SC	22/07/2020	72 - 85	88 - 56	55,6	11,1	1,35	8,2	0,7	T1 - 3
Val Late	US 812	22/07/2020	72 - 84	88 - 56	56,4	12,0	1,50	8,0	1,7	T1 - 3

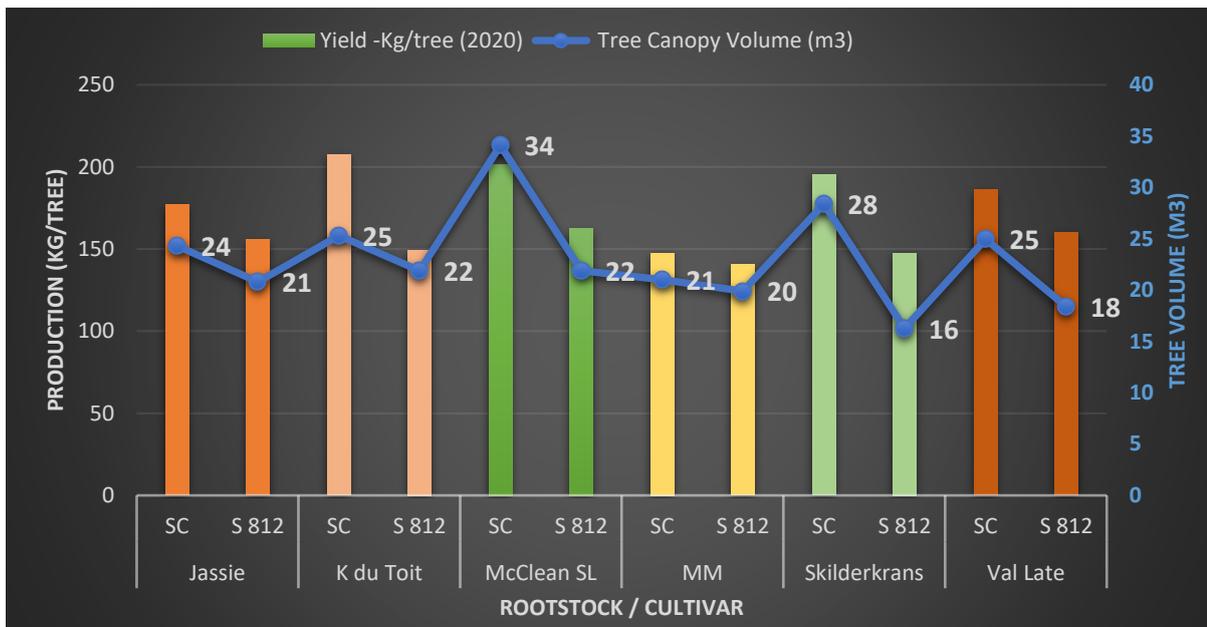
**Table 5.4.11.3.** Fruit size distribution for Valencia selections at Crocodile Valley (Nelspruit) during the 2020 season.

Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Jassie	Sunki 812	> 48	0,55	Jassie	SC	> 48	0,41
Jassie	Sunki 812	48	4,93	Jassie	SC	48	5,70
Jassie	Sunki 812	56	17,59	Jassie	SC	56	20,86
Jassie	Sunki 812	72	24,89	Jassie	SC	72	27,08
Jassie	Sunki 812	88	41,39	Jassie	SC	88	37,40
Jassie	Sunki 812	105/125	10,65	Jassie	SC	105/125	8,54
Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
McClean SL	Sunki 812	> 48	0,85	McClean SL	SC	> 48	5,51
McClean SL	Sunki 812	48	3,87	McClean SL	SC	48	3,17

McCleen SL	Sunki 812	56	12,47	McCleen SL	SC	56	12,98
McCleen SL	Sunki 812	72	22,99	McCleen SL	SC	72	23,30
McCleen SL	Sunki 812	88	50,41	McCleen SL	SC	88	40,83
McCleen SL	Sunki 812	105/125	9,41	McCleen SL	SC	105/125	14,21
<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>	<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>
K du Toit	Sunki 812	> 48	0,11	K du Toit	SC	> 48	0,00
K du Toit	Sunki 812	48	2,83	K du Toit	SC	48	0,42
K du Toit	Sunki 812	56	12,33	K du Toit	SC	56	6,15
K du Toit	Sunki 812	72	25,99	K du Toit	SC	72	23,09
K du Toit	Sunki 812	88	51,63	K du Toit	SC	88	59,65
K du Toit	Sunki 812	105/125	7,12	K du Toit	SC	105/125	10,70
<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>	<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>
Val Late	Sunki 812	> 48	3,57	Val Late	SC	> 48	0,17
Val Late	Sunki 812	48	4,71	Val Late	SC	48	4,18
Val Late	Sunki 812	56	16,48	Val Late	SC	56	11,07
Val Late	Sunki 812	72	24,08	Val Late	SC	72	25,84
Val Late	Sunki 812	88	42,26	Val Late	SC	88	47,65
Val Late	Sunki 812	105/125	8,90	Val Late	SC	105/125	11,09
<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>	<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>
Skilderkrans	Sunki 812	> 48	4,73	Skilderkrans	SC	> 48	0,36
Skilderkrans	Sunki 812	48	26,40	Skilderkrans	SC	48	5,30
Skilderkrans	Sunki 812	56	15,76	Skilderkrans	SC	56	16,91
Skilderkrans	Sunki 812	72	20,29	Skilderkrans	SC	72	24,70
Skilderkrans	Sunki 812	88	26,50	Skilderkrans	SC	88	36,73
Skilderkrans	Sunki 812	105/125	6,33	Skilderkrans	SC	105/125	16,01
<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>	<b>Cultivar</b>	<b>Rootstock</b>	<b>Size</b>	<b>% Fruit</b>
MM	Sunki 812	> 48	1,28	MM	SC	> 48	11,59
MM	Sunki 812	48	30,56	MM	SC	48	24,50
MM	Sunki 812	56	17,79	MM	SC	56	23,83
MM	Sunki 812	72	18,93	MM	SC	72	21,46
MM	Sunki 812	88	26,05	MM	SC	88	17,04
MM	Sunki 812	105/125	5,38	MM	SC	105/125	1,58

**Table 5.4.11.4.** Production per tree for Valencia selections at Crocodile Valley (Nelspruit) during the 2020 season.

<b>Cultivar</b>	<b>Rootstock</b>	<b>Kg/tree (2019)</b>	<b>Tree Volume (m<sup>3</sup>)</b>	<b>Kg/tree (2020)</b>	<b>Tree Volume (m<sup>3</sup>)</b>
Jassie	Sunki 812	123,1	18,9	155,6	20,8
Jassie	SC	180,4	22,1	177,0	24,3
McCleen SL	Sunki 812	149,1	19,5	162,3	21,9
McCleen SL	SC	217,0	31,0	201,1	34,1
K du Toit	Sunki 812	120,9	19,9	149,4	21,9
K du Toit	SC	212,7	23,0	207,4	25,3
Val Late	Sunki 812	107,0	16,7	160,2	18,4
Val Late	SC	148,6	22,7	186,1	25,0
Skilderkrans	Sunki 812	93,6	14,8	147,6	16,3
Skilderkrans	SC	139,5	25,8	195,3	28,4
MM	Sunki 812	116,6	18,1	140,5	19,9
MM	SC	116,6	19,1	147,6	21,0



**Figure 5.4.11.1.** Average production per tree (kg/tree) versus tree canopy volume (m<sup>3</sup>) of seven Valencia cultivars grafted on Swingle citrange (SC) and Sunki citrandarin (S 812) in the Nelspruit area.

#### 5.4.12 PROGRESS REPORT: Evaluation of Grapefruit on different rootstocks in a semi-desert production area (Kakamas)

Project 922 by J. Joubert and W. Swiegers (CRI)

##### Summary

Visual evaluations of Star Ruby and Nelruby bud-unions indicated that the unions were still in good condition and the combinations compatible (need to confirm once the trial comes to an end). Sunki 812 is a hybrid rootstock cross between a Sunki mandarin and Beneke trifoliolate (US812). The tree size of this combination is described as medium (similar to Carrizo tree size and growth rate). In combination with Star Ruby, the tree was smaller compared to Citrange 35 (C35) and similar to Benton citrange (BC) (dwarfing rootstocks). Nelruby tree size in combination with US812 was the smallest compared to C35 and BC. Yield production was up this season for Nelruby, while only certain combinations with Star Ruby were up in yield production. Nelruby outperformed Star Ruby during the season. Both selections' fruit size ranged with counts 56 – 27.

Seed counts on the Nelruby fruit were higher compared to Star Ruby, being virtually seedless. Colour development on both selections and all the rootstock combinations was good. The dwarfing rootstock, Citrange 35 performed very well, bearing in mind the impact of the high pH of the soil. Other rootstocks also starting to perform well were BC, Terra Bella (TB), Swingle Citrumelo (SC) and US812. Future evaluations will determine the adaptability of these rootstocks.

Evaluations to date show that these rootstocks could be of value to citrus producers, particularly Sunki 812, should high pH levels and calcareous soils be a problem. Sunki 812 was selected for its high tolerance to *Phytophthora*, citrus nematodes and tristeza, as well as better tolerance of high pH and calcareous soils.

##### Opsomming

Visuele evaluasies van die Star Ruby en Nelruby entlas, met 'n gesonde entlas verbinding, het bewys dit is verenigbaar met die kombinasies (moet bevestig word wanneer die proef tot 'n einde kom). Sunki 812 is 'n hibried onderstam kruising tussen Sunki mandaryn en Beneke trifoliaat (US 812). Die boomgrootte van hierdie kombinasie word as medium beskou (vergelyk met Carrizo boomgrootte en groeikragtigheid). In kombinasie met Star Ruby was die boomgrootte bietjie kleiner as Citrange 35 en min of meer dieselfde grootte as Benton citrange (verdwergde onderstamme). Nelruby in kombinasie met US812 was die kleinste boom in vergelyking met C 35 en BC. Die oes produksie het baie verbeter hierdie seisoen vir Nelruby, terwyl net sekere kombinasies

met Star Ruby verbeter het met hulle produksie. Nelruby het beter presteer hierdie seisoen as Star Ruby in hierdie proef. Albei seleksies se vruggrootte het gepiek tussen grootte 56 – 27.

Saad tellings op die Nelruby vrugte was hoër in vergelyking met Star Ruby wat feitlik saadloos toets. Kleur ontwikkeling op albei seleksies en al die onderstam kombinasies was goed. Die verdwergde onderstam, Citrange 35 het baie goed presteer wanneer die impak van die hoë pH van die grond in ag geneem word. Ander onderstamme wat ook begin goed presteer is BC, TB, SC en US812. Verdere evaluasies sal die aanpasbaarheid van hierdie onderstamme bevestig.

Evaluasies tot op datum toon aan dat hierdie onderstamme waardevol kan wees vir die sitrus produsente; meer spesifiek Sunki 812, waar hoë pH vlakke en kalkagtige gronde voorkom. Sunki 812 was vir sy hoë verdraagsaamheid teen Phytophthora, sitrus aalwurms en tristeza, asook beter weerstand vir hoë pH en kalkagtige gronde, geselekteer.

### Objectives

- To investigate the performance of Star Ruby and Nelruby Grapefruit on suitable rootstocks in a hot citrus growing area on replant soils.
- To improve production, internal quality, rind colour and fruit size count distributions.

### Materials and methods

Seeds of Benton Citrange, Citrange 35, Carrizo Citrange, Rough Lemon, Sunki 812, Swingle Citrumelo, Terrabella and X639 were propagated by OSK Nursery, a CIS accredited nursery in the Kakamas region of the Western Cape.

Star Ruby and Nelruby grapefruit were budded onto the following rootstocks at OSK nursery in 2010: Benton Citrange, Citrange 35, Carrizo Citrange, Rough Lemon, Sunki 812, Swingle Citrumelo, Terra Bella and X639. The trees were planted at Karsten in March 2012.

**Table 5.4.12.1.** The number of trees per rootstock in the Star Ruby and Nelruby Grapefruit trial at Kakamas.

Selection	Rootstock	No. of trees
Star Ruby	BC	6
Star Ruby	C35	6
Star Ruby	CC	6
Star Ruby	RL	3
Star Ruby	Sunki 812	6
Star Ruby	SC	6
Star Ruby	TB	6
Star Ruby	X639	5
Nelruby	BC	6
Nelruby	C35	5
Nelruby	Sunki 812	5
Nelruby	SC	6
Nelruby	TB	6
Nelruby	X639	6

### Results and discussion

The Grapefruit trial was harvested for the fifth time this season with a fair to good crop on the trees.

#### Star Ruby

The lowest crop production for the 2020 season was in combination with CC yielding 46.2 kg/tree (2019; BC – 73 kg/tree) and the best on TB yielding 139.8 kg/tree (2019; RL – 105.9 kg/tree), (Table 5.4.12.4). The second highest crop was produced on C35 with 114.8 kg/tree, and the average yield for the Star Ruby trial was 89.7 kg/tree (2019 – 90.9 kg). Internally fruit quality was good with Brix ranging from 8.7 with CC up to 10.0 with C35 and TB (average 9.5) and juice levels below 50% except for in combination with BC 50.6% (Table 5.4.12.2).

The acid content remained fairly high this season at 1.41% average (slightly higher than 2019), The Brix:acid ratio ranged between 6.0 (CC) - 7.0 (TB) (compare to 2019 ratio 7). Fruit size count on BC, C35 and X639 peaked between counts 48 – 32, on CC fruit size count peaked between 36 – 27, on RL counts peaked between 40 – 27 and on US 812 the fruit size count peaked between counts 56 - 48. On TB fruit size counts peaked between 56 – 36.

### Nelruby

Nelruby juice content was low the previous season below 50%. The lowest Brix:acid ratio of 6.75 was in combination with SC. The highest Brix:acid ratio 7.83 followed was on BC. All ratios is lower compared to 2019 season ratios, (Table 5.4.12.2). C35 had the highest Brix 11.4 followed by BC and TB. The external colour development on all 6 rootstock combinations peaking at T1 to T3. Most of the combinations peaked at count 48 (2019 peaked at count 64), followed by counts 36 and 56. The best crop on the Nelruby trees was in combination with SC (123.2 kg/tree), followed by TB (119.4 kg/tree) and BC (116.6 kg/tree).

### **Conclusions**

The seed content in the Star Ruby fruit remained significantly lower in comparison with the Nelruby fruit. Fruit size distribution from Star Ruby had a big range between the different combinations, and Nelruby fruit size count peaked between (counts 48, 36 and 56). Nelruby cropped a better yield on the trees (average 112.3 kg/tree versus 89.7 kg) compared to Star Ruby this season. Star Ruby had improved colour development (deeper red blush on rind) where Nelruby was more “yellowish”.

Star Ruby and Nelruby were evaluated on eight and six rootstocks, respectively, the most important combination of the above mentioned was Sunki 812 (Sunki mandarin x Beneke trifoliolate). Sunki 812 was selected for replanting conditions, very specific high pH and calcareous soils. The first and second evaluations and harvest indicated that the other rootstocks outperformed Sunki 812, but Sunki 812 is starting to perform better and future evaluations will be crucial to determine the best combination in these semi-desert conditions.

**Table 5.4.12.2.** Internal fruit quality of Star Ruby and Nelruby Grapefruit on different rootstocks at Karsten Boerdery (Kakamas) on 10 June 2020.

<b>Cultivar</b>	<b>Root-stock</b>	<b>Juice (%)</b>	<b>Brix °</b>	<b>Acid (%)</b>	<b>Ratio</b>	<b>Avg. seed</b>	<b>Colour</b>
Nelruby	BC	46,1	10,7	1,37	7,83	1,5	T 1 - T 2
Nelruby	C35	45,0	11,4	1,53	7,47	1,6	T 1 - T 3
Nelruby	SC	42,8	9,4	1,39	6,75	0,3	T 1 - T 3
Nelruby	Sunki 812	44,3	10,2	1,42	7,16	0,0	T 1 - T 2
Nelruby	TB	42,2	10,6	1,46	7,25	0,5	T 1 - T 3
Nelruby	X639	43,0	9,3	1,35	6,88	1,4	T 1 - T 2
Star Ruby	BC	50,6	9,4	1,33	7,06	0,2	T 1 - T 3
Star Ruby	C35	48,6	10,0	1,48	6,75	0,0	T 1 - T 3
Star Ruby	CC	46,3	8,7	1,43	6,09	0,3	T 1 - T 3
Star Ruby	RL	44,1	9,3	1,39	6,69	0,0	T 1 - T 3
Star Ruby	SC	48,3	9,5	1,40	6,78	0,5	T 1 - T 3
Star Ruby	Sunki 812	49,4	9,8	1,42	6,88	0,6	T 1 - T 3
Star Ruby	TB	46,4	10,0	1,36	7,33	0,0	T 1 - T 2

Star Ruby	X639	46,6	9,0	1,43	6,29	0,0	T 2 - T 3
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**Table 5.4.12.3.** Fruit size distribution at Karsten Boerdery during the 2020 season.

Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Star Ruby	BC	27	1,99	Nelruby	BC	27	1,15
Star Ruby	BC	32	16,65	Nelruby	BC	32	8,80
Star Ruby	BC	36	17,24	Nelruby	BC	36	19,84
Star Ruby	BC	40	25,79	Nelruby	BC	40	12,90
Star Ruby	BC	48	34,86	Nelruby	BC	48	43,72
Star Ruby	BC	56	3,46	Nelruby	BC	56	13,61
Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Star Ruby	C35	27	4,81	Nelruby	C35	27	0,36
Star Ruby	C35	32	17,58	Nelruby	C35	32	4,75
Star Ruby	C35	36	16,42	Nelruby	C35	36	17,28
Star Ruby	C35	40	26,88	Nelruby	C35	40	14,97
Star Ruby	C35	48	31,17	Nelruby	C35	48	45,57
Star Ruby	C35	56	3,14	Nelruby	C35	56	17,06
Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Star Ruby	CC	27	40,59	Nelruby	Sunki 812	27	0,25
Star Ruby	CC	32	35,29	Nelruby	Sunki 812	32	3,31
Star Ruby	CC	36	12,06	Nelruby	Sunki 812	36	11,04
Star Ruby	CC	40	7,35	Nelruby	Sunki 812	40	12,04
Star Ruby	CC	48	3,82	Nelruby	Sunki 812	48	50,66
Star Ruby	CC	56	0,88	Nelruby	Sunki 812	56	22,71
Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Star Ruby	RL	27	22,83	Nelruby	SC	27	14,39
Star Ruby	RL	32	44,09	Nelruby	SC	32	25,79
Star Ruby	RL	36	11,02	Nelruby	SC	36	22,29
Star Ruby	RL	40	13,39	Nelruby	SC	40	10,95
Star Ruby	RL	48	7,87	Nelruby	SC	48	21,84
Star Ruby	RL	56	0,79	Nelruby	SC	56	4,73
Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Star Ruby	Sunki 812	27	0,27	Nelruby	TB	27	2,81
Star Ruby	Sunki 812	32	2,29	Nelruby	TB	32	13,94
Star Ruby	Sunki 812	36	5,92	Nelruby	TB	36	21,36
Star Ruby	Sunki 812	40	8,48	Nelruby	TB	40	11,41
Star Ruby	Sunki 812	48	53,57	Nelruby	TB	48	36,42
Star Ruby	Sunki 812	56	29,48	Nelruby	TB	56	14,05
Cultivar	Rootstock	Size	% Fruit	Cultivar	Rootstock	Size	% Fruit
Star Ruby	SC	27	17,19	Nelruby	X639	27	3,56
Star Ruby	SC	32	35,51	Nelruby	X639	32	13,50
Star Ruby	SC	36	16,15	Nelruby	X639	36	20,03
Star Ruby	SC	40	17,47	Nelruby	X639	40	12,55
Star Ruby	SC	48	12,46	Nelruby	X639	48	36,36
Star Ruby	SC	56	1,23	Nelruby	X639	56	14,01
Cultivar	Rootstock	Size	% Fruit				
Star Ruby	TB	27	1,70				
Star Ruby	TB	32	9,62				
Star Ruby	TB	36	17,22				
Star Ruby	TB	40	11,60				

Star Ruby	TB	48	43,77
Star Ruby	TB	56	16,08
Cultivar	Rootstock	Size	% Fruit
Star Ruby	X639	27	8,95
Star Ruby	X639	32	27,45
Star Ruby	X639	36	17,77
Star Ruby	X639	40	23,28
Star Ruby	X639	48	19,73
Star Ruby	X639	56	2,82

**Table 5.4.12.4.** Production per tree of Star Ruby and Nelruby Grapefruit trees on different rootstocks at Karsten Boerdery (Kakamas) during the 2020 season.

Cultivar	Rootstock	Kg/tree (2017)	Kg/tree (2018)	Kg/tree (2019)	Kg/tree (2020)
Nelruby	BC	75,3	28,1	86,8	116,6
Nelruby	C35	80,2	74,4	73,4	105,2
Nelruby	Sunki 812	51,8	31,7	76,6	116,0
Nelruby	SC	83,9	48,7	114,6	123,2
Nelruby	TB	62,3	7,9	92,2	119,4
Nelruby	X639	62,1	35,1	74,1	93,4
Star Ruby	BC	49,9	34,3	73,0	99,2
Star Ruby	C35	58,4	45,2	86,8	114,8
Star Ruby	CC	61,7	30,7	99,9	46,2
Star Ruby	RL	62,0	66,5	105,9	47,8
Star Ruby	Sunki 812	58,5	40,8	95,9	95,3
Star Ruby	SC	67,8	32,7	89,0	95,5
Star Ruby	TB	58,2	20,9	96,4	139,8
Star Ruby	X639	58,6	39,4	80,0	78,8

#### 5.4.13 PROGRESS REPORT: Cultivar characteristics and climatic suitability of Satsuma mandarins in a cold production region (Sundays River Valley)

Project 57B by W. Swiegers and Z. Zondi (CRI)

##### Summary

The trees were topworked in 2012 to the following selections which were also the order of ripening: Miho Wase, Ueno, Dobashi Beni Aoshima, and the season was finished off with Imamura. Picking periods for Satsumas should be limited to 2-3 weeks to ensure good internal quality and avoid puffiness. Satsuma selections need degreening after harvest as the internal quality is ahead of the colour development.

##### Opsomming

Die bome was in 2012 getopwerk na die volgende seleksies toe, wat ook dien as die volgorde van rypwording; Miho Wase, Ueno, Dobashi Beni Aoshima en die seisoen was afgesluit met Imamura. Pluk periodes vir Satsumas sal strek van 2-3 weke aangesien vrugte se sure vinnig daal en die skil powerig raak. Vrugte se kleur is laat teenoor die interne kwaliteit en ontgroening sal moet gedoen word.

##### Objectives

- To select Satsuma cultivars with improved and consistent productivity, fruit size, rind colour, and internal fruit quality (Brix, acidity and ratio).

- To extend the harvest period (both earlier and later maturity).
- To describe the characteristics of new Satsuma cultivars and determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on early to late Satsuma selections from the Sundays River Valley part of the Eastern Cape. The following selections were evaluated: Miho Wase, Ueno, Aoshima, Imamura, and Dobashi Beni.

For Satsuma mandarins, a ratio of 9:1 is considered to be the build-up towards peak maturity of 10:1. After reaching the peak, the ratio increases to 11:1, after which the fruit are considered over mature. This process is approximately three weeks long. Fruit harvested before and after this period would result in higher instances of quality and rind issues.

**Table 5.4.13.1.** List of Satsuma selections evaluated at Invercloy (Kirkwood) during 2020.

Selection	Rootstock	Topworked
Aoshima	Carrizo	2012
Miho Wase	Carrizo	2012
Ueno	Carrizo	2012
Dobashi Beni	Carrizo	2012
Imamura	Carrizo	2012

## Results and discussion

### Aoshima

Aoshima was the second last selection to reach peak maturity. The fruit size count for Aoshima was count 1x - 1xxx (big fruit). Aoshima juice percentage was just below 55% (low). In all 5 seasons Aoshima had a low juice %. Brix was 8.5° towards peak maturity peaking at 9° when over mature. The selection started with a low acid %. The fruit was seedless. The external colour development of the Aoshima was not very good, with a T6 - T7 on the colour plate. T 6 on the colour plate while the fruit was over mature and very pebbly.

### Miho Wase

Miho Wase was the first selection to mature to peak maturity this year. The selection reached peak maturity very early. Miho Wase are also used as the control. The selection had a big fruit size count of 1xx - 1xxx. Juice percentage for Miho Wase was higher with 54.1% this season compared to last season's 50.9% juice. Miho Wase had a Brix° of 9.2° and acid percentage of 0.56% with a 16.4 ratio (over mature). The colour was T5 - T6 on the colour plate. The fruit had no seeds and the external colour development was once again behind the internal development. The internal colour was a deep orange.

### Ueno

Ueno is a mid to late maturing selection. At this Satsuma trial site, it reached peak maturity more towards the beginning of the mid-maturing selection range. Ueno's fruit size count was big, ranging from count 1xx to 1xxx. Count 1xx was towards build-up to peak maturity and count 1xxx was when the fruit was over mature. The juice percentage was the same this season around 52%, compared to last season. Ueno Brix° was 7.8° and the acid percentage was 0.82% at a ratio of 9.5. Ueno had no seeds and the colour of Ueno on the colour plate was a T7 towards peak maturity and a T5 when it was over mature. The fruit was flat and peelability was easy.

### Imamura

Imamura is one of the late-maturing selections for this Satsuma trial site, and it was the last selection to reach peak maturity. Imamura normally reaches peak maturity beginning to end of May in cool production regions. The juice percentage for Imamura was the third highest, being 53.8%. Imamura Brix° was 10.4° the highest of

all the selections and a very good acid of 0.88% at a ratio of 11.8. Seed count was seedless. The colour development was T3 – T5 on the colour plate, one of the best compared to the other selections, but still delayed. The internal colour was deep orange and the fruit rind varied from smooth to coarse.

#### Dobashi Beni

Dobashi Beni are the control selection for the mid to late maturing Satsuma selections. Dobashi Beni was the third selection to reach peak maturity. Fruit size count was very good with count 1 – 1x. The juice percentage towards peak maturity, was 54.4%. Brix° was 8.5° and acid % was 0.90% towards peak maturity. Internal colour is deep orange, rind is smooth and peelability is easy. Dobashi Beni was seedless. This selection had a delayed external colour development being T5 on the colour plate during at 15.1 ratio being over mature.

#### **Conclusion**

Aoshima, Miho Wase, Imamura and Ueno had a big fruit size i.e., count 1xxx. Dobashi Beni had a good fruit size count ranging from 1 to 1x. Dobashi Beni had the best juice percentage being 54.4 % followed by Imamura 53.8%. Imamura had the highest Brix° at peak maturity being 10.4 and Ueno had the lowest Brix° being 7.8° of all the Satsuma selections. All the selections were seedless.

**Table 5.4.13.2.** Internal fruit quality data for Satsuma selections in the Addo and Kirkwood region of the Eastern Cape during the 2020 season.

Date	Cultivar	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-03-11	Aoshima	CC	1xx	53.2	7.9	1.05	7.5	0.0	8
2020-03-24	Aoshima	CC	1x	51.4	8.5	0.98	8.7	0.0	7
2020-04-20	Aoshima	CC	1xxx	54.1	9.0	0.60	15.0	0.0	6
2020-03-11	Dobashi Beni	CC	1	52.9	7.5	0.83	9.0	0.0	8
2020-03-24	Dobashi Beni	CC	1x	54.4	8.5	0.90	9.4	0.0	7
2020-04-20	Dobashi Beni	CC	1x	53.1	9.2	0.61	15.1	0.0	5
2020-03-24	Imamura	CC	1xx	55.3	9.5	1.33	7.1	0.0	7
2020-04-20	Imamura	CC	1xxx	53.8	10.4	0.88	11.8	0.0	5
2020-05-14	Imamura	CC	1xxx	54.5	10.5	0.89	11.8	0.2	3
2020-03-11	Miho wase	CC	1xxx	54.1	9.2	0.56	16,4	0.0	6
2020-03-24	Miho wase	CC	1xx	54.1	8.7	0.54	16.1	0.0	5
2020-03-11	Ueno	CC	1xx	50.3	7.5	1.09	6.9	0.0	8
2020-03-24	Ueno	CC	1xx	52.6	7.8	0.82	9.5	0.0	7
2020-04-20	Ueno	CC	1xxx	51.0	8.1	0.60	13.5	0.0	5

#### 5.4.14 **PROGRESS REPORT: Cultivar characteristics and climatic suitability of Mandarin hybrids in a cold production region (Sundays River Valley)** Project 997B by W. Swiegers and Z. Zondi (CRI)

#### **Summary**

The mandarin trial is divided into two different trial sites. Two of the sites are in the Kirkwood region of the Sundays River Valley. In the Dunbrody trial site some of the selections have an interstock and it is the third harvest for those selections. The selections in Dunbrody trial site are as follows and this is also the order of ripening: Saint Andre, RHM, Samba, Edit x Nova, Tango, Tasty 1, Leanri, Gold Nugget, Mor 26, IRM 2, IRM 1

and Tanor Late. At the other site in the Kirkwood region, we evaluated the following selections in their order of ripening: Etna, Sirio, Tasty 1 and Tanor Late and they were all directly topworked onto the rootstock.

## Opsomming

Die mandaryn proef is opgedeel in 2 verskillende proef persele. Die twee persele is in Sondagsrivier Vallei n.l. Kirkwood. By die Dunbrody proef perseel in die Kirkwood area is van die seleksies op 'n tussenstam getopwerk. Die volgorde van ryppwording by die perseel was as volg: Saint Andre, RHM, Samba, Edit x Nova, Tango, Tasty 1, Leanri, Gold Nugget, Mor 26, IRM 2, IRM 1 en Tanor Late. By die ander perseel in Kirkwood is al die seleksies direk op die onderstam en was die volgende seleksies geevalueer in hulle orde van ryppwording: Etna, Sirio, Tasty 1 en Tanor Late.

## Objectives

- To select mandarin hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new mandarin hybrid cultivars and to determine the climatic suitability of these cultivars in cold production regions

## Materials and methods

Field evaluations and laboratory analyses were conducted on mandarin hybrid selections from the Sundays River Valley. A range of new mandarin hybrids has been added to this area. The following varieties were evaluated: Edit x Nova, Saint Andre, Leanri, Gold Nugget, RHM, Samba, Tanor Late, Tango, IRM 1, IRM 2, Mor 26, Etna, Sirio, and Tasty 1.

A ratio of 11:1 for mandarin hybrids is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.14.1.** List of Mandarin hybrid selections from Kirkwood region of the Sundays River Valley (Dunbrody) during the 2020 season.

Selection	Rootstock	Topwork
Saint Andre	Carrizo	2013
Edit x Nova	Carrizo with Midnight interstock	2015
Leanri	Carrizo with Midnight interstock	2015
Gold Nugget	Carrizo with Midnight interstock	2015
RHM	Carrizo with Midnight interstock	2015
Samba	Carrizo with Midnight interstock	2015
Tanor Late	Carrizo with Midnight interstock	2015
Tango	Carrizo	2013
Tasty 1	Carrizo with Midnight interstock	2015
IRM 1	CC	
IRM 2	CC	
Mor 26	CC	

**Table 5.4.14.2** List of Mandarin hybrid selections from Kirkwood region of the Sundays River Valley (Invercloy) during the 2020 season.

Selection	Rootstock	Topwork
Etna	Carrizo	2012
Sirio	Carrizo	2012
Tanor Late	Carrizo	2012
Tasty 1	Carrizo	2012

## Results and discussion

### RHM

RHM had a very good fruit size count of 2 - 1. Acids start low and drop quickly before it stabilise, the acid percentage towards peak maturity was 0.96% at ratio 9.7. The external colour development was T7 on the colour plate towards peak maturity. The selection has fair Brix° just below 10° and acid percentage just below 1.0% towards peak maturity. Juice percentage for RHM was very high around 56%. This selection was virtually seedless. The fruit is firm with a smooth rind and a deep orange internal colour, with a thin rind and good flavour.

### Edit x Nova

Edit x Nova is an early to mid-maturing mandarin hybrid. It had a good fruit size count ranging from 3 – 1, smaller compared to last season (1x – 1xxx) due to a heavier crop. Edit x Nova hangs very well on the tree. The sugars and acid percentage don't change much close to and at peak maturity, while the external colour development improves. This could be due to good internal quality. The selection has good Brix° close to peak maturity of 12.7° and acid percentage just above 1.00%. This internal quality contributes to good fruit flavours. Juice percentage for Edit x Nova this season was 56.8%. The selection was seedless. Towards peak maturity the colour was a T5 on the colour plate. The rind colour developed a deep orange colour when the fruit was left to see how long they can hang.

### Saint Andre

Saint Andre was the first selection to reach peak maturity. At peak maturity the Saint Andre has a very good juice percentage peaking at 58.4%. Fruit size of Saint Andre was good, ranged from 1 to 1xx fruit size count. The sugar was good at peak maturity as expected with the acid percentage stabilizing around 0.80%. Brix° was 11.0° at peak maturity. During the evaluations, there were virtually no seeds. The Saint Andre had an external colour of T6 on the colour plate at peak maturity. The fruit did reach T1 on the colour plate when they were left to hang and the acids were still around 0.80% with the Brix also going up to 13.6. Rind is slightly pebbly and flesh is deep orange. The fruit is flat to round and peelability is easy and the flavour very good. The trees had a good crop on them.

### Gold Nugget

Gold Nugget fruit size count was good with 1 - 1x count. The juice percentage of Gold Nugget ranged between 50% - to just above 55%. Towards peak maturity, the Gold Nugget had a T5 – T6 colour on the colour plate. Brix° toward peak maturity was around 10°. Acid percentage did decrease a bit, but the acid percentage was still good at 1.00% at peak maturity. Gold Nugget's good sugars and acid contribute to this tasty fruit. There were no seeds in the Gold Nugget. Peelability of the fruit is easy. The fruit is round and pebbly.

### Tango

Tango is mid to late maturing, seedless selection. Tango was T4 on the colour plate towards peak maturity. Juice percentages (around 60.0%) towards peak maturity. The fruit size for Tango was excellent count 2 - 1. Tango had very good Brix° and acid percentage this season towards peak maturity. Brix° was 11.7° and acid percentage was 1.05% towards peak maturity. The rind is shiny and smooth and the flesh has a deep orange colour.

### Etna

Etna is an early maturing mandarin hybrid. Etna reached peak maturity first on this trial site. External colour development was delayed, T5 on the colour plate at peak maturity. Etna will be able to degreen. Fruit size count is 1xx – 1xxx. Juice percentage for Etna was just below 55% at peak maturity. Etna had 0.2 – 1.0 seeds per fruit during all the evaluations. Brix:Acid ratio 12.3, Brix° was 13.0° and acid percentage was 0.65%. Etna has a deep orange internal colour.

#### Sirio

Sirio is also an early maturing mandarin hybrid. It was the second selection to reach peak maturity at this trial site. Fruit size count for Sirio was count 1xx - 1xxx. Juice percentage was above 55% for the selection at peak maturity. Seed count was low 0.0 – 0.8 seeds per fruit. Rind colour development was good with a T1 at the colour plate (peak maturity). Sirio's rind colour is a deep orange as well as the flesh. Peelability was not easy, but the fruit flavour is good.

#### Tanor Late

Tanor Late is a late-maturing mandarin hybrid. Fruit size count for the selection was large with a 1xxx count on both sites and with an interstock. Tanor Late had a fair juice percentage, below 55% (peak maturity) directly on the rootstock and good juice percentage above 55% with an interstock and was seedless. Brix: Acid ratio towards peak maturity was excellent, the Brix: Acid ratio was better directly on the rootstock. The selection with the interstock is 3 years younger than the selection directly on the rootstock. The good internal quality contributes to better shelf life for the fruit as well as the good flavour of the fruit. External colour development was very good being T1 on the colour plate towards peak maturity. Tanor Late rind colour is a beautiful dark orange colour and internally the colour is an excellent deep orange colour. Tanor Late had a very good crop on the trees. Peelability for Tanor Late is easy but there are small thorns on the bearing branches.

#### Samba

Samba is an early to mid-maturing mandarin hybrid selection. Samba on Carrizo rootstock with Midnight interstock produced a good crop with good internal quality. Trees are fast growing and thornless. Colour development was early in the season (deep orange) with a smooth rind texture on the fruit. T4 on the colour plate towards peak maturity and T1 at peak maturity. Fruit were virtually seedless this season in the combined trial block and fruit size peaked (count 2 to 1x) very good fruit size. Juice percentage at peak maturity was also very good around 60%. Brix was at 12° at peak maturity and acid 1.00%. This good internal quality give Samba its unique and good flavour.

#### Leanri

Leanri is also an early to mid-maturing mandarin hybrid. It had a fruit size count ranging from 1 - 1xx. Leanri has a very good juice percentage. Juice percentage was just above 60% towards and at peak maturity. The sugars and acid percentage was one of the highest at peak maturity. The Brix° was 13.5° and acid percentage of 1.13% when the ratio was 12.0. This internal quality contributes to the good fruit flavour. The selection was virtually seedless during the evaluations with seed count of 0.0 – 0.2 seeds per fruit. Towards peak maturity the colour was already a T1 on the colour plate. The rind colour was deep orange as well as the internal colour.

#### Tasty 1

Tasty 1 is a mid-maturing selection. Tasty 1 fruit size count was 1xx – 1xxx (big fruit), with the fruit on the interstock being a size smaller 1xx. Juice percentage on the rootstock as well as interstock was below 50% (very low). Brix 10.9° and acid percentage was 0.93% towards peak maturity in combination with the interstock, while directly on the rootstock the Brix was 11.2° and acid percentage 0.99%. Colour development was delayed for Tasty 1 being T5 on the colour plate directly on the rootstock, but with the interstock combination it was T1 on the colour plate towards peak maturity. The selection seed count was lower directly on the rootstock compared with the interstock.

#### IRM 1&2

IRM 1 external colour development was better compared to IRM 2, although IRM 2 had a deeper orange colour. IRM 1&2 produced very good internal quality fruit. IRM 2 juice percentage was close to 60% at peak maturity compared to IRM 1 juice percentage around 55% at peak maturity. IRM 1 had the highest Brix towards peak maturity with Brix of 16.7 and acids of 1.44% compared to IRM 2 Brix of 13.8 and acids % of 1.09%. IRM

1 reached T1 on the colour plate before peak maturity. Seed counts for IRM 2 was lower compared with IRM 1.

#### Mor 26

The fruit size count was very good, with count 2 - 1. The external colour development peaked between T4 - T1. Mor 26 internal quality was good with juice levels just below 55% (peak maturity), Brix above 14.0° and fairly high acid levels (around 1.0%). The seed count peaked at 6.5 seeds in the fruit.

#### **Conclusion**

The following selections had the largest fruit size count with a 1xxx count: Tanor Late, Tasty 1, Etna and Sirio. The selections with the highest juice percentage above 60% were Saint Andre, Leanri, Samba and Tango. The selections with the highest °Brix above 13 was IRM 1&2, Mor 26, Tanor Late, Samba, Leanri and Saint Andre. IRM 1&2, Tasty 1 and Mor 26 were the selections that had the most seeds per fruit. IRM 1 & 2, Leanri, Mor 26, Saint Andre, Samba, Tanor Late, Tasty 1 and Sirio reached a colour T1 on the colour plate.

**Table 5.4.14.3.** Internal fruit quality data for Mandarin hybrid selections from Kirkwood region of the Sundays River Valley (Dunbrody) during the 2020 season.

Date	Selection	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-05-25	Edit x Nova	CC with Valencia Interstock	2	56.8	12.7	1.17	10.9	0.0	T 5
2020-06-01	Edit x Nova	CC with Valencia Interstock	2	56.6	12,7	1.25	10.2	0.0	T 5
2020-07-15	Edit x Nova	CC with Valencia Interstock	3	58,6	12,9	0,77	16,9	0,08	T 1 - T 2
2020-06-01	Gold Nugget	CC with Valencia Interstock	1x	53.9	9.7	1.09	8.9	0.0	T 6
2020-06-15	Gold Nugget	CC with Valencia Interstock	1	50.0	10.4	1.01	10.3	0.0	T 6
2020-06-30	Gold Nugget	CC with Valencia Interstock	1x	56.5	10.5	1.02	10.3	0.0	T 5
2020-07-15	Gold Nugget	CC with Valencia Interstock	1x	54,9	10,6	0,63	16,9	0.0	T 2 - T 4
2020-06-30	IRM 1	CC	1x	53.7	15.4	2.03	7.6	3.0	T 3
2020-07-15	IRM 1	CC	2	55,5	16,7	1,44	11,6	5,92	T 1 - T 2
2020-06-15	IRM 2	CC	1	58.4	11.8	1.66	7.1	2.4	T 5
2020-06-30	IRM 2	CC	1	59.4	12.9	1.45	8.9	2.2	T 4
2020-07-15	IRM 2	CC	1	59,6	13,8	1,09	12,7	4,42	T 1 - T 4
2020-06-01	Leanri	CC with Valencia Interstock	1x	62.2	11.9	1.12	10.6	0.0	T 1
2020-06-15	Leanri	CC with Valencia Interstock	1xx	60.4	12.4	1.06	11.7	0.0	T 1

2020-06-30	Leanri	CC with Valencia Interstock	1xx	63.1	13.5	1.13	12.0	0.2	T 1
2020-07-15	Leanri	CC with Valencia Interstock	1	65,4	13,9	0,78	17,8	0,17	T 1
2020-06-01	Mor 26	CC	2	53.5	13.6	1.74	7.8	2. 2	T 5
2020-06-15	Mor 26	CC	1	54.8	14.1	1.36	10.4	1.8	T 4
2020-07-15	Mor 26	CC	2	54,6	15,8	1,04	15,2	6,58	T 1
2020-04-20	RHM	CC with Valencia Interstock	2	56.7	9.3	0.96	9.7	0.1	T 7
2020-05-11	RHM	CC with Valencia Interstock	1	56.0	9.8	0.72	13.6	0.2	T 6
2020-06-05	RHM	CC with Valencia Interstock	1	60.1	10.8	0.66	16.4	0.0	3--4
2020-04-20	Saint Andre	CC	1xx	58.4	11.0	0.86	12.8	0.0	T 6
2020-05-11	Saint Andre	CC	1x	61.2	11.8	0.84	14.0	0.3	T 5
2020-05-25	Saint Andre	CC	1	64.0	12.7	0.82	15.5	0.0	T 1
2020-06-01	Saint Andre	CC	1x	62.7	13.6	0.95	14.3	0.0	T 1
2020-04-20	Samba	CC with Valencia Interstock	2	59.9	11.4	1.12	10.2	0.0	T 6
2020-05-11	Samba	CC with Valencia Interstock	1	61.2	12.0	1.00	12.0	0.0	T 4
2020-05-25	Samba	CC with Valencia Interstock	1x	61.0	13.7	0.89	15.4	0.3	T 1
2020-05-11	Tango	CC	2	58.7	10.8	1.05	10.3	0.0	T 6
2020-06-01	Tango	CC	1	59.9	11.7	1.05	11.1	0.0	T 4
2020-06-15	Tango	CC	1	60.5	12.2	1.21	10.1	0.0	T 4
2020-08-04	Tanor Late	CC with Valencia Interstock	1xxx	55.3	13.5	1.23	11.0	0.0	T 1
2020-09-01	Tanor Late	CC with Valencia Interstock	1xxx	58.0	13.5	1.17	11.5	0.0	T 1
2020-09-10	Tanor Late	CC with Valencia Interstock	1xxx	53.7	14.1	1.05	13.4	0.0	T 1
2020-05-11	Tasty 1	CC with Valencia Interstock	1xx	42.9	10.0	1.02	9.8	3.2	T 6

2020-06-01	Tasty 1	CC with Valencia Interstock	1xx	43.9	10.5	0.91	11.5	1.6	T 4
2020-06-15	Tasty 1	CC with Valencia Interstock	1xx	48.2	10.9	0.93	11.7	0.4	T 1
2020-07-15	Tasty 1	CC with Valencia Interstock	1x	49,7	11,9	0,60	19,7	3,25	T 1

**Table 5.4.14.4.** Internal fruit quality data for Mandarin hybrid selections from Kirkwood region of the Sundays River Valley (Invercloy) during the 2020 season.

Date	Selection	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-04-20	Etna	CC	1xx	54.0	13.0	0.65	12.3	1.0	T 5
2020-05-11	Etna	CC	1xxx	48.7	8.5	0.58	14.7	0.6	T 4
2020-04-20	Sirio	CC	1xx	56.4	10.5	0.92	11.4	0.2	T 4
2020-05-11	Sirio	CC	1xxx	53.2	11.2	0.87	12.9	0.8	T 1
2020-05-25	Sirio	CC	1xxx	51.9	11.3	0.75	15.1	0.0	T 1
2020-08-04	Tanor Late	CC	1xxx	53.2	13.5	1.24	10.9	0.0	T 1
2020-09-01	Tanor Late	CC	1xxx	53.7	14.0	1.11	12.6	0.3	T 1
2020-09-10	Tanor Late	CC	1xxx	53.4	14.0	1.08	13.0	0.0	T 1
2020-05-11	Tasty 1	CC	1xx	47.1	11.2	0.99	11.3	0.8	T 5
2020-05-25	Tasty 1	CC	1xx	48.8	11.8	0.84	14.0	1.5	T 2
2020-06-01	Tasty 1	CC	1xxx	48.0	12.3	0.82	15.0	0.7	T 1

#### 5.4.15 PROGRESS REPORT: Cultivar characteristics and climatic suitability of Mandarin hybrids in a cold production region (Gamtoos River Valley)

Project 997C by W. Swiegers and Z. Zondi (CRI)

##### Summary

Loerie is the main trial site in the Gamtoos River Valley area, but there is a new site on the way with all of the latest selections in Patensie. Unfortunately the area is currently going thru drought. The first evaluations for the new site will start in 2021 season. Both trial sites will form part of the Gamtoos River Valley. At Loerie the season started off with St Andre and was followed up by Etna, Sirio, Samba, Tasty 1, Nadorcott, Tango, Gold Nugget and the season ended with Tanor Late.

##### Opsomming

Loerie is die hoof perseel in die Gamtoos Rivier Valleie, maar daar is 'n nuwe perseel in Patensie wat al die nuwe seleksies gaan bevat. Ongelukkig gaan die area deur erge droogte. Die eerste evaluasies van die perseel begin in 2021. Albei persele maak deel uit van die Gamtoos Rivier Valleie. By Loerie het die seisoen begin met St Andre gevolg deur Etna, Sirio, Samba, Tasty 1, Nadorcott, Tango, Gold Nugget en die seisoen het geëindig met Tanor Late.

##### Objectives

- To select mandarin hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).

- To describe the characteristics of new mandarin hybrid cultivars and to determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on mandarin hybrid selections from the Gamtoos River Valley. A range of new mandarin hybrids had been added to this area. The following cultivars were evaluated: Saint Andre, Etna, Sirio, Tanor Late, Tasty 1, Tango, Gold Nugget, Samba and Nadorcott.

A ratio of 11:1 for mandarin hybrids is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.15.1** List of experimental mandarin hybrid selections evaluated in the Loerie (N. Ferreira) region of the Gamtoos River Valley during the 2020 season.

Selection	Rootstock	Topwork
Etna	Carrizo	2012
Sirio	Carrizo	2012
Saint Andre	Carrizo	2012
Tasty 1	Carrizo	2012
Gold Nugget	Carrizo	2012
Tango	Carrizo	2012
Tanor Late	Carrizo	2012
Nadorcott	Carrizo	2012
Samba	Carrizo	2017

## Results and discussion

### Gold Nugget

The fruit size for Gold Nugget was count 1 – 1x. Fruit for this selection is pebbly and peelability is easy. At peak maturity the external colour development was T1 on the colour plate. For this selection it is a yellow orange colour. There were thorns on the bearing branches, but they get smaller as the tree gets older. Gold Nugget is a seedless cultivar. At peak maturity the internal juice percentages were 58.1%. Internal quality for the selection is good with high sugars above 14 and good acids around 1.22%, this also contributes to the good flavour of Gold Nugget.

### Tango

Tango had a very good fruit size (count 1) and was seedless, as it is a seedless variety. The selection had good external colour development T1 on the colour plate at peak maturity. Tango rind and internal colour is a deep orange. The rind also has a natural shine and peels very easily, and the rind oil doesn't bother. Tango juice percentage was around 60%. Tango's Brix and acid ratios were good, indicating that the fruit will have a good shelf life and give Tango a good flavour.

### Tanor Late

Tanor Late is a late maturing mandarin hybrid. Fruit size count for the selection was extra-large with a 1xxx count. Tanor Late juice percentage, around 50% (peak maturity). Tanor Late was seedless. Brix: Acid ratio towards peak maturity was good. The good internal quality contributes to better shelf life for the fruit and the good flavour of the fruit. External colour development was very good, being T1 on the colour plate towards peak maturity.

### Saint Andre

Saint Andre was the first selection to reach peak maturity in this production region. The fruit size count was between 2 and 1. The juice percentage this season was very good just above 60%. At peak maturity the colour was delayed T6 on the colour plate. The acids and sugars remained stable during the production season for Saint Andre. At peak maturity Brix 10.9° and Acid percentage was 0.90%. Internal quality was good and it contributes to the good flavour. The selection had a seed count peaking at 0.3 seeds per fruit, (virtually seedless) during the evaluations.

#### Etna

Etna is an early maturing mandarin hybrid. The fruit size count for the Etna this season was the same compared to last season, count 1x – 1xx. The juice percentage for Etna was good, above 60% juice at peak maturity. Compared with Sirio, Etna's juice percentage was higher. The Brix and Acid levels of Etna were slightly lower than Sirio towards peak maturity. Sirio had a slightly higher Brix and acid. Etna reached peak maturity before Sirio in the mandarin range of new experimental cultivars. Etna had a T1 on the colour plate range at peak maturity. Etna's seed count peaked at 0.1 seeds per fruit, lower than Sirio.

#### Sirio

Sirio also an early maturing mandarin hybrid. Sirio had a large - extra-large fruit size count of 1x - 1xx. Sirio developed a lower juice percentage compared to Etna. The juice percentage increased towards peak maturity to around 55%, before it drop slightly. The Brix and Acid levels of Sirio were slightly higher than Etna at peak maturity. Sirio external colour development was slightly delayed, being T2 on the colour plate at peak maturity. Sirio seed count ranged between 0.3 – 1.1 seeds per fruit.

#### Tasty 1

Tasty 1 developed an extra-large fruit size and peaked at count 1xx. The external colour development was delayed with a T3 – T4 on the colour plate at peak maturity. The juice percentages were not good for Tasty 1, being below 50%. Seed count were 1.4 – 2.1 seeds per fruit. At peak maturity the Brix was 10.7° with acid percentage of 0.82%.

#### Nadorcott

Nadorcott developed a favourable fruit size (count 2 - 1) for this trial site. The selection was seedless during the evaluations. There was a good colour development (T1) at peak maturity. Rind texture was very smooth with a natural shine. The selection developed good internal quality with juice levels (above 60%), Brix averaging 11.6° and acceptable acids (0.88%).

#### Samba

Samba produced a good first crop with good internal quality. Colour development was good with early colour break, reaching T 1 on the colour plate at peak maturity. The rind colour is a deep orange. Fruit size for Samba was also favourable with medium – large fruit size count 2 – 1. Internal quality was very good with juice percentage around 60% at peak maturity, with Brix of 11.9° and acid percentage of 0.86%. Brix:Acid ratio was 13.8. Seed count peaked at 0.4 seeds per fruit. Peelability is easier than Nova, with a good flavour fruit.

### **Conclusion**

All of the selections had very good external colour development (T1) at peak maturity except St Andre and Tasty 1. The following selections had the largest fruit size (count 1xx - 1xxx); Tanor Late and Tasty 1, Sirio and Etna. Nadorcott St Andre, Samba and Tango cropped the smallest fruit size (count 2 - 1). Etna, Nadorcott, Samba, Tango and St Andre developed juice percentages above 60%. Tasty 1 had juice percentages below 50%. Gold Nugget, Tango and Tanor Late had the highest Brix level above 13°. The selection with the highest seed count was Tasty 1.

**Table 5.4.15.2.** Internal fruit quality data for experimental mandarin hybrid selections from the Loerie (N. Ferreira) region of the Gamtoos River Valley region during the 2020 season.

Date	Selection	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-04-21	Etna	CC	1xx	62.7	9.3	0.93	10.0	0.1	T5
2020-05-04	Etna	CC	1x	61.8	9.5	0.89	10.7	0.0	T4
2020-05-20	Etna	CC	1x	60.1	9.7	0.71	13.7	0.1	T1
2020-06-10	Gold Nugget	CC	1x	55.5	11.9	1.24	9.6	0.0	T5
2020-08-11	Gold Nugget	CC	1	58.1	14.1	1.22	11.6	0.0	T1
2020-08-26	Gold Nugget	CC	1	58.8	15.5	1.15	13.5	0.0	T1
2020-06-10	Nadorcott	CC	2	60.8	10.5	1.06	9.9	0.0	T4
2020-06-23	Nadorcott	CC	1	64.4	11.6	0.88	13.2	0.0	T1-2
2020-04-21	Saint Andre	CC	1	61.9	10.9	0.90	12.1	0.3	T6
2020-05-04	Saint Andre	CC	1	63.0	11.4	0.82	13.9	0.3	T5
2020-05-20	Saint Andre	CC	2	62.8	11.5	0.78	14.7	0.3	T2
2020-04-21	Samba	CC	1	61.0	10.4	0.95	10.9	0.4	T5
2020-05-04	Samba	CC	1	58.9	10.4	0.93	11.2	0.4	T4
2020-05-20	Samba	CC	2	59.6	11.9	0.86	13.8	0.0	T1
2020-04-21	Sirio	CC	1xx	53.2	10.3	0.91	11.3	0.3	T4
2020-05-04	Sirio	CC	1x	55.8	10.5	0.84	12.5	0.6	T2
2020-05-20	Sirio	CC	1xx	53.7	11.0	0.81	13.6	1.1	T1
2020-06-10	Tango	CC	1	61.8	11.1	1.08	10.3	0.0	T4
2020-07-28	Tango	CC	1	57.4	13.4	1.14	11.0	0.1	T1
2020-08-26	Tango	CC	1	63.8	14.0	1.23	11.4	0.2	T1
2020-07-28	Tanor Late	CC	1xxx	50.6	12.4	1.08	11.5	0.0	T1
2020-08-26	Tanor Late	CC	1xxx	51.5	13.5	0.90	15.1	0.1	T1
2020-09-08	Tanor Late	CC	1xxx	52.8	13.8	0.93	14.8	0.0	T1
2020-05-20	Tasty 1	CC	1xx	49.5	10.5	0.94	11.2	2.1	T5
2020-06-10	Tasty 1	CC	1xx	49.3	10.7	0.82	13.1	1.4	T3-4

**5.4.16 PROGRESS REPORT: Cultivar characteristics and climatic suitability of mandarin hybrids in a cold production region (Western Cape)**  
Project 997D by W. Swiegers (CRI)

**Summary**

The trial site in Citrusdal consists of a cultivar block with a selection of all the new experimental cultivars from early-maturing to late maturing selections. Cross pollination was high in this block due to all the different selections present. The season started with Tami 2/65 and then RHM followed by, Samba, Leanri, Etna, Nadorcott ARC, Sirio, IRM 2, Tango, Or 4, Gold Nugget, Furr, Mor 26, Nadorcott and IRM 1.

**Opsomming**

Die proef perseël in Citrusdal bevat meeste van die nuwe eksperimentele seleksies van vroeg tot laat rypwordend. Die kruisbestuiwing in hierdie proef perseël is baie hoog weens al die verskillende seleksies teenwoordig. Die orde van rypwording was as volg gewees Tami 2/65 gevolg deur RHM, Samba, Leanri, Etna, Nadorcott ARC, Sirio, IRM 2, Tango, Or 4, Gold Nugget, Furr, Mor 26, Nadorcott en IRM 1.

**Objectives**

- To select mandarin hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new mandarin hybrid cultivars and determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on mandarin hybrid selections from Citrusdal region of the Western Cape. The following selections were evaluated: Tami 2/65, Sirio, RHM, Etna, Samba, Mor 26, Or 4, IRM 1, IRM 2, Furr, Tango, Gold Nugget, Nadorcott ARC, Nadorcott en Leanri.

A ratio of 11:1 for mandarin hybrids is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.16.1.** List of experimental mandarin hybrid selections evaluated in the Citrusdal region of the Western Cape during the 2020 season.

Selection	Rootstock	Topwork	Planted
Furr (Clemcott)	CC	2011	
Gold Nugget	CC	2010	
ARC Nadorcott	CC	2010	
Nadorcott	CC		2009
RHM	CC		2013
Tango	CC	2010	
Leanri	CC	2015	
Sirio	CC	2012	
Etna	CC	2012	
Samba	CC	2012	
IRM 1	CC		2009
IRM 2	CC	2010	
Mor 26	CC		Unknown
Or 4	CC		Unknown
Tami 2/65	CC	2010	

## Results and discussion

### Tami 2/65

Tami 2/65 is an early maturing mandarin hybrid. Tami 2/65 was the first selection to reach peak maturity at the Citrusdal site. The fruit size for Tami 2/65 was good with a fruit size count range between 3 – 1x. Internal juice percentage was good above (55%). Internal colour is a deep orange. The fruit peels easily. The selection was virtually seedless. Rind colour development was not good with T7 on the colour plate at peak maturity. The selection doesn't have high acid and it tends to drop quickly leaving you with a short harvesting period. Brix is between 9 – 10°. Crop on the trees looked good. Further test need to be done to determine the potential of the cultivar.

### Furr (Clemcott)

Furr is used as a control for the mid-maturing mandarin selections. The juice content was good, just below 60% (peak maturity). The fruit size count was range from 1x - 1xxx. Furr has a very good eating quality. Due

to the high cross pollination in the mixed trial block, Furr produced a number of seeds per fruit; up to 1.2. Furr's external colour development was very good this season; T1 on the colour plate range at peak maturity. Brix: Acid ratio for Furr was very good. High sugars and good acid give Furr its good flavour.

#### Etna

Etna is an experimental early mandarin hybrid. Etna reached peak maturity more to the mid-maturing range. The fruit size count for the Etna this season ranged from count 3 – 1xx. Etna had a juice percentage above 60% towards peak maturity. Internal quality at peak maturity for Etna was good. Brix was around 10 and acid percentage around 0.9%. Etna had a T1 – T2 on the colour plate at peak maturity. Etna has a deep orange internal colour. Etna seed count was between 0.4 – 0.5 seeds per fruit.

#### Sirio

Sirio is also an early experimental mandarin hybrid, but it also reached peak maturity more to the mid-maturing range the same as Etna. Fruit size count for Sirio was the same as for Etna 3 – 1xx. Internal quality for Sirio was better than that of Etna, with Brix of 13° and acid above 1.0%. Sirio also has better flavour than Etna. Juice percentage for Sirio was lower compared to Etna. Sirio seed count was 0.3 – 1.1 seeds per fruit. Sirio had no external colour development problems, being fully coloured at peak maturity. Sirio has internally as well externally deep orange colour but peelability is not easy.

#### Mor 26

Fruit size for Mor 26 was medium – extra large with count 3 – 1xx. The juice percentage for Mor 26 was very good, 60.7% juice percentage at peak maturity. Rind colour development was good to reach T1 on the colour plate at peak maturity. The seed count was 0.1 – 0.7 seeds per fruit. Internal quality was good at peak maturity Brix° above 13° and acid percentage was just above 1.0%. This good Brix: Acid ratio will contribute to good eating fruit with good flavour and shelf life.

#### Or 4

The size count for this selection was also medium – extra large count 3 - 1xx. The fruit is round to oblate. Juice percentage at peak maturity was very good at 60.7%. Internal quality for Or is very good. Brix is high, above 14° and the acids were just below 1.0% even when the fruit was over mature. This good Brix: Acid ratio will contribute to excellent eating fruit with great flavour and shelf life. Peelability is easy and oily. Or reached T1 on the colour plate at peak maturity and average seed count was 0.5 seeds per fruit.

#### Gold Nugget

Tree manipulation is necessary to control the strong vegetative and upright growth habit. Gold Nugget developed good tasting fruit with a high Brix: acid ratio. The fruit peaked internally with Brix around 13.1°. Due to the good quality of the fruit, it will be possible to hang the fruit longer on the trees with an extended shelf life. Gold Nugget's fruit size was at count 3 – 1x. At peak maturity and fully coloured (T1- T2). The juice percentage for this selection was around 52%. Gold Nugget is seedless.

#### Nadorcott & Nadorcott ARC

Nadorcott ARC is an induced Nadorcott selection to minimise the average seeds per fruit. Nadorcott ARC has the same growth habit and characteristics as the Nadorcott. Fruit size for Nadorcott ARC ranged between counts 3 – 1xx and for Nadorcott it ranged between 2 – 1xx. The internal juice percentages were around 55% at peak maturity for both selections. The Nadorcott selections developed good Brix above 12° with acids around 1.0%, ensuring a good balance and eating quality as well as shelf life. The fruit was fully coloured (T1) before peak maturity. Both selections were seedless. Fruit have a natural shine on them. Nadorcott ARC reached peak maturity first, followed by Nadorcott.

#### Samba

Samba is an early – mid maturing experimental low seeded mandarin hybrid. The selection was seedless during the evaluation. Samba has a favourable fruit size count that ranges between counts 4 – 2. Crop was good. Fruit is round to oblate with halo. Peelability is easier for Samba than Nova and Samba rind can be oily. Long before peak maturity was achieved, Samba was a T 1 on the colour plate. Samba has an exceptional deep orange external colour and a very deep internal colour. At peak maturity; Samba has high Brix and good

acids and these give Samba its unique and excellent flavour. The juice percentage at peak maturity for this selection was 61.7%.

### Tango

Tango developed a very smooth rind texture, similar to Nadorcott, with a natural shine. The fruit had a very good colour development in the cooler areas (colour plate T1) at peak maturity. Tango was seedless. The fruit size peaked at count 3 – 1x in Citrusdal. Internally the average juice percentage for Tango was around 60%. At the trial site the Brix: acid ratio was good, with Brix 13° and acid 0.90%.

### IRM 1 & IRM 2

The IRM 1 is a late maturing experimental mandarin hybrid. IRM 2 is a mid to late maturing experimental mandarin hybrid. IRM 1 reached peak maturity 2 - 3 weeks after IRM 2. The fruit size counts for IRM 2 were 3 – 1xx, for IRM 1 it was 2 – 1xx. IRM 1 had higher juice content between the 2 selections. Internal quality for IRM 1 was better than that of IRM 2. IRM 1, Brix was 14.4° and acid was 1.17% at peak maturity. IRM 2 close to peak maturity was Brix 11.1° and acid 1.06%. IRM 1 seed count peaked at 0.9 seeds per fruit and IRM 2 seed count peaked at 0.7 seeds per fruit. External colour development was good for both selections IRM 1 & 2 (T 1 – T2 on the colour plate) at peak maturity. IRM 1 & 2 are prone to ribbing.

### Leanri

Leanri is an early – mid maturing mandarin hybrid selection. It was the fourth selection to reach peak maturity. The fruit had a good colour development (T1- T2) at peak maturity. The rind colour was orange and the internal colour was deep orange. Leanri had a good internal quality, juice percentage at 60.3%, Brix (11.7°) with acids (1.00%) at peak maturity. The flavour was good and peelability easy. The selection's seed count ranged from 0.2 – 1.0 seeds per fruit. Fruit size count ranged 2 – 1xxx.

### RHM

RHM was second to reach peak maturity at the trial site. The fruit size (count 4 – 1xx). Acid dropped quickly but stayed stable around 0.80% at peak maturity in cold production regions and with the good sugars it contributed to good flavour and eating quality. The external colour development was delayed with T6 on the colour plate when the fruit was at peak maturity. The selection has good Brix° at 10.9° and acid percentage of 0.85%. Juice percentage for RHM was very good, above 60%. There were seed for this selection during evaluations with a count of 3.1 seeds per fruit. The fruit is firm with a smooth rind and a deep orange internal colour. Some of the fruit tends to split.

## **Conclusion**

Leanri and Furr had the largest fruit size (1xxx). Samba had the smallest fruit size with a count 4 – 2. RHM had the most seeds per fruit on average (1.9 seeds per fruit). Samba, Nadorcott and Nadorcott ARC were the only selections that were completely seedless. The following selections had a juice percentage over 60% at peak maturity IRM 1, Mor 26, Nadorcott, Or 4, Tango, RHM, Etna, Samba and Leanri. Most of the selections were a T1 on the colour plate range (good colour development) before or at peak maturity.

**Table 5.4.16.3.** Internal fruit quality data for experimental mandarin hybrid selections from the Citrusdal region of the Western Cape during the 2020 season.

Date	Cultivar	Rootstock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-07-01	IRM 1	CC	2 - 1xx	61,0	12,2	1,18	10,3	0,3	T 2 - T 3
2020-07-29	IRM 1	CC	2 - 1x	64,0	14,4	1,17	12,3	0,9	T 1 - T 2
2020-08-24	IRM 1	CC	1 - 1xx	62,3	12,6	1,05	12,0	0,4	T 1 - T 2
2020-06-03	IRM 2	CC	3 - 1	55,5	9,9	1,17	8,4	0,0	T 5
2020-07-01	IRM 2	CC	1 - 1xx	47,6	11,1	1,06	10,5	0,7	T 2
2020-07-29	IRM 2	CC	2 - 1	58,5	13	0,75	17,3	0,1	T 1 - T 2

2020-05-14	Leanri	CC	2 - 1xxx	45,0	11	0,90	12,3	0,4	T 4 - T 5
2020-06-03	Leanri	CC	1 - 1xx	60,3	11,7	1,00	11,7	1,0	T 1 - T 2
2020-07-01	Leanri	CC	2 - 1x	51,5	12,4	1,04	11,9	0,2	T 1
2020-07-01	Mor 26	CC	3 - 1x	49,0	12,8	1,17	11,0	0,1	T 2 - T 3
2020-07-29	Mor 26	CC	1 - 1xx	60,7	13,5	1,01	13,4	0,7	T 1
2020-08-24	Mor 26	CC	2 - 1xx	64,1	13,3	1,04	12,8	0,4	T 1
2020-06-02	Murcott	CC	1x - 1xx	63,1	11,5	1,23	9,4	0,7	T 4
2020-07-01	Murcott	CC	5 - 1xx	49,0	12,3	1,09	11,3	0,8	T 1
2020-07-29	Murcott	CC	1x - 1xxx	57,6	12,6	0,94	13,3	1,2	T 1
2020-07-01	Nadorcott	CC	2 - 1 xx	58,3	12,5	1,36	9,2	0,0	T 1
2020-07-29	Nadorcott	CC	2 - 1xx	62,5	13,5	1,01	13,4	0,0	T 1
2020-08-24	Nadorcott	CC	2 - 1x	56,8	13,4	1,09	12,3	0,0	T 1
2020-06-03	Nadorcott ARC	CC	3 - 2	57,5	9	1,24	7,3	0,0	T 5
2020-07-01	Nadorcott ARC	CC	2 - 1x	48,0	12,1	1,06	11,4	0,0	T 2
2020-07-29	Nadorcott ARC	CC	2 - 1xx	55,3	14	0,84	16,6	0,0	T 1
2020-07-01	Or	CC	3 - 1	46,9	14	1,34	10,4	0,5	T 1
2020-07-29	Or	CC	2 - 1xx	60,7	14,2	0,98	14,5	0,3	T 1
2020-08-24	Or	CC	1 - 1xx	56,2	13,7	0,82	16,7	0,8	T 1
2020-04-20	RHM	CC	4 - 2	56,3	10	0,92	10,9	0,6	T 8
2020-05-14	RHM	CC	4 - 1x	60,7	10,9	0,85	12,8	2,1	T 6
2020-06-02	RHM	CC	2 - 1xx	63,5	11,5	0,80	14,3	3,1	T 3 - T 4
2020-04-20	Tami 2/65	CC	3 - 1x	58,1	9,2	0,80	11,5	0,1	T 7
2020-05-14	Tami 2/65	CC	3 - 1	52,0	10,1	0,67	15,0	0,0	T 5
2020-07-01	Tango	CC	3 - 1	54,9	12,5	1,19	10,5	0,0	T 1
2020-07-29	Tango	CC	2 - 1x	66,0	13	0,90	14,5	0,0	T 1
2020-08-24	Tango	CC	3 - 2	58,9	13	0,85	15,3	0,0	T 1
2020-05-13	Etna	CC	3 - 1xx	49,5	10,1	0,97	10,4	0,5	T 4
2020-06-02	Etna	CC	2 - 1	61,9	10	0,90	11,2	0,4	T 1 - T 3
2020-07-01	Gold Nugget	CC	3 - 1	54,9	11,7	1,23	9,5	0,1	T 2
2020-07-30	Gold Nugget	CC	2 - 1x	52,6	13,1	0,96	13,6	0,0	T 1 - T 2
2020-05-13	Samba	CC	4 - 2	61,7	12,8	1,14	11,3	0,0	T 1 - T 2
2020-06-02	Samba	CC	3 - 2	60,2	12,7	1,14	11,2	0,0	T 1
2020-06-02	Sirio	CC	2 - 1xx	51,2	11,5	1,17	9,8	1,1	T 1
2020-07-01	Sirio	CC	3 - 2	50,8	13,2	1,23	10,7	0,3	T 1

#### 5.4.17 PROGRESS REPORT: Cultivar characteristics and climatic suitability of mandarin hybrids in a cold production region (South West Cape)

Project 997E by W. Swiegers (CRI)

#### Summary

This is a fairly new trial site in South West Cape. The trial trees had their fourth crop during the 2020 season. It's a cultivar block with a selection of all the new experimental cultivars from early maturing to late maturing selections. South West Cape is well suited for soft citrus. There is cross pollination in this block due to all the different selections that are present. A new site is going to be added to this site to cover more of the new

selections. The order of ripening was as follows: Tami 2/65, RHM, Goldup, Edit x Nova, Or 4, Leanri, Or 1, Taylor Lee LS, IRM 1, IRM 2 and the season ended with Mor 26.

## Opsomming

Dit is 'n nuwe proef perseël in die Suid Wes Kaap. 2020 was die vierde seisoen met vrugte op die bome. Die meeste van die nuwe eksperimentele seleksies van vroeg tot laat rypwordend kom in die perseël voor. Die Suid Wes Kaap is goed geskik vir sagte sitrus verbouing. Die kruisbestuiwing in hierdie proef perseël is hoog weens al die verskillende seleksies teenwoordig. Daar gaan nog 'n perseël bykom wat ook van die nuutste seleksies sal bevat. Die orde van rypwording was as volg gewees: Tami 2/65, RHM, Goldup, Edit x Nova, Or 4, Leanri, Or 1, Taylor Lee LS, IRM 1, IRM 2 en Mor 26 wat die seisoen afgesluit het.

## Objectives

- To select mandarin hybrid cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new mandarin hybrid cultivars and determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on mandarin hybrid selections from Buffeljagsrivier region of the South West Cape. The following selections were evaluated: Goldup, RHM, Tami 2/65, Edit x Nova, Or 4, Or 1, Leanri, Taylor Lee LS, Mor 26, IRM 2 and IRM 1.

A ratio of 11:1 for mandarin hybrids is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.17.1.** List of experimental mandarin hybrid selections evaluated in the Buffeljagsrivier region of the South West Cape during the 2020 season.

Selection	Rootstock	Topwork
Edit x Nova	CC	2014
IRM 1	CC	2014
IRM 2	CC	2014
Mor 26	CC	2014
Tami 2/65	CC	2014
Taylor Lee LS	CC	2014
Goldup	CC	2014
Or 1	CC	2014
Or 4	CC	2014
RHM	CC	2014
Leanri	CC	2014

## Results and discussion

### Tami 2/65

Tami 2/65 is an early maturing experimental mandarin. Fruit size for Tami 2/65 was small to large (count 4 – 1) compared to 2019 season fruit size medium – extra-large (count 2 – 1xx). The internal juice percentage at

peak maturity was good. Rind is smooth and the colour is a deep orange. The fruit peels easily. The selection seed count peaked at 1.7 seeds per fruit during the evaluations. Rind colour development was slightly delayed with T1 – T3 on the colour plate at peak maturity. The selection doesn't have high acid to start with but it had a good acid percentage ,0.90% at peak maturity with a Brix 13.8°.

#### Edit x Nova

Edit x Nova is an early to mid-maturing experimental mandarin hybrid. Edit x Nova had very good Brix: Acid ratio at peak maturity, Brix° was 13.2° and acid percentage was 1.07% at ratio 12.4. This will give Edit x Nova its good flavour. The seed count peaked at 0.2 seeds per fruit. Edit x Nova were fully coloured at peak maturity. Fruit size for Edit x Nova was favourable with 3 – 1x count.

#### IRM 1 & 2

The IRM 1 & 2 are late-maturing experimental mandarin hybrids. IRM 1 reached peak maturity before IRM 2 this season, but normally it is the other way around. IRM 1 & 2 are prone to ribbing and alternate bearing. The fruit size count for IRM 1 was very good (count 3 – 1), IRM 2 was slightly smaller (count 4 – 1). Internally the juice content for both selections increased towards peak maturity to above 60%. Brix: Acid ratio at peak maturity was very similar for IRM 1 & 2, Brix was high above 16° and acid was good above 1.0%. Seed count peaked around 3.0 seeds per fruit. IRM 1 & 2 were a T1 on the colour plate before peak maturity. The rind was smooth and peelability easy.

#### Mor 26

Mor 26 fruit size count is smaller compared to the 2019 season, 2020 (count 4 – 2); 2019 (count 2 – 1x). Towards peak maturity, Mor 26 juice percentage was 59.9%. Mor 26 internal quality was one of the best of all the selections. Very high sugars and good acids. Towards peak maturity with ratio 10.9 the Brix was already at 17.9° and acid 1.64%. The average seed count for this selection peaked at 2.9 seeds per fruit. Rind colour development was good to reach T1 colour on the colour plate towards peak maturity.

#### Taylor Lee LS

Taylor Lee LS is mid to late maturing experimental mandarin hybrid. The trees bore medium - large fruit on the trees with count 3 – 1x, and at peak maturity the juice content was 64%. Brix: Acid ratio at 12.7 the selection had Brix 16.9° and acid of 1.33%. The selection seed counts were on average 4.0 seeds per fruit. Taylor Lee LS reached T1 colour on the colour plate before peak maturity.

#### Leanri

Leanri is an experimental early – mid maturing mandarin hybrid. The average seed count for Leanri ranged between 3.2 – 3.3 seeds per fruit. Leanri had a good Brix° and acid ratio towards peak maturity (ratio 11.0), the Brix was 15.4° and acid was 1.41%. This gives Leanri its good flavour. The juice percentage for Leanri was very good around 60%. The fruit was fully coloured up before peak maturity. Fruit size count was (count 3 – 1x).

#### RHM

The fruit size count range from medium to extra-large (count 2 – 1xxx) and the acid dropped quickly towards peak maturity to around 0.80% but it stayed stable at around 0.80% until over mature. The external colour development was delayed with T2 – T3 on the colour plate when the fruit was at peak maturity. At peak maturity the selection had good Brix° at 11.2 and acid percentage of 0.86%. Juice percentage for RHM was good 57%. There were seeds in this selection during evaluations with an average count of 7.0 seeds per fruit. The fruit is firm with a smooth rind and a deep orange internal colour.

#### Or 1 & 4

Or 1 & 4 is a mid - late maturing mandarin hybrids. The size count for Or 1 ranged from medium to large count 3 – 1x and fruit size count for Or 4 was also medium to large 3 - 1. Juice percentage increase towards peak maturity to around 60%, with Or 1 being slightly higher. Internal quality for the two Or selections is very good. Brix is high above 15° and the acids was still above 1.00% at peak maturity. This good Brix: Acid ratio will contribute to excellent eating fruit with great flavour and shelf life. Peelability is easy but oily. Or 1 & 4 reached T1 on the colour plate towards peak maturity. Or 4 had a slightly higher seed count.

## Goldup

Goldup is an early maturing experimental mandarin hybrid. It reached peak maturity third in the trial block. Ratio of 11.8 was reached on 15 May. Internal quality was good. Juice percentage was the highest in the trial block with 67.1%, with 10.8° Brix and acid percentage of 0.91%. The trees are still very young and the internal quality improves as the trees get older. This selection does tend to have a low acid to start with and it tends to drop quickly, but stabilises. Colour development was slightly delayed at peak maturity of T2 on the colour plate. Fruit size ranged between 3 – 1x (medium - large). Goldup was virtually seedless during evaluations. Fruit shape is flattish and has a natural shine. Peelability is easy and the rind has a prominent aroma when it gets peeled. Rind oil is high.

## Conclusion

RHM had the largest fruit size peaking at count (2 - 1xxx) and Mor 26 had the smallest fruit size count (4 - 2). RHM had the most seeds per fruit on average (7.0). None of the selections were completely seedless. Most of the selections had a juice percentage above 60% at peak maturity and all of them were above 55%. Only RHM didn't reach T 1 on the colour plate before or at peak maturity. Selections that didn't reach Brix above 14° towards peak maturity and at peak maturity were Goldup, Tami 2/65 and RHM.

**Table 5.4.17.2.** Internal fruit quality data for experimental mandarin hybrid selections from the Buffeljagsrivier region of the Western Cape during the 2020 season.

Date	Cultivar	Rootstock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-05-15	Edit X Nova	CC	2 - 1x	46,5	12,6	1,11	11,4	0,0	T 2 - T 3
2020-06-05	Edit X Nova	CC	2 - 1x	62,1	13,2	1,07	12,4	0,2	T 1
2020-07-02	Edit X Nova	CC	3 - 2	58,9	14,3	1,09	13,1	0,0	T 1
2020-04-22	Goldup	CC	3 - 1	46,4	10,7	1,07	10,0	0,3	T 1 - T 2
2020-05-15	Goldup	CC	2 - 1x	67,1	10,8	0,91	11,8	0,0	T 2
2020-07-02	IRM 1	CC	3 - 1	46,5	14,8	1,76	8,4	3,0	T 2
2020-07-31	IRM 1	CC	2 - 1	60,6	16	1,35	11,9	3,2	T 1
2020-07-02	IRM 2	CC	4 - 1	55,4	15,6	1,91	8,2	3,7	T 2
2020-07-31	IRM 2	CC	3 - 1	63,4	16,1	1,46	11,0	2,7	T 1 - T 2
2020-06-05	Leanri	CC	2 - 1x	59,6	14,1	1,35	10,4	3,2	T 1
2020-07-02	Leanri	CC	3 - 1	61,2	15,4	1,41	11,0	3,2	T 1
2020-07-30	Leanri	CC	3 - 1	60,4	17	1,27	13,4	3,3	T 1
2020-07-02	Mor 26	CC	4 - 2	59,3	15,2	1,82	8,3	2,4	T 2
2020-07-31	Mor 26	CC	4 - 2	59,9	17,9	1,64	10,9	2,9	T 1
2020-07-02	Or 1	CC	3 - 2	55,8	14,9	1,57	9,5	1,3	T 1 - T 2
2020-07-31	Or 1	CC	2 - 1x	61,6	15,6	1,18	13,2	2,5	T 1
2020-07-02	Or 4	CC	3 - 2	56,0	15	1,56	9,6	2,7	T 2
2020-07-31	Or 4	CC	3 - 1	58,2	16,4	1,18	13,8	1,3	T 1
2020-04-22	RHM	CC	2 - 1x	55,5	10,4	0,96	10,8	5,2	T 3 - T 6
2020-05-15	RHM	CC	2 - 1xxx	57,0	11,2	0,86	13,0	11,1	T 2 - T 3
2020-06-05	RHM	CC	2 - 1xx	54,5	11,5	0,82	14,0	4,7	T 1
2020-04-22	Tami 2/65	CC	3 - 1	53,3	11,5	1,07	10,7	0,5	T 5 - T 6
2020-05-15	Tami 2/65	CC	4 - 1	57,6	12,4	0,90	13,8	1,7	T 1 - T 3
2020-06-05	Taylor Lee	CC	3 - 1x	61,7	15,2	1,68	9,0	5,3	T 1
2020-07-02	Taylor Lee	CC	2 - 1	61,7	15,9	1,39	11,4	3,4	T 1
2020-07-31	Taylor Lee	CC	3 - 1	64,0	16,9	1,33	12,7	3,9	T 1

#### 5.4.18 **PROGRESS REPORT: Cultivar characteristics and climatic suitability of navel oranges in a cold production region (Sundays River Valley)**

Project 998B by W. Swiegers and Z. Zondi (CRI)

##### **Summary**

There are 3 trial sites in the Sundays River Valley. They are all between Kirkwood and Addo. The trial site at Endulini is a new site with early to late maturing navel selections that had their third crop on them. For this site the season started with: Sunrise Early, Trosky Early, De Wet 1, KS Early, Lethaba Early, Navelina, Painter Early 2, Cara Cara, Kirkwood Red, Washington, Clark, Glen Ora Late 2, Glen Ora Late, Lazyboy, Witkrans, Suitangi, Carninka Late, Gloudi, HE Late, Caloma and Lane Late ended the season. The other trial site at Arundel is a late maturing trial and the season kicked off with Clark, followed by Hutton, Autumn Gold, Barnfield Summer, Summer Gold, Gloudi, Cambria, Chislett, Lane Late and the season finished with Glen Ora Late. The third site at Invercloy also had an early maturing selection. Fischer was evaluated there.

##### **Opsomming**

Daar is 3 proef persele in die Sondagsrivier vallei. Hulle is tussen Kirkwood and Addo gelee. Die nuwe proef perseel by Endulini het hulle derde drag opgehad in die (2020) seisoen. Die proef bevat die meeste vroeë tot laat rypwordende nawel seleksies. Die seisoen het begin met Sunrise Early, Trosky Early, De Wet 1, KS Early, Lethaba Early, Navelina, Painter Early 2, Cara Cara, Kirkwood Red, Washington, Clark, Glen Ora Late 2, Glen Ora Late, Lazyboy, Witkrans, Suitangi, Carninka Late, Gloudi, HE Late, Caloma en klaar gemaak met Lane Late. Die ander proef perseel is by Arundel en dit is net laat rypwordende seleksies. Daar het die seisoen begin met Clark, gevolg deur Hutton, Autumn Gold, Barnfield Summer, Summer Gold, Gloudi, Cambria, Chislett, Lane Late en Glen Ora Late het die seisoen afgesluit. Die derde proef perseel is by Invercloy en die perseel bevat 'n vroeë rypwordende seleksies. Fischer was daar geevalueer.

##### **Objectives**

- To select navel cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new navel cultivars and to determine the climatic suitability of these cultivars in cold production regions.

##### **Materials and methods**

Field evaluations and laboratory analyses were conducted on navel selections from the Sundays River Valley region of the Eastern Cape. The following early to late maturing selections were evaluated: Sunrise Early, Trosky Early, De Wet 1, KS Early, Lethaba Early, Navelina, Painter Early 2, Cara Cara, Kirkwood Red, Washington, Clarke, Glen Ora Late 2, Glen Ora Late, Lazyboy, Witkrans, Suitangi, Carninka Late, Gloudi, HE Late, Caloma, Hutton, Autumn Gold, Barnfield Summer, Summer Gold, Gloudi, Cambria, Chislett, Lane Late, and Fischer

For navels, a ratio of 9:1 is considered to be the build-up towards peak maturity of 10:1. After reaching the peak, the ratio increases to 11:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in greater instances of quality and rind issues.

**Table 5.4.18.1.** List of navel selections evaluated at Sundays River Valley (Endulini) during 2020.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
Cara Cara	CC	2015
Carninka Late	CC	2015
Clarke	CC	2015
De Wet 1	CC	2015
Glen Ora Late	CC	2015
Glen Ora Late 2	CC	2015
Gloudi	CC	2015
HE Late	CC	2015
Kirkwood Red	CC	2015
KS Early	CC	2015
Caloma	CC	2015
Letaba Early	CC	2015
Lane Late	CC	2015
Lazyboy	CC	2015
Navelina	CC	2015
Painter Early 2	CC	2015
Suitangi	CC	2015
Sunrise Early	CC	2015
Trosky Early	CC	2015
Washington	CC	2015
Witkrans	CC	2015

**Table 5.4.18.2.** List of navel selections evaluated at Sundays River Valley (Arundel) during 2020.

<b>Selection</b>	<b>Rootstock</b>	<b>Planted</b>
Autumn Gold	CC	1997
Clarke	CC	1997
Chislett	CC	1997
Hutton	CC	1997
Lane Late	CC	1997
Cambria	CC	1997
Gloudi	CC	1997
Glen Ora Late	CC	1997
Barnfield Summer	CC	1997
Summer Gold	CC	1997

**Table 5.4.18.3.** List of navel selections evaluated at Sundays River Valley (Invercloy) during 2020.

<b>Selection</b>	<b>Rootstock</b>	<b>Topworked</b>
Fischer	CC	2012

## Results and discussion

### Summer Gold

Summer Gold is a mid - late maturing navel. Fruit size was large and fruit size count peaked at (count 56). Summer Gold internal quality was good, high Brix and good acid percentage. At ratio 10.9 which is consider over mature the Brix was 11 and acid 1.01%. Juice percentage was low, below 50%. Colour development was delayed compared to the internal quality, being T5 – T6 on the colour plate.

### Trosky Early

Trosky Early is an experimental early maturing navel. The fruit size count was good, count 56. Trosky Early had a delayed colour development (colour plate T5 – T6) at over maturity. The selection's juice percentage was 50.8%. Internal quality was good at Brix:Acid ratio of 11.3, Brix was 10.1° and acid percentage was 0.89%.

### Autumn Gold

The external colour development was delayed (colour plate T5) at peak maturity. The selection bore favorably sized fruit and peaked at count 56. Autumn Gold had a fair juice percentage of 50.8% at peak maturity. Internal quality was very good at peak maturity with high Brix 12.3 and acid percentage (1.06%). The navel ends were small.

### Barnfield Summer

Barnfield Summer is a late maturing navel. Barnfield Summer had a very good and preferred fruit size (count 56) for export. The juice percentages were very low 43.6% for this selection. Barnfield Summer has a delayed external colour development, being a T6 at ratio 11.0. The acid was fair 0.83% at over maturity and it was supported with fair Brix of 9.1°. The navel opening was small.

### Glen Ora Late & Glen Ora Late 2

The fruit size count of Glen Ora Late 2 was very good with a 56 count preferred export size compared to Glen Ora Late that had fruit size count 48 - 56. The juice percentage for both selections was above 55%, close to 60% at peak maturity and over maturity. Glen Ora Late external colour development was also slightly better T4 – T5 on the colour plate compared to Glen Ora late 2, T5 – T6. Both selections had very good Brix levels just below 11.0° with high acids just below 0.90% at peak maturity, indicating that the fruit can hang slightly longer on the trees. At Arundel site where the older trees are, the Brix and acids were higher compared to the younger trees. The rind is smooth to slightly coarse with a small to closed navel end.

### Lane Late

Lane Late is the control for the late maturing selections. Lane Late was the last selection to reach peak maturity at the Endulini trial site, and the second last selection to reach peak maturity at the Arundel trial site. Lane Late fruit size count was 56, great size for export. Lane Late had good juice percentage around 55% at peak maturity. Endulini trial site trees, (younger trees) had the higher juice percentage. The external colour development was also delayed on Lane Late, (T4 – T5) by peak maturity. Lane Late also kept its acids quite well being around 1.0% (good shelf life), older trees having a slightly higher acid. The Brix was high 11° at Arundel trial site (older trees) compared to the younger trees at Endulini with Brix 10°. The flavour was good. Lane Late had small protruding navel-end on the fruit.

### Chislett Summer

Chislett Summer had a slightly delayed color development, T3 at peak maturity. Fruit size count peaked at count 56, large fruit size count. Juice percentage was on the lower side. The Brix: Acid ratio for Chislett Summer at peak maturity was very good, Brix 12.4° and acid percentage 1.11%. The flavour of the selection was very good.

### Clarke

Clarke's fruit size count was 56. The young trees at Endulini had a slightly higher juice percentage at peak maturity compared to Arundel's older trees. Internal quality was fair at both sites with Brix below 10° and acid of 0.9%. The external colour development of Clarke was delayed compared to the internal quality. The external colour was T5 - T6 on the colour plate.

### Gloudi

Gloudi is a promising late maturing navel. The juice percentage of Gloudi was still good when the fruit was over mature, just over 50.0%. Gloudi had a fruit size count peaking at 56 count. With a ratio of just below 11.0 which is considered towards over maturity, internal quality was still good with above Brix 10.0° and acid percentage around 1.0%. Gloudi's acid levels remained stable (good shelf life). Colour development was delayed being T5 – T6 on the colour plate. Gloudi had a close to small navel end with a good crop.

### Witkrans 3

Witkrans is also a very promising late maturing navel. The crop was good. The external colour development was delayed with a T6 on the colour plate at peak maturity. The fruit size count for Witkrans was good, peaking at a count of 56, great for packing and export. The juice percentage for Witkrans was high and very good, around 55% and acids were still around 0.90% at peak maturity. Brix was also good and along with the acid gave Witkrans great flavour. It has a closed to small navel end.

### Cambria

Cambria is a well-known mid-late navel selection with very good internal quality. The selection was used as control for the mid to late maturing navel trial in the Sundays River Valley. The fruit shape was more elongated compared to the other navel selections. Cambria fruit size count 56. The selection had a slight delay in colour development being at colour plate T4 at peak maturity. The juice was low just above 50% at peak maturity and Brix was 10.8° with acids above 1.05%.

### De Wet 1

De Wet 1 is an experimental mid-maturing navel that has produced a good crop consistently every year. Manipulation is necessary to control fruit size because over cropping results in smaller fruit. The selection developed a fairly soft rind; one of the characteristics of the De Wet selection. De Wet 1 had a closed navel end on the fruit without having to spray 2,4-D and developed a small internal navel. The selection had good fruit size and peaked at count 56. Fruit shape was round. The internal quality was good with fair juice content of just over 50%. At peak maturity, the external colour peaked at colour plate T7. The Brix remained low around 9° and acid percentage around 0.72% when the fruit was considered overmature with a ratio 11.3.

### Caloma

Caloma is an experimental late maturing navel with potential. It bore fruit with large fruit size and peaked at count 56. The crop was good. The external colour development of Caloma was delayed with a T6 – T7 on the colour plate at peak maturity. Acids kept quite well, the Brix was high even when the fruit was over mature. Juice content was around 55% at peak maturity. Flavour was great. Most of the fruit had a small - closed navel end.

### Washington

Washington is the control for the mid-maturing navel selections. The external colour development was behind the internal quality of the fruit (T6) on the colour plate standards. Washington fruit size count peaked at count 56. The juice content of Washington tested around 53.0%. Brix levels above 10° with acids of 0.79% when the fruit was already over mature.

### Fischer

Fischer is a very good early to mid-maturing control. The acid levels on Fischer navel were good, averaging around 1.0% for the season. Juice and Brix at peak maturity were 42.8% and 10.3°, respectively. Externally the fruit colour development was delayed, and peaked at T5. Fruit size was large, fruit size (count 56 - 48). Very good crop on the trees.

### Hutton

Hutton had the preferred fruit size for navel production and export (count 56). The juice percentage was around 50.0% at peak maturity. Navel ends were open and the rind was coarse. Peak maturity was reached in May. The Brix was around 10° and acid around 0.90%, with T5 colour on the colour plate at peak maturity.

### Lazyboy

Lazyboy is a late maturing navel with good internal quality. Brix was 10.9° and the fruit hung well on the tree with acid percentage of 0.90%. Even when the fruit is over mature the acid is still stable before it drops. Juice percentage is just above 50%. Fruit size is a uniform medium to medium large peaking at count 56. Navel ends are mainly small to closed with occasional fruit having more open navels. Fruit shape is round and rinds are smooth. Internal colour is orange. Externally the colour development was delayed; T6 – T7 at peak maturity. The flavour is very good.

### Suitangi

Suitangi was one of the late maturing experimental navel selections evaluated, the external colour development was delayed, T6 on the standard colour plate. The selection normally has deep orange rind colour and it had a fairly small navel end. Suitangi peaked at count 56. Suitangi internal quality was fair with juice content 51%; Brix levels around 8.9° with acids around 0.80% achieved at a Brix:Acid ratio of 11.3.

### Cara Cara

Cara Cara is the control for pigmented navels. The cultivar reached peak maturity very early in the season due to low acids. Internal quality was as follows when the Brix: Acid ratio of 13.5 was achieved; juice percentage of 50.2%, Brix 9.6° and acid percentage 0.71%. The external appearance was delayed at T6 on the colour plate. Fruit size is uniform large, (count 56). Navel ends are small - medium. Fruit shape is round and rinds are smooth. Internal flesh colour is an intermediate red, and flavour is very good.

### Carninka Late

Carninka late is a late maturing experimental navel. Peak maturity was reached even earlier this season mid-May compared to 2019 season mid-July. Carninka late had a good fruit size, count 56. It also had good internal fruit quality at peak maturity, juice percentage of 53.9%, Brix 10.1° and good acid percentage (0.90%). Fruit is firm and has a smooth peel. Colour development was delayed, reaching T6 on the colour plate at peak maturity. Navel end is closed to small and flavour very good.

### HE Late

HE Late is an experimental late maturing navel. Peak maturity on the young trees was reached end May to early June. It reached the preferred fruit size peaking at count 56 (large fruit). HE Late internal quality was very good. Juice percentage was above 55% and the Brix around 10° with an acid percentage of around 1% at peak maturity. Future evaluations will determine the exact maturity period and if the internal quality will stay good. With the acid percentage that stayed stable the fruit can hang on the tree to colour up.

### Kirkwood Red

Kirkwood red is a red pigmented navel. The fruit sometimes has external blush and internal colour pigment is dark red. The colour development was delayed being T6 on colour plate at peak maturity. The cultivar matured slightly later than Cara Cara navel. Kirkwood Red fruit has a relatively good juice content 54.8%. At over maturity the Brix was 9.7° and acid was 0.75%. Peak maturity was also reached earlier than normal. The fruit is small with closed end navel and fruit sized peaked at count 56. The tree is more compact compared to Cara Cara. Fruit shape is round to slightly oval.

### Letaba Early

It was the third crop on the trees for this selection; future evaluations will give us more info about this experimental cultivar. Peak maturity was reached in mid-April, again earlier this season compared to the previous 2 seasons. Fruit size count peaked at count 48. At peak maturity (ratio 10) the internal quality was as follows: juice percentage below 55%, Brix around 9° and acid percentage around 0.90%. Colour development was delayed reaching T6 on the colour plate at peak maturity.

### Sunrise Early

Sunrise Early is an early maturing experimental navel. Sunrise Early was the first selection to reach peak maturity at this new trial site. Future evaluations will give us a better indication how early the selections are. Fruit size was good with count 56. On 23.04.2020 the ratio was 12.6 over maturity. Juice percentage was 49%, the Brix was low (9.5°) and acid 0.77%. Colour development was delayed at T3 – T4 on the colour plate.

### Navelina

Navelina is a mid maturing navel like Washington and not an early maturing navel like Lina. In this trial site Navelina reached peak maturity before Washington. The tree is not as vigorous as the Washington tree. Fruit are medium to large (counts 56) with a small to closed navel. The external fruit colours, with T7 at peak maturity were behind the internal quality. Fruit shape is slightly elongated, and rinds are smooth. The flavour is very good with good sugars, acid levels and a ratio of 9.9:1

### Painter Early 2

An early - mid maturing navel. External colour development was delayed at T7 on the colour plate. Fruit size is a uniform large, count 56. Navel ends are small. Fruit rinds are smooth. Internal flesh colour is orange. The flavour is good with good internal quality. Juice above 50%, Brix at 10° and acids at 1% at peak maturity. The acids stayed stable and good, long after peak maturity.

### KS Early

KS Early is an experimental mid-maturing navel. It shows good potential. KS Early has a smooth rind and very small to closed navel end. Fruit is firm. KS Early fruit size count was favourable, count of 56. Colour basically just reaches T7 on the colour plate at peak maturity. Juice percentage was just above 50%. Brix and acid percentage at peak maturity were moderate. As the trees get older the external colour and internal quality will improve.

### **Conclusion**

The Addo area is well suited for navel production in South Africa. Most of the selections had a very good fruit size and peaked at count 56, with Glen Ora Late, Letaba Early and Fischer peaking at count 48. All of the selections had delayed colour development. The following selections had juice percentage above 55%: Witkrans 3, Lane Late, Glen Ora Late & 2, HE Late. De Wet 2, Gloudi, Chislett, Summer Gold, Autumn Gold and Lane Late had the highest Brix above 11.0° for this trial at peak maturity. All the navel selections were seedless.

**Table 5.4.18.4.** Internal fruit quality data for early to late Navel selections from the Addo (Arundel) region of the Sundays River Valley during the 2020 season.

Date	Selection	Root stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Colour	Avg Seed
2020-06-01	Autumn Gold	CC	56	50.8	12.3	1.06	11.6	0.0	T5
2020-06-01	Barnfield Summer	CC	56	43.6	9.1	0.83	11.0	0.0	T6
2020-06-01	Cal Lane Late	CC	56	50.5	10.4	1.13	9.2	0.0	T6
2020-06-15	Cal Lane Late	CC	56	54.0	11.4	1.14	10.0	0.0	T4
2020-06-01	Cambria	CC	56	51.9	10.8	1.05	10.3	0.0	T4
2020-06-15	Cambria	CC	56	52.4	11.6	0.96	12.1	0.0	T4
2020-06-15	Chislett	CC	56	43.6	12.4	1.11	11.2	0.0	T3
2020-05-11	Clark	CC	56	46.0	9.4	0.93	10.1	0.0	6
2020-05-25	Clark	CC	56	48.5	10.6	0.98	10.8	0.0	T5
2020-06-01	Clark	CC	56	51.5	10.7	0.92	11.6	0.0	T5
2020-06-01	Glen Ora Late	CC	56	48.9	12.8	1.31	9.8	0.0	T5
2020-06-15	Glen Ora Late	CC	56	59.3	13.2	1.22	10.8	0.0	T4
2020-06-15	Glen Ora Late	CC	48	41.8	12.5	1.13	11.2	0.0	T5
2020-06-01	Gloudi	CC	56	50.4	11.2	1.03	10.9	0.0	T5
2020-06-15	Gloudi	CC	56	53.9	11.8	0.93	12.7	0.0	T3-T4
2020-06-01	Hutton	CC	56	48.9	9.9	0.78	12.7	0.0	T5
2020-06-01	Summer Gold	CC	56	48.3	11.0	1.01	10.9	0.0	T6
2020-06-15	Summer Gold	CC	56	47.8	11.7	0.98	11.9	0.0	T5

**Table 5.4.18.5.** Internal fruit quality data for early to late Navel selections from the Addo/Kirkwood (Endulini) region of the Sundays River Valley during the 2020 season.

Date	Selection	Root stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg Seed	Colour
2020-06-15	Cal Lane Late	CC	56	57.3	10.2	0.95	10.7	0.0	T5
2020-05-11	Cara Cara	CC	56	50.2	9.6	0.71	13.5	0.0	T6
2020-06-01	Carninka Late	CC	56	53.9	10.1	0.90	11.2	0.0	T6
2020-06-15	Carninka Late	CC	56	56.2	11.6	0.88	13.2	0.0	T4
2020-05-11	Clark	CC	56	51.4	9.4	0.90	10.4	0.0	T6
2020-05-25	Clark	CC	56	53.2	9.8	0.84	11.7	0.0	T6
2020-06-01	Clark	CC	56	54.2	9.7	0.79	12.2	0.0	T6
2020-04-20	De Wet 1	CC	56	50.7	8.1	0.72	11.3	0.0	T7
2020-06-01	Glen Ora Late	CC	56	56.7	10.4	0.86	12.1	0.0	T5
2020-06-15	Glen Ora Late	CC	56	59.6	10.9	0.87	12.5	0.0	T5
2020-06-01	Glen Ora Late 2	CC	56	56.2	10.7	0.88	12.2	0.0	T6
2020-06-15	Glen Ora Late 2	CC	56	58.1	11.3	0.89	12.7	0.0	T5
2020-06-01	Gloudi	CC	56	51.9	10.1	0.94	10.7	0.0	T6
2020-06-15	Gloudi	CC	56	55.4	10.1	0.77	13.1	0.0	T6
2020-06-01	HE Late	CC	56	55.4	10.2	0.98	10.4	0.0	T6
2020-06-15	HE Late	CC	56	56.8	10.8	0.86	12.6	0.0	T5
2020-05-11	Kirkwood Red	CC	56	54.8	9.7	0.75	12.9	0.0	T6
2020-04-23	Sunrise Early Navel	CC	56	49.0	9.5	0.77	12.6	0.0	3--4
2020-05-06	Sunrise Early Navel	CC	56	52.5	10.3	0.74	13.9	0.0	T4
2020-04-20	KS Early	CC	56	51.4	9.3	0.90	10.3	0.0	T7
2020-05-25	KS Early	CC	56	54.8	9.7	0.72	13.5	0.0	T5
2020-05-11	Caloma	CC	56	54.6	9.7	0.98	9.9	0.0	T6
2020-06-01	Caloma	CC	56	54.8	10.5	1.01	10.4	0.0	T6-T7
2020-06-15	Caloma	CC	56	59.4	10.7	0.80	13.4	0.0	T4-T5
2020-06-01	Lazyboy	CC	56	50.7	10.7	0.90	11.9	0.0	T7
2020-06-15	Lazyboy	CC	56	50.5	11.7	1.01	11.6	0.0	T6
2020-07-15	Lazyboy	CC	56	49,6	10,9	0,50	22,0	0.0	T 1 - T 3
2020-04-20	Letaba Early	CC	48	53.7	9.2	0.91	10.1	0.0	T6
2020-05-11	Letaba Early	CC	56	54.4	9.6	0.81	11.9	0.0	T5
2020-04-20	Navelina	CC	56	54.7	9.0	0.91	9.9	0.0	T7
2020-05-11	Navelina	CC	56	56.8	9.8	0.83	11.8	0.0	T5
2020-04-20	Painter Early 2	CC	56	51.9	10.1	1.05	9.6	0.0	T7
2020-05-11	Painter Early 2	CC	56	53.4	10.6	0.94	11.3	0.0	T6
2020-05-25	Painter Early 2	CC	56	53.7	10.7	0.80	13.4	0.0	T4 -T5
2020-06-01	Suitangi	CC	56	51.0	8.9	0.79	11.3	0.0	T6
2020-04-20	Trosky Early	CC	56	50.8	10.1	0.89	11.3	0.0	T6
2020-05-11	Trosky Early	CC	56	49.6	10.9	0.84	13.0	0.0	T5
2020-05-11	Washington	CC	56	53.0	10.0	0.79	12.7	0.0	T6
2020-06-01	Witkrans 3	CC	56	55.7	10.3	0.90	11.4	0.0	T6
2020-06-15	Witkrans 3	CC	56	57.2	11.0	0.85	12.9	0.0	T5
2020-08-04	Witkrans 3	CC	56	55.4	12.0	0.79	15.2	0.0	T1

**Table 5.4.18.6.** Internal fruit quality data for early to late Navel selections from the Kirkwood (Invercloy) region of the Sundays River Valley during the 2020 season.

Date	Selection	Root stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Colour	Avg Seed
2020-04-20	Fischer Navel	CC	48	42.8	10.3	1.05	9.8	0.0	T5
2020-05-11	Fischer Navel	CC	56	49.1	12.3	0.89	13.8	0.0	T1

#### 5.4.19 PROGRESS REPORT: Cultivar characteristics and climatic suitability of experimental navel oranges in a cold production region (Gamtoos River Valley)

Project 1001B by W. Swiegers and Z. Zondi (CRI)

##### Summary

The trial consists of a few experimental early, mid and late navel selections. Painter Early 2 and Addo Early started the season as the two early navel selections for evaluation. De Wet 1 is a mid-maturing navel producing a round fruit shape. The fruit developed a closed navel end. The late maturing selections evaluated in order of ripening consisted of Lazyboy, Suitangi and Caloma. This region is going through severe drought.

##### Opsomming

Hierdie proef bestaan uit 'n paar eksperimentele vroeë-, middel- en laat navel seleksies. Orde van rypwording was Painter Early 2 gevolg deur Addo Early, dit is die 2 vroeë seleksies wat gevalueer was. De Wet 1 is 'n mid-rypwordende navel met 'n ronde vrugvorm. Die vrugte het 'n toe navel-ent. Die laat navel seleksie wat ge-evalueer was en orde van rypwording bestaan uit Lazyboy, Suitangi en Caloma. Die streek gaan gebuk onder erge droogte.

##### Objectives

- To select navel cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new navel cultivars and to determine the climatic suitability of these cultivars in cold production regions.

##### Materials and methods

Field evaluations and laboratory analyses were conducted on navel selections from regions of the Gamtoos River Valley. The following selections were evaluated: DeWet 1, Caloma, Lazyboy, Painter Early 2, Addo Early and Suitangi

A ratio of 9:1 is considered to be the build-up towards peak maturity for selections. When the ratio between sugar and acid is 10:1, the fruit is considered to be at peak maturity. After reaching the peak, the ratio increases to 11:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in higher instances of quality and rind issues.

**Table 5.4.19.1.** List of navel selections evaluated at Loerie site in the Gamtoos River Valley, Eastern Cape during the 2020 season.

Selection	Rootstock	Topworked	Planted
De Wet 1	Carrizo	2012	

Addo Early	Carrizo		2016
Caloma	Carrizo	2012	
Lazyboy	Carrizo	2013	
Painter Early 2	Carrizo	2012	
Suitangi	Carrizo		2016

## Results and discussion

### De Wet 1

De Wet 1 is a mid-maturing navel. Production is consistently good every year. The selection have a fairly soft rind. A fruit characteristic of De Wet 1 is that it has a closed navel end without having to spray 2,4-D and develops a small internal navel. The selection had good fruit size count 56; perfect for navel production and export. The internal quality was moderate with juice content around 50%. At peak maturity, the external colour peaked at colour plate T6. The Brix remained around 9° and acid percentage around 0.9%.

### Caloma

Caloma is a promising experimental late maturing navel. The fruit crop was good. Caloma bore fruit with a large fruit size at count 56. Caloma fruit is round and firm. Caloma's internal quality was good. The selection's internal quality was as follows at peak maturity, good juice percentage of 58.0%, good Brix 10.0° and a good acid just around 0.90%. The external colour development of Caloma was delayed compared with the internal development, T5 on the colour plate at ratio 11.2. Caloma's rind is smooth and the navel ends are small to close. Flavour of Caloma is excellent.

### Addo Early

Addo Early was the second selection at peak maturity in this trial site. It is an experimental early maturing Navel. The navel end on the fruit was small. This selection had the biggest fruit size of counts 56 - 48 at peak maturity in the trial site. The external colour development range was delayed T7 (colour plate). Internal quality was moderate, hopefully it improves as the tree gets older.

### Painter Early 2

Painter Early 2 was the first selection to mature for this navel trial. Large sized fruit was on the trees with count 56. Painter Early 2 was at T6 on the colour plate at peak maturity. The juice percentage of Painter Early 2 increased towards peak maturity to 53.6%. Painter Early 2 had fair Brix and acid levels at peak maturity. Fruit shape is round with smooth rind and small navel ends.

### Suitangi

Suitangi is one of the late maturing experimental navel selections evaluated. It was the second crop on the trees. Crop improved compared to last season in 2019. The external colour development was delayed; T7 on the standard colour plate at peak maturity. The selection had a small navel end. Suitangi peaked at count 56. Suitangi internal quality was moderate at peak maturity with juice content around 50%. Brix levels around 10° with acids of 0.90% assured good tasting fruit with good flavour.

### Lazyboy

Lazyboy is a late maturing navel with good internal quality. Brix was 10.4° and the fruit hangs well on the tree with acid percentage of 0.94%. Even when the fruit is over mature the acid is still stable before it drops. Fruit size peaked at count 56. Navel ends are mainly small to closed with occasional fruit having more open navels. Fruit shape is round and rinds are smooth. Externally the colour development was delayed; T6 at peak maturity. The flavour is very good.

## Conclusion

The fruit size of all the navel selections peaked at count 56 at peak maturity, except Addo Early. The Navel selection with the highest juice percentage was Caloma (58%). All of the selections had a delayed external

colour development on the colour plate at peak maturity. Caloma and Lazyboy developed the highest Brix values for this trial.

**Table 5.4.19.2.** Internal fruit quality data for Experimental Navel selections from the Gamtoos River Valley region of the Eastern Cape during the 2020 season.

Date	Selection	Rootstock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-04-20	Addo Early	CC	56	47.1	7.5	1.01	7.4	0.0	T7
2020-05-04	Addo Early	CC	48	44.1	7.8	0.85	9.2	0.0	T7
2020-05-04	De Wet 1	CC	56	51.7	8.1	0.82	9.9	0.0	T7
2020-05-20	De Wet 1	CC	56	51.7	8.8	0.87	10.1	0.0	T6
2020-06-10	De Wet 1	CC	56	52.5	9.0	0.69	13.0	0.0	T3
2020-05-20	Caloma	CC	56	55.2	9.8	1.07	9.2	0.0	T6
2020-06-10	Caloma	CC	56	58.0	10.0	0.89	11.2	0.0	T5
2020-06-23	Caloma	CC	56	57.7	10.2	0.83	12.3	0.0	T4-T5
2020-05-20	Lazyboy	CC	56	50.0	10.4	0.94	11.1	0.0	T6
2020-06-10	Lazyboy	CC	56	47.0	10.5	0.74	14.2	0.0	T5
2020-04-21	Painter Early 2	CC	56	53.6	8.9	0.88	10.1	0.0	T6
2020-05-04	Painter Early 2	CC	56	55.3	9.4	0.84	11.2	0.0	T5
2020-05-20	Suitangi	CC	56	49.7	9.1	0.91	10.0	0.0	T7
2020-06-10	Suitangi	CC	56	52.8	9.3	0.78	11.9	0.0	T6

**5.4.20 PROGRESS REPORT: Cultivar characteristics and climatic suitability of experimental navel oranges in a cold production region (Western Cape)**  
Project 998D by W. Swiegers (CRI)

**Summary**

Citrusdal is probably one of the best regions to farm Navels in the country. The trial consists of most of the recent selections and a few newer ones will be added. The trial consists of a few experimental early, mid and late navel selections in 2 trial sites. Fukumoto and Lina are the controls for early maturing navels. Fischer was used as control for the early-mid selections, Washington as control for the mid-maturing navel selections and Cambria for the late navel sections. Cara Cara was used as the control for the pigmented navels. Most of the trees are older and have big tree volumes. Tibshreany Early, Rayno Early, Gerhard Early, Lina, Fukumoto and Palmer started the season as the early navel selections for evaluation. The mid navel selections that were evaluated in order of ripening were as follows: Summer Gold, Cara Cara, Fischer, Washington, Navelina, Hutton, Kirkwood Red, Chislett Summer, Carninka and Clarke. The late selections that were evaluated and were last to reach peak maturity were Cambria, Gloudi, Barnfield Summer, Glen Ora Late, Lane Late, Glen Ora Late 2 and Witkrans.

**Opsomming**

Citrusdal is seker een van die beste streke in die land vir Navels. Die proef het die meeste van die nuwe seleksies. Daar gaan nog uitgebrei word op hulle. Hierdie spesifieke proef bestaan uit 'n paar eksperimentele vroe-, middel- en laat nawel seleksies in 2 proef persele. Fukumoto en Lina is as kontrole gebruik vir die vroe seleksies. Fischer is as kontrole gebruik vir die vroe - mid seleksies, Washington word as kontrole gebruik vir die middel seleksies en Cambria dien as kontrole vir die laat nawel seleksies. Cara Cara word as kontrole gebruik vir die gepigmenteerde seleksies. Die meeste van die bome is al ouer en die bome het 'n groot boom volume. Die volgorde van rypwording was beginnende met die vroe seleksies Tibshreany Early, Rayno Early, Gerhard Early, Lina, Fukumoto en Palmer gevolg deur die middel seleksies se volgorde, Summer Gold, Cara Cara, Fischer, Washington, Navelina, Hutton, Kirkwood Red, Chislett Summer, Carninka en Clarke. Die

seisoen was afgesluit met die laat seleksies se orde van ryppwording Cambria, Gloudi, Barnfield Summer, Glen Ora Late, Lane Late, Glen Ora Late 2 and Witkrans.

## Objectives

- To select navel cultivars with improved and consistent productivity, fruit size, rind colour, peelability, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the characteristics of new navel cultivars and to determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on navel selections from regions of the Citrusdal Valley. The following selections were evaluated: Tibshreany Early, Rayno Early, Gerhard Early, Lina, Fukumoto, Palmer, Summer Gold, Cara Cara, Fischer, Washington, Navelina, Hutton, Kirkwood Red, Chislett Summer, Carninka, Clarke, Cambria, Gloudi, Barnfield Summer, Glen Ora Late, Lane Late, Glen Ora Late 2 and Witkrans.

A ratio of 9:1 is considered to be the build-up towards peak maturity for selections. When the ratio between sugar and acid is 10:1, the fruit is considered to be at peak maturity. After reaching the peak, the ratio increases to 11:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in greater instances of quality and rind issues.

**Table 5.4.20.1.** List of navel selections evaluated at various sites in the Citrusdal, Western Cape during the 2020 season.

Selection	Rootstock	Planted	Topworked
Cara Cara	Carrizo	2009	
Fischer	Carrizo	2009	
Fukumoto	Carrizo	2009	
Gerhard Early	Carrizo	2009	
Glen Ora Late	Carrizo	2009	
Gloudi	Carrizo	2009	
Kirkwood Red	Carrizo	2009	
Barnfield Summer	Carrizo		2016
Chislett Summer	Carrizo	2009	
Clarke	Carrizo		2010
Glen Ora Late 2	Carrizo		2014
Hutton	Carrizo	2009	
Lina	Carrizo		2016
Lane Late	Carrizo	2009	
Summer Gold	Carrizo		2010
Washington	Carrizo	2009	
Carninka	Carrizo		2010
Navelina	Carrizo		2016
Rayno Early	Carrizo	2009	
Tibs Early	Carrizo		2014
Witkrans	Carrizo		2011
Cambria	Carrizo		2011
Palmer	Carrizo		2011

## Results and discussion

### Barnfield Summer

Barnfield Summer is a late maturing navel, and it was the fourth last selection to reach peak maturity, it was the selection's second crop. Barnfield Summer fruit size ranged count 88 – 40 (medium to big fruit). The juice percentages were below 50% for this selection. Barnfield Summer has a fair external colour development, being a T4 at ratio 10.4. The acid remained good 0.9% until over maturity and it was supported with good Brix peaking at 10. The navel end was small.

### Clarke

Clarke's fruit size was medium – large, count 88 - 48. The juice percentage was low. Internal quality was good at over maturity, having a Brix of 10.5 and acid of 0.92%. The external color development of Clarke was delayed T3 – T4 on the color plate compared to the internal quality.

### Fukumoto

Fukumoto was the fifth selection to reach peak maturity. Internal quality at peak maturity (ratio 10.2), the Brix was 9.7° and good acid levels (0.95%). The good Brix: Acid ratio give Fukumoto its great flavour. It is also a great eating fruit. The color development was delayed, with color plate T5 at peak maturity. The acids remain stable even when the fruit was over mature (around 0.90% for 3 weeks) fruit will hang well and have a good shelf life. Fukumoto produced a small - medium size fruit which peaked at count 56. The navel-end on the fruit was medium open and protruding, one of the characteristics of the selection.

### Chislett Summer

Chislett Summer had a delayed color development T4 at peak maturity. Fruit size count peaked at count 72 - 56, medium - large fruit size count. Juice percentage was slightly on the lower side. The Brix: Acid ratio for Chislett Summer at peak maturity was good, Brix around 9° and acid percentage 0.83%. The flavour of the selection was very good.

### Gerhard Early

Gerhard Early is an experimental early maturing navel. Gerhard Early was third of the early selections to reach peak maturity at the trial site. Gerhard Early trees bore medium to large sized fruit that peaked at count 56. Gerhard Early had delayed color development (color plate T5) when the fruit was at peak maturity. Sugars and acids were good at peak maturity, Brix around 10° and acid around 0.90%.

### Fischer

Fischer (control) had a delayed colour development with colour plate T5 – T6 at peak maturity. Fischer had a good fruit size which peaked at count 48. Fischer internal quality was very good at peak maturity, Brix around 10° and acid around 0.90%. The flavour was very good. The navel end for Fischer was small to closed and the fruit had a smooth rind. The crop was very good.

### Glen Ora Late & Glen Ora Late 2

Glen Ora Late & Glen Ora Late 2 is late maturing navels with a very good flavour. Glen Ora Late 2 trees are younger than Glen Ora Late. The fruit size count of Glen Ora Late peaked at 48 count (big fruit) while Glen Ora Late 2 was slightly bigger peaking 40 count. The external colour development for Glen Ora Late and Glen Ora Late 2 was delayed (T3 – T4 on the plate) as peak maturity. Both Glen Ora Late selections acids stayed stable after reaching peak maturity. Along with the good and stable acids the Brix was also good around 10°. The rind is smooth to slightly coarse with small to close navel end. Juice percentage for both selections were below 50%. Glen Ora Late reached peak maturity before Glen Ora Late 2.

### Gloudi

Gloudi is a promising late navel selection. The fruit shape was round and the fruit firm with a small navel end. Gloudi had a very good fruit size peaking counts 56 the preferred count for navel production and exports. The selection had a delayed colour development being at colour plate T4 at peak maturity, degreening would have to be done. Gloudi Brix was good 9.7° with acids around 0.90%. Even when the fruit was well over mature the acid was at 0.92% (good shelf life).

### Hutton

Hutton had medium - large fruit size for navel production (count 88 - 56). Navel ends were open and the rind was coarse. Peak maturity was reached in June later than last seasons May. The Brix was around 9° and acid around 0.85%, with T5 colour on the colour plate at peak maturity.

### Washington

Washington was used as control for the mid-maturing navel, and it was the fourth to reach peak maturity of the mid maturing selections. The external colour development was behind the internal quality of the fruit (T5 – T6) on the colour plate at peak maturity. Washington fruit size peaked at count 56. Brix levels around 9.0° with acids of 0.90%. Navel ends were medium for Washington.

### Cara Cara

Cara Cara is the control for pigmented navels. Cara Cara is a mid-maturing pigmented navel. Compared to last season's colour development of T5 – T6 on the colour plate at peak maturity, this season the colour development was T5 at peak maturity. The fruit size ranged between counts 105 - 64. Fruit shape was round with smooth rind. The navel ends were small. The flavour was good. Internal colour was an intermediate red in the beginning of the season and as the season went on the red flesh became a bit deeper red.

### Kirkwood Red

Kirkwood Red is a mid-maturing pigmented navel. Peak maturity was reached later than Cara Cara navel. The colour development for Kirkwood Red was better than last season T4 – T6, being T3 – T4 this season. In the 2020 season the colour was delayed T3 – T4, but the good acids allowed us to keep the fruit longer on the trees and it did colour up to T1 on the colour plate. Internal quality for Kirkwood Red was good. The flavour was excellent. Fruit size for Kirkwood Red was small (count 105) to large (count 56). Flesh colour was deep red; even the fruit stem was red.

### Carninka Late

Carninka Late is a late maturing experimental navel. Peak maturity was reached in June compared to 2019 season July. Carninka late had fruit size, count ranged between (count 88 – 64). The internal fruit quality at 11.7 ratio, was good, Brix just below 10° and good acid percentage (0.84%). Fruit is firm and has a smooth peel. Colour development was T3–T4 on the colour plate at peak maturity. Fruit can hang on the trees for longer periods. Navel end is close to small and flavour very good.

### Navelina

Navelina is a mid-maturing navel. It was the selection's second crop. The tree is not as vigorous as the Washington. Fruit size peaked at large fruit (counts 48 with a very small to closed navel). The fruit had a delayed colour (T6) at peak maturity. Fruit shape is slightly elongated, and rinds are smooth. Internal quality was moderate.

### Lina

Lina was the fourth to reach peak maturity at this trial site and also one of the early maturing controls. The selection had a delayed colour development with a colour plate of T4 – T5 when it was at peak maturity. The selection had a good fruit size and peaked at count 56. The fruit shape was more elongated with a large navel-end (fairly open). Internal quality at peak maturity for Lina was good; Brix above 10° and acids around 0.90%.

### Lane Late

Lane Late is the control for the late maturing selections. It reached peak maturity third last in the trial block. Fruit size was medium to big, fruit size count were count 72 – 48. The internal quality at peak maturity was good with juice percentage just above 50%, Brix 9.4° and acid percentage 0.95%. The colour development were slightly delayed being T3 – T4 on the colour plate.

### Rayno Early

Rayno Early is a very early maturing experimental navel. It was the second selection to reach peak maturity at the trial site. Fruit size was small to medium with counts 125 – 72. For an early maturing navel, it managed to

get very good Brix above 10° at peak maturity with stable and good acids around 0.90%. T7 was the colour on the colour plate when the fruit was over mature. Crop was fair.

#### Summer Gold

Summer Gold is one of the mid-maturing navels in the trial site. Fruit size was large and fruit size count peaked at (count 56). Summer Gold internal quality was good, high Brix and good acid percentage. At ratio 10.8 which is consider over mature the Brix was 10.1° and acid 0.93%. Colour development was delayed compared to the internal quality, being T6 – T7 on the colour plate.

#### Tibshreany Early

Tibshreany Early is also an experimental early maturing navel. The crop was fair. It was the first selection to reach peak maturity at the trial site. Peak maturity was reached at the end of March – beginning April, Tibshreany Early fruit size count ranged from 125 (small fruit) - 56 (large fruit). At over maturity the Brix was around 9.1° with acid percentage of 0.84. Tibshreany Early also kept its acid stable. Colour development was delayed with T6 at a ratio of 11:1.

#### Witkrans

Witkrans is a very promising late maturing navel. The trees also had a good crop on them. The external colour development was delayed with a T3 - T4 on the colour plate at peak maturity. The fruit size count for Witkrans was good, peaking at a count of 56 which is great for navel production and export. Witkrans acids were around 0.9% at peak maturity. Brix was good and along with the good acid it will give Witkrans great flavour. It has a closed to small navel end.

#### Cambria

Cambria is a well-known late navel selection with very good internal quality. Cambria was used as control for the late selections. Cambria had small navel end openings. The fruit shape was more elongated compared to the other navel selections. Small to medium sized fruit and peaked at count 64. The Brix was 10.8° with acids, 0.99% and Brix:acid ratio 10.9. Juice was below 50%. Colour development was delayed.

#### Palmer

The external colour development of the selection was delayed (colour plate T6) towards peak maturity. The selection had a good fruit size and peaked at count 56. The acids were good at peak maturity but Brix and Juice percentage were low.

### **Conclusion**

Most of the fruit were smaller in 2020 season compared to the 2019 season. The following selections (Gerhard Early, Chislett Summer, Fukumoto, Gloudi, Hutton, Kirkwood Red, Lina, Palmer, Summer Gold, Tibs Early, Washington and Witkrans were the only selections that peaked at fruit size count 56. The selections were seedless. The best colour development was from Witkrans, Lane Late, Kirkwood Red, Glen Ora Late, Glen Ora Late 2, Clarke and Carninka Late. The selections with Brix above 10.0° at peak maturity were Gerhard Early, Glen Ora Late, Kirkwood Red, Lina, Summer Gold, Clarke, Rayno Early, Cambria and Witkrans. Gloudi had the best juice percentage.

**Table 5.4.20.2.** Internal fruit quality data for Experimental Navel selections from the Citrusdal region of the Western Cape during the 2020 season.

Date	Selection	Rootstock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-06-03	Barnfield Summer	CC	72 - 56	45,2	8,1	0,98	8,2	0	T 6
2020-07-01	Barnfield Summer	CC	64 - 40	47,1	9,3	0,90	10,4	0	T 4

2020-07-29	Barnfield Summer	CC	88 - 56	53,9	10,1	0,88	11,5	0	T 3
2020-04-20	Cara Cara	CC	88 - 64	45,2	8,8	1,01	8,7	0	T 6
2020-05-14	Cara Cara	CC	105 - 64	34,8	9,1	0,85	10,7	0	T 5
2020-06-03	Carninka	CC	88 - 64	45,6	8,7	1,01	8,6	0	T 6
2020-07-01	Carninka	CC	88 - 64	47,3	9,8	0,84	11,7	0	T 3 - T 4
2020-07-29	Carninka	CC	72 - 64	51,5	9,2	0,74	12,4	0	T 3 - T 4
2020-06-03	Chislett Summer	CC	72 - 56	42,4	9,1	1,01	9,0	0	T 6
2020-07-01	Chislett Summer	CC	72 - 56	38,1	9,9	0,83	11,9	0	T 4
2020-06-03	Clarke	CC	88 - 48	35,8	7,8	0,97	8,0	0	T 6
2020-07-01	Clarke	CC	88 - 64	49,0	10,5	0,92	11,4	0	T 3 - T 4
2020-04-20	Fischer	CC	88 - 64	48,9	9,3	1,01	9,2	0	T 6
2020-05-14	Fischer	CC	88 - 48	52,0	9,7	0,91	10,6	0	T 5 - T 6
2020-06-02	Fischer	CC	88 - 64	50,9	10,5	0,88	11,9	0	T 4
2020-03-16	Fukumoto	CC	88 - 64	41,2	8,6	1,06	8,1	0	T 6
2020-04-20	Fukumoto	CC	105 - 56	45,7	9,7	0,95	10,2	0	T 5
2020-05-14	Fukumoto	CC	88 - 64	45,5	10,8	0,90	12,0	0	T 3 - T 4
2020-04-20	Gerhard Early	CC	88 - 56	50,5	9,5	0,88	10,8	0	T 5 - T 6
2020-05-14	Gerhard Early	CC	88 - 64	51,2	10,3	0,89	11,6	0	T 5
2020-06-03	Glen Ora Late	CC	72 - 48	48,1	8,9	1,04	8,6	0	T 5 - T 6
2020-07-01	Glen Ora Late	CC	88 - 64	48,8	10	0,98	10,2	0	T 3 - T 4
2020-07-29	Glen Ora Late	CC	72 - 48	53,8	10,6	0,82	12,9	0	T 1 - T 4
2020-06-03	Glen Ora Late 2	CC	72 - 48	45,9	8	0,96	8,3	0	T 6
2020-07-01	Glen Ora Late 2	CC	72 - 48	48,4	9,6	0,97	9,9	0	T 3 - T 4
2020-07-29	Glen Ora Late 2	CC	56 - 40	50,7	10,1	0,78	12,9	0	T 2 - T 3
2020-06-03	Gloudi	CC	88 - 56	48,8	8,9	1,10	8,1	0	T 6
2020-07-01	Gloudi	CC	88 - 56	53,3	9,7	0,90	10,8	0	T 4
2020-07-29	Gloudi	CC	88 - 56	53,1	11	0,92	12,0	0	T 2 - T 3
2020-05-14	Hutton	CC	88 - 56	29,9	8,6	0,98	8,8	0	T 6 - T 7
2020-06-02	Hutton	CC	72 - 56	44,9	9,1	0,85	10,7	0	T 5
2020-05-14	Kirkwood Red	CC	105 - 64	52,7	10,2	1,26	8,1	0	T 5
2020-06-02	Kirkwood Red	CC	72 - 56	49,7	10	0,97	10,3	0	T 3 - T 4
2020-07-01	Kirkwood Red	CC	88 - 64	59,0	11,4	1,03	11,1	0	T 1
2020-06-03	Lane Late	CC	72 - 48	50,0	8,4	0,96	8,8	0	T 5
2020-07-01	Lane Late	CC	72 - 56	50,9	9,4	0,95	9,9	0	T 3 - T 4
2020-07-29	Lane Late	CC	72 - 48	53,9	10	0,74	13,5	0	T 2 - T 3
2020-03-16	Lina	CC	125 - 56	43,3	8,9	1,07	8,3	0	T 7
2020-04-20	Lina	CC	105 - 64	50,5	10,2	0,94	10,8	0	T 4 - T 5
2020-05-14	Lina	CC	72 - 56	32,5	10,2	0,85	12,1	0	T 5
2020-04-20	Navelina	CC	105 - 48	46,4	8,4	0,91	9,2	0	T 7
2020-05-14	Navelina	CC	88 - 40	38,1	8,7	0,87	10,0	0	T 6
2020-06-02	Navelina	CC	72 - 56	51,6	8,9	0,81	11,0	0	T 4 - T 5
2020-04-20	Palmer	CC	105 - 56	45,3	8,5	0,97	8,8	0	T 6 - T 7
2020-05-14	Palmer	CC	72 - 56	39,6	9,3	0,85	11,0	0	T 6
2020-03-16	Rayno Early	CC	125 - 72	50,5	8,9	1,01	8,8	0	T 6
2020-04-20	Rayno Early	CC	125 - 72	46,1	10,2	0,91	11,2	0	T 7
2020-04-20	Summer Gold	CC	105 - 72	45,7	9,2	1,15	8,0	0	T 8

2020-05-14	Summer Gold	CC	125 - 64	49,7	10,1	0,93	10,8	0	T 6 - T 7
2020-06-03	Summer Gold	CC	88 - 56	47,2	9,6	0,88	10,9	0	T 6
2020-03-16	Tibs Early	CC	125 - 64	38,6	8,8	1,04	8,4	0	T 6 - T 7
2020-04-20	Tibs Early	CC	105 - 56	48,4	9,1	0,82	11,1	0	T 6
2020-04-20	Washington	CC	105 - 64	46,3	8,7	1,08	8,1	0	T 6 - T 7
2020-05-14	Washington	CC	72 - 64	40,9	9,3	0,90	10,4	0	T 5 - T 6
2020-06-02	Washington	CC	88 - 56	55,5	9,3	0,85	11,0	0	T 5
2020-07-01	Witkrans	CC	88 - 64	43,4	9,7	1,04	9,3	0	T 3 - T 4
2020-07-29	Witkrans	CC	88 - 56	57,0	10,4	0,89	11,7	0	T 3 - T 4
2020-06-02	Cambria	CC	125 - 88	45,6	9,4	1,09	8,6	0	T 6
2020-07-01	Cambria	CC	88 - 64	45,9	10,8	0,99	10,9	0	T 4
2020-07-30	Cambria	CC	105 - 88	54,5	11,5	0,74	15,5	0	T 1 - T 3

#### 5.4.21 **PROGRESS REPORT: Cultivar characteristics and climatic suitability of Valencia oranges in a cold production region (Sundays River Valley)**

Project 1097A by W. Swiegers and Z. Zondi (CRI)

#### **Summary**

The Valencia discussed in this trial were top worked in the 2011 season. The trees produced their first crop in the 2015 season. There is a possibility for a new trial site. The early maturing selection for the trial site is Turkey with Midnight as control. The mid-maturing Valencia selections are Alpha, Gusocora, Bennie 2, Henrietta and Midnight 1. The late maturing Valencia selections will be McClean SL, Lavalle and Lavalle 2. At this trial site the season started with Turkey, followed by Midnight, Midnight F17, Midnight 2, Midnight 1, Alpha, Bennie 2, Louisa, Henrietta, Gusocora, Ruby Valencia, Mc Clean SL, Lavalle and the season ended off with Lavalle 2.

#### **Opsomming**

Die Valencia wat bespreek word in hierdie proef was in die 2011 seisoen getopwerk. Die bome het hulle eerste drag in die 2015 seisoen gehad. Daar is 'n moontlikheid om 'n perseel te begin. Die vroeë seleksie vir die proef perseel bestaan uit Turkey en Midnight wat as kontrole dien. Die mid seleksies is Alpha, Gusocora, Bennie 2, Henrietta en Midnight 1. Die laat rypwordende Valencia seleksies was as volg; McClean SL, Lavalle en Lavalle 2. Die proef perseel se seisoen het begin met Turkey, gevolg deur by Midnight, Midnight F17, Midnight 2, Midnight 1, Alpha, Bennie 2, Louisa, Henrietta, Gusocora, Ruby Valencia, Mc Clean SL, Lavalle en die seisoen het afgeëindig met Lavalle 2. Die volgorde van rypwording kan beïnvloed word deur die area wat n intermediere area is vir Valencia.

#### **Objective**

To select Valencia cultivars with improved and consistent productivity, fruit size, rind colour, peelability, and internal fruit quality (seedlessness, ratio), and extended harvest period (both earlier and later maturity).

To describe the cultivar characteristics of new Valencia cultivars and to determine the climatic suitability of these cultivars in a cold production region.

#### **Materials and methods**

Field evaluations and laboratory analyses were conducted on Turkey, Midnight, Midnight F17, Midnight 2, Midnight 1, Alpha, Bennie 2, Louisa, Henrietta, Gusocora, Ruby Valencia, Mc Clean SL, Lavalle and Lavalle 2.

**Table 5.4.21.1.** Internal fruit quality minimum export requirements for Valencia types.

Cultivar	Juice %	Brix	Min Acid	Max Acids	Ratio	Colour
Valencia EU	48	8.5	0.6	1.8%	7.5:1	Colour plate 3 of set no. 34
Midknight	52	9.5	0.85	1.8%	7.5:1	Colour plate 3 of set no. 34
*Turkey	50	10.0	0.85	1.5%	7.5:1	Colour plate 3 of set no. 34

\*Interim internal fruit quality standards.

**Table 5.4.21.2.** List of Valencia selections evaluated at Panzi (Kirkwood) during 2020 season.

Selection	Rootstock	Topwork
Alpha	CC	2011
Bennie 2	CC	2011
Gusocora G5	CC	2011
Henrietta	CC	2011
Louisa	CC	2011
Lavalle	CC	2011
Lavalle 2	CC	2011
McClellan SL	CC	2011
Midknight	CC	2011
Midknight 1	CC	2011
Midknight 2	CC	2011
Midknight F17	CC	2011
Ruby Valencia	CC	2011
Turkey	CC	2015

## Results and discussion

### Alpha

Alpha bore medium size fruit this season on the trees, peaking at count 72. Alpha Valencia were virtually seedless and the fruit shape remained fairly round with a slightly pebbly rind. The external colour development peaked at T1 – T2 with good internal quality, high Brix just below 11° supported with good acids by the time of maturity. Juice content peaked at 59% at peak maturity.

### Bennie 2

The fruit size peaked from count 56 this season, a good Valencia export fruit size. Bennie 2 has good acids for the fruit to hang on the trees longer to harvest at a later time, resulting in fewer rind problems (pitting). The selection had a seed count 2.9 – 3.2. There was no delay in external colour development (T1 – T2) at peak maturity. The rind colour was deep orange with a smooth to coarse rind. The flesh was orange and the fibre strength was soft compared to the other Valencia selections. Bennie 2 developed a very good internal quality juice percentage just above 60%, Brix:Acid ratio of this selection gave it its good flavour. The crop was very good.

### Gusocora

There were no delay in external colour development on the fruit (T1). When the selection reached a T1 the Brix was around 11.7 with an acid of 1.18%. Gusocora was completely seedless and will be regarded as a seedless selection. The juice content of Gusocora this season was high 62.6% and the fruit size ranged between counts 72 – 64 smaller, the same as 2019 season. The fruit was firm with a round shape and a smooth rind.

### Lavalle & Lavalle 2

Lavalle reached peak maturity second last and Lavalle 2 reached peak maturity last. Lavalle & Lavalle 2 had a very good export fruit size at count 56. Lavalle also had fruit with fruit size count 64. Both Lavalle selections had high juice percentage, with Lavalle being slightly higher and it also had 0.4 seeds during one evaluation. All the rest of the evaluations on both selections were virtually seedless. Brix: acid ratio for both selections was good, Brix above 11 (Lavalle 2 being higher) and the acid percentage were above 1.3% (Lavalle 2 being higher). There was no problem with the external colour development for Lavalle 2, Lavalle had a delayed colour development T3 on the colour plate range at peak maturity. The fruit was reasonably easy to peel and the internal colour was orange with a slightly softer flesh. The flavour was also very good.

#### Midnight, Midnight 1, 2 & F17

Midnight was used as control in this trial site. All the Midnight selections that were evaluated, had no seeds except Midnight 2. The fruit size count for all the selections peaked at count 72, smaller compared to 2019 season count 56. The juice content for all the Midnight selections met the minimum export standards, Midnight 1 had the highest juice content above 60%. Midnight reached T5 on the colour plate at peak maturity, while the other selections were T4 on the colour plate. Comparing Brix and acid content between Midnight (control) and the Midnight selections. The Midnight selections had a slightly higher Brix and acid content. Brix and acid content was very good.

#### McClellan SL

Fruit shape for McClellan SL is a fairly round fruit with a soft fibre strength that peels easily, containing low rind oil levels. All the fruit evaluated remained completely seedless. The trees bore medium sized fruit (count 72 to 64). The internal quality was good with high juice levels for this trial site (62.1%), Brix 11.8 and acid around 1%. Juice content increased as the fruit hung but not by much. There was no delay in external colour development being a T1, before peak maturity.

#### Turkey

Fruit size for Turkey was perfect for export with fruit size peaking at count 64. Turkey was the first selection to reach peak maturity. Turkey juice content increased towards peak maturity above 55%; great for export. Brix was above the 10° for export. The external colour of this selection was T1 at peak maturity. Turkey seed count peaked at 2.9 seeds per fruit during the evaluations. Fruit shape was round with coarse rind. The rind colour was deep orange.

#### Henrietta

Henrietta juice levels peaked above 60% with high Brix (up to 11°) and acids 1.55% at peak maturity. The external colour development was T2 – T3 for the season. The average seeds per fruit were 0.3 seeds per fruit.

#### Louisa

The fruit was virtually seedless and internal quality was good with juice (59.2%) and good Brix levels (10.7°). The rind colour was yellow by the time of peak maturity between T2 – T3. Fruit size peaked at medium size fruit, count 72 to count 64.

#### Ruby Valencia

Ruby Valencia bore medium fruit that peaked at count 64. The juice content of Ruby Valencia at peak maturity was around 60%. At peak maturity Ruby's external colour development was T1 on the colour plate range. Fruit seed count ranged between 0.9 – 1.8 seeds per fruit. At peak maturity the internal quality was good. Brix was just above 11° and acid content around 1.3%. The colour of the flesh was red, and the selection had a good flavour.

### **Conclusions**

None of the Valencia selections had a problem with external colour development, all of them reached T1 on the colour plate at peak maturity. All of the selections' internal and external qualities complied with the minimum export requirement for Valencia types. Bennie 2 had the highest count of 3.2 seeds per fruit followed by Turkey 2.9 seeds per fruit. All the other selections were virtually seedless, except Midnight 2 and Ruby Valencia. All of the selections had a fruit size count ranging from 72 - 56. The following selections developed a juice content

above 60% at peak maturity; Bennie 2, Gusocora, Henrietta, Lavalle, Lavalle 2, Mc Clean SL, Midnight 1, Ruby Valencia and Turkey.

**Table 5.4.21.3.** Internal fruit quality data for Valencia selections at Panzi (Sundays River Valley) during the 2020 season.

Date	Cultivar	Root stock	Count	Juice %	Brix°	Acid %	Ratio	Avg. Seed	Colour
2020-06-15	Alpha	CC	72	62.0	10.3	1.50	6.9	0.2	T4
2020-07-22	Alpha	CC	88	59.0	10,7	0,98	10,9	0.1	T 1 - T 2
2020-06-15	Bennie 2	CC	56	59.1	9.6	1.45	6.6	2.9	T4
2020-07-22	Bennie 2	CC	72	61.7	10	0,95	10,5	3.2	T 1 - T 2
2020-08-19	Gusocora (G5)	CC	72	61.1	11.3	1.74	6.5	0.0	T1
2020-09-01	Gusocora (G5)	CC	72	62.6	11.7	1.18	9.9	0.0	T1
2020-09-28	Gusocora (G5)	CC	64	61.8	11.6	0.90	12.9	0.0	T1
2020-08-04	Henrietta	CC	72	60.8	11.4	1.55	7.4	0.4	T2- T3
2020-09-01	Henrietta	CC	64	61.0	10.6	1.23	8.6	0.2	T1
2020-09-09	Lavalle	CC	56	61.1	11.1	1.35	8.2	0.2	T3
2020-09-28	Lavalle	CC	56	60.6	11.3	1.20	9.4	0.1	T1
2020-10-07	Lavalle	CC	64	59.9	11.3	1.16	9.7	0.4	T1
2020-09-09	Lavalle 2	CC	56	60.9	12.1	1.58	7.7	0.0	T1
2020-09-28	Lavalle 2	CC	56	60.3	11.9	1.37	8.7	0.0	T1
2020-10-07	Lavalle 2	CC	56	60.4	11.9	1.30	9.2	0.1	T1
2020-08-04	Louisa	CC	72	59.2	10.7	1.21	8.8	0.3	T2 - T3
2020-09-01	Louisa	CC	64	57.8	11.4	1.08	10.6	0.0	T1
2020-09-09	Mc Clean SL	CC	64	62.1	11.8	1.06	11.1	0.0	T1
2020-09-28	Mc Clean SL	CC	72	64.8	11.9	0.99	12.0	0.0	T1
2020-10-07	Mc Clean SL	CC	72	63.5	12.1	0.91	13.3	0.0	T1
2020-06-01	Midnight	CC	72	57.5	9.3	1.23	7.6	0.0	T5
2020-06-15	Midnight	CC	72	59.3	10.0	1.18	8.5	0.0	R 3
2020-07-22	Midnight	CC	105	60.2	10,4	0,8	13,0	0.2	T 1
2020-06-15	Midnight 1 I 15	CC	72	62.3	10.0	1.27	7.9	0.0	4
2020-09-01	Midnight 1 I 15	CC	72	62.1	11.1	0.99	11.8	0.0	T1
2020-06-15	Midnight 2 H14	CC	72	55.9	10.5	1.30	8.1	2.2	T4
2020-08-04	Midnight 2 H14	CC	72	57.2	11.5	1.19	9.7	2.2	T1
2020-09-01	Midnight 2 H14	CC	105	56.8	12.5	1.08	11.6	1.1	T1
2020-06-01	Midnight F 17	CC	72	56.8	9.4	1.25	7.5	0.0	T4
2020-06-15	Midnight F 17	CC	72	57.9	10.2	1.23	8.3	0.0	T4 - T5
2020-08-04	Midnight F 17	CC	72	58.3	11.3	1.11	10.2	0.0	T1
2020-09-01	Midnight F 17	CC	88	59.4	12.1	0.96	13.5	0.0	T1
2020-09-01	Ruby Valencia	CC	64	61.7	11.1	1.31	8.5	1.8	T1
2020-09-09	Ruby Valencia	CC	64	60.1	10.5	1.10	9.5	1.5	T1
2020-09-28	Ruby Valencia	CC	64	61.4	10.9	1.13	9.6	1.4	T1
2020-10-07	Ruby Valencia	CC	64	61.1	10.6	1.06	10.0	0.9	T1
2020-06-01	Turkey	CC	72	55.4	11.1	1.23	9.0	2.9	T1
2020-06-15	Turkey	CC	64	56.1	11.6	1.11	10.5	2.6	T1
2020-07-22	Turkey	CC	88	59.3	12,4	0,74	16,8	2.9	T 1 - T 2

## 5.4.22 PROGRESS REPORT: Cultivar characteristics and climatic suitability of Valencia oranges in a cold production region (Citrusdal)

Project 1097B by W. Swiegers (CRI)

### Summary

The climate and the soil make this region an intermediate region to farm Valencia oranges. The Valencia fruit tends to get high sugars, but acids also stay high in this region. It gives fruit with good flavour and shelf life. Most of the trees were planted in 2009 and consist of early-, mid- and late maturing selections. The ripening order was as follows starting with Turkey, McClean SL, Gusocora, Midnight H14, Midnight, Delta, Henrietta, Alpha, Bennie, Alpha 2, Ruby Val, Val Late, Midnight F17, Bennie 3, Louisa and the season finished off with Lavelle and Lavelle 2.

### Opsomming

Die klimaat en die grond maak die verbouing van Valencia 'n intermediere area. Hoë suikers word verkry, maar die suur bly hoog. Dit maak vrugte met goeie geure en hou vermoë. Die meeste bome is in 2009 geplant en bestaan uit vroeë-, mid- en laat rypwordende seleksies. Die orde van rypwording was as volg, Turkey, McClean SL, Gusocora, Midnight H14, Midnight, Delta, Henrietta, Alpha, Bennie, Alpha 2, Ruby Val, Val Late, Midnight F17, Bennie 3, Louisa en die seisoen is afgesluit met Lavelle en Lavelle 2.

### Objective

- To select Valencia cultivars with improved and consistent productivity, fruit size, rind colour, peelability, and internal fruit quality (seedlessness, ratio), and extended harvest period (both earlier and later maturity).
- To describe the cultivar characteristics of new Valencia cultivars and to determine the climatic suitability of these cultivars in a cold production region.

### Materials and methods

Field evaluations and laboratory analyses were conducted on Turkey, McClean SL, Gusocora, Midnight H14, Midnight, Delta, Henrietta, Alpha, Bennie, Alpha 2, Ruby Val, Val Late, Midnight F17, Bennie 3, Louisa, Lavelle and Lavelle 2.

**Table 5.4.22.1.** Internal fruit quality minimum export requirements for Valencia types.

Cultivar	Juice %	Brix	Min Acid	Max Acids	Ratio	Colour
Valencia EU	48	8.5	0.6	1.8%	7.5:1	Colour plate 3 of set no. 34
Midnight	52	9.5	0.85	1.8%	7.5:1	Colour plate 3 of set no. 34
*Turkey	50	10.0	0.85	1.5%	7.5:1	Colour plate 3 of set no. 34

\*Interim internal fruit quality standards.

**Table 5.4.22.2.** List of Valencia selections evaluated at Kweekkraal (Citrusdal) during 2020 season.

Selection	Rootstock	Topwork
Alpha	CC	2009
Alpha 2	CC	2016
Bennie	CC	2009
Bennie 3	CC	2016
Henrietta	CC	2010
Delta	CC	2009

Gusocora	CC	2009
Turkey	CC	2009
Valencia Late	CC	2009
McClellan SL	CC	2009
Midnight	CC	2009
Ruby Valencia	CC	2009
Lavalle	CC	2009
Lavalle 2	CC	2011
Midnight H14	CC	2011
Midnight F17	CC	2016
Louisa	CC	2011

## Results and discussion

### Alpha & Alpha 2

It was Alpha 2's second crop. The internal quality for Alpha was better than Alpha 2, this could be that Alpha is an older tree. Alpha internal quality at peak maturity were good with juice content 55.4%, Brix 11° and acid 1.39% compared to Alpha 2 internal quality that was moderate with juice content 50.8%, Brix 9.4° and acid 1.27%. Fruit size for Alpha varied from count 105 to 64 compared to Alpha 2 count 88 to 64, but still good for Valencia production and export. External colour peaked on T1 for both selections. As the fruit hung on the trees the internal quality improved for export fruit of higher standards. Both selections were seedless during the evaluations.

### Bennie & Bennie 3

The fruit size count peaked for Bennie at count 88 to 56 (medium – large) fruit size and a good Valencia export size. For Bennie 3 fruit size was count 105 – 48 (small to big), but it is due to the age of the tree. Bennie had a soft fibre strength compared to the other Valencia selections. But due to the high acid the fruit was left to hang so that the acids can come down to 1.1% and Brix rise to above 10°. This made the selection mature much later but also gave it a good flavour. The selection had a much better internal quality after the fruit was left to hang a bit. Seed count for Bennie ranged between 0.0 – 1.7 seeds per fruit, while Bennie 3 was virtually seedless. There was no delay in external colour development peaking at (T1) at peak maturity. Bennie and Bennie 3 developed a moderate juice content around 55% as the fruit matured and was left on the tree a bit longer.

### Gusocora

The fruit had a delayed colour development with T4 on the colour plate at peak maturity. The Brix and juice percentage also went up and the acids also dropped to get a fruit with very good export internal quality. The Brix peaked at 11° and acids were still good at 1.07% with good juice content above 55%. Gusocora was completely seedless and will be regarded as a seedless selection for future plantings. Fruit size peaked at count 125 - 88.

### Henrietta

The peelability of Henrietta was quite easy and the fruit shape was round. The rind texture was smooth with a medium rind oil on the fruit. The average seed count was 1.3 seeds per fruit. Fruit size count ranged from count 125 – 64. Henrietta had a slight delay in external colour development (T2 – T3) at peak maturity. Henrietta produced a good juice content above 55%, but good Brix:Acid ratio.

### Lavalle and Lavalle 2

There was no major difference between Lavalle and Lavalle 2. Lavalle fruit size this season was slightly larger peaking at count 48 compared to Lavalle 2, count 56. Lavalle selections were seedless and the juice content of these selections increased towards peak maturity to above 55% with a Brix: acid ratio around 8. Brix was also good along with the high acids. Lavalle 2 had a slightly higher Brix. There was a delay with the external colour development when Lavalle developed T2 -T3 on the colour plate range at peak maturity and Lavalle 2 developed T3 – T4 on the colour plate. The fruit was reasonably easy to peel and the internal colour was

orange with a slightly softer flesh. The flavour was also very good. Lavalley reached peak maturity slightly earlier than Lavalley 2.

#### Louisa

Fruit size count for Louisa was very good ranging from count 105 – 64. The fruit has smooth rinds, but rind oil is high. Fruit shape is round to slightly elongate. Louisa's internal quality complies with all the export standards. Low juice content of 52.2%, Brix at 9.3° and acid 1.22%. Colour development was delayed being a T3 – T4 on the colour plate. Fruit rind colour is more of a yellow colour. Louisa was completely seedless.

#### Valencia Late

Valencia Late was the control for the late maturing selections. The Valencia Late produced small – medium size fruit at count 105 - 64. The internal and external quality was moderate, the juice content was around 55%, and Brix started off good at 10° with acid content 1.23%, but colour development was delayed T3 – T2. Seed counts were between 0.3 – 0.7 seeds per fruit.

#### Delta

Delta, as the control cultivar, produced completely seedless fruit and a good yield on the trees. Fruit size peaked between count 125 – 72. Smaller fruit due to the big crop. Moderate internal quality (end of July), Brix of 9.7° and acid content of 1.22% and juice content 56.4%. The external colour of the fruit was T6 – T3. The fruit was round with a smooth rind and peeled fairly easily.

#### McClellan SL

McClellan SL tree bore fruit with fruit size count ranging from count 125 - 64. The trees bore a big crop. The selection was seedless. External colour development were delayed T2 – T4 on the colour plate at peak maturity. Brix was good (above 10°) peaking at 11.2:1 and acids remained stable towards the end of the season around 1%, resulting in a very good Brix: Acid ratio. The fruit is firm with a round to elongated fruit shape with a smooth rind. Externally as well as internally the colour is deep orange.

#### Turkey

Fruit size did vary for Turkey with count ranging between 72 – 56. Brix was around 10° and acids around 1.15%. This will meet the export standards as well as the external colour that was at T1 – T3 on the colour plate. Turkey was virtually seedless. Fruit characteristics for Turkey were round fruit shape, with a very good flavour, soft rag, fairly thin rind and easy peeling. The internal colour was light yellow, and externally the fruit remained yellow. This selection has the qualities of a mid-season orange; for instance, the exceptionally soft fruit, and the soft rind that can result in rind problems if managed incorrectly.

#### Midnight, Midnight F17 and Midnight H14

Midnight was used as control for this trial site but also as control for the other two Midnight selections. The fruit size development for Midnight peaked at count 56 followed by Midnight H14 count 64 and Midnight F17 peaking at count 72. The smallest fruit size count for Midnight and Midnight F17 was count 105, and Midnight H14 was count 125. All the Midnight selections bore round fruit with a medium to coarse rind, fibre strength was fairly soft and the fruit peeled easily. The Midnight selections were virtually seedless. The colour development at peak maturity was slightly delayed for all the selections. The trees produced well. All the Midnight selections had a good Brix of more or less the same around 10°. Midnight H14 was first to reach peak maturity, followed by Midnight and Midnight F17 was the last selection to reach peak maturity.

#### Ruby Valencia

Ruby Valencia bore small - large fruit that peaked at count 105 - 56. The juice content of Ruby Valencia at peak maturity was above 55%. At peak maturity Ruby's external colour development was T2 – T4 on the colour plate range. Fruit seed count ranged between 0.0 – 1.7 seeds per fruit. At peak maturity the internal quality was moderate. Brix was at 10° and acid content around 1.35%. The colour of the flesh was red, and the selection has a unique taste.

## **Conclusions**

All of the selections had problems with their external colour development, except Alpha, Alpha 2, Bennie 3 and Turkey that were able to reach T1 on the colour plate when the fruit was left to hang. All the selections met the minimum export standards. Bennie, Henrietta, Valencia Late and Turkey were the selections on average with the highest number of seeds per fruit. All the other selections were completely seedless. The fruit size varied quite a lot between selections, but all of them were good enough for export and peaked around count 64 - 56. All the selections produced a Brix around 10°, except Alpha with Brix around 11°. Acid content for the selections varied around 1.2% but when the fruit was left to hang, it reached 1.1%. Lavalley had the highest juice content at 56.5%.

**Table 5.4.22.3.** Internal fruit quality data for Valencia selections at Kweekkraal (Citrusdal) during the 2020 season.

Date	Cultivar	Root stock	Count	Juice %	Brix°	Acid %	Ratio	Avg. Seed	Colour
2020-07-29	Alpha	CC	105 - 64	55,4	11	1,39	7,9	0,0	T 1 - T 2
2020-08-24	Alpha	CC	88 - 64	53,4	11,3	1,36	8,3	0,0	T 1 - T 2
2020-09-08	Alpha	CC	88 - 72	54,6	11,9	1,25	9,5	0,0	T 1 - T 2
2020-09-22	Alpha	CC	72 - 64	53,8	11,9	1,14	10,4	0,0	T 1
2020-07-29	Alpha 2	CC	72 - 64	50,8	9,4	1,27	7,4	0,0	T 1 - T 3
2020-08-24	Alpha 2	CC	88 - 64	54,0	10	1,20	8,3	0,0	T 1 - T 2
2020-09-08	Alpha 2	CC	88 - 64	55,1	10,1	1,13	8,9	0,0	T 1
2020-09-22	Alpha 2	CC	88 - 64	56,0	10,1	1,07	9,5	0,0	T 1
2020-07-29	Bennie	CC	88 - 56	52,6	9,1	1,20	7,6	1,7	T 1 - T 3
2020-08-24	Bennie	CC	88 - 72	54,0	9,5	1,22	7,8	0,1	T 1 - T 2
2020-09-08	Bennie	CC	88 - 72	54,7	9,5	1,13	8,4	0,6	T 1 - T 2
2020-09-22	Bennie	CC	88 - 64	53,2	10,1	1,09	9,3	0,0	T 1 - T 3
2020-07-29	Bennie 3	CC	88 - 48	56,3	9,3	1,34	7,0	0,0	T 1 - T 3
2020-08-24	Bennie 3	CC	105 - 64	51,5	9,2	1,21	7,6	0,6	T 1 - T 2
2020-09-08	Bennie 3	CC	88 - 64	53,1	9,5	1,13	8,4	0,3	T 1
2020-09-22	Bennie 3	CC	88 - 72	57,5	10,4	1,17	8,9	0,0	T 1 - T 2
2020-07-29	Delta	CC	125 - 72	56,4	9,7	1,22	7,9	0,0	T 3 - T 6
2020-09-08	Delta	CC	125 - 88	57,2	10,1	1,11	9,1	0,0	T 2 - T 3
2020-07-29	Gusocora	CC	125 - 88	55,8	10,5	1,25	8,4	0,0	T 4
2020-08-24	Gusocora	CC	105 - 72	55,6	10,9	1,20	9,1	0,0	T 1 - T 3
2020-09-08	Gusocora	CC	105 - 88	56,4	10,7	1,07	10,0	0,0	T 2
2020-09-22	Gusocora	CC	105 - 88	57,3	11,2	1,07	10,4	0,0	T 2 - T 3
2020-07-29	Henrietta	CC	125 - 72	55,1	9,9	1,26	7,9	1,2	T 2 - T 3
2020-08-24	Henrietta	CC	105 - 64	57,0	10,2	1,25	8,2	0,5	T 2 - T 4
2020-09-08	Henrietta	CC	105 - 72	56,1	9,9	1,20	8,2	0,8	T 2 - T 3
2020-09-22	Henrietta	CC	105 - 64	57,7	10,5	1,09	9,6	1,3	T 1 - T 3
2020-08-24	Lavalle	CC	88 - 48	53,0	9,3	1,45	6,4	0,0	T 2 - T 5
2020-09-08	Lavalle	CC	88 - 56	59,5	10,3	1,20	8,6	0,0	T 2 - T 3
2020-09-22	Lavalle	CC	88 - 56	57,4	10,8	1,25	8,7	0,0	T 3 - T 4
2020-09-08	Lavalle 2	CC	88 - 56	55,2	10,9	1,33	8,2	0,0	T 3 - T 4
2020-09-22	Lavalle 2	CC	88 - 56	56,2	11,4	1,43	8,0	0,0	T 4 - T 5
2020-07-29	Louisa	CC	105 - 72	52,4	8,8	1,36	6,5	0,0	T 2 - T 5
2020-08-24	Louisa	CC	105 - 72	52,2	9,3	1,22	7,6	0,0	T 3 - T 4
2020-09-08	Louisa	CC	105 - 64	49,8	8,5	1,04	8,1	0,0	T 2 - T 3

2020-09-22	Louisa	CC	105 - 72	53,9	9,6	1,03	9,3	0,0	T 3 - T 4
2020-07-29	McClellan SL	CC	105 - 72	57,2	10,3	1,22	8,5	0,0	T 2 - T 4
2020-08-24	McClellan SL	CC	105 - 64	57,8	10,1	1,11	9,1	0,0	T 2 - T 3
2020-09-08	McClellan SL	CC	105 - 64	57,7	9,8	1,07	9,2	0,0	T 1 - T 3
2020-09-22	McClellan SL	CC	125 - 72	58,9	11,2	1,03	10,9	0,0	T 2 - T 3
2020-07-29	Midnight	CC	105 - 64	52,9	9,8	1,22	8,0	0,0	T 2 - T 3
2020-08-24	Midnight	CC	88 - 64	56,2	10,1	1,16	8,7	0,1	T 1 - T 2
2020-09-08	Midnight	CC	88 - 56	53,0	10,3	1,09	9,5	0,0	T 2
2020-09-22	Midnight	CC	105 - 72	57,8	11,3	1,12	10,1	0,1	T 2
2020-07-29	Midnight F 17	CC	105 - 72	51,0	9,1	1,19	7,6	0,0	T 3 - T 5
2020-08-24	Midnight F 17	CC	105 - 72	55,8	9,6	1,21	7,9	0,0	T 2
2020-09-08	Midnight F17	CC	105 - 72	55,8	10,2	1,04	9,8	0,0	T 2
2020-09-22	Midnight F17	CC	105 - 64	53,3	10,3	0,96	10,7	0,6	T 1 - T 2
2020-07-29	Midnight H14	CC	105 - 72	56,0	10,1	1,25	8,1	0,0	T 3
2020-08-24	Midnight H14	CC	125 - 72	53,8	10	1,25	8,0	0,2	T 2 - T 3
2020-09-08	Midnight H14	CC	88 - 72	51,1	9,7	1,04	9,4	0,0	T 1 - T 3
2020-09-22	Midnight H14	CC	105 - 72	55,1	10,5	0,96	10,9	0,0	T 1 - T 2
2020-07-29	Ruby Valencia	CC	105 - 64	56,1	10	1,35	7,4	0,0	T 2 - T 4
2020-09-08	Ruby Valencia	CC	105 - 56	51,4	9	1,05	8,5	1,7	T 2 - T 3
2020-07-01	Turkey	CC	88 - 56	46,7	9,7	1,35	7,2	0,0	T 3
2020-07-29	Turkey	CC	72 - 56	52,6	10,1	1,15	8,8	0,3	T 1 - T 3
2020-08-24	Turkey	CC	72 - 64	50,0	10,6	1,26	8,4	0,0	T 2
2020-09-08	Turkey	CC	72 - 56	51,0	11,6	1,10	10,5	0,1	T 1
2020-08-24	Valencia Late	CC	105 - 72	55,8	10,1	1,23	8,2	0,3	T 2 - T 3
2020-09-08	Valencia Late	CC	105 - 64	54,0	10,5	1,14	9,2	0,7	T 1 - T 2
2020-09-22	Valencia Late	CC	88 - 64	55,2	10,8	1,03	10,5	0,6	T 1 - T 3

#### 5.4.23 PROGRESS REPORT: Cultivar characteristics and climatic suitability of Clementine mandarins in a cold production region (Sundays River Valley)

Project 1000B by W. Swiegers and Z. Zondi (CRI)

#### Summary

Only some of the selections were evaluated. The open selections will be used as controls for the new selections in the future. For the Sundays River Valley there are two Clementine sites with most of the selections. There is a new exciting site that will come into production in a few years with all the latest selections. Some of the selections are on interstock. The season started with Basol, Nules, followed by Esbal, Early Esbal and ended with Large Esbal.

#### Opsomming

Die seleksies wat geëvalueer was die seisoen is net 'n paar van die seleksies. Die oop seleksies dien as kontroles vir die nuwe seleksies. Vir die Sondags Rivier Vallei is daar 2 Clementine persele wat die meeste seleksies bevat. Daar kom 'n nuwe opwindende perseel by met al die nuwe seleksies. Van die seleksies is op 'n tussen stam. Die seisoen het begin met Basol, Nules gevolg deur Esbal, Early Esbal en ge-eindig met Large Esbal.

#### Objectives

- To select Clementine cultivars with improved and consistent productivity, fruit size, rind colour, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the cultivar characteristics of new Clementine cultivars and to determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on Clementine selections from the Sundays River Valley region of the Eastern Cape; planted 2012. The following cultivars were evaluated: Basol, Early Esbal, Large Esbal, Esbal and Nules.

A ratio of 11:1 Clementines is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.23.1.** List of Clementine selections evaluated at Invercloy (Kirkwood) during 2020.

Selection	Rootstock	Planted
Esbal	Carrizo	2012
Nules	Carrizo	2012

**Table 5.4.23.2.** List of Clementine selections evaluated at Dunbrody (Kirkwood) during 2020.

Selection	Rootstock	Topworked
Basol	Carrizo with Midnight as interstock	2015
Early Esbal	Carrizo with Midnight as interstock	2015
Large Esbal	Carrizo with Midnight as interstock	2015

## Results and discussion

### Basol

Basol is the earliest maturing Clementine selection. Basol trees tend to develop galls on the trunk. The Basol trees with the navel interstock don't show galls on the trees yet. This one is with an interstock. Fruit size for Basol was small with count 5. Internal quality was excellent for Basol at ratio 11.6:1 the juice content was 61.2%, Brix 11.9° and acid 1.03%. This selection was seedless. External colour break was delayed, T6 on the colour plate. The fruit peels easily. Basol tends to have a very short harvest period before the fruit is over mature and starts to granulate and get puffy.

### Esbal & Early Esbal & Large Esbal

Esbal was used as control for the two experimental Esbal selections. Early Esbal is selected to mature before Esbal and Large Esbal is selected to crop bigger fruit than Esbal. The order of ripening for the 3 selections were Esbal first, followed by Early Esbal and Large Esbal finished the season. Factors that can contribute to the order of ripening is: Esbal trees are older and were topworked directly onto CC rootstock. Early – and Large Esbal trees were topworked onto Valencia interstock with CC as rootstock, and it was also the third crop for these trees. Early Esbal had slightly smaller fruit size ranging count 3 - 1 while Esbal were count 1 and Esbal Large fruit size ranged at count 2 - 1. Esbal had the best internal quality, highest juice content, and the best Brix:acid ratio towards peak maturity. Esbal's internal quality was excellent. Early Esbal were completely seedless, Large Esbal seed count peaked at 1.1 seeds per fruit and Esbal seed count peaked at 0.3 seeds per fruit. All three Esbal selections had a delayed colour development at peak maturity. Fruit was round to oblate with a smooth to pebbly rind

### Nules

Nules were the second selection to reach peak maturity. The selection had a fair juice percentage (50.8%) at peak maturity. Nules had a favourable fruit size count 1. Internal quality for Nules at peak maturity was good; Brix (11.2°) and acid (0.93%). Nules also kept its acids well. This contributes to Nules good flavour. Nules average seed count was 0.5 seeds per fruit. Rind colour development was not good with T7 on the colour plate at peak maturity. Peelability is easy and the internal colour is orange.

## Conclusion

On average Nules, Esbal and Large Esbal had the highest seed count of all the selections that were evaluated, around 0.4 seeds per fruit. Most of the selections had delayed colour development at peak maturity except Early Esbal and Esbal with T1 on the colour plate when the fruit was left to hang a bit. Degreening practices will be essential after harvesting to ensure optimal colour development. Basol, Esbal and Nules had the highest Brix (11 - 12°). Basol had the smallest fruit size count 4 - 5. Basol and Esbal had the highest juice percentage: around 60%.

**Table 5.4.23.3.** Internal fruit quality data for Clementine selections in the Sundays River Valley region of the Eastern Cape during the 2020 season.

Date	Cultivar	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-03-11	Basol	CC	5	61.2	11.9	1.03	11.6	0.0	T6
2020-03-24	Basol	CC	4	64.3	12.2	0.88	13.9	0.0	T5
2020-04-20	Basol	CC	5	60.8	12.3	0.82	15.0	0.0	T2
2020-04-20	Early Esbal	CC	3	55.6	9.5	0.93	10.2	0.0	T5
2020-05-11	Early Esbal	CC	1	50.9	9.9	0.90	11.0	0.0	T4
2020-05-25	Early Esbal	CC	1	49.3	10.7	0.99	10.8	0.0	T1
2020-04-20	Large Esbal	CC	2	50.6	10.4	1.14	9.1	1.1	T7
2020-05-11	Large Esbal	CC	1	53.0	10.9	1.10	9.9	0.1	T5
2020-05-25	Large Esbal	CC	1	50.2	11.7	1.12	10.4	0.0	T3 - T4
2020-04-20	Esbal	CC	1	61.2	11.1	0.97	11.4	0,3	T5
2020-05-25	Esbal	CC	1	59.1	12.7	0.93	13.7	0.3	T1
2020-04-20	Nules	CC	1	50.8	11.2	0.93	12.0	0.8	T7
2020-05-11	Nules	CC	1	57.8	12.7	0.92	13.8	0.1	T5
2020-05-25	Nules	CC	1	53.8	12.5	0.80	15.6	0.5	T4

### 5.4.24 PROGRESS REPORT: Cultivar characteristics and climatic suitability of Clementine mandarins in a cold production region (Western Cape)

Project 1000D by W. Swiegers (CRI)

#### Summary

There were two trial sites in Citrusdal where we evaluated Clementines this season. The open selections are used as controls in the trial sites. The selections that were evaluated were early-, mid- and late selections. The early selections will play an important role as they start to overlap the Satsumas. Basol started the season in Citrusdal followed by Basol with interstock, Octubrina, Clemenluz, Early Esbal, Nules, Large Esbal and Esbal finished the season.

#### Opsomming

Daar is 2 proef persele in Citrusdal waar Clementines geëvalueer was die seisoen. Die oop seleksies dien as kontroles vir die proef persele. Die seleksies wat geëvalueer word is vroeë-, mid – en laat seleksies. Die vroeë seleksies gaan nog 'n belangrike rol speel in die toekoms soos wat dit begin oorvleuel met Satsumas. Basol het

die seisoen in Citrusdal begin, gevolg deur Basol met tussenstam, Octubrina, Early Esbal, Nules, Large Esbal, en Esbal het die seisoen afgesluit.

## Objectives

- To select Clementine cultivars with improved and consistent productivity, fruit size, rind colour, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the cultivar characteristics of new Clementine cultivars and to determine the climatic suitability of these cultivars in cold production regions.

## Materials and methods

Field evaluations and laboratory analyses were conducted on Clementine selections from various regions in the Western Cape. The following varieties were evaluated: Nules, Esbal, Basol, Basol with interstock, Early Esbal, Large Esbal, Octubrina, and Clemenluz.

A ratio of 11:1 Clementines is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.24.1.** List of Clementine selections evaluated at Kweekkraal and Stargrow (Citrusdal) during 2020.

Selection	Rootstock	Planted	Topworked
Nules	Carrizo	2009	
Basol	Carrizo		2010
Basol interstock	Carrizo		2011
Esbal	Carrizo	2009	
Early Esbal			2011
Large Esbal			2011
Octubrina			2016
Clemenluz			2009

## Results and discussion

### Basol and Basol interstock

Basol is one of the earliest maturing Clementine selections. Basol trees tend to develop galls on the trunk but the Basol trees with the navel interstock tend to develop galls a lot slower. Fruit size on Basol was bigger compared to Basol on the interstock. Basol fruit size was small (count 5 – 3) compared to Basol interstock fruit size small with count (5). Basol with interstock had better internal quality compared to Basol on CC at peak maturity. The selection on CC had a lower seed count 0.0 seeds per fruit and Basol with interstock had 0.3 seeds per fruit. External colour break for Basol on CC at peak maturity was T5 compared to Basol with interstock T6 on the colour plate. Basol has a very short harvest period before the fruit is over mature and starts to granulate.

### Esbal & Early Esbal & Large Esbal

Esbal was used as control for the 2 experimental Esbal selections. Early Esbal is selected to mature before Esbal and Large Esbal is selected to crop bigger fruit than Esbal. The order of ripening for the 3 selections was Early Esbal first, followed by Large Esbal and Esbal finished the season at this trial site. Early Esbal had slightly smaller fruit peaking count 4 while Esbal Large fruit size peaked at count 1 and Esbal fruit size peaked at count 2. Esbal had the highest juice content around 60% followed by Early Esbal at around 55% juice content and Large Esbal juice content was below 55%. Esbal had the best internal quality, the best Brix:acid ratio towards peak maturity Brix 11.4 and acid 1.05% (ratio 10.9:1), with juice percentage 63.8%. All three selections

were virtually seedless. All 3 selections had delayed colour development at peak maturity, Esbal had the best colour development T2.

### Nules

Nules was used as control. At peak maturity fruit size count was 5 – 2 (small - medium). The internal quality was good with Brix (11.7°) and acid (0.98%) at peak maturity ratio 11:9. Acids stayed stable at 0.8% even when the fruit was well over mature. Internal colour development was delayed, being T5 on colour plate at peak maturity. Those acids will give the fruit good shelf life and the high sugars with acids give Nules its good flavour. The rind is smooth and thin and it peels easily. The seed count was 1.2 – 1.3 seeds per fruit.

### Octubrina

Octubrina is a new experimental early maturing Clementine. It must have sweet orange as an interstock. It reaches peak maturity about 2 – 3 weeks before Nules. It was the second crop for the trees and the crop was good. Fruit size range was very good for Clementine production, with fruit size count 3 – 2. At peak maturity (ratio 11:8), the Brix was 11.7 and acid percentage was 0.99%. Acids stayed stable after peak maturity, which is very good. Seedless during evaluations. External colour development very good towards peak maturity, being T1 on the colour plate. The selection does degreen very well. The fruit is round to flattish and peelability is easy.

### Clemenluz

Clemenluz is an early maturing Clementine selection. Nules was used as a control for this section. The Clemenluz reached peak maturity before Nules according to the ratio. Compared to Nules, Nules and Clemenluz internal quality is very close to each other. Clemenluz developed higher juice percentage, higher Brix and better acids at peak maturity. Clemenluz seed count peaked at 3.5 seeds per fruit. Colour development was delayed at peak maturity. Rind colour is a yellow orange and the peelability of the fruit is easy.

### **Conclusion**

None of the selections were completely seedless, except Basson and Octubrina, because they were planted in mixed trial blocks. Clemenluz had the highest seed count 1.5 – 3.5 seeds per fruit. Most of selections had delayed colour development. Degreening practices will be essential after harvesting to ensure optimal colour development. Octubrina was the only selection to reach T1 on the colour plate at peak maturity. Basol with CC had the highest Brix (12.6°) and acid (0.77%) at over maturity. Basol had the smallest fruit size and peaked at count 5.

**Table 5.4.24.2.** Internal fruit quality data for Clementine selections in the Citrusdal region (Kweekkraal and Stargrow) of the Western Cape during the 2020 season.

Date	Cultivar	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-03-16	Basol	CC/NAV	5	65,5	11,9	0,82	14,6	0,3	T 6
2020-03-16	Basol	CC	5 - 3	56,7	12,6	0,77	16,3	0,0	T 5
2020-04-20	Early Esbal	CC	below 5 - 4	55,6	10,6	0,99	10,7	0,3	T4
2020-03-16	Esbal	CC	5 - 4	55,1	9,6	0,93	10,3	0,5	T 7
2020-05-14	Esbal	CC	4 - 2	61,5	10,6	0,99	10,7	0,4	T 4 - T 5
2020-06-02	Esbal	CC	4 - 3	63,8	11,4	1,05	10,9	0,8	T 2
2020-04-20	Large Esbal	CC	5 - 3	52,8	11,4	1,37	8,3	0,3	T 7
2020-05-14	Large Esbal	CC	4 - 1	52,2	12,1	1,21	10,0	0,3	T 4 - T 5
2020-04-20	Nules	CC	5 - 2	57,9	10,9	1,04	10,5	1,2	T 7

2020-05-14	Nules	CC	3 - 2	48,2	11,7	0,98	11,9	1,3	T 5
2020-04-10	Octubrina	CC	3 - 2	58,5	11,7	0,99	11,8	0,0	T 1
2020-03-16	Clemenluz	CC	5 - 3	59,1	9,2	1,09	8,5	3,5	T 7
2020-04-20	Clemenluz	CC	3 - 1x	50,9	11	0,95	11,6	3,0	T 5

#### 5.4.25 PROGRESS REPORT: Cultivar characteristics and climatic suitability of Clementine mandarins in a cold production region (South West Cape)

Project 1000E by W. Swiegers (CRI)

##### Summary

The trial site doesn't have a wide range of selections at the moment. Buffeljagsrivier region will be one of the biggest Clementine trial sites in the future with another site on the way. The season started with Basol, followed by Nules, Early Esbal, Esbal and ended with Large Esbal.

##### Opsomming

Die proef perseel het nie op die oomblik 'n wye verskeidenheid van seleksies nie. Buffeljagsrivier area gaan in die toekoms een van die grootste Clementine proef persele word. Daar is nog 'n perseel op pad. Die seisoen het begin met Basol, gevolg deur Nules, Early Esbal, Esbal en ge-eindig met Large Esbal.

##### Objectives

- To select Clementine cultivars with improved and consistent productivity, fruit size, rind colour, internal fruit quality (Brix, acidity and ratio), seedlessness and extended harvest period (both earlier and later maturity).
- To describe the cultivar characteristics of new Clementine cultivars and to determine the climatic suitability of these cultivars in cold production regions.

##### Materials and methods

Field evaluations and laboratory analyses were conducted on Clementine selections from the Buffeljagsrivier region of the South West Cape; the planting date was 2014. The following cultivars were evaluated: Basol, Esbal, Early Esbal, Large Esbal and Nules.

A ratio of 11:1 Clementines is considered to be the build-up towards peak maturity of 12:1. After reaching the peak, the ratio increases to 13:1, after which it is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in a greater instance of quality and rind issues.

**Table 5.4.25.1.** List of Clementine selections evaluated at Olivedale (Buffeljagsrivier) during 2020 season.

Selection	Rootstock	Planted
Basol	Carrizo	2014
Esbal	Carrizo	2014
Nules	Carrizo	2014
Early Esbal	Carrizo	2014
Large Esbal	Carrizo	2014

##### Results and discussion

###### Basol

Basol is an early maturing Clementine selection. Fruit size count for Basol was 4 - 1. Basol juice percentage towards peak maturity started with 49.1%. There were barely seeds in this selection. This selection reached

T4 – T5 towards peak maturity. Basol had the Brix at 11.0° and acid at 1.08% towards peak maturity. The fruit peels easily. Basol's rind colour is deep orange. Basol has a very short harvest period before the fruit is over mature and starts to granulate.

#### Esbal & Early Esbal & Large Esbal

Esbal was used as control for the experimental Early Esbal & Large Esbal Clementine. Early Esbal was selected to reach peak maturity earlier than Esbal, while Large Esbal was selected for bigger fruit size. In this trial site Early Esbal reached peak maturity before Esbal and Esbal matured before Large Esbal. Fruit size count for Esbal was 4 – 2, slightly bigger than Early Esbal fruit size count 5 – 2 and Large Esbal fruit size 4 – 1x. Internal quality for Early Esbal was the best with juice content above 55%, Brix above 12° and acid around 1%. Large Esbal had the second best internal quality with a slightly lower Brix. Esbal had good Brix and acid percentage, just a low juice content below 50%. Rind colour development was also slightly better for Large Esbal T1 – T3 compared to Early Esbal T2 – T5 and Esbal T4 – T5 on (colour plate) when the fruit was at peak maturity. Fruit is round to oblate. Rind is smooth to pebbly. Peelability is easy but rind oil a bother. Seed count for Esbal peaked 2.8 seeds per fruit compared to Early Esbal seed count that peaked at 0.7 seeds per fruit and average Large Esbal seed count were 0.9 seeds per fruit.

#### Nules

Nules was the second selection to reach peak maturity. The selection's juice percentage increased towards peak maturity. Internal quality for Nules at peak maturity was fair. Brix (11.4°) and acid (0.94%) and juice content 51.3%. Nules seed count ranged from 4.6 to 6.6 seeds per fruit. Rind colour development was not good for Nules with a T4 – T5 on the colour plate at peak maturity. Nules had a good fruit size at count 3 – 1x (peak maturity). Yields were good for Nules. Peelability is easy and internal colour is orange.

#### **Conclusion**

Basol had the lowest seed count and Nules had the highest seed count. Large Esbal were the only selections to reach T1 on the colour plates. Degreening practices will be essential after harvesting to ensure optimal colour development for Nules. Early Esbal had the highest Brix (12.1°). Early Esbal had the smallest fruit size count 5 - 2. Large Esbal and Nules had the biggest fruit size count 3 – 1x. Early Esbal had the best juice percentage.

**Table 5.4.25.2.** Internal fruit quality data for Clementine selections in the Buffeljagsrivier region (Olivedale) of the South West Cape during the 2020 season.

Date	Cultivar	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg. Seed	Colour
2020-03-18	Basol	CC	4 - 1	49,1	11	1,08	10,2	0,2	T 4 - T 5
2020-04-22	Early Esbal	CC	5 - 2	56,8	12,1	1,06	11,4	0,7	T 2- T 5
2020-04-22	Esbal	CC	4 - 2	49,7	11,1	1,09	10,2	2,8	T 4 - T 5
2020-04-22	Large Esbal	CC	4 - 3	58,3	11,2	1,17	9,6	0,7	T 5 - T 6
2020-05-15	Large Esbal	CC	3 - 1x	53,0	11,7	1,02	11,4	1,1	T 1 - T 3
2020-04-22	Nules	CC	3 - 1x	51,3	11,4	0,94	12,1	6,6	T 4 - T 5
2020-05-15	Nules	CC	3 - 1x	56,5	12,5	0,83	15,0	4,6	T 2 - T 4

#### 5.4.26 **PROGRESS REPORT: Cultivar characteristics and climatic suitability of Satsuma mandarins in a cold production region (Western Cape)**

Project 57D by W. Swiegers (CRI)

#### **Summary**

The trial location is in an area well suited for Satsuma production. Most of the trees were planted in 2012. The trees look good with medium to large tree canopies. The order of ripening was as follows; Miyagawa Wase started the season, followed by, Miho Wase, Sugiyama, Ueno, Imamura, and Bela was the selection to finish the season.

Picking periods for Satsumas should be limited to 2-3 weeks to ensure good internal quality and avoid puffiness. Satsuma selections need degreening after harvest as the internal quality is ahead of the colour development.

### Opsomming

Die proef se ligging is goed geskik vir Satsuma produksie. Die meeste bome is geplant in 2012. Die bome lyk goed met goeie boom volume. Die orde van rypwording was as volg: Miyagawa Wase het die seisoen begin gevolg deur Miho Wase, Sugiyama, Ueno, Imamura, en Bela het die seisoen klaargemaak.

Pluk periodes vir Satsumas sal strek van 2-3 weke aangesien vrugte se sure vinnig daal en die skil powwerig raak. Vrugte se kleur is laat teenoor die interne kwaliteit en ontgroening sal moet gedoen word.

### Objectives

- To select Satsuma cultivars with improved and consistent productivity, fruit size, rind colour, and internal fruit quality (Brix, acidity and ratio).
- To extend the harvest period (both earlier and later maturity).
- To describe the characteristics of new Satsuma cultivars and determine the climatic suitability of these cultivars in cold production regions.

### Materials and methods

Field evaluations and laboratory analyses were conducted on Satsuma selections from the Citrusdal region of the Western Cape. The following selections were evaluated: Imamura, Miho Wase, Miyagawa Wase, Sugiyama, Ueno, and Bela.

For Satsuma mandarins, a ratio of 9:1 is considered to be the build-up towards peak maturity of 10:1. After reaching the peak, the ratio increases to 11:1, after which the fruit is considered over mature. This process from start to the end of the peak is approximately three weeks long. Fruit harvested before and after this period would result in greater instances of quality and rind issues.

**Table 5.4.26.1.** List of Satsuma selections evaluated at Kweekkraal and Stargrow (Citrusdal) during 2020.

Selection	Rootstock	Topworked
Imamura	Carrizo	2012
Miho Wase	Carrizo	2011
Miyagawa Wase	Carrizo	2012
Sugiyama	Carrizo	2012
Ueno	Carrizo	2012
Bela	Carrizo	2012

### Results and discussion

#### Miho Wase

Miho Wase was the second selection to mature in this trial site. The rind was smooth, and the fruit peeled easily. Fruit size for Miho Wase was mostly count 3 – 1x. The selection was seedless. Fruit colour on the colour plate was T4 – T5 at peak maturity. Fruit matured internally, rind colour development was delayed. Sugar at

peak maturity was around 9.5° with an acid percentage of around 0.79%. Juice content decreased towards peak maturity.

#### Imamura

Imamura is a late maturing Satsuma. In this cold production region, it reached peak maturity from end-May. It was the second last selection to reach peak maturity. For a Satsuma, Imamura had a good Brix: Acid ratio, 10° and 1.07% respectably (ratio 9.4 towards peak maturity). One of the selections with a low juice percentage. Seed count was 0.2 – 0.5 seeds per fruit. External colour development was T5 on the colour plate. Internal colour was deep orange.

#### Bela

Bela is a new late maturing Satsuma selection. This was also the last selection to reach peak maturity in beginning of July. The fruit is large with a fruit size count of 1 – 1xxx. The rind was smooth to pebbly and peelability was easy. Internal colour is an excellent deep orange. At peak maturity the internal quality was good for Bela with Brix around 10.2° and acid above 0.95%. External colour development was delayed compared to the internal maturity with T3 – T4 on the colour plate. Bela was virtually seedless.

#### Miyagawa Wase

Miyagawa Wase was the first selection to reach peak maturity. The fruit size of Miyagawa Wase at peak maturity was count 3 – 1xx. Brix: acid ratio 13.2:1; Brix was 9.1° and acid was 0.69%. Colour development was not good and delayed compared to internal maturity. The colour on the colour plate at peak maturity was T5. This selection was virtually seedless and the juice percentage increased towards peak maturity. The fruit was smooth and flat and the internal colour was deep orange.

#### Ueno

This selection is a mid to late maturing selection for this trial site. It had a low highest juice content medium to extra-large fruit size count with a 1 – 1xxx count. The Brix° and acid percentage for Ueno at peak maturity were very good, 10.1° and 0.87% respectively. There were 0.1 – 1.8 seeds per fruit and Ueno colour on the colour plate at peak maturity was T5. Fruit peeled easily.

#### Sugiyama

Sugiyama is a mid to late maturing Satsuma. At this trial site it reached peak maturity in end-April. Sugiyama fruit size count 2 - 1xxx. The Brix° and acid percentage of Sugiyama were around 9° and 0.72% respectively at over maturity. Sugiyama had the highest juice percentage. Seed count for this selection ranged 0.7 – 1.8 seeds per fruit. There was also a delay in colour development with a T7 – T8 on the colour plate towards peak maturity.

### **Conclusion**

Most of the selections peaked with a large to extra-large fruit size (count 1 - 1xxx). Miho Wase had the smallest fruit size count peaking at count 1x. Bela had the highest Brix° of all the Satsuma selections above (10°). Sugiyama and Ueno had the highest seed count with 1.8 seeds per fruit. Rind colour development was not good for any of the selections at peak maturity. Bela had the best internal colour. Sugiyama had the highest juice percentage.

**Table 5.4.26.2.** Internal fruit quality data for Satsuma selections in the Citrusdal region of the Western Cape during the 2020 season.

Date	Cultivar	Root-stock	Count	Juice (%)	Brix°	Acid (%)	Ratio	Avg, Seed	Colour
2020-06-02	Bela	CC	1 - 1xxx	44,5	9,4	1,16	8,1	0,2	T 6
2020-07-01	Bela	CC	1xx - 1xxx	36,3	10,2	0,95	10,8	0,0	T 3 - T 4
2020-04-20	Imamura	CC	2 - 1xx	49,5	9,5	1,22	7,8	0,2	T 7 - T 8

2020-05-13	Imamura	CC	3 - 1xx	43,7	10	1,07	9,4	0,5	T 5
2020-03-16	Miho Wase	CC	3 - 1	56,1	8,8	0,98	9,0	0,0	T 6 - T 7
2020-04-20	Miho Wase	CC	2 - 1x	49,4	9,5	0,79	12,0	0,0	T 4 - T 5
2020-03-16	Miyagawa Wase	CC	3 - 1x	52,6	8,8	1,19	7,4	0,0	T 7
2020-04-20	Miyagawa Wase	CC	1 - 1xx	53,5	9,1	0,69	13,2	0,1	T 4 - T 6
2020-04-20	Sugiyama	CC	2 - 1xx	57,2	8,9	0,99	9,0	1,8	T 7 - T 8
2020-05-13	Sugiyama	CC	1 - 1xxx	37,2	9,3	0,72	13,0	0,7	T 3 - T 4
2020-04-20	Ueno	CC	1 - 1xx	53,3	9,1	1,16	7,9	0,1	T 7 - T 8
2020-05-13	Ueno	CC	1 - 1xxx	40,4	10,1	0,87	11,6	1,8	T 5

#### 5.4.27 **PROGRESS REPORT: Studies into the high incidence of chimeras of Valencia orange cultivars, specifically Valencia Late.**

Project 1185 (2019/20 - 2021) by P.J.R. Cronje, J. Joubert, W. Swiegers, J. Niemann and V. White (CRI)

#### **Summary**

During the 2020 season, the experiments addressing relevant questions related to the occurrence of chimera in Valencia Late were repeated and expanded. The first experiments revolved around quantifying chimera incidence in the same trees in the subsequent season. The second aspect reported on, is the results of topworking done on Valencia Late buds from various sources, and lastly repeat observations of chimera incidence in a climatically different region to the other areas in which research was done. The results indicated that a slight decrease in incidence was seen in some orchards. However, the incidence and severity remain to a large extent constant. The first results of the top working experiment indicated a variation in incidence depending on the source of budwood. Lastly, it was confirmed that chimeras occur in all Valencia Late orchards, although considerable variation in incidence exists in these orchards planted in Citrusdal. The variation in incidence in these orchards established pre-citrus improvement programme could offer the opportunity to identify new low incidence selections of Valencia Late. These preliminary results in this challenging project are slowly adding information to this poorly understood genetic event in citriculture and will be continued for the foreseeable future.

#### **Opsomming**

Gedurende die 2020 seisoen was eksperimente voortgesit en uitgebrei wat na relevante aspekte kyk wat moontlik bepalend kan wees tot die voorkoms van chimeras in Valencia Late. Die drie hoof aspekte wat ondersoek word, fokus op die kwantifisering van voorkoms in die selfde bome oor meer as een seisoen om 'n patroon te dokumenteer. In die tweede proef is verslag gedoen oor oorwerk van onderstamme met Val Late materiaal van verskeie bronne. Laastens word daar verslag gedoen oor die opvolg observasies gemaak oor die voorkoms van chimera in 'n produksie streek met 'n uiteenlopende klimaat tot die res van die proewe. Daar was bevind dat daar in van die bome en boorde 'n effense verlaging in voorkoms en graad was, maar oor die algemeen blyk dit of die voorkoms en graad van chimeras konstant bly. In die eerste resultate uit die oorwerk proef was daar drastiese variasie in chimera voorkoms tussen die verskillende bronne. Laastens was bevestig dat alle Val Late boorde in Citrusdal chimera vrugte ontwikkel maar dat die voorkoms drasties verskil. Hierdie variasie in voorkoms kan die geleentheid bied om 'n nuwe lae voorkoms chimera lyn te selekteer. Hierdie voorlopige resultate van die uitdagende projek dra stuksgewys inligting by, en poog om lig te werp op die problematiese genetiese gebeurtenis in sitrus en gaan voortgesit word.

#### **Introduction**

In modern citriculture, somatic mutations are the mechanism responsible for the mutation (and therefore chimeras) found in commercial orchards and breeding programs. The diversity seen in cultivars of sweet

oranges illustrates how somatic mutations cause diversity in cultivated varieties (cultivars). However, most mutations that develop and are visually identified are degenerative and lead to corrugation, variegation and dry or seedy fruit. The chance or propensity of the citrus genome, as well as factors responsible for mutations and development of chimeras in the *Citrus* genus, is not well understood.

The reason for the increased interest in mutations and chimera in the South African citrus industry has been the result of the last five years of high levels (10-40%) of chimeras in some commercial Valencia orchards, leading to drastic financial losses. The high incidence of chimeric fruit in specific selections of the old clone Valencia, i.e., 'Late' and 'McClean' and to a lesser degree 'Alpha' and 'Du Roi' remain problematic and unresolved. This ongoing project involves a high degree of recording and observational research in orchards as well as grafting trials of "affected" material and is structured to gain insight into various aspects of this problem.

**The project involves various experimental work, some of which will be long term evaluations;**

1. Quantification of chimera sensitivity and incidence.
2. Testing possibility of transfer of chimeric properties via grafting.

#### **1. Quantification of Chimera incidence in commercial orchards**

##### **Methods and materials**

During February 2021, the same orchards and grading system as previously used (CRI annual report 2020) was used to quantify the incidence of chimeras in the Limpopo and Mpumalanga production areas (Table 5.4.27.1). In short, ten trees per orchard were evaluated at the end of February for incidence and severity of chimeras. For each of the ten trees selected per orchard, three shoots (left, middle and right) were selected at two height intervals (<1.5 m and >1.5 m), repeated on both the east and west-facing sides of the row. For each selected shoot, all the fruit were graded according to a severity scale as a class: 0 being normal and 3 being a severe chimera affected fruit. Tagged branches from the previous season was re-evaluated and individual shoots with chimera fruit were tagged to better track chimera incidence next season.

In 2020, chimera incidence and severity was the highest at Riverside, Malalane. Accordingly, twenty trees, from this experimental site were selected, and branches with high chimera incidence were removed. Ten trees were treated with NAA (naphthalene-acetic-acid) and ten trees served as control trees. Briefly, one branch was removed from both the east and west facing side and treated with an NAA, mineral oil and PVA mixture (0.75:6:0.5), control trees were only treated with a mineral oil and PVA mixture. Apical branches of similar diameter (1.5 – 3 cm) at a height of 1.5 m were removed. The mixtures were applied evenly around a 10 cm strip of the removed branch. Shoot re-emergence and chimera incidence will be monitored in the new season from October 2021 onwards.



**Photo 5.4.27.1.** Comparison of shoot regrowth, NAA treated shoots (left) and control shoot (right).

**Table 5.4.27.1.** Farms used for detail investigation of chimera incidence in Limpopo and Mpumalanga.

Production area	Farm	Cultivar	Rootstock	Plant date	Row direction	Block #
Malelane	Riverside	Val Late	SC	2013	N-S	V11A
Hoedspruit	Moriah	Val Late	CC	2006	N-S	Bloukraal 4
Hoedspruit	Moriah	Val Late	SC	2006	N-S	Bloukraal 5
Letsitele	Letaba Estate	Val Late	SC	2007	N-S	N27B
Letsitele	Mahela	Val Late	SC	2012	N-S	TJ16 Junction

To confirm the incidence of chimeras on Late Valencia cultivars in a climatically different production area, an estimate of incidence in the same orchards as in 2020 was done in Citrusdal (Table 5.4.27.4).

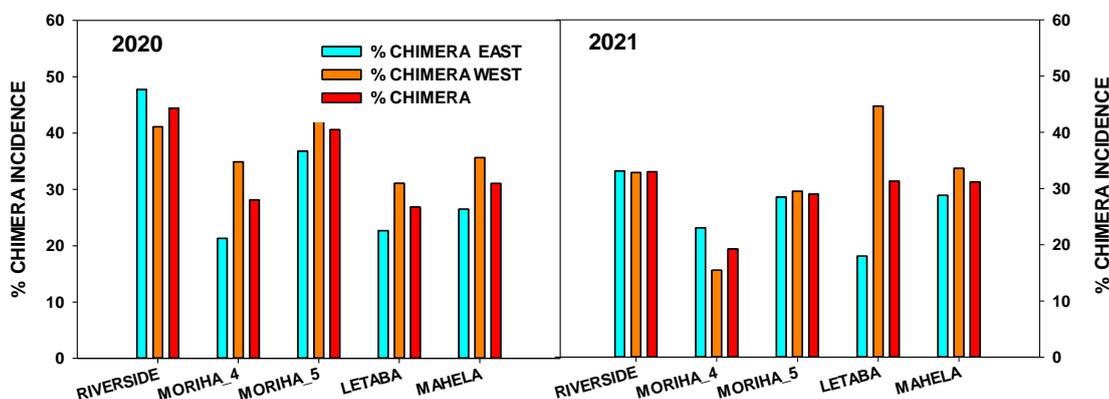
### Results and discussion

Similar numbers of fruit were analysed per orchard for season two compared to season one, with slightly less fruit being evaluated in season two (Table 5.4.27..2). It was however noted that not all the tagged branches supported fruiting shoots and an effort was made to tag individual fruit bearing shoots to assist with tracking chimera incidence at a branch level. The chimera incidence decreased at Riverside and Moriah 4/5 and showed a slight increase at Mahela and Letaba (Fig 5.4.27.1). There is area also a change in severity at the different sites, however from these preliminary results of only two season no pattern is evident. In general, it is still however too early to make any definite conclusion on the occurrence of chimera in a tree, branch or shoot level.

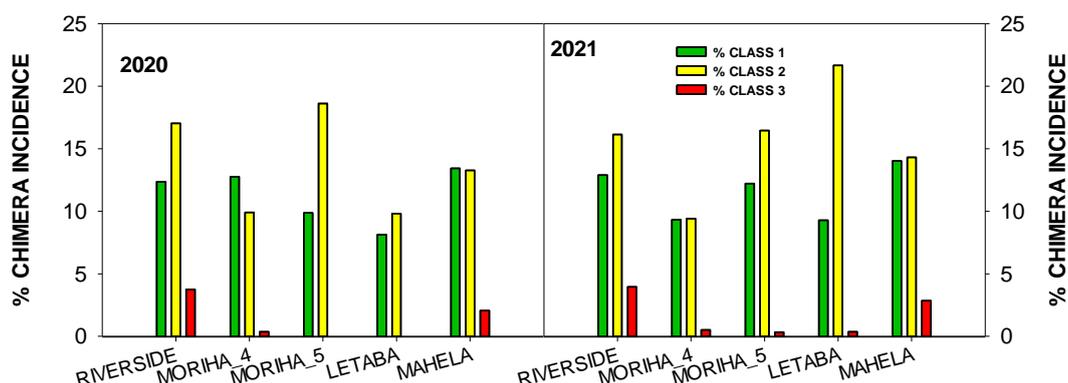
To try and develop practical management actions to decrease chimera occurrence, it is thought that suppressing re-growth with NAA at the branching point of a confirmed chimera shoot could be feasible (Photo 5.4.27.1). Results on this technique will be reported on in 2022.

**Table 5.4.27.2.** Comparison of number of fruit evaluated for both seasons i.e., 2020 and 2021.

FARM	AMOUNT OF FRUIT EVALUATED	
	SEASON ONE (2020)	SEASON TWO (2021)
RIVERSIDE	499	467
MORIAH_4	445	362
MORIAH_5	401	220
LETABA	380	406
MAHELA	460	438



**Figure 5.4.27.1.** Chimera incidence expressed as mean percentage of fruit. Chimera incidence given for both aspect and total occurrence per orchard, 2020 and 2021 seasons.



**Figure 5.4.27.2.** Chimera severity in 2020 and 2021 season, expressed as % incidence of each class (1 to 3) evaluated for two sides of ten trees per orchard.

**Table 5.4.27.4.** Summary of evaluating chimera incidence in Citrusdal over two years.

Producer	Establishment / Replacement	Nursery	Cultivar	CFB code	Rootstock	Estimated average % chimera per tree (Mar. 2020)	Estimated average % chimera per tree (Mar. 2021)
SPG du Plessis	2008	Stargrow	Valencia Late	V/03/072/G8	SC	9	20 - 30
SPG du Plessis	2010	Stargrow	Valencia Late	V/03/057/12	SC	9	20 - 30
A vd Merwe	1945		Valencia Late		RL	4.5	5%
Ouwerf Boerdery	1950		Valencia Late		RL	5	Orchard remove
Ouwerf Boerdery	Plant Replacement & 2011	Cederberg	Valencia Late	V/03/057/12	RL	6	20 - 30
Ouwerf Boerdery	1986	Goede Hoop	Valencia Late		RL	3	3
Ouwerf Boerdery	2015	Cederberg	Valencia Late	V/03/057/P06	CC	11	20 - 30
Mouton Citrus	1950 replacement tree +	Replacement trees: Cederberg/ Stargrow	Valencia Late	Replacement trees all different coded	RL (old) RL + CC new trees	6	3%

In general, a higher chimera incidence was seen in Citrusdal during 2020; however, the severity of the chimeras differed widely. It is thought that the lack of concern for chimeras in this area stems from two factors: firstly, those fruit with severe chimera symptoms will abscise during the late summer as rapid fruit growth occurs and secondly, that fruit with a chimera presenting as a colour segment change, will not be seen as problematic for these fruits destined for fresh juicing, during sorting in the packhouse (Photo 5.4.27.2).



**Photo 5.4.27.2.** Example of the lesser severe chimera from Citrusdal region.

### **General conclusions**

Due to the problematic nature of studies on chimera and especially in efforts to determine the reason why certain situations result in higher-than-average incidence, limited conclusions will be made. The status and aim of this project are to systematically gather descriptive data on how the mutations expressed in branched, trees and orchards. This will be done over consecutive seasons to define if a seasonal pattern is influential. The grafting/topwork results which are yielding results will add a new layer of information for the 2021/2022 season from which certain new experimental directions can be chosen.

### **Further research**

#### **Selection of new low chimera Val Late**

In addition to the continuation of the various experiments, a process of selecting and evaluating low chimera incidence on late Valencia's will be started in 2021. This will be done from two sources viz. orchards established pre 2000 and the ARC and CRI genebank. Only selected buds behind fruit with visually confirmed lack of chimeras will be included in the selection process. No buds from only vegetative shoots will be cut. An additional contribution will be to cut buds behind confirmed chimera and non-chimera fruit from the ARC and CRI genebank trees. The budwood will go through shoot tip grafting and pre-immunised with LMS6 (Lime Mildstrain 6) and Grapefruit Mildstrain 12 (GFMS12). Trees will be established on Swingle rootstock in large containers at CRI-CRC for evaluation purposes or established in commercial orchard.

#### **Molecular analysis of chimera tissue**

Chimera formation can be due to species mixtures, genome mutations and rearrangements, and variation in ploidy levels. A new development in the project is a molecular analysis aiming to perform a proof of principle experiment to investigate the possible genetic origin of chimera formation in Late Valencia. The aim is to determine definitively that the chimera is not a species chimera with *P. trifoliata*, although unlikely it remains to be confirmed. Secondly, to determine if the chimera is possibly due to ploidy level variation. During January, samples of various severity and symptoms of chimera were taken from a Late Valencia orchard in Malalane. The whole-genome sequencing (re-sequencing) will be used to perform a genome-wide association study (GWAS) that will hopefully enable us to identify the origin of chimera formation. Different sectorial regions of chimeric fruit will be sequenced and used for the comparative data analyses. The results from this part of the project will be reported on in the 2022 annual report.

## **6 CITRUS IMPROVEMENT SCHEME (CIS)**

P.H. Fourie, J.B. Meyer, M. le Roux, M.J. Nell, G. Cook (CRI) and E. Jooste (ARC-TSC)

### **Summary**

The South African Citrus Improvement Scheme (CIS) strives to ensure a profitable citrus industry that is established with high quality citrus trees that are free from diseases and horticulturally true to type. Certified rootstock seed and budwood are supplied from the Citrus Foundation Block (CFB) outside Kariega. A total of 6.5 million buds were supplied by the CFB or authorised for cutting in certified nurseries. Mandarin (35.8%) was the most popular citrus type, followed by Valencia (25.0%), lemon (11.9%), navel (9.3%), grapefruit (7.8%) and Clementine (7.5%). CFB's ability for primary supply improved to 78.4%; this can be attributed to a decrease in demand, new multiplication trees of high demand cultivars that came into production, and increased demand

for lemons. Budwood stock of the 502 cultivar lines at CFB must be constantly managed to meet demand of sought-after varieties. In 2020/21, 17 000 new multiplication trees were produced, 4 000 redundant trees were removed. The new rootstock seed orchards of high demand rootstock cultivars started to contribute to the seed harvest and 2020 yielded a record seed harvest of >8 000 L.

## **Opsomming**

Die doelwit van die Suid-Afrikaanse Sitrus Verbeteringskema (SVS) is om die winsgewendheid van die suider-Afrikaanse sitrusbedryf te verbeter deur te verseker dat die industrie gevestig word met hoë kwaliteit, siektevrye kwekerybome wat tuinboukundig tipe-eg is. Gesertifiseerde okuleerhout en saad word voorsien vanaf die in Sitrus Grondvesblok buite Uitenhage. 'n Totaal van 6.5 miljoen ogies is deur die Grondvesblok en in samewerking met gesertifiseerde kwekerye verskaf. Mandarin (35.8%) was die mees populêre sitrus tipe, gevolg deur Valencia (25.0%), suurlemoen (11.9%), nawel (9.3%), pomelo (7.8%) en Clementine (7.5%). Te midde van die verhoogde aanvraag na okuleerhout en saad het die primêre verskaffing vanaf die Grondvesblok 78.4% verbeter. Hierdie kan toegeskryf word aan die daling in aanvraag, nuwe vermeerderingsbome wat in produksie gekom het, asook 'n hernude aanvraag na suurlemoene. Okuleerhout voorraad van 502 kultivar lyne moet konstant bestuur word om soveel as moontlik van die hoë aanvraag kultivars te kan verskaf. In 2020/21 is 17 000 nuwe vermeerderingsbome gemaak, 4 000 onnodige bome verwyder. Die nuwe saadbron boorde met hoë-aanvraag onderstam kultivars het tot die oes bygedra en die 2020 saad-oes was 'n rekord van >8 000 L.

### **6.1 Introduction**

The purpose of the CIS is to enhance the standard of the South African citrus industry by ensuring that only horticulturally superior plants, which are free of viruses, diseases and pests, are supplied to growers and certified. The Citrus Growers Association of southern Africa (CGA) is responsible for the CIS and delegated its authority to CRI. In order to achieve this objective, close co-operation is required between CRI, the Agricultural Research Council - Tropical and Subtropical Crops (ARC-TSC), DALRRD's Directorate of Plant Health (DPH) and citrus nurseries represented by the South African Citrus Nurserymen's Association (SACNA). The organisations and committees, as well as all participating role players in the CIS are represented on the CIS Advisory Committee (CISAC), which advises CRI on the CIS operations as specified in its Procedural Guide. Additionally, Cultivar and Pathology sub-committees co-ordinate the respective CIS activities.

Budwood and rootstock seed is produced and supplied from the CRI Citrus Foundation Block (CFB) outside Kariega. The phytosanitary status of propagation material certified by the CIS is ensured by virus-elimination and diagnostic services of cultivars prior to CIS introduction and routinely confirmed through re-indexing of mother trees as well as multiplication blocks. This report summarises budwood and rootstock seed production and supply, nursery and tree certification services, and the pathogen elimination and diagnostic support services by ARC-TSC and CRI's CRC in Nelspruit.

### **6.2 Budwood**

This report summarises the seasonal supply of budwood from 1 July 2020 to 30 June 2021. Following the record numbers of 7.15, 7.04 and 6.41 million buds in 2017/18, 2018/19, 2019/20, respectively, 6.50 million buds were supplied by the Citrus Foundation Block (CFB) and authorised for cutting in certified nurseries (BCIN) in 2020/21. During this period 19 035 buds were exported to neighbouring countries. Budwood demand was mostly from Western Cape (41.4%), Limpopo (30.2%), followed by the Eastern Cape (14.0%), Mpumalanga (9.1%) and the other provinces ranging from 1.5% to 0.8% (Table 6.2.1).

Mandarin (35.8%) was the most popular citrus type, followed by Valencia (25.0%), lemon (11.9%), navel (9.3%), grapefruit (7.8%) and Clementine (7.5%); in 2019/20 this proportion was 41.8%, 21.3%, 11.6%, 8.4%, 6.3% and 7.8%, respectively (Table 6.2.2). All other variety types accounted for 2.5% of the total budwood supply (2.1% in 2019/20). A break-down of buds per variety type to nurseries in different provinces are given in Table 6.2.3.

Mandarin supply remained high at 2.33 million buds, but was down from the 3.42-million peak in 2018/19 (Fig. 6.2.1). Valencia demand was stable from 2014/15 – 2016/17 with a 3-year average of 560 thousand buds; however, in the past four seasons, supply increased to 1.3 million buds supplied per year for 2017/18, 2018/19, 2019/20 and 1.6 million in 2020/21 (18.8% increase) (Fig. 6.2.2), and surpassed lemon demand. Supply of lemon budwood increased from 435 thousand buds in 2018/19 to around 760 thousand buds per season in 2019/20 and 2020/21 (Fig. 6.2.3), with Eureka ranking as the second most popular cultivar in 2019/20 and 2020/21 (Table 6.2.4). Navel demand increased from 536 thousand buds in 2019/20 to 603 thousand buds in 2020/21 (Fig. 6.2.4). The high demand for Clementine buds remained at around 500 thousand buds per season (Fig. 6.2.5), which is significant, considering that the 10-year average in 2013/14 was 62 thousand buds. Grapefruit demand has been low for a number of years and increased from a low base of 45-, 77-, and 144 thousand buds in 2014/15, 2015/16 and 2016/17, respectively, to 336-, 329- 405 and 504 thousand buds supplied in 2017/18, 2018/19, 2019/20 and 2020/21, respectively (Fig. 6.2.6). This high demand is significant and recent supply figures are comparative to the 10 year average of 535 thousand buds during the 1990's grapefruit boom.

The top 30 varieties comprised 90.5% of total number of buds supplied. ARC Nadorcott LS (ARCCIT9) was the most popular cultivar, followed by Eureka, Midnight, Star Ruby, Tango, Jassie, Turkey, Bennie 2, Nules, Leanri and Nova (Table 6.2.4). ARC Nadorcott LS supply levels have increased year on year from 29 160 to 267 422 to 513 582 to 1 080 328 to 1 112 123 in 2018/19, slightly decreasing to 960 851 in 2019/20 and 903 749 in 2020/21. The protected mandarin varieties in the top 10 (ARC Nadorcott LS (ARCCIT9), Tango and Leanri) contributed to 23.5% to the total budwood supply, with a large proportion (32.0%) of these buds BCIN supplied in 2020/21: ARC Nadorcott LS (34.4%), Tango (40.2%) and Leanri (5.7%). BCIN supply decreased for ARC Nadorcott LS (from 51.2% in 2019/20 to 34.4% in 2020/21) and Midnight (from 28.8% to 24.1%), but increased for Star Ruby (24.9% to 44.8%) and Tango (29.7% to 40.2%). The top 10 cultivars comprised 65.3% (4.23 million) of all of the budwood supplied, of which 72.7% were supplied from the CFB (Table 6.2.4). The top 30 varieties supplied for the past 3 seasons are graphically presented in Fig. 6.2.7.

Primary CFB supply significantly improved from 63.2% and 73.9% in 2018/19 and 2019/20, respectively, to 78.4% in 2020/21 (Fig. 6.2.8). BCIN proportion per variety type: mandarins (43.9% of which ARC Nadorcott LS (ARCCIT9), and Tango comprised of 77.4%, and Sigal, PE1, Nova, Nadorcott 1, Leanri and RHM a further 20.7%), Valencia's (32.1% of which Midnight, Bennie 2, Turkey and Jassie comprised 98.4%), grapefruit (14.9%, comprised mostly of Star Ruby), Clementine (4.5%, comprising of Octubrina 76.5% and Nules 18.8%), navel (2.2%) and other (2.4%).

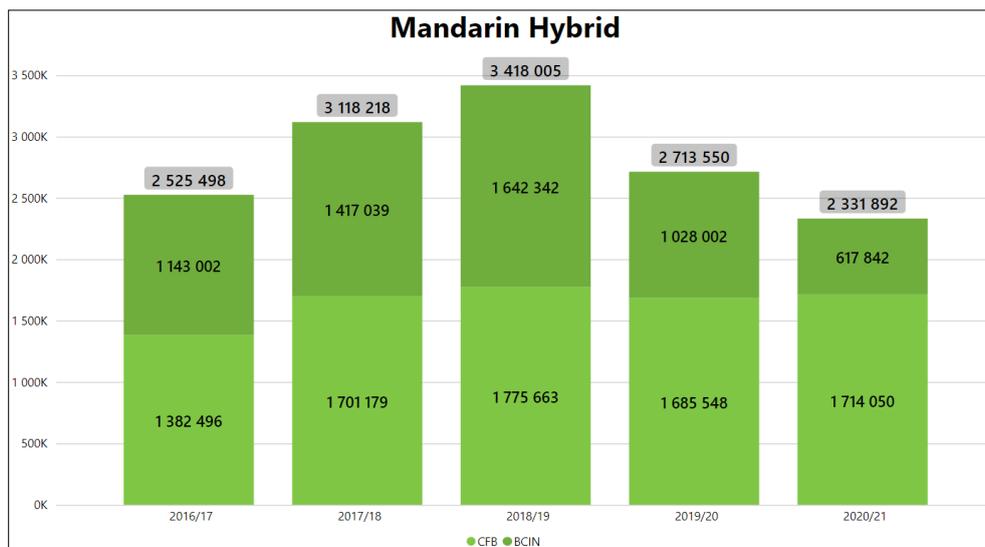
**Table 6.2.1.** Buds supplied during the period July to June 2018/19 – 2020/21.

Area	2018/19	Dist %	2019/20	Dist %	2020/21	Dist %
<b>Local</b>	<b>7 012 269</b>	<b>99.6%</b>	<b>6 364 443</b>	<b>99.3%</b>	<b>6 477 908</b>	<b>99.7%</b>
Eastern Cape	1 063 259	15.1%	1 084 102	16.9%	910 923	14.0%
Gauteng	245 575	3.5%	174 475	2.7%	99 574	1.5%
KwaZulu-Natal	17 840	0.3%	58 100	0.9%	54 500	0.8%
Limpopo	2 008 942	28.5%	1 980 863	30.9%	1 960 510	30.2%
Mpumalanga	567 054	8.1%	659 658	10.3%	590 761	9.1%
North West	125 610	1.8%	156 410	2.4%	97 070	1.5%
Northern Cape	299 629	4.3%	76 200	1.2%	75 710	1.2%
Western Cape	2 684 360	38.1%	2 174 635	33.9%	2 688 860	41.4%
<b>International</b>	<b>27 450</b>	<b>0.4%</b>	<b>44 329</b>	<b>0.7%</b>	<b>19 035</b>	<b>0.3%</b>
Botswana	500	0.0%		0.0%		0.0%
India	1 250	0.0%	1 250	0.0%		0.0%
Nigeria	1 000	0.0%		0.0%		0.0%
Swaziland		0.0%		0.0%	320	0.0%
USA		0.0%	339	0.0%		0.0%
Zambia		0.0%	1 100	0.0%		0.0%
Zimbabwe	24 700	0.4%	41 640	0.6%	18 715	0.3%

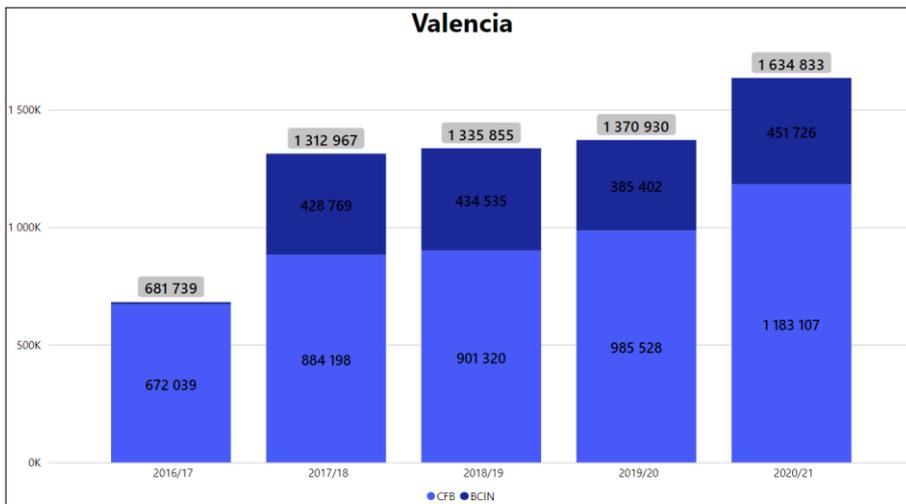
<b>Total</b>	<b>7 039 719</b>	<b>100.0%</b>	<b>6 408 772</b>	<b>100.0%</b>	<b>6 496 943</b>	<b>100.0%</b>
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**Table 6.2.2.** Buds supplied during the period July to June 2018/19 – 2020/21.

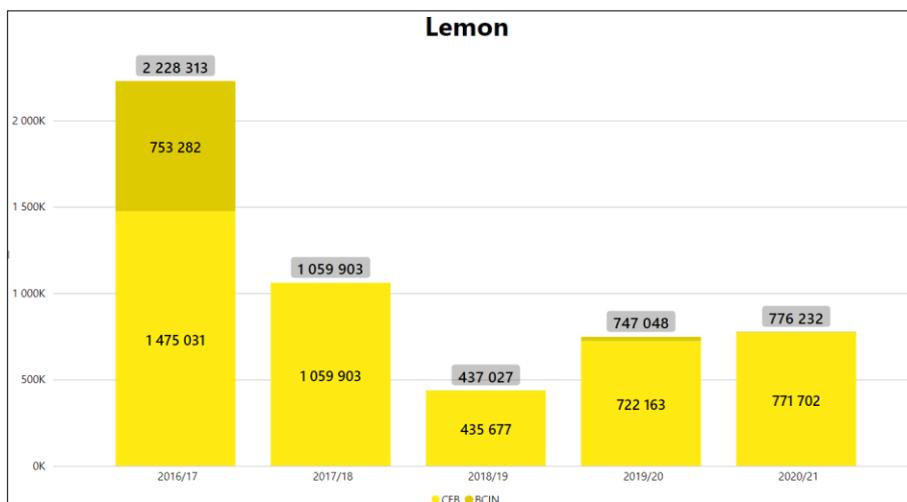
Variety Type	2018/19	Dist %	2019/20	Dist %	2020/21	Dist %
<b>Local</b>	<b>7 012 269</b>	<b>99.6%</b>	<b>6 364 443</b>	<b>99.3%</b>	<b>6 477 908</b>	<b>99.7%</b>
Mandarin Hybrid	3 408 005	48.4%	2 675 810	41.8%	2 324 792	35.8%
Valencia	1 322 755	18.8%	1 366 930	21.3%	1 624 213	25.0%
Lemon	434 677	6.2%	745 698	11.6%	775 932	11.9%
Navel	859 540	12.2%	536 023	8.4%	603 160	9.3%
Grapefruit	329 889	4.7%	405 715	6.3%	503 924	7.8%
Clementine	537 010	7.6%	501 375	7.8%	486 346	7.5%
Lime	25 385	0.4%	36 560	0.6%	65 026	1.0%
Satsuma	35 130	0.5%	44 248	0.7%	39 866	0.6%
Midseason	33 110	0.5%	20 169	0.3%	20 066	0.3%
Kumquat	12 355	0.2%	14 590	0.2%	16 890	0.3%
Diverse	8 128	0.1%	9 070	0.1%	12 737	0.2%
Pummelo	4 925	0.1%	7 685	0.1%	3 476	0.1%
Rootstock	1 360	0.0%	470	0.0%	1 480	0.0%
Ellendale		0.0%	100	0.0%		0.0%
<b>International</b>	<b>27 450</b>	<b>0.4%</b>	<b>44 329</b>	<b>0.7%</b>	<b>19 035</b>	<b>0.3%</b>
<b>Total</b>	<b>7 039 719</b>	<b>100.0%</b>	<b>6 408 772</b>	<b>100.0%</b>	<b>6 496 943</b>	<b>100.0%</b>



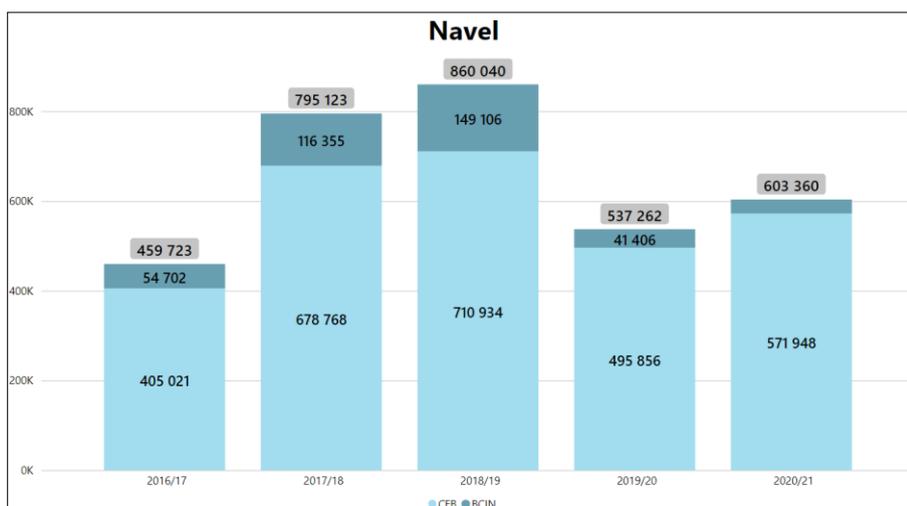
**Figure 6.2.1.** Mandarin hybrid budwood (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2015/16 – 2020/21.



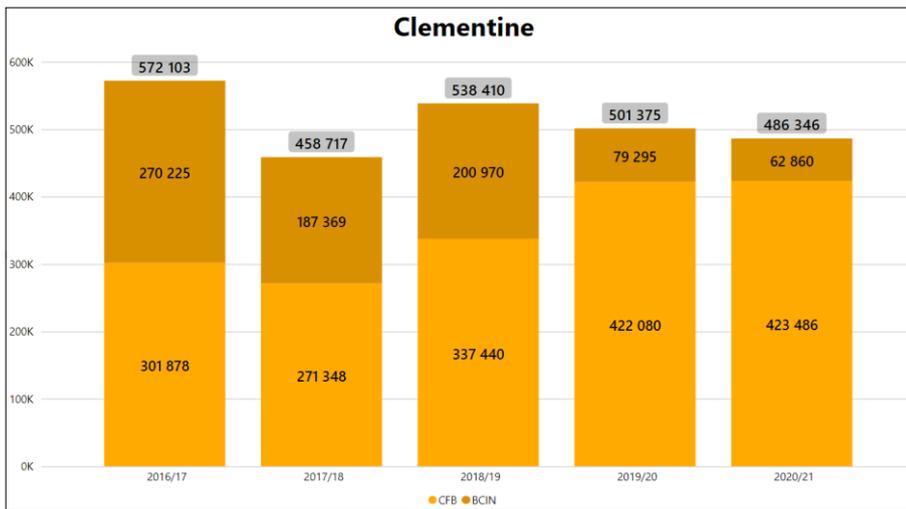
**Figure 6.2.2.** Valencia budwood (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2015/16 – 2020/21.



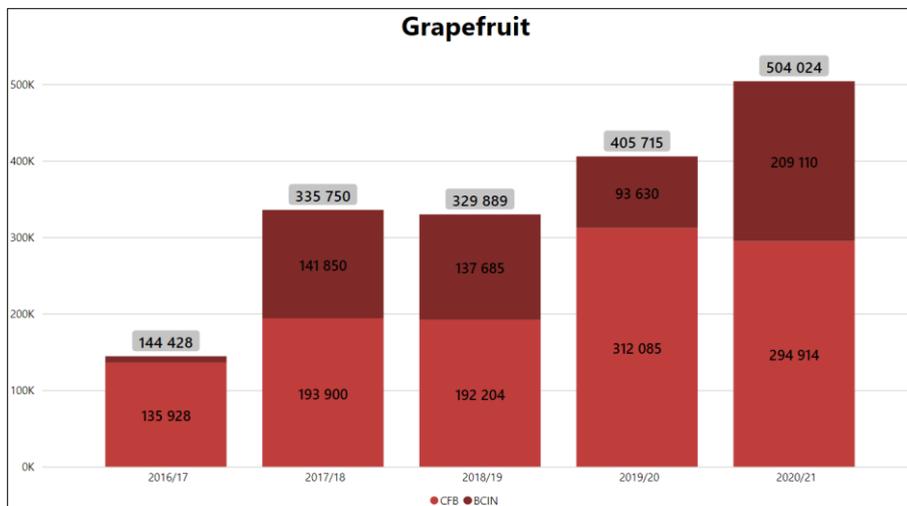
**Figure 6.2.3.** Lemon budwood (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2015/16 – 2020/21.



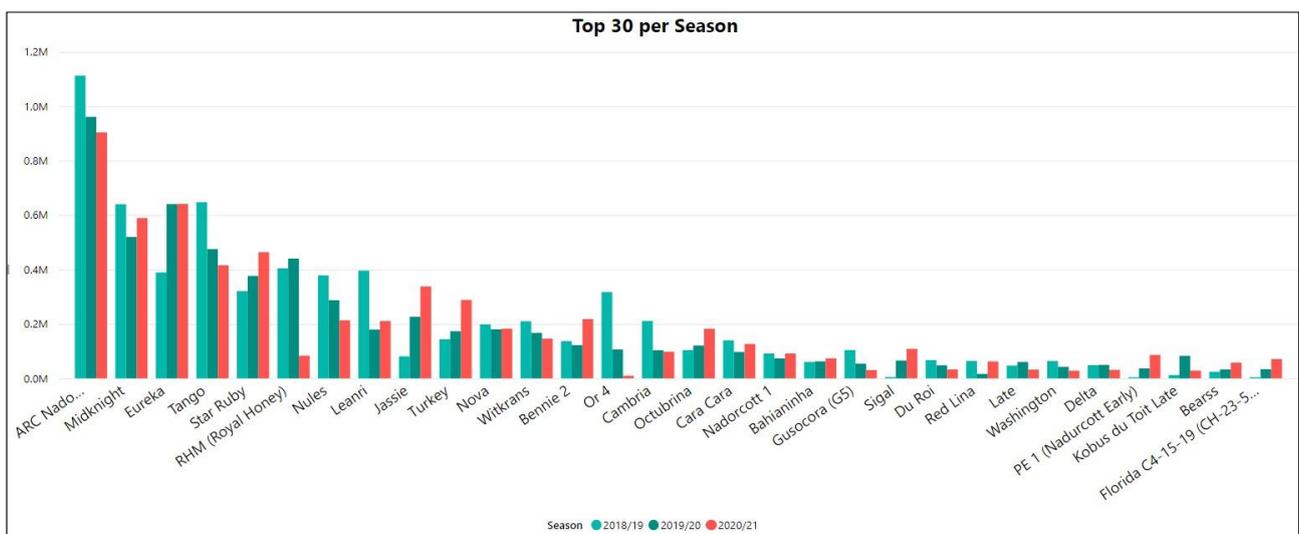
**Figure 6.2.4.** Navel budwood (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2015/16 – 2020/21.



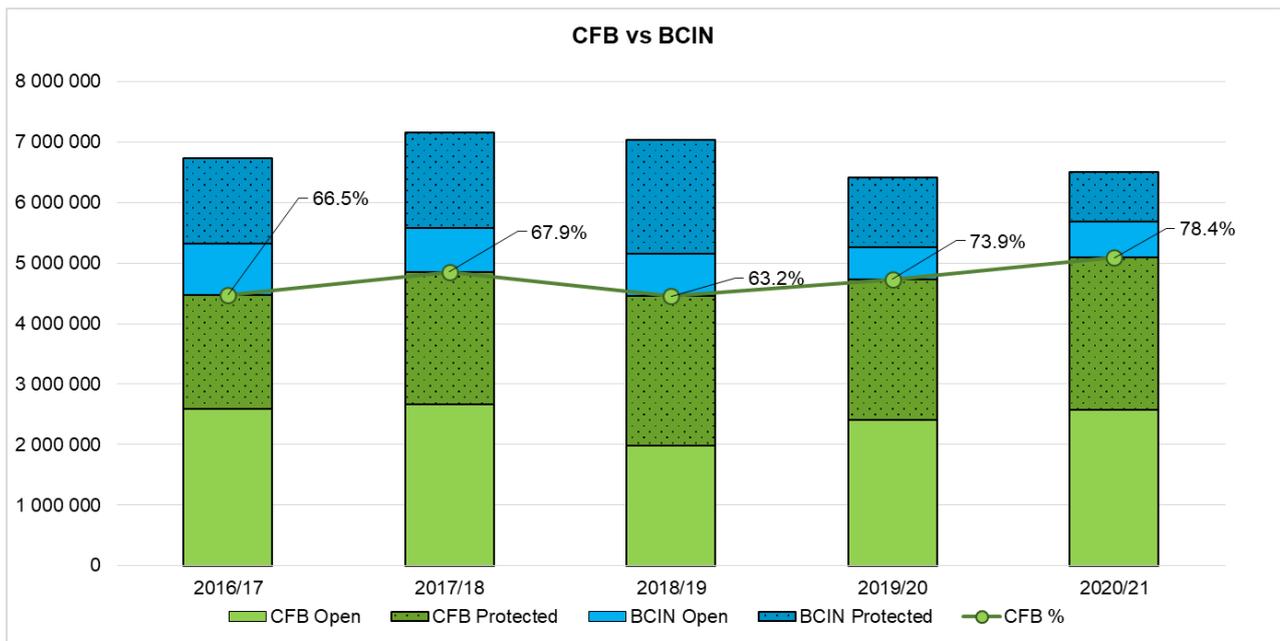
**Figure 6.2.5.** Clementine budwood (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2015/16 – 2020/21.



**Figure 6.2.6.** Grapefruit budwood (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2015/16 – 2020/21.



**Figure 6.2.7.** Budwood supply (BCIN/CFB) of the other variety types in the top 30 in 2018/19 – 2020/21.



**Figure 6.2.8.** Budwood of open and protected cultivars (total number of buds per season) supplied by the CFB and authorised for cutting in nurseries (BCIN) during the periods July to June from 2016/17 – 2020/21.

**Table 6.2.3.** Buds supplied per variety type per area (total number of buds per season) during the periods July to June from 2018/19 – 2020/21.

Variety Type	Season	EC	GP	KZN	LP	MPU	NW	NC	WC	EXPORTS	Total
<b>Clementine</b>	2018/19	43 490	24 500		19 680		4 000	18 600	426 740	1 400	538 410
	2019/20	43 644	25 500		19 800	6 150	2 000		404 281		501 375
	2020/21	26 795	200		27 380	7 006			424 965		486 346
<b>Diverse</b>	2018/19	5 758	430		750				1 190		8 128
	2019/20	500	5 430		2 380	40			820		9 170
	2020/21	130	7 230		1 330	1 114	150	10	2 773	540	13 277
<b>Grapefruit</b>	2018/19	20 720	29 254		126 042	76 273		17 410	64 620		329 889
	2019/20	2 800	3 000	2 000	219 340	79 075	19 470	2 900	72 700		405 715
	2020/21	14 896	13 300		317 873	77 120	17 470	38 010	25 255	100	504 024
<b>Kumquat</b>	2018/19	1 000	1 285	2 000	1 700	600	5 000		770		12 355
	2019/20	900	5 050		1 730		3 000	500	3 410		14 590
	2020/21	20	5 700		2 750	2 000	1 130		5 290		16 890
<b>Lemon</b>	2018/19	213 050	7 960	7 320	42 850	23 287	15 000	39 000	86 210	2 350	437 027
	2019/20	245 682	35 820	29 500	266 500	15 017	29 700	8 000	115 479	1 350	747 048
	2020/21	204 899	20 550	13 000	251 325	94 458	18 250	6 800	166 650	300	776 232
<b>Lime</b>	2018/19	2 350	1 795		3 900	8 880	600		7 860	100	25 485
	2019/20	2 210	6 515		4 550	14 500	2 075	500	6 210		36 560
	2020/21	4 970	5 900		6 600	25 386	2 360	10	19 800	25	65 051
<b>MandarinHybrid</b>	2018/19	368 197	6 668	20	1 298 065	230 839	28 940	106 271	1 369 005	10 000	3 418 005
	2019/20	446 082	21 300		697 435	355 858	43 450	10 000	1 101 685	37 740	2 713 550
	2020/21	293 173	5 040	5 500	434 619	113 796	17 420	8 000	1 447 244	7 100	2 331 892
<b>Midseason</b>	2018/19	250				240			32 620		33 110
	2019/20	550					400		19 219		20 169
	2020/21	9 160			600	66	500	20	9 720	150	20 216
<b>Navel</b>	2018/19	283 023	7 840	3 500	88 200	37 370	34 710	30 900	373 997	500	860 040
	2019/20	91 486	31 260	2 500	146 540	32 124	24 900	39 800	167 413	1 239	537 262
	2020/21	84 041	23 750	11 300	76 620	45 817	19 620	7 750	334 262	200	603 360
<b>Pummelo</b>	2018/19				3 750	1 100			75		4 925
	2019/20				7 600				85		7 685
	2020/21	18	100		300	2 408			650		3 476
	2018/19		200	500				450	210		1 360

<b>Satsuma</b>	2019/20	250							220		470
	2020/21	720	720	200							1 480
<b>Valencia</b>	2018/19	19 770	19 770		2 500	7 250	400				35 130
	2019/20	17 203	17 203		1 000	6 500		2 000	3 500		44 248
	2020/21	19 910	19 910		8 500	3 220	4 006	2 000			39 866

**Table 6.2.4.** Top 30 cultivars based on total number of buds supplied for seasons July to June from 2018/19 – 2020/21.

2018/19			2019/20			2020/21		
Cultivar	BCIN	CFB	Cultivar	BCIN	CFB	Cultivar	BCIN	CFB
ARC Nadorcott LS MAN *	681 240	430 883	ARC Nadorcott LS MAN *	492 278	468 573	ARC Nadorcott LS MAN *	311 341	592 408
Tango MAN	346 000	301 198	Eureka LEM	20 305	619 939	Eureka LEM	2 000	638 881
Midnight VAL	256 825	382 985	Midnight VAL	149 508	369 797	Midnight VAL	141 770	446 954
RHM (Royal Honey) MAN	341 738	62 535	Tango MAN	141 100	333 742	Star Ruby GFT	207 430	256 090
Leanri MAN	150 344	245 507	RHM (Royal Honey) MAN	314 893	125 211	Tango MAN	166 915	248 368
Eureka LEM		389 072	Star Ruby GFT	93 630	282 685	Jassie VAL	156 716	180 893
Nules CLE	145 000	233 133	Nules CLE	63 065	223 889	Turkey VAL	57 620	190 935
Star Ruby GFT	137 685	182 724	Jassie VAL	121 324	104 955	Bennie 2 VAL	88 550	129 076
Or 4 MAN	46 600	270 249	Nova MAN	16 200	163 811	Nules CLE	11 800	201 295
Cambria NAV	68 310	142 707	Leanri MAN	8 545	170 603	Leanri MAN	12 000	198 659
Witkrans NAV	14 950	194 782	Witkrans NAV	14 419	152 423	Nova MAN	21 400	160 967
Nova MAN	16 900	181 149	Turkey VAL	26 145	126 545	Octubrina CLE	48 110	133 830
Turkey VAL	50 998	92 670	Bennie 2 VAL	20 360	101 718	Witkrans NAV	8 412	137 371
Cara Cara NAV	65 846	73 621	Octubrina CLE		120 602	Cara Cara NAV	22 000	104 183
Bennie 2 VAL	31 433	105 100	Or 4 MAN		106 099	Sigal MAN	33 388	74 900
Gusocora (G5) VAL	19 176	84 825	Cambria NAV	9 487	93 647	Cambria NAV		97 645
Octubrina CLE	53 570	49 853	Cara Cara NAV	17 500	79 285	Nadorcott 1 MAN	18 200	72 943
Nadorcott 1 MAN	8 500	80 484	Kobus du Toit Late VAL	47 703	35 000	PE 1 MAN	30 798	55 261
Jassie VAL	24 903	55 842	Nadorcott 1 MAN		73 185	RHM (Royal Honey) MAN	12 000	71 158
Queen MAN		67 629	Sigal MAN	17 846	47 469	Bahianinha NAV		73 459
Du Roi VAL	33 050	33 676	Bahianinha NAV		62 350	Florida C4-15-19 MAN #	9 000	61 875
Red Lina NAV		63 597	Late VAL		59 970	Red Lina NAV		61 984
Washington NAV		63 370	Andes 1 Clemenz CLE	4 700	50 600	Limoneira 8A LEM		61 669
Bahianinha NAV		60 120	Gusocora (G5) VAL	6 000	47 790	Bearss LIM	18 176	39 294
Lea MAN	49 320	5 995	Delta VAL		49 490	Ma'ayana MAN ®		49 521
Delta VAL		48 379	Du Roi VAL	9 362	38 438	Marsh GFT	1 200	36 219
Late VAL		46 925	Furr (Clem x Murcott) MAN		45 975	Du Roi VAL	1 700	30 934
Gold Nugget MAN		29 800	Washington NAV		42 010	Late VAL		32 259
Kirkwood Red NAV		28 213	PE 1 MAN	10 880	25 710	Furr (Clem x Murcott) MAN	2 800	28 771
Miho Wase SAT	12 950	11 310	Genoa LEM		36 354	Delta VAL		31 288

<b>Top 30</b>	<b>2 555 338</b>	<b>4 018 333</b>	<b>Top 30</b>	<b>1 605 250</b>	<b>4 257 865</b>	<b>Top 30</b>	<b>1 383 326</b>	<b>4 499 090</b>
<b>&gt; Top</b>	<b>32 540</b>	<b>433 508</b>	<b>&gt; Top</b>	<b>70 480</b>	<b>475 177</b>	<b>&gt; Top</b>	<b>23 030</b>	<b>591 497</b>
<b>Total</b>	<b>2 587 878</b>	<b>4 451 841</b>	<b>Total</b>	<b>1 675 730</b>	<b>4 733 042</b>	<b>Total</b>	<b>1 406 356</b>	<b>5 090 587</b>
	<b>36.8%</b>	<b>63.2%</b>		<b>26.1%</b>	<b>73.9%</b>		<b>21.6%</b>	<b>78.4%</b>

\* ARCCIT9 (ARC Nadorcott LS) MAN

# Florida C4-15-19 (CH-23-56) MAN

@ Ma'ayana (Edit X Nova) MAN

### 6.3 Seed

CFB is the primary supplier of rootstock seed in SA, supplied around 95.9% of certified seed. A significant decrease in fruit yield, as well as number of seed per fruit yield were experience during 2018/19, leading to a reduction in supply of most rootstock varieties in that season. In 2019/20, yields were in line with the estimated yield potential. The harvest in 2020/21 was a new record at the CFB for all seed varieties with >8000 L seed harvested. Carrizo citrange seed yield was 6.6% higher than the record year of 2017/18 and 19.8% higher than 2019/20; C35 citrange was 41.9% higher than 2019/20 and US812 (SXB), Swingle citrumelo, Rough Lemon and X639 respectively 38.2%, 9.6%, 32.9% and 80.9% higher for the same period.

During May to April 2021, 6574 litres of seed were supplied locally (Table 6.3.1) and 114 litres of seed were exported to SADC countries (Table 6.3.1). Carrizo citrange remains the most popular rootstock (36.8%), followed by X639 (20.0%), Swingle citrumelo (14.4%), C35 citrange (10.7%), Rough lemon (5.4%), Minneola x Trifoliata (3.1%) and other rootstock cultivars (5.5%) (Table 6.3.2).

**Table 6.3.1.** Seed (litres) supplied by the CFB during the periods May to April 2018/19 – 2020/21.

Area	2018/19	Dist %	2019/20	Dist %	2020/21	Dist %
<b>Local</b>	<b>5 330</b>	<b>98.6%</b>	<b>6 329</b>	<b>99.3%</b>	<b>6 574</b>	<b>98.3%</b>
Eastern Cape	750	13.9%	750	11.8%	873	13.0%
Gauteng	54	1.0%	205	3.2%	76	1.1%
KwaZulu Natal	35	0.6%	136	2.1%	25	0.4%
Limpopo	2 157	39.9%	2 350	36.9%	2 660	39.8%
Mpumalanga	345	6.4%	435	6.8%	522	7.8%
North West	83	1.5%	136	2.1%	213	3.2%
Northern Cape	457	8.5%	425	6.7%	164	2.5%
Western Cape	1 449	26.8%	1 891	29.7%	2 042	30.5%
<b>International</b>	<b>74</b>	<b>1.4%</b>	<b>42</b>	<b>0.7%</b>	<b>114</b>	<b>0.7%</b>
Botswana		0.0%		0.0%	3	0.0%
Dem. Rep. of Congo	2	0.0%	1	0.0%	11	0.2%
Swaziland	14	0.3%		0.0%	12	0.2%
Zambia	9	0.2%	0	0.0%		0.0%
Zimbabwe	49	0.9%	41	0.6%	88	1.3%
<b>Total</b>	<b>5 404</b>	<b>100.0%</b>	<b>6 371</b>	<b>100.0%</b>	<b>6 688</b>	<b>100.0%</b>

**Table 6.3.2.** Seed (litres) supplied by the CFB during the periods May to April 2018/19 – 2020/21.

Rootstock cultivar	2018/19	Dist %	2019/20	Dist %	2020/21	Dist %
<b>CFB</b>	<b>4 714</b>	<b>87.2%</b>	<b>5 479</b>	<b>86.0%</b>	<b>6 412</b>	<b>95.9%</b>
Benton citrange (BC)	15	0.3%	11	0.2%	41	0.6%
C35 citrange (C35)	1 021	18.9%	529	8.3%	717	10.7%
Carrizo citrange (CC)	1 434	26.5%	2 736	42.9%	2 459	36.8%
Mineola X Trifoliata (MXT)	151	2.8%	60	0.9%	204	3.1%
Rough lemon (RL)	313	5.8%	289	4.5%	363	5.4%
Swingle citrumelo (SC)	919	17.0%	1 129	17.7%	961	14.4%
Troyer citrange (TC)	288	5.3%	336	5.3%	152	2.3%
US-812 Sunki X Benecke (SXB)	70	1.3%	43	0.7%	59	0.9%
Volckameriana (VA)	141	2.6%	116	1.8%	80	1.2%
X639	304	5.6%	212	3.3%	1 338	20.0%
Yuma citrange (YC)	15	0.3%	6	0.1%	10	0.1%
Other	45	0.8%	14	0.2%	29	0.4%
<b>Imported</b>	<b>190</b>	<b>3.5%</b>	<b>501</b>	<b>7.9%</b>	<b>21</b>	<b>0.3%</b>
<b>SPIN**</b>	<b>500</b>	<b>9.3%</b>	<b>391</b>	<b>6.1%</b>	<b>255</b>	<b>3.8%</b>
<b>Total</b>	<b>5 404</b>	<b>100.0%</b>	<b>6 371</b>	<b>100.0%</b>	<b>6 688</b>	<b>100.0%</b>

\*\*Seed produced in nurseries

## 6.4 Production

**Budwood:** CFB presently maintains more than 149 thousand multiplication trees of 502 cultivar lines with a potential annual budwood stock of >10 million buds. In the past season, the STG facilities at CRI-Nelspruit released 12 new cultivars to the CFB and re-introduced 8 existing cultivar lines, and ARC-TSC introduced 19 new cultivars and re-introduced 5 existing cultivars to the CFB (Table 6.4.1). Introduced cultivars are budded to rootstock seedlings in CFB's rapid multiplication tunnels, 1 145 new multiplication trees were made from these releases.

In order to timeously address budwood demand, CIS obtains budwood demand estimates from the CIS Cultivar Committee, private cultivar owners/agents and nurseries. This feedback is considered with historical supply and BCIN records, and multiplication tree stocks are managed accordingly. In 2020/21, 17 340 new multiplication trees, representing 45 cultivar lines, were made. To address space constraints, 4 096 multiplication trees from less popular cultivars were removed. In 2021/22 a specific effort will be made to remove multiplication blocks of category 4-L cultivars. Two trees of the category 4-L cultivars will be preserved in the dedicated bay for pre-immunised trees in the new polycarbonate nucleus block.

CIS needs a lead time of 2 years to meet demand levels, and considering that stocks of many different cultivars must be managed, and that budwood supply of newly introduced cultivars are limited, supply levels realistically requires a number of years to meet high demand levels. Cases of short supply, and reliance on the BCIN system in such cases, is therefore a reality. Budwood shortages were experienced for 23 cultivars in the top 30; however, reliance on the BCIN system declined from 26.1% in 2019/20 to 21.6% in 2020/21.

A total of 165 virus free accessions was received from the CRI's nucleus block facility and duplicated in two bays of the polycarbonate nucleus block facility. Accessions from the ARC nucleus block will be duplicated in spring of 2021.

Thirty new cultivars were added to the existing 392 cultivars established in the evaluation block for true to type evaluation purposes. Another 32 will be planted in spring of 2021.

**Table 6.4.1.** Cultivar introductions from 2016/17 – 2020/21.

Source	2016/17	2017/18	2018/19	2019/20	2020/21
ARC: New introductions	4	14	9	10	19
ARC: Re-introductions	2	14	5	6	5
CRI: New introductions	6	7	7	22	12
CRI: Re-introductions		21		2	5
CFB: Re-multiplication of existing cultivar lines	35	84	57	51	65

**Seed:** During 2020/21 the CFB seed harvest was well above the forecasted yields for most cultivars. Flowering conditions were optimal during the 2019/20 season contributing to the record harvest of >8 000L in total. The resulting Carrizo harvest was the highest ever recorded and the full demand of X636 orders was met. Surplus C35, Carrizo, Swingle and Troyer seed was made available. Although the yields for RL and US 812 (SXB) were also higher than previous two seasons, full demand could not be met.

To rejuvenate trees in some of the older orchards, pruning in the form of staghorn, removing central leaders and selective light pruning was implemented after the harvest season, taking historic yield data into consideration. Two rounds of gibberellic acid were applied, one after two-thirds petal fall (10 ppm) and another 1 week later (10 ppm) to all the commercially important cultivars. Beehives were again brought in during the flowering season to aid with pollination.

Removal of all trees outside insect-secure structures from CFB is a necessary measure to ensure the sustained

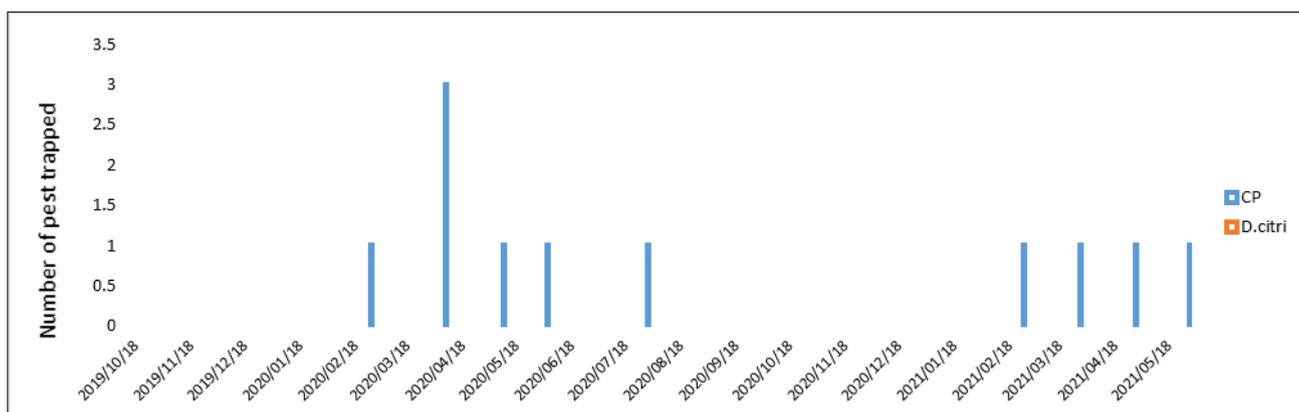
biosecurity of the CFB budwood sources, considering the threat of exotic pests and diseases and the potential quarantine implications. After approval of the CAPEX proposal to acquire and develop a seed farm, 3 412 rootstock trees were budded on X639. Approximately 2 600 more trees will be budded in summer to complete the total of trees needed for the planned 12 hectare seed farm development.

**CFB pest monitoring:** Pest monitoring at the Citrus Foundation Block (CFB) includes weekly scouting and the use of yellow sticky traps in insect-secure structures and seed orchards. Monitoring includes pests such as aphids (AP), Asian (ACP, *Diaphorina citri*) and African (*Trioza erytreae*, CP) citrus psyllids, and other citrus production pests. Both psyllid species are vectors of Citrus Greening bacteria while many aphid species are vectors for *citrus tristeza virus*. Trapping was implemented from 2017 and the CFB was examining the yellow sticky traps until September 2019, whereafter trap reading was conducted by CRI's biosecurity division at the CRC in Nelspruit. Monitoring of traps on the 5-km perimeter of the buffer zone surrounding the CFB was implemented since April 2020. Thus far, 9 locations are routinely monitored.

With scouting and trapping no CP or ACP species were detected in any of the insect-secure structures. Furthermore, no sign of CP damage or life stages were detected in the closed structures. In 2020, aphids were recorded only once in June in one greenhouse. An immediate spray was performed to eradicate the pest. No aphids have been detected in 2021 in any of the structures. CFB has intensified the monitoring of insect-security of structures on a weekly basis and is keeping written and photographic records of breaches, as well as the action taken to rectify a breach.

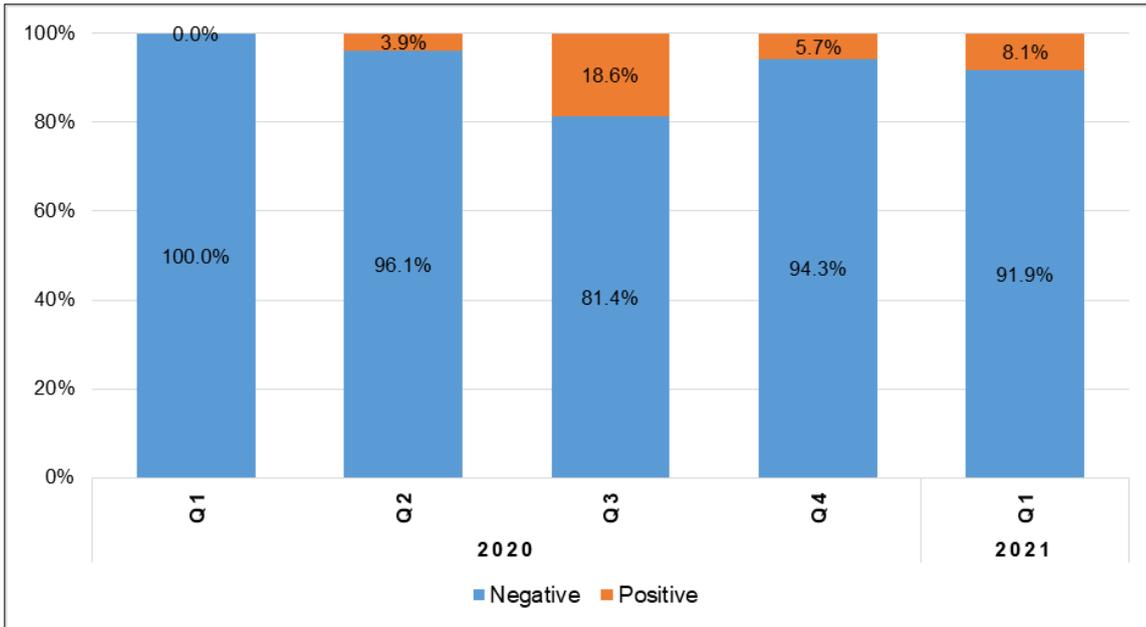
No ACP was detected at the CFB or in the surrounding buffer zone. Results show that there was a very low pest pressure recorded at the CFB during 2020 and 2021. No psylla damage was evident during scouting. Control strategies (systemic insecticides) were implemented to protect flush before spring and thereafter to ensure effective pest control. The products used were carefully selected to limit the effects on pollinators in seed source orchards.

With trapping, low numbers of CP were recorded in each season in the orchards (Fig. 6.1.1). Only a single CP insect was found per trap and not more than one per orchard in both 2020 and 2021. CP was trapped from GVB 6 in February; GVB88-2 & GVB 3 in April and SB15B in June. In 2021, CP was trapped from GVB 91-7, GVB88-2 and GVB5. On the 5-km perimeter monitoring traps, CP was trapped at CFB005 in April and CFB009 in July; traps at both locations are in non-citrus host trees. ACP was never trapped.



**Figure 6.4.1** Total number of *Trioza erytreae* (CP) and *Diaphorina citri* (D. citri) trapped on a total of 45 traps from all seed orchards and 9 traps from the 5-km perimeter.

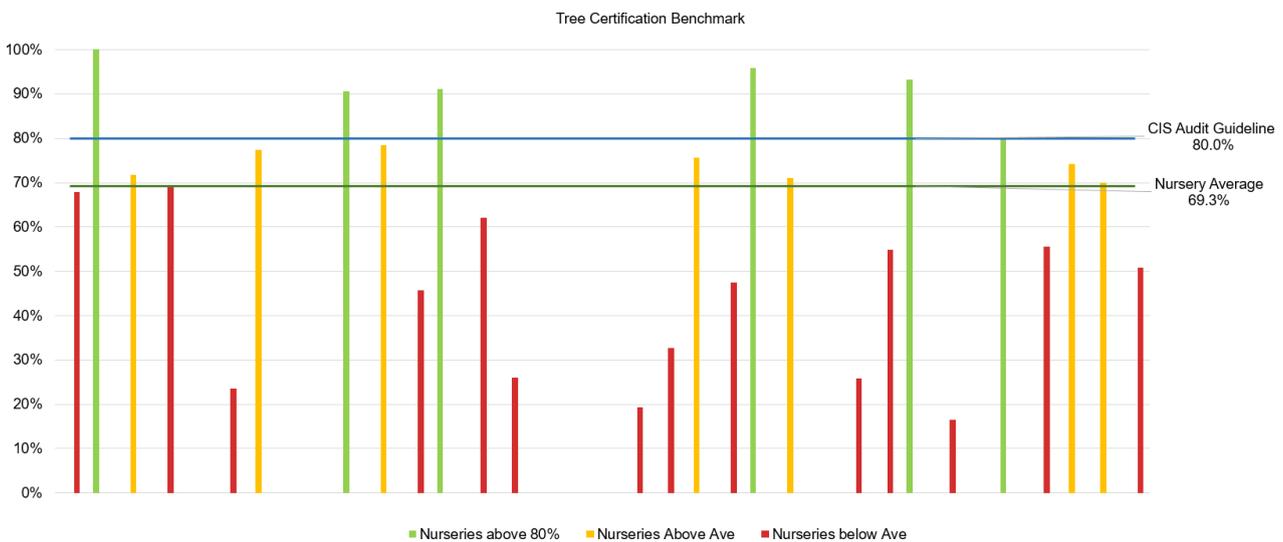
**Phytophthora monitoring:** Annual quarterly samples from increase trees, the evaluation block and mother trees were taken for analysis of the soil- and water-borne *Phytophthora* species and submitted to CRI's Diagnostic Centre at the CRC in Nelspruit. Only *Phytophthora citrophthora* was detected. In the third quarter (spring) the percentage positive blocks was high at 18.6%. Treatment was effective and thereafter the percentage *P. citrophthora* was kept under 10%. The rapid multiplication tunnels were identified as an important control point. Irrigation of the smaller rapid multiplication pots are being changed from micro-sprinklers to drip-irrigation, which should reduce the suitability of conditions for *Phytophthora*. The tunnels are also treated by drenching the floors with copper from time to time (Figure 6.4.2).



**Figure 6.4.2** Quarterly results for *Phytophthora citrophthora* detected in all potted trees at the CFB.

### 6.5 Tree Certification

In total, 3 415 473 trees were certified during April to March 2021; 71 619 of the trees presented for certification, did not meet the certification criteria (Table 6.5.1). Figure 6.5.1 indicates the nursery tree certification criterion of trees certified as a percentage of budwood supplied during the 2-year period prior to the maximum age certifiable age of trees in the nursery (30 months after budding). Of the 36 certified nurseries, 6 nurseries scored above the 80% benchmark, 7 nurseries above the nursery average of 69.3%, and 23 nurseries below this average (note that new nurseries might not have certified trees in this period yet). This criterion was included in the updated nursery certification guideline and nurseries were penalised if they did not meet the 80% benchmark.



**Figure 6.5.1** Nursery tree certification as a percentage of budwood supplied during the 2-year period prior to the maximum age certifiable age of trees in the nursery (30 months after budding). The updated CIS audit guidelines require a tree certification percentage of >80%.

### 6.6 Nursery Certification

The May 2020 self-audit (due to Covid lockdown) and the two physical nursery audits in November 2020 and

May 2021 were conducted using the newly updated audit criteria. The majority of nurseries welcomed the updated criteria and proposed “star-rating” of nurseries based on the final audit scores. The audit guidelines were progressively updated following the audits and feedback from nurseries.

During the November 2020 audits, by Thys du Toit, Michael Nell and Paul Fourie, appointments were made for 3-4 hours, compared to the normal 2-3 hours allocated per nursery. The extra time ensured that all 37 nurseries that were visited, had a sufficient understanding of the new criteria. This was also the first audit round attended by the new CIS Nursery advisor, Michael Nell, and was the last audit round by Thys du Toit. The overall nursery standard was high and the average score during the November 2020 audit was 67.8%.

Changes to the audit guidelines for the May 2021 audit, included better description of audit scores for certain criteria, simplifying and combining certain sections, and expanding the tree quality criterion (“Seedling and tree status”) into three separate criteria: “Seedling status”, “Tree status” and “Root development status”, each with a high score weighting with the aim to emphasize the importance of growing a strong and healthy tree that meets the CIS Tree Standard.

The May 2021 nursery audits have been completed with 34 certified nurseries and 3 prospective CIS nurseries visited by Michael Nell and Jacolene Meyer. This audit round took place across 6 provinces over a 5-week period. The audit team was happy to see that the nurseries were more acquainted with the new criteria and that there have been efforts to make improvements since November 2020. Audits ran smoothly and an immense amount of value was added by the “Root development status” criterion. A minimum of 10 trees were randomly selected and removed from their bags to inspect root health and root formation. A lot was learnt about the characteristics of various growth mediums and the massive influence that irrigation management has on root health. The implications of the HLB/ACP Action Plan on nurseries were discussed during audits, and it was good to see that nurseries have a positive attitude towards the system and are planning their transformations towards HLB Safe structures. Since November 2020 a few more nurseries have acquired insect-secure structures. In general, the nurseries had a good attitude towards the audit criteria and the value it adds to nurseries. The average audit score was 69.2%, an improvement since the previous round. Further minor adjustments to the criteria will be made before finalising the certification and audit guidelines.

**Table 6.5.1.** Total number of trees certified, and not meeting the certification criteria, from April to March for 2018/19, 2019/20 and 2020/21 per area and variety type.

Variety Type	Season	EC	FS	GP	KZN	LP	MP	NC	NW	WC	EXPORTED	TOTAL
Clementine	2018/19	91 159		2 800		35 652	6 180		3 466	280 400	10 900	430 557
	2019/20	37 096				6 213	6 980		1 515	172 108	3 617	227 529
	2020/21	15 975		7 500		20 730	7 564	2 500	1 998	154 759	2 083	213 109
Diverse	2018/19	75				265						340
	2019/20					110						110
	2020/21	50				247						297
Grapefruit	2018/19	4 914		14 913	18 000	117 947	59 761	20	7 310		4 320	227 185
	2019/20	5 903		4 760	44 200	51 813	17 899		6 575	4 600	11 550	147 300
	2020/21	2 999		132	39 830	116 697	41 728				24 720	226 106
Kumquat	2019/20					895						895
	2020/21					762				200	25	987
Lemon	2018/19	152 635	100	19 060	6 770	98 188	58 709	19 950	13 050	111 047	10 005	489 514
	2019/20	48 815		778	2 400	47 064	10 277	317	7 000	44 962	5 070	166 683
	2020/21	173 940		3 032	10 500	174 768	15 000	3 000		7 135	21 776	409 151
Lime	2018/19	1 467				2 964				1 612	2 695	8 738
	2019/20	1 765		100		1 312				900		4 077
	2020/21	300			450	15 560				550	1 352	18 212
Mandarin Hybrid	2018/19	412 160	220	8 010	1 300	607 541	442 117	26 394	1 380	392 112	32 628	1 923 862
	2019/20	166 836		200		595 995	290 236	14 577	17 250	354 378	23 818	1 463 290
	2020/21	346 283		28 028	2 150	467 413	180 091			386 399	38 321	1 448 685
Midseason	2018/19									3 348		3 348
	2019/20									570		570
	2020/21					7 353				32		7 385
Navel	2018/19	155 914	1660	20 610	1 960	41 036	12 050	21 905	2 390	63 015	20 010	340 550
	2019/20	160 691	50	3 220		55 442	50 171	24 758	5 000	45 431	20 234	364 997
	2020/21	99 655		9 350	200	44 213	75 415	5 347	10 050	52 635	11 793	308 658
Pummelo	2018/19	500				2 515						3 015
	2019/20					10						10
	2020/21	700				1 945	530				30	3 205
Satsuma	2018/19	8 907				1 813				12 294		23 014
	2019/20	6 135								2 661		8 796
	2020/21	639				500				9 516	30	10 685

<b>Valencia</b>	2018/19	161 010		3 104	9 996	236 678	105 837	6 167	3 050	71 755	18 220	615 817
	2019/20	92 313		3 900	26 850	296 806	54 529	2 836	44 283	76 452	31 096	629 065
	2020/21	107 576		28 221	28 950	441 428	59 901	10 000	1 200	54 555	37 162	768 993
<b>TOTAL CERTIFIED</b>	2018/19	988 741	1 980	68 497	38 026	1 144 599	684 654	30 646	74 436	935 583	98 778	4 065 940
	2019/20	519 554	50	12 958	73 450	1 055 660	430 092	81 623	42 488	702 062	95 385	3 013 322
	2020/21	748 117		76 263	82 080	1 291 616	380 229	13 248	20 847	665 781	137 292	3 415 473
<b>TOTAL NOT CERTIFIED</b>	2018/19	15 035			107	204	1 321	3 030		11 196		30 893
	2019/20	30 870				359	1 622			17 016	2 298	52 165
	2020/21	40 353				19 691	3 000			4 521	4 054	71 619

A summary of the amount of nurseries that fall under each star rating is shown in Table 6.6.1, and the updated nursery list in Table 6.6.2.

**Table 6.6.1.** Star-rated CIS Certified following the November 2020 and May 2021 audits.

Nursery star rating	Final audit score	Audit round	
		Nov-20	May-21
5-star	>80%	4	6
4-star	70-79%	12	10
3-star	60-69%	15	15
2-star	50-59%	4	5
1-star	<50%	1	0
<b>Nurseries certified</b>		<b>36</b>	<b>36</b>

**Table 6.6.2.** CIS Certified Nurseries in May 2021

Nursery	Town / Province		Contact Person	Tel	Cell	Email
Apapanzi	Kirkwood	EC	Nellis Meiring	042 230 1483	082 550 6210	nellis@srvalley.co.za
Attwell Citrus	Kirkwood	EC	Wayne Attwell	042 230 1560	072 463 7118	mandy@attwellcitrus.co.za
Augsburg	Clanwilliam	WC	Alta Laing	082 952 8127	079 527 0316	admin@augsburnnursery.co.za
BF Joubert	Kirkwood	EC	Francois Joubert	042 230 0309	084 951 1922	bfjkweek@srvalley.co.za
Cape Grow	Kraaifontein	WC	Eugene Nepgen		084 416 0184	eugene@cgrow.co.za
Casmar	Mooi-nooi	NW	Neville Wenholtz Jnr		082 881 4189	casmarnursery@absamail.co.za
Cederberg Tree	Citrusdal	WC	Patricia Willemsse	022 921 3526	076 622 7007	trish@cederbergtreenursery.co.za
Dodhill	Chegutu	ZIM	Pete Breitenstein		+263 77 222 1046	dodhill@iwayafrica.co.zw
Du Roi	Letsitele	LP	Zylon McGaffin	015 345 1650	076 227 6704	zylon@duroi.co.za
Du Roi Halls	Nelspruit	MPU	Arve Grindstad	013 004 0462	071 411 0131	arve@duroi-halls.co.za
Esselen	Malalane	MPU	Louis Esselen	013 790 0160	078 803 7010	esselenkwekery@gmail.com
Gamtoos	Patensie	EC	Keuler Engela	042 283 0506	072 260 9813	keuler@rikusld.co.za
Groot Patrysvlei	Clanwilliam	WC	EP van Niekerk	027 482 2619	082 456 0135	epvn@capspanfarms.co.za
H J Joubert	Montagu	WC	Herman Joubert	023 614 2237	082 578 5747	hopewell@breede.co.za
Henley Citrus	Letsitele	LP	André Swanepoel	015 386 0211	084 513 8649	productionmanager@bigday.co.za
Heuers Wholesale	Brits	NW	David Seewald	012 253 2097	082 887 4269	david@heuers.co.za
Hoedspruit	Hoedspruit	LP	Lafras Tremper		083 652 2167	tremper@hdsnursery.com
Komati Fruit	Malalane	MPU	Milanie vd Merwe	013 790 3004	082 418 7693	milanie@riversidefarm.co.za
Lafranette	Kirkwood	EC	Francois Nel		082 412 1739	conel@srvalley.co.za
Letsitele	Letsitele	LP	Elzanne Engelbrecht	015 345 1600	074 472 2919	elzanne@mahela.co.za
Mistkraal	Kirkwood	EC	Tyna Ferreira	042 230 0614	082 789 5150	beans@srvalley.co.za
Montana	Nelspruit	MPU	Dane Ross	079 871 6175	082 808 5661	dane@montananurseries.co.za
Moorland Seedlings	Loerie	EC	Rian Moore	042 286 0605	082 2860 604	rian@moorland.co.za
Ngwenya	Malalane	MPU	Milanie vd Merwe	013 790 3004	082 418 7693	milanie@riversidefarm.co.za
Nouvelle la Cotte	Letsitele	LP	Riaan Lemmer	015 386 9995	083 253 1586	riaan@nouvellecotte.co.za
Oranjerivier Sitrus	Kakamas	NC	Blom Rossouw	054 441 0183	083 306 0622	osk@vodamail.co.za
Paksaam	Patensie	EC	Adri Ferreira	042 283 0201	082 923 4412	paksaam@gamtoos.co.za
Parma (Hoedspruit)	Hoedspruit	LP	Albert Horn	087 806 5649	072 022 4356	parma@global.co.za
Parma (Lutzville)	Lutzville	WC	Boetie Mouton		082 896 5066	parma@namaquanet.co.za
Rietvlei	Tzaneen	LP	Lucas McLean	083 630 3236	083 630 3236	rietvlei@global.co.za
Sondagsrivier Hillside	Kirkwood	EC	Willem Truter	042 230 0349	083 227 6655	willem@srvalley.co.za
Stargrow	Citrusdal	WC	Andries vd Westhuizen	022 921 2232	082 873 3336	andries@stargrow.co.za
Tulbagh	Tulbagh	WC	Bredell Roux	023 230 0694	082 214 2520	admin@tulbaghnursery.co.za
Tweeling	Kirkwood	EC	Jan Potgieter	042 230 1408	082 560 2179	tweeling@srvalley.co.za
Waterfall	Adelaide	EC	Rudy van der Meulen	046 684 0738	082 695 3433	rudyvdmeulen@gmail.com
Witkrans	Boshhoek	NW	Linda Grobler	014 573 3036	082 414 4739	Witkrans1@mweb.co.za

## 6.7 Statutory Improvement Scheme

The new Plant Improvement Act (PIA) came into force in 2018, which stipulates that public Schemes must be converted to statutory schemes. The Citrus Improvement Scheme schedule was updated accordingly. A Memorandum of Understanding between the Minister of DALRRD and the designated authority, CGA, was approved by stakeholders at CISAC-2019. In the face of imminent biosecurity challenges, the urgency in proceeding with promulgation was stressed at CISAC-2019. All stakeholders finally agreed on 19 June 2020 that the CIS be promulgated as a compulsory statutory CIS. The Registrar of the Plant Improvement Act was requested to proceed with the necessary steps toward promulgation.

## 6.8 Protective zone surrounding the Citrus Foundation Block

The legislation, declaring a radius of 5 km around the CFB as a citrus free area, was published in the Government Gazette on 21 January 2011. Orders to remove all citrus trees were issued by DALRRD. Most residents have removed their citrus trees. DALRRD and CRI-CIS have made several follow-up visits to one owner refusing to remove trees. DALRRD has laid a case at the Uitenhage Police station, and the owner eventually agreed to remove the citrus trees. CFB assisted with the removal. CRI's Biosecurity Division and DALRRD will repeat surveys and follow up on orders issued for tree removal at other properties with citrus trees.

## 6.9 Shoot tip grafting and CIS diagnostic services at CRI-Nelspruit

Project 1144 by J. H. J. Breytenbach, C. Steyn, R. de Bruyn and G. Cook (CRI)

### Objectives

- A. Cultivar introduction (administration, establishment, STG, diagnostics, pre-immunisation, submission to the CFB and Nucleus Block)
- B. Maintenance of the virus-free gene source
- C. Biological and molecular re-indexing of mother trees and multiplication blocks at the CFB
- D. Collaboration and duplicate indexing with ARC-TSC laboratory
- E. *Ad hoc* diagnostics for GTDs for growers and external institutions
- F. *Ad hoc* investigations as required by CIS
- G. Facility management

### Materials and methods

#### **A. Cultivar introduction (administration, establishment, STG, diagnostics, pre-immunisation, submission to CFB and Nucleus Block)**

*In vitro* cultured rootstocks: The standard method used for *in vitro* cultured rootstocks is to expose the cotyledons by removing the seed coat of Troyer citrange or Rough lemon seed and surface sterilise in a 20% solution of household bleach, which contains 3.5% sodium hypochlorite (NaOCl), for 10 minutes followed by three rinses in sterile distilled water. Three to four seeds are planted in growth tubes containing sterile Murashige and Skoog (MS) agar medium. Germination takes place in an incubator at a constant temperature of 28°C in continuous darkness. When the seedlings have reached a height of 30 to 40 mm, they are stored at 4°C in darkness.

#### Scion preparation:

*Method 1:* Buds of the source plant are budded on a standard rootstock in the glasshouse. After bud growth and maturation (approximately 3 to 4 months) the source plant is defoliated by hand to induce flushing. Ten to 14 days later the new shoots are harvested and surface sterilised on a flow bench for 5 minutes in a 7.5% solution of household bleach and then rinsed three times in sterile distilled water.

*Method 2:* If sufficient budwood of the source plant is supplied, numerous buds are budded to a few rootstocks each with 2 buds. As shoots develop from the buds they are used directly for STG and only one bud is left to grow as the reference source plant. The time from budding to STG is reduced by at least 3 months. Shoot harvesting and preparation for STG is done as above.

**STG:** The rootstock seedling is aseptically decapitated about 50 mm above the cotyledons. The cotyledons and their auxiliary buds are removed and a balcony incision is made, 1 mm horizontally and 1 – 2 mm diagonally approximately 10 mm from the top. The cuts are made through the cortex to reach the cambium. A shoot tip consisting of the apical meristem and 2 to 3 leaf primordia is excised from the growth point of the collected shoots under a stereo microscope. The growth tip is placed on the horizontal cut surface of the incision on the rootstock. The grafted plant is transferred to sterile MS liquid medium and cultured at constant 28°C exposed to 16 hours of light per day.

**STG plant propagation:** The shoot tip normally starts growing 3 to 4 weeks after STG. The shoot tip is micro-grafted with the seedling rootstock onto a vigorous-growing virus-free rootstock in the glasshouse. After micro-grafting, the graft is covered with a plastic bag for 8 days to prevent desiccation. Once the graft has sufficiently grown, the plant is screened for GTD pathogens.

**Virus indexing:** The micro-graft is pre-screened for CTV and CVds by RT-PCR. If the plant is negative, buds are taken for biological indexing, but if the plant is positive, it is the new source plant from which further STGs are done. Elimination of GTD pathogens is confirmed by biological indexing on sensitive indicators. Biological indexing results are thereafter verified using molecular diagnostics to detect CVd, CTV, CPsV, CTLV and *Liberibacter* spp that cause Citrus Greening and Huanglongbing.

On average, it takes 24 to 30 months to obtain a virus-free accession, which includes the biological indexing to confirm the virus-free status of the cultivar. However, delays can occur with elimination of some pathogens. The reason for these “difficult to remove” cases is unknown.

#### **B. Maintenance of the virus-free gene source**

Virus-free STG plants are established on virus-free rootstocks and maintained in an insect-free tunnel. Material derived from the gene source is pre-immunised with suitable CTV cross-protection sources, prior to release to the CFB at Uitenhage. Two trees of each selection are maintained in the gene source and trees have to be re-budded to new rootstocks every 5 years as part of the routine maintenance. Photo records of fruit from each cultivar/selection are kept on the database to confirm cultivar identifications. The purpose is to ensure that the correct citrus fruit type is produced from each accession and as an additional confirmation that no mix-ups have occurred. Cultivar identification of the gene source accessions at the ARC are also done with the assistance of a CRI cultivar evaluator.

#### **C. Biological and molecular re-indexing of mother trees and multiplication blocks at the CFB**

All CFB mother trees are re-indexed every second year to establish if the CTV pre-immunized source is maintained. Re-indexing of CFB mother trees for CVds is done simultaneously every second year. All CFB mother trees and seed source trees are inspected annually for symptoms of citrus greening disease by ARC-TSC and CRI virologists. PCR and/or biological indexing are conducted on plants showing suspicious symptoms. Most other citrus viruses are transmitted by infected budwood only, minimizing the infection potential at the CFB. Re-indexing of CFB mother trees for CTLV, CPsV and coguviruses including CiVA and CCGaV is done every 10 years.

Screening of CFB multiplication trees for CVds is done yearly on a third of the multiplication trees. Therefore all the CFB multiplication trees are screened every third year. The screening is done by direct RT-PCR of pooled samples of each cultivar. Each pooled sample consists of 20 leaves. A leaf is taken from every third tree and a pooled sample is therefore representative of a block of 60 trees. Each cultivar is sampled separately and the number of sub-samples of a cultivar is proportional to the size of the block. Each sample is tested with viroid-specific tests for citrus bent leaf viroid (CBLVd), hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bark cracking viroid (CBCVd), citrus viroid V (CVd V) and citrus exocortis viroid (CEVd).

#### **D. Collaboration and duplicate indexing with ARC-TSC laboratory**

Shoot tip grafting for the CIS is done at both CRI and ARC-TSC laboratories. To confirm the pathogen-free status of new accessions prior to release to the CFB, duplicate molecular testing is done by both laboratories.

### E. *Ad hoc* diagnostics for GTDs for growers and external institutions

Field material received for diagnostics is generally budded to 3 indicator host plants. The plants are cut back to force new growth and maintained in glasshouses at various temperatures required for symptom expression depending on the suspected disease being indexed. The indicators are monitored for symptoms for a minimum of 3 months post inoculation. Direct molecular tests are also done, depending on the diagnostic requirement.

### F. *Ad hoc* investigations as required by CIS

Diseases of unknown aetiology or outbreaks of graft transmissible pathogens are occasionally encountered and require investigation. Investigations may include biological and molecular indexing for the presence of graft transmissible diseases, surveys, trials or other analyses.

### G. Facility management

Routine maintenance and improvements at the CIS Nelspruit facilities are done to ensure the safekeeping of accessions.

## Results and discussion

Objective / Milestone	Achievement
A. Cultivar introduction and STG pipeline	9 accessions in STG pipeline 3 new accessions received 10 accessions released to the CFB
B. Maintenance of the virus-free gene source	410 cultivars maintained Renewal of gene source is 98% completed Citrus type verification of fruiting trees confirmed 31 cultivars this season
C. Biological and molecular re-indexing of mother trees and multiplication blocks at the CFB	Plant preparation done for CPsV and coguvirus biological indexing of the CFB Mother trees for inoculation in May 2021 Screening multiplication blocks for viroids: greenhouses 2 completed (636 samples processed, representing ~40 000trees) A real-time PCR to simultaneously detect citrus coguviruses, CiVA and CCGaV was implemented CFB mother trees in GH5 from rows 10-19 were indexed for CVds, CTV, CTLV, CPsV and coguviruses. A replacement CTV pre-immunization source for grapefruititis being phased in
D. Collaboration and duplicate indexing with ARC-TSC laboratory	All accessions sent to the CFB were duplicate tested prior to release.
E. <i>Ad hoc</i> diagnostics for GTDs for growers and external institutions	Approx. 120 <i>ad hoc</i> samples analysed
F. <i>Ad hoc</i> investigations as required by CIS	SSR markers used to verify the Argentinian rootstock lines and to assist in other cultivar verifications
G. Facility management	A temperature monitoring system was installed in all growth rooms and tunnels Ongoing insect monitoring in all CRI growth rooms and tunnels as stipulated in HLB and ACP action plan Physical breaches in tunnels and glasshouses were monitored and sealed as detected A new UV water sterilisation system was installed and old system was serviced for a backup An office cubicle was built inside the STG facility to

	accommodate the STG technician The steam steriliser was serviced A new water pump was installed in the small tunnel A new fan motor was installed in the Nucleus Block A tunnel New timers were fitted for the glasshouse lights. One self-assessment CIS audit and one external audit conducted
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**A. Cultivar introduction (administration, establishment, STG, diagnostics, pre-immunisation, submission to CFB and Nucleus Block)**

Introductions for STG and subsequent release to the CFB (phase 6) from 2016 to date are summarised in Table 6.9.1. Three new selections of three variety types were submitted for STG in this report period. Nineteen accessions are at various stages in the STG pipeline including long term biological indexing. A total of 400 STGs were done within this period, including failed grafts. Ninety-two STGs were successfully micro-grafted, which is a 23% STG success rate.

In the 2017 annual cycle, nine cultivars in the nucleus block (NB) tested positive for GTD pathogens, which were re-introduced to the STG pipeline for pathogen removal. Seven of these cultivars went through STG of which three were interim released to the CFB and three tested viroid- and CTV-free by direct RT-PCR, the remainder will be processed in 2021.

To facilitate a faster turn-around with the STG process, new introductions are tested directly via RT-PCR prior to STG to determine the original pathogen status and then again directly after STG as soon as sufficient material is available for testing. These additional steps allow quicker detection of pathogens not eliminated by the initial STG step. Re-STG can therefore commence quicker rather than waiting for completion of the biological indexing. This process does, however, not replace the final biological indexing and PCR to confirm the pathogen-free status prior to final release of the accession. These additional tests are routinely done and samples processed are not reported.

Biological indexing of ten successful STGs for CTV, CTLV and CVds were finalised and confirmed negative by RT-PCR. Biological indexing of 9 STGs for CTV, CTLV and CVds and 19 for CPsV and coguviruses are ongoing. Ten cultivars were interim-released to the CFB.

**Table 6.9.1.** STG submissions in the pipeline for graft transmissible disease elimination and indexing (phases 1 to 5). Interim release (phase 6) is reported as released to CFB.

Variety type <sup>2</sup>	STG introductions and releases 2016 to 2020 <sup>1</sup>																
	2016/17			2017/18			2018/19			2019/20			2020/21				
	Bf	New Introductions	Releases to CFB	Bf	New Introductions	Releases to CFB	Bf	New Introductions	Releases to CFB	Bf	New Introductions	Releases to CFB	Bf	New Introductions	Releases to CFB	Withdrawn	Balance
C	3	0	1	2	1	1	2	2	1	3	0	2	1	0	0	0	1
G	0	1	0	1	0	0	1	0	0	1	0	1	0	1	0	0	1
L	1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
Li	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0
Mi	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0
Ma	5	2	5	2	3	3	2	1	1	2	3	1	4	0	2	0	2
N	13	1	4	10	3	1	12	2	3	11	1	9	3	1	2	1	1
V	8	2	1	9	2	1	10	1	2	9	4	7	6	0	3	1	2

Or	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rs	0	0	0	0	2	0	2	0	0	2	1	2	1	1	0	0	2
<b>Total</b>	<b>30</b>	<b>6</b>	<b>11</b>	<b>25</b>	<b>11</b>	<b>6</b>	<b>30</b>	<b>6</b>	<b>7</b>	<b>29</b>	<b>11</b>	<b>22</b>	<b>18</b>	<b>3</b>	<b>10</b>	<b>2</b>	<b>9</b>

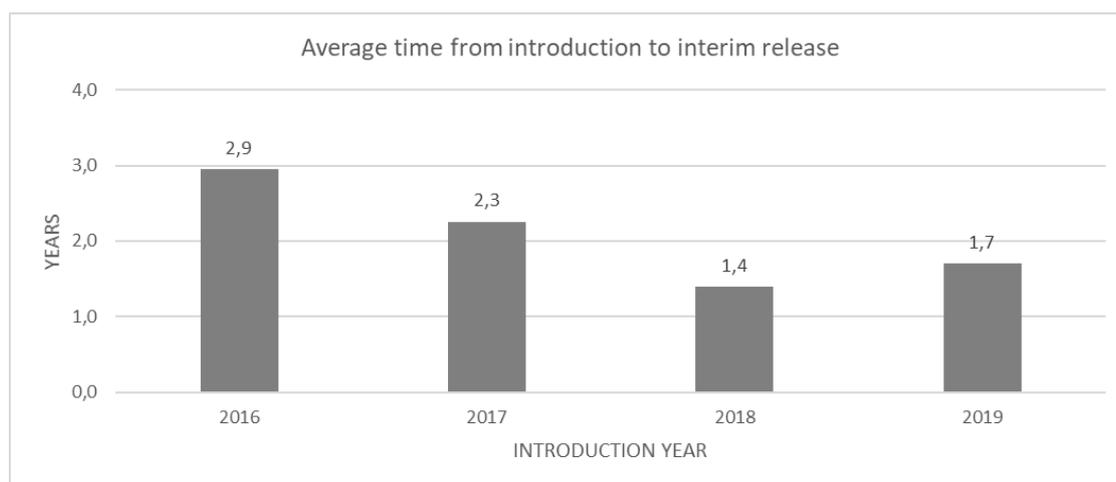
<sup>1</sup> Bf = Brought forward from previous year; Balance = Balance for the current reporting year.

<sup>2</sup> Variety type: C = Clementine; G = Grapefruit; L = Lemon; Li = Lime; Mi = Midseason; Ma = Mandarin; N = Navel; R = Reticulata; V = Valencia; Or = Ornamental; Rs = Rootstock.

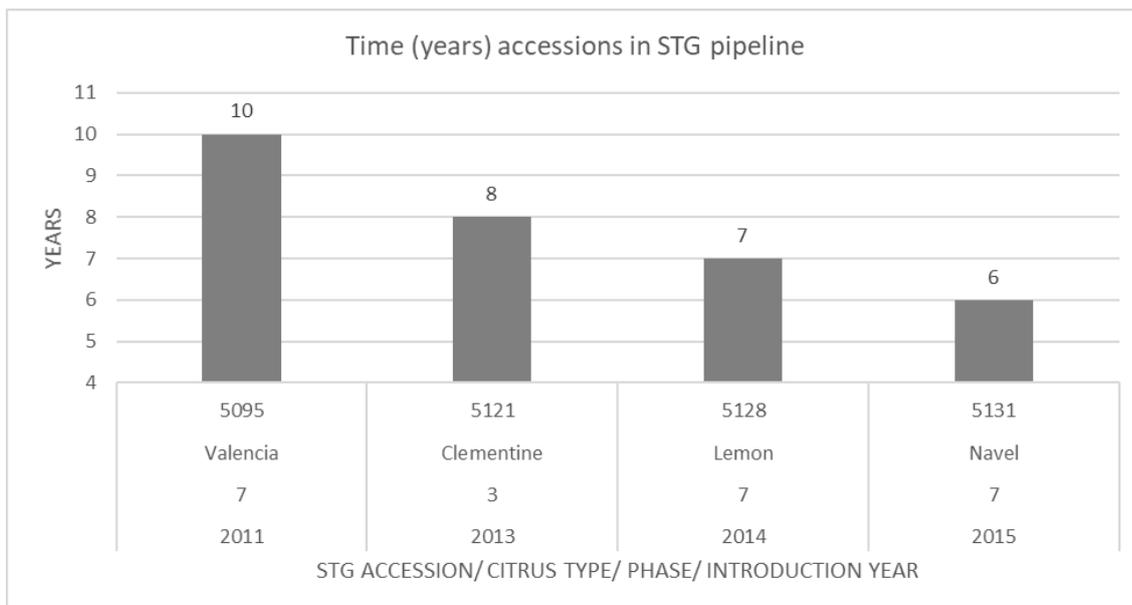
The supply of new cultivars allows the industry to remain competitive in a global market. The STG process was recognised as a hindrance as some cultivars remained in the STG pipeline for lengthy periods. Improvements to the STG process are assessed in how quickly accessions are released to the CFB. To gauge the efficiency of the STG progress over time, some parameters are graphically presented in Figures 6.9.1 to 4, which provide additional information to the numbers provided in Table 6.9.1.

Figure 6.9.1 shows the average time from introduction of cultivars to interim release to the CFB since 2016. A significant reduction in the average STG pipeline time was achieved since 2017. These averages are for 5, 10, 3 and 10 accessions in 2016, 2017, 2018 and 2019, respectively. However, problem accessions are occasionally encountered from which pathogen elimination is problematic; these accessions remain in the STG pipeline for longer periods. The reasons for these failures are not always clear. A conviron (growth chamber) was acquired to aid pathogen elimination. Controlled, fluctuating light and temperature regimes in the conviron influences plant growth and simultaneously reduces pathogen titres close to the meristem, allowing for improved pathogen elimination during STG.

The graph of Figure 6.9.2 shows 4 accessions that have been in the STG pipeline for up to 10 years. Accession 5095 originally tested negative for both CTV and CVds by direct RT-PCR, but after biological indexing the accession tested positive for CTV and was resubmitted for STG. The CTV was eliminated in this second round and the accession was interim released to the CFB in 2021. To date 81 STGs were done for accession 5121 and 4 micro-grafts were obtained. A micro-graft obtained in 2020 was negative for pathogens in initial tests. Biological indexing for CTV, CTLV, CVds, CPsV and coguviruses have commenced. Accession 5128 contained citrus tatter leaf virus (CTLV), which is notoriously difficult to eliminate. Seventy-four STGs were performed on this accession. An ex-plant from one STG, still containing CTLV, was placed in the conviron to assist with the elimination. STGs done from this plant generated 6 ex-plants, which were all negative for CTLV. The accession was interim released to the CFB in 2021. Thirty four STGs were performed from accession 5131 and 6 ex-plants were obtained. A micro-graft obtained in 2020 was negative for pathogens in initial tests and results were confirmed with biological indexing. Accession 5131 was interim released to the CFB in 2021.



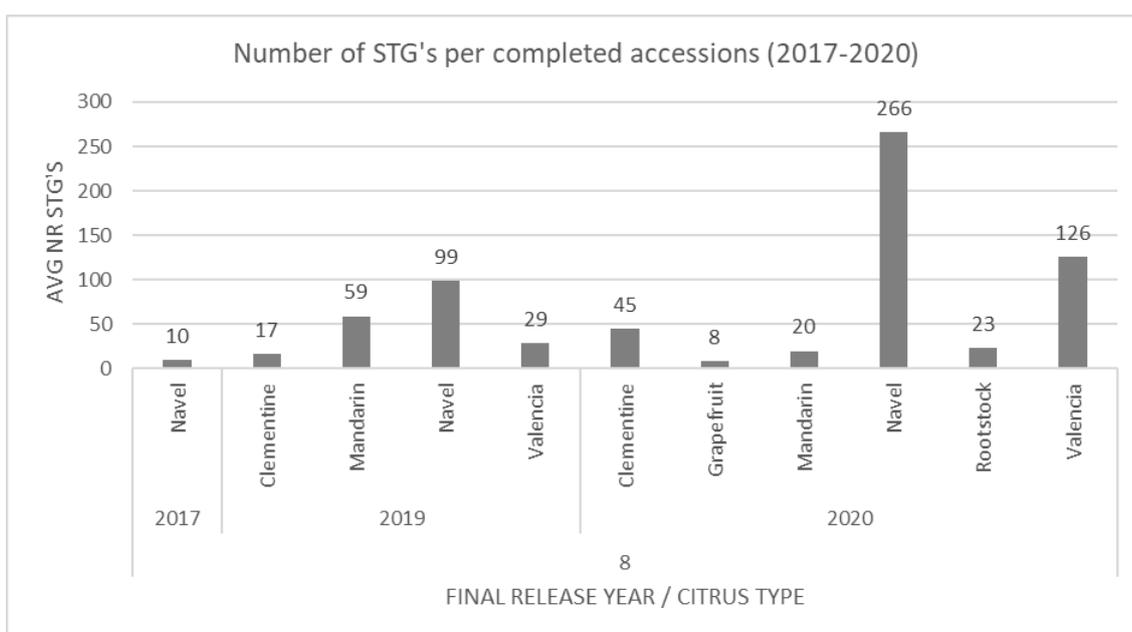
**Figure 6.9.1.** The average time (years) that accessions were in the STG pipeline from introduction to interim release is shown per introduction year for the period 2016-2019.



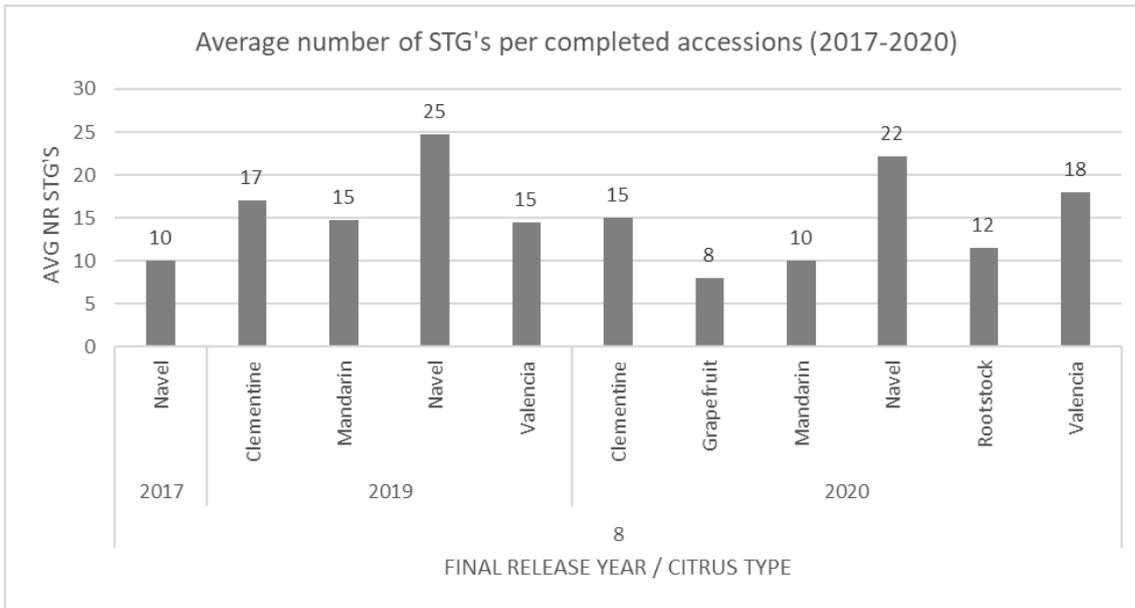
**Figure 6.9.2.** Problematic accessions in the STG pipeline.

As a measure of productivity, the number of STGs performed on finalised accessions, per citrus type and per year finalised, is shown in Figure 6.9.3. A total of 400 STGs were done for the period April 2020 - March 2021. The efficiency and unpredictability of the STG process can be assessed by the number of STG's that was done to obtain a pathogen-free accession. Figure 6.9.4 shows the average number of STGs performed per citrus type for accessions released per year. The values indicate that the STG efficiency has remained constant and the number of STGs required to obtain a successful plant is within the expected range.

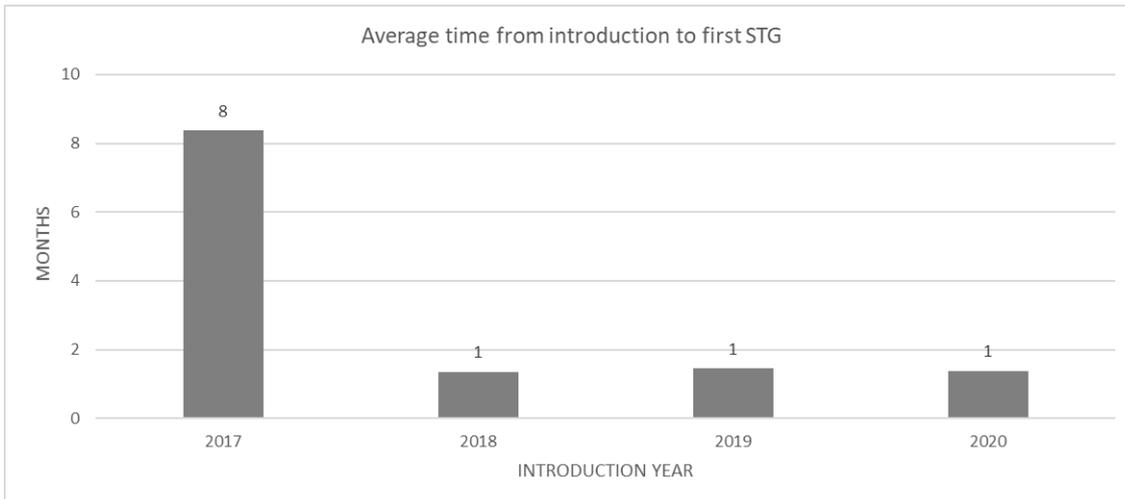
A major improvement to the STG process was the use of all additional buds. Extra buds are budded to available rootstocks and the bud-shoots are used directly for STG. This eliminates the time required for a nurse plant to grow sufficiently before defoliation can occur. Figure 6.9.5 shows that since implementing this approach the average time before the first STG can be performed was reduced to 1 month.



**Figure 6.9.3.** The number of STGs done for finalized accessions per citrus type and per year in which the accessions were final released to the CFB.



**Figure 6.9.4.** The average number of STGs performed per citrus type for accessions released per year.



**Figure 6.9.5.** The average period (months) from receipt of accession to the first STG.

### B. Maintenance of the virus-free gene source

The nine accessions which tested positive for CVds and/or CTV during the routine indexing in 2017 are currently in the STG pipeline for pathogen elimination. The CRI gene source currently comprises 410 accessions and the number of selections per variety type is shown in Table 6.9.2. A Phytophthora outbreak in the CRI Nucleus Block was addressed by a total renewal of all accessions. As a result of the Phytophthora root rot and losses during re-budding, some accessions were lost and were replaced from the ARC nucleus block (20 cultivars). Twelve cultivars were re-STGd from CFB lines and these tested negative for pathogens in initial tests. Biological indexing for CTV and CVds have commenced and the accessions will be re-introduced into the gene source once virus-free status is confirmed in 2021. A photo record is kept of fruit produced on the NB trees each year and kept in a database. The database is used to confirm the citrus fruit type of each accession to ensure that no potential mix-ups have occurred. Thirty-one additional accessions were verified during the 2020 season. One accession was not true to type and was resubmitted from the true to type CFB for STG. The number of accessions confirmed to be the correct citrus type is presented in Table 6.9.2.

**Table 6.9.2.** The number of accessions per variety type maintained in the CRI Nucleus Block and the number of accessions confirmed to date as the correct citrus type.

Variety Type	No. of cultivars at CRI	Citrus type confirmed by fruit
Clementine	34	12
Diverse (Citron, Sour orange, etc.)	2	1
Ellendale	4	-
Grapefruit	24	18
Kumquat	2	2
Lemon	24	22
Lime	5	2
Mandarin hybrid	69	34
Midseason	35	17
Navel	99	29
Ornamental	4	4
Pumelo	8	6
Rootstock	25	22
Satsuma	8	6
Valencia	68	27
<b>Total</b>	<b>410</b>	<b>202</b>

### C. Biological and molecular re-indexing of mother trees and multiplication blocks at the CFB

A real-time PCR to simultaneously detect citrus coguviruses, including CiVA and CCGaV, developed in project 1241, was implemented as a CIS diagnostic to detect viruses previously detected by biological indexing only.

CFB mother trees in GH5 from rows 10-19 were indexed for CVds, CTV, CTLV, CPsV and coguviruses, according a 10-year cycle schedule. This constituted half of the mother block. Apart from one Clementine cultivar this batch tested negative for the panel of GTD pathogens. CiVA was detected in the mother trees and the nucleus block trees of this cultivar. CiVA is a newly described virus and specific detection assays were only recently developed. It is probable that this infection was not detected by the initial biological screening following STG, which was the only detection method previously available. This cultivar was resubmitted for STG.

The mother trees were also screened for the presence of CTV to determine whether the pre-immunisation source is present. This indicated that either some cultivars were not pre-immunised or that the CTV is not retained over time. Sixty-seven percent of grapefruit and 66% of sweet orange trees were positive for CTV, whereas only 35% of soft citrus trees were positive. Research is ongoing to better understand cross-protection and CTV strain interaction with the citrus hosts.

The screening of the CFB multiplication blocks for citrus viroids in greenhouse structure 2 was completed. This entailed a representative sub-sampling of 40 000 trees and processing of 636 samples. Citrus dwarfing viroid (CDVd) was detected in one accession and citrus bark cracking viroid (CBCVd) in another.

We are in the process of evaluating an alternate CTV pre-immunisation source, B390-5, for commercial grapefruit cultivars. Commercial cultivars containing the previous CTV source, GFMS35, and the new source will be maintained in parallel at the CFB until it is confirmed that the new source shows no adverse effects and a comparative analysis of the two sources is completed. Six of the commercial grapefruit cultivars were pre-immunised with B390-5 and sent to the CFB for establishment in this report period. A comparative glasshouse trial is also underway and trees of most grapefruit accessions were prepared and pre-immunised with the 2 CTV sources. Pre-immunisations were confirmed with RT-PCR and the plants will be evaluated for stem pitting in the next report period. A research field trial is also in preparation to compare the effect of these sources on tree health and production under normal field conditions.

#### D. Collaboration and duplicate indexing with ARC-TSC laboratory

Shoot tip grafting for the CIS is done at both the CRI and ARC-TSC laboratories. To confirm the pathogen-free status of new accessions prior to release to the CFB, duplicate molecular testing is done by both laboratories. The number of ARC accessions tested for specific pathogens are presented in Table 6.9.3.

**Table 6.9.3.** Sample numbers of duplicate tests of ARC introductions, for various pathogens.

Pathogen	ARC-TSC accessions
CTV*	44
CVd	16
CTLV	25
CPsV & coguviruses	7
'Ca' Liberibacter species	10

\*Includes testing to confirm CTV pre-immunization

#### E. *Ad hoc* diagnostics for GTDs for growers and external institutions

- Approximately 120 samples were received for various *ad hoc* analyses in this report period. Analyses were done for various pathogens including CVds, CTV, coguviruses and also SSR analyses to verify cultivars and rootstocks.
- Diagnostic samples submitted for citrus greening verification are first tested with a real-time assay for universal detection of all citrus affecting 'Candidatus Liberibacter' species. Positive samples are further tested to determine the Liberibacter species. Fifteen suspect samples were received and all were negative for 'Ca. L. asiaticus'.

#### F. *Ad hoc* investigations as required by CIS

- SSR markers were used to differentiate 10 Argentinian rootstocks and to verify that the sources established at the CFB are correct by comparing profiles to those of seed batches obtained for the verification process.

#### G. Facility management

- Routine maintenance and internal audits were done on a weekly basis. A self-assessment CIS internal audit was conducted in June and an external audit was conducted by the CIS manager in November 2020 and May 2021.
- Insect monitoring in all CRI growth rooms and tunnels is ongoing as stipulated in the action plan for HLB and ACP.
- Physical breaches in tunnels and glasshouses were monitored and sealed as detected.
- A new water sterilisation system was installed and the old UV system was serviced to be used as a backup.
- An office cubicle was built inside the STG facility to accommodate the STG technician.
- The steam steriliser was serviced.
- A new water pump was installed in the small tunnel.
- A new fan motor was installed in the Nucleus Block A tunnel.
- New timers were fitted for the glasshouse lights.

6.10 **Diagnostic and technical services for the Citrus Improvement Scheme by the ARC-TSC**  
Project P03000127 by E. Jooste, N. Hlalele and Z. Theledi

#### Summary

During the 2020/2021 reporting period, the backlog of accessions in the PEQ pipeline was cleared except for one accession, which is still in the STG phase. The success rate of STG increased to 23% and this contributed

to accessions progressing quicker in the pipeline. Twenty-four accessions were interim-released after 6 months biological indexing and a further twenty-eight were released after completing the long biological indexing. In addition, the average years for an accession in the pipeline decreased to 2 years for accessions released in 2019; this was last achieved in 2013 confirming the progress made to improve and streamline the PEQ processes. The average time from introduction to first STG was also improved to 2 months, despite the large number of accessions in the pipeline. The ARC nucleus block currently has 701 trees of 533 citrus accessions. To date, variety type verifications were done on 322 of these accessions.

## Introduction

The disease management unit at the Agricultural Research Council's Tropical and Subtropical Crops, Nelspruit campus (ARC-TSC) is a role player in the mandate of the Citrus Improvement Scheme (CIS) to ensure the supply of pathogen-free propagation material to the South African citrus industry. The Post Entry Quarantine (PEQ) function, assigned by the Department of Agriculture, Land Reform and Rural Development (DALRRD) to the ARC-TSC, is critical to ensure no foreign pathogens are introduced via imported budwood. Budwood imports are subjected to shoot tip grafting (STG), to eradicate graft transmissible pathogens, *i.e.* viruses, viroids and bacteria. Once STG is completed, the material is indexed on biological indicators and molecular tests to detect various pathogens. Seed imports are subjected to grow out tests and subsequent molecular diagnosis for pathogens as per the quarantine requirements.

The different phases of the STG process are summarized in Annexure 6 (CIS Procedural Guide). When a selection is cleared from Plant Quarantine Services, Stellenbosch, the budwood arrives at the ARC-TSC facility the next day. The selection is received and is then in **Phase 1**, where a unique number is assigned to a selection and source plants are established. The unique number is used in all further processes to track the accession and all relevant information is captured in a database. If a problem with the quality of the budwood is encountered, the importer is informed.

**Phase 2** is the shoot tip grafting (STG) phase where the top 0.15 mm of a meristem of a shoot tip is grafted to an etiolated rootstock. Success of the procedure is variable and therefore a number of STG attempts are required for each selection and are reported. Many factors contribute to the STG success rate and may include the pathogen status of the plant, the size of meristem tip and various unknown biological factors that may influence the growth of the initial meristem.

Successful growth of the shoot-tip can be seen within 2 to 3 weeks and the *in vitro* plant is then micro-grafted to a rootstock to establish a nurse-plant. This is **Phase 3** of the procedure. The nurse plant is left to grow until bark are mature enough for biological indexing, which is **Phase 4** in the process. Prior to biological indexing, the nurse plant is pre-screened for pathogens with molecular techniques. If a nurse plant tests positive for any pathogen, the STG procedure is repeated (Phase 2). Biological indexing for various pathogens and diseases are conducted on a range of citrus hosts and evaluated over various time-frames for symptom development.

If the nurse plant indexes free of pathogens, **Phase 5** commences and includes pre-immunisation with an approved mild-CTV source depending on the citrus type. CTV pre-immunisation is done simultaneously with the biological indexing if sufficient buds are available, to save time in the process.

Accessions progress to the interim release phase, or **Phase 6**, when both the 6-month biological indexing and duplicate molecular confirmation tests are negative and CTV pre-immunisations are confirmed. Budwood is then released to CFB if the client wishes to introduce the accession into the CIS. At this stage, the selection can be released to the client for trial purposes.

Once interim released, the accession is in **Phase 7** and includes a 12-month biological evaluation for Citrus Greening/HLB, Impietratura and Psorosis-like pathogens. If the biological evaluations are negative, duplicate molecular testing for the pathogens is done in the relevant diagnostic laboratories at ARC and CRI to confirm the negative status of the plants.

**Phase 8** is the final release of the accession to the client and introduction to the CIS if required.

## 1. PEQ for citrus propagation material, including pathogen-therapy using shoot-tip-grafting(STG), conventional and molecular diagnostics

PEQ introductions were established in the quarantine glasshouse at ARC-TSC and the following outputs are recorded for April 2020-March 2021:

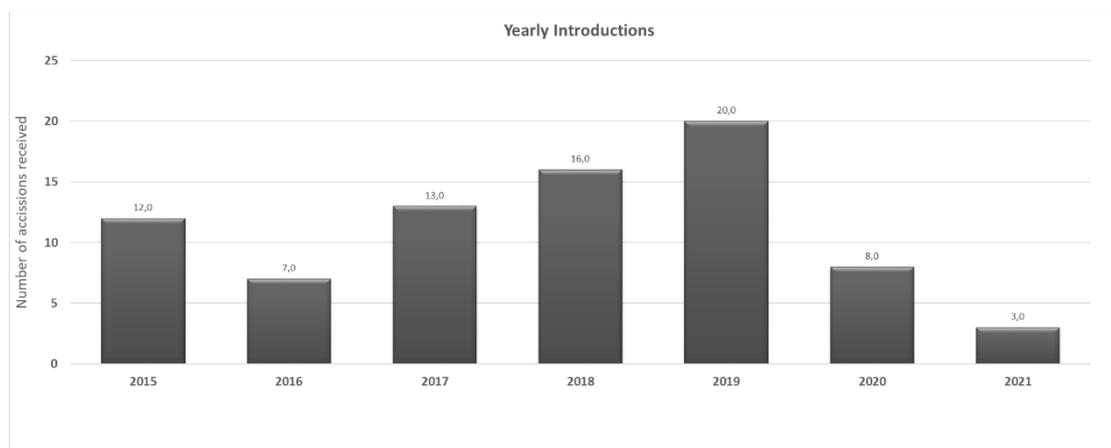
### **STG success rate vs. number of STGs done:**

During the reporting period STG was performed on 32 selections in the pipeline. Fifty-six successful STG's were obtained from 25 selections from April 2020 to December 2020. The STG success rates varied between 6.25% to 50% per individual selection with an average success rate of 19%. For some cultivars, a higher STG success rate was recorded, especially with the lemon and finger lime varieties. For the period January 2021 to March 2021, a 23% STG success rate was achieved with 16 successes from 69 STGs. Three mandarin varieties and one sweet orange variety had STG success rates above 20%. All of the established nurse plants were pathogen-free after STG.

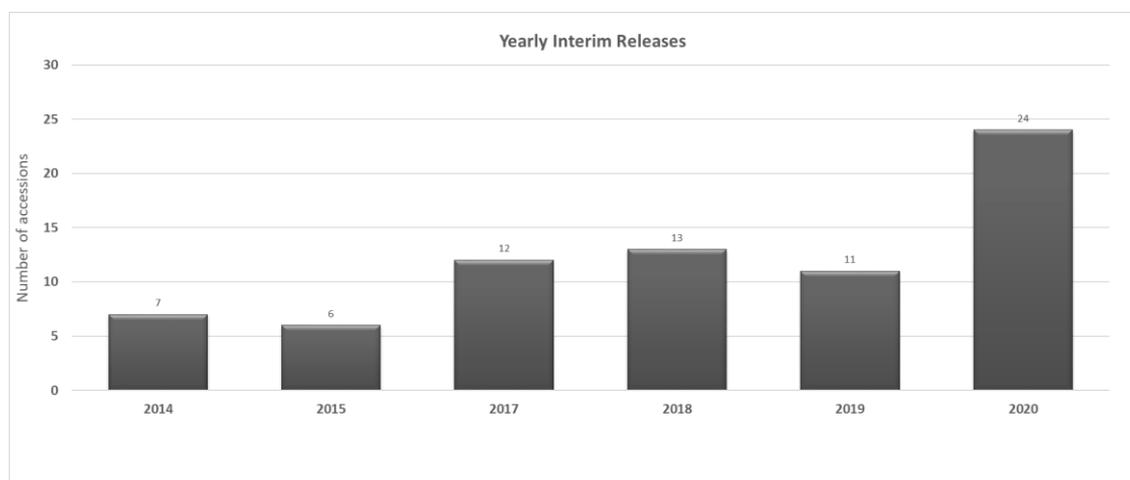
### **Analysis of introductions, processes and timeframes relating to citrus PEQ**

#### *Yearly introductions*

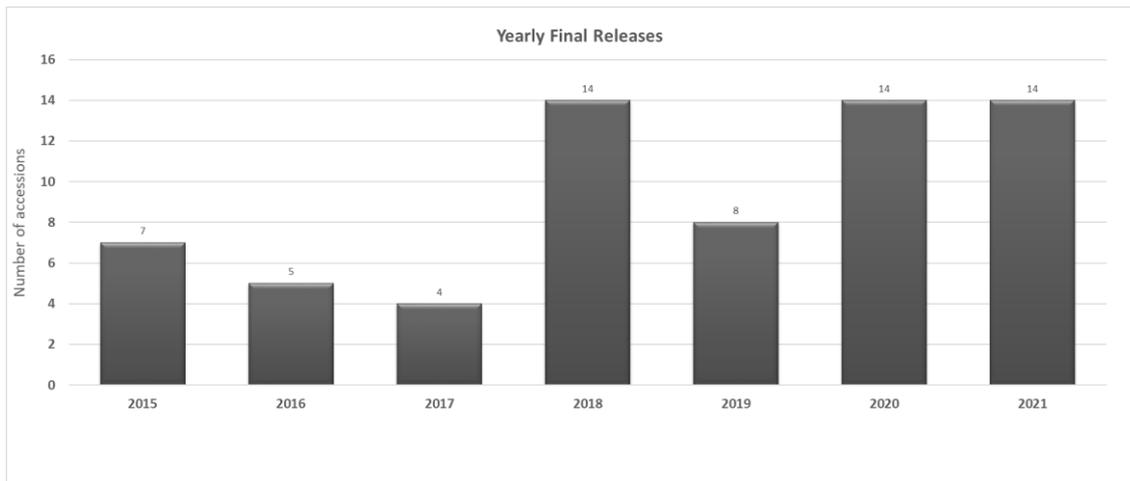
The number of accessions introduced to the PEQ pipeline from 2015 to 2021 is shown in Figure 6.10.1. Two new international budwood introductions (ARC 2105 and ARC 2106) were received from April 2020 to March 2021. The other six accessions shown on the 2020 graph were received before April 2020. ARC 2105 are already in the biological indexing phase. From March 2021 to May 2021, three new introductions were captured. Figure 6.10.2 and 3 show the number of accessions released for interim (phase 6) and final (phase 8) release. A total of 24 interim releases and 28 final releases were documented for 2020/2021.



**Figure 6.10.1.** Yearly introductions from 2015 to March 2021



**Figure 6.10.2.** Yearly interim releases from 2014 to 2020



**Figure 6.10.3.** Yearly final releases from 2015 to 2021

### ***Accessions in pipeline***

The progress on the total of 57 accessions, including 8 local ARC accessions, which are currently in the pipeline is summarized in Figure 6.10.4. The phases in the pipeline are summarised as follows:

- Phase 1: Introduction
- Phase 2: STG phase
- Phase 3: Successful STG micrografted to rootstock seedling
- Phase 4: Biological indexing phase
- Phase 5: Line tested virus-free and CTV pre-immunisation commences
- Phase 6: Interim release to CFB
- Phase 7: 12-month biological indexing
- Phase 8: Final release

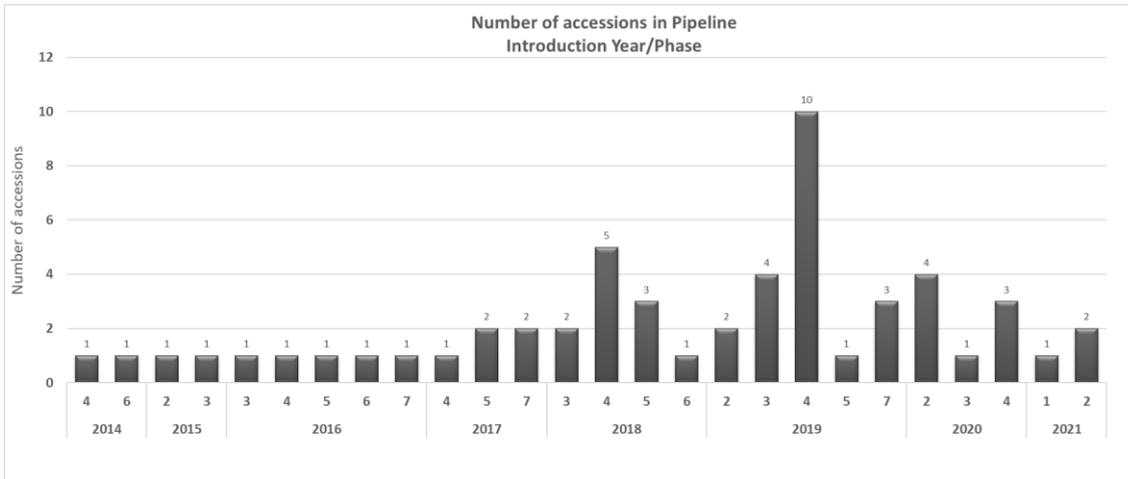
The backlog reported in previous years were cleared during the 2020/2021 reporting year, except for one accession, introduced in 2015, that is still in the STG phase (Phase 2). Priority are given to this accession. The nurse plant from an accession from 2015 are growing and will start biological indexing when bark materials mature (Phase 3). Additionally, accession 1917, which was introduced in 2012, was final released (Phase 8).

From the 2016 introductions, , all proceeded to different phases in the pipeline, including one micro-grafted, two currently in the biological indexing phase (Phase 4 and 5) and three selections were released (Phase 6). Introductions in 2017 all went through and three accessions are currently in the biological indexing phase (Phase 6) and two selections in the long indexing phase (Phase 7). Eight introductions from 2018 are currently in the biological indexing phase (Phases 4 and 5) and one accession was released (Phase 6). STG are done on two accessions introduced in 2019, four accessions are growing to be included in biological indexing, and eleven accessions are currently in the biological indexing phase (Phase 6) and three accessions in the long indexing phase (Phase 7). Four accessions from 2020 are currently in the STG phase (Phase 2) and three accessions in the biological indexing phase (Phase 4). Two accessions introduced in 2021 are in the STG phase (Phase 2).

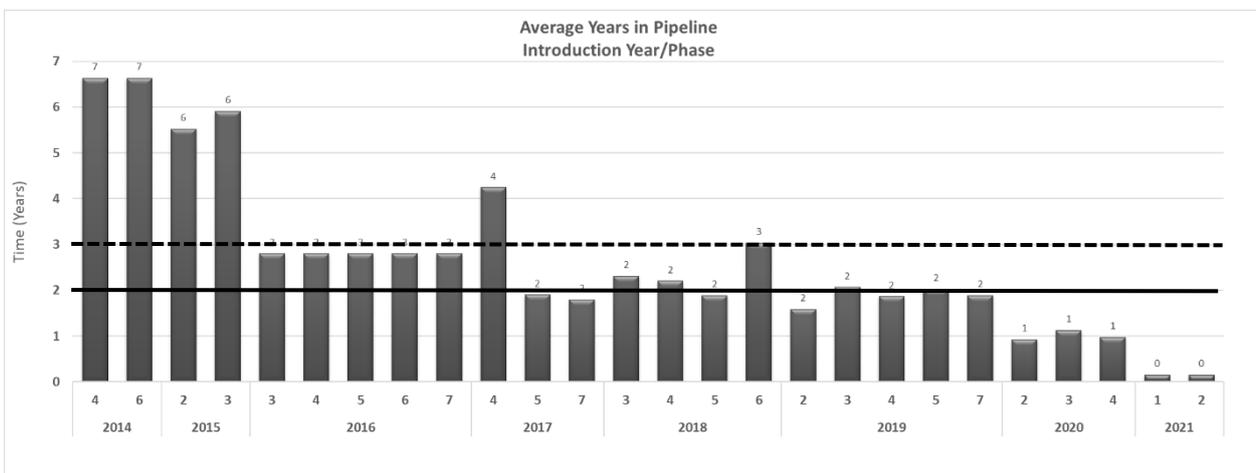
The average time for accessions in the pipeline is shown in Figure 6.10.5. Four accessions, introduced in 2014 and 2015, were flagged by the Pathology Committee as problem cases and with intense interventions three accessions, two pummelos and one mandarin, proceeded to biological indexing (Phase 4), interim release (Phase 6) and growing to maturity for inclusion in biological indexing. Four accessions introduced in 2016 will potentially go over the long-term average time. A decrease in the average time of accessions introduced in later years are shown over time.

Figure 6.10.6 shows the average time of accessions from introduction to interim release for accessions introduced in 2013-2019. The dashed line indicates the long-term average period to release accessions from

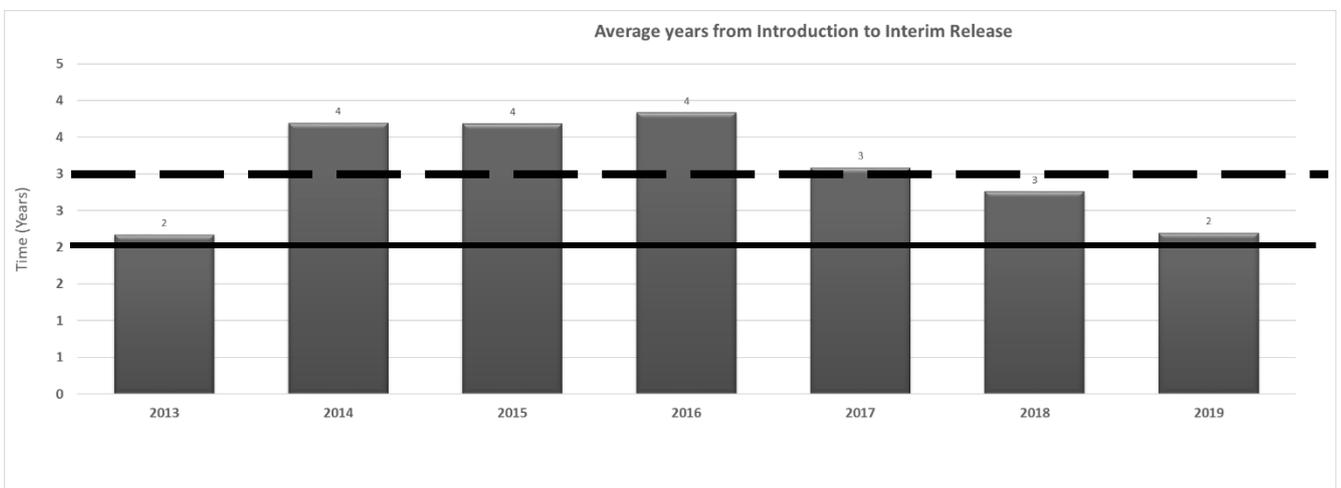
PEQ. Previously, thirty-eight accessions were used to compile a baseline average of 3 years. In the past, twelve accessions took longer to reach the interim release phase and represent 30% of the accessions from this period. From the graph (Figure 6.10.6), it is clear that accessions introduced in 2017 proceeded quicker to the interim release phase. A clear improvement in average release times are shown.



**Figure 6.10.4.** Number of accessions currently in the pipeline. The introduction year and number of samples in the different STG phases are indicated.



**Figure 6.10.5.** The average time of accessions in the pipeline indicating year of introduction and the phase. The expected- and long-term average time for PEQ completion are indicated with a solid line and dashed line, respectively.



**Figure 6.10.6.** Average time (years) from introduction to interim release

## Number of STGs required establishing a nurse plant

STG is not always successful at the first attempt. The average number of STGs per cultivar, released in 2020 and 2021, is summarised in Figure 6.10.7. The high STG number required for the pummelo group (156) represent one accession, 1978, which were referred to in the previous discussion as being a problematic accession but was released in 2020. The success rate is unpredictable, but we have recorded a higher STG success rate in the past year. We implemented the bigger cut during STG, but it is not the only solution as it also depends on compatibility with the rootstock and on biology.

On average, in our experience, mandarin and grapefruit varieties generally need more STGs before success. The finger limes are tricky to work with because of their shoot size, but to date we managed to get five finger lime accessions to the biological indexing phase within an average time of 5 months, *i.e.* STG (Phase 2), micrografted the nurse plant (Phase 3) and time for the nurse plant to grow to have mature bark for biological indexing (Phase 4).

The average time from introduction to first STG attempt is presented in Figure 6.10.8. An improvement in this time is seen from 2017 with a significant improvement visible in 2019, 2020 and 2021.

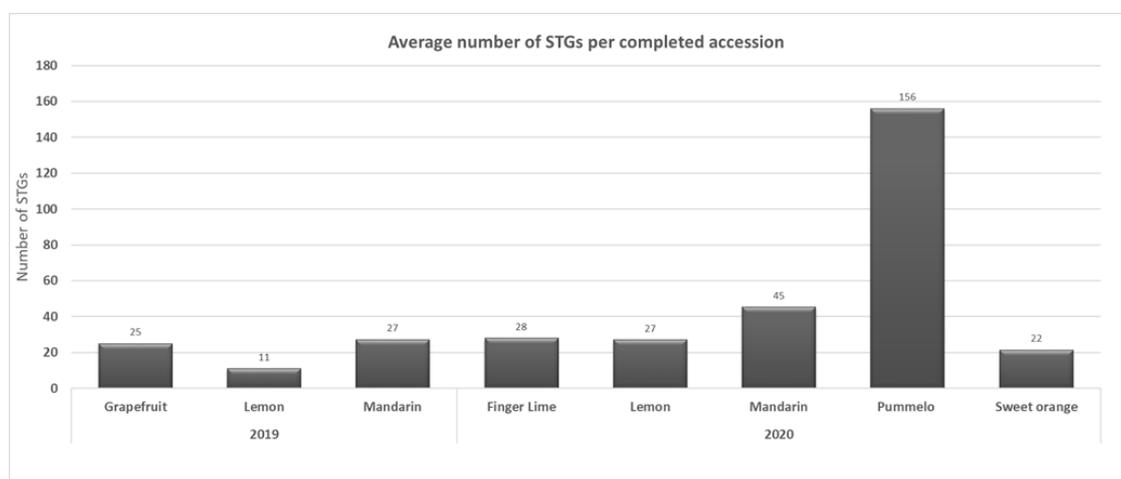


Figure 6.10.7. Average number of STGs for completed accessions released in 2019 and 2020 per citrus group

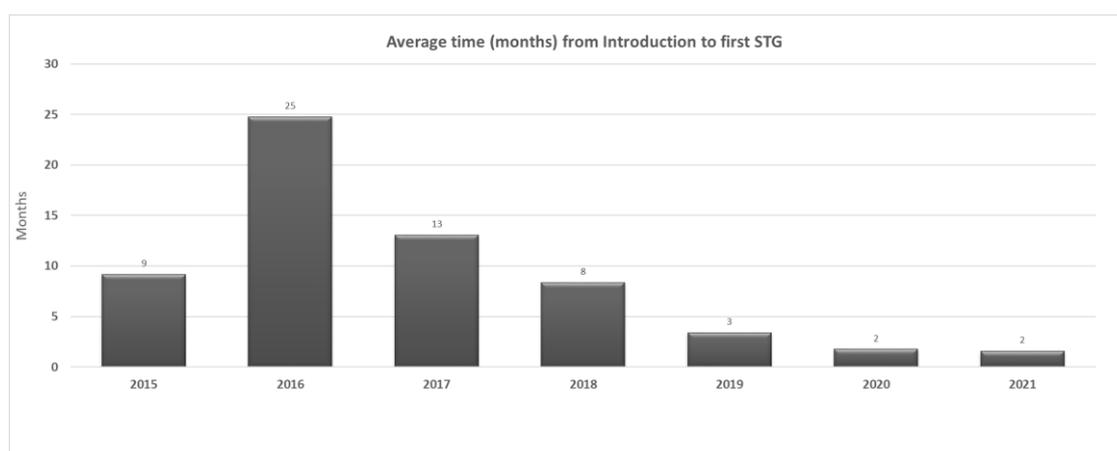


Figure 6.10.8. Average time from introduction to first STG

## 2. Maintenance of a virus-free nucleus block of citrus cultivars and germplasm

Currently the ARC nucleus block has 701 trees of 533 accessions (Table 6.10.1). Mistakes during repotting/relabeling in the past, have led to some mix-ups, which became apparent during trueness-to-type (TtT) evaluation of exported, field or CFB Evaluation Block trees. Citrus type verifications are done annually with assistance of Johan Joubert from CRI, with photo records for each accession. These inspections were done in May, August 2020 and February 2021. In total, 335 cultivars have been confirmed as the expected

citrus type in 2020 and 2021. These included 13 pre-immunised sources. The pre-immunised sources represent the original tree of an accession and type confirmation on these accessions ensures early type verifications. In this reporting period, one accession was detected as not true-to-type. The selection was from an older ARC breeding line.

**Table 6.10.1.** Summary of cultivar type identifications from fruiting trees in the ARC nucleus block

Variety Type	ARC accessions	Citrus type confirmed by fruit
Clementine	42	23
Diverse (Citron, Sour orange, etc.)	9	4
Ellendale	5	1
Grapefruit	29	24
Kumquat	3	3
Lemon	41	29
Lime	4	2
Finger lime	1	0
Mandarin hybrid	141	80
Midseason	40	18
Navel	78	42
Pumelo	15	11
Rootstock	54	31
Satsuma	17	16
Valencia	54	38
<b>Total</b>	<b>533</b>	<b>322</b>

### 3. Introduction of cultivars to the CIS Citrus Foundation Block following *Citrus tristeza virus* pre-immunisation

During this period, 20 imported accessions and 4 local ARC accessions were interim released after biological indexing and molecular verifications (Figure 6.10.2). Plant material from 7 virus-free selections was sent to cultivar owners to use in trials. Twenty-eight accessions that completed the long biological indexing phase (Phase 7) were released. The final releases included 3 local ARC selections (Figure 6.10.3).

### 4. CIS diagnostic support for CRI-Nelspruit, including validation of diagnostic tests and improvement of diagnostic protocols

During this reporting period, 18 accessions in the PEQ pipeline moved to the biological indexing phase (Phase 4). Confirmation of the disease-free status of nurse plants was done using molecular detection as pre-screening tool (described in Annexure 6C). Diagnostic support of duplicate tests for ARC and CRI accessions are summarised in Table 6.10.2 and 3. Diagnostic support was also given in the validation of PCR test results of CFB mother block material.

**Table 6.10.2.** ARC molecular screening of PEQ accessions for release to CFB

Pathogen	Number of accessions	Number of tests
CTV-Pre-immunisations	25	25
CTV-indexed	26	26
CTLV	30	30
Greening	30	30
Viroids	23	138
CPsV and CiVA	34	68

**Table 6.10.3.** CRI molecular screening of accessions for release to CFB

Pathogen	Number of accessions	Number of tests
CTV-Pre-immunisations	11	11
CTV-indexed	13	13
CTLV	18	18
Greening	12	12
Viroids	13	78

#### **Technical CIS support through participation on CISAC and CIS-Pathology committees**

- Jooste, AEC. Participated in the 20th annual CISAC meeting, Teams meeting, July 2020
- Jooste, AEC. Participated in the Citrus Post Entry Quarantine Workshop, Teams meeting, May 2020
- Jooste, A.E.C, Hlahlele, N and Theledi, Z participated in the CIS Pathology meeting, Teams meeting, 25 November 2020 and the CIS facility audit, ARC-TSC, 17 November 2020.
- CIS technical support (Ms Hlahlele and Ms Theledi) assisted with preparation for the above meetings.

#### **Acknowledgment**

ARC-TSC's PEQ laboratory thank CRI for the financial, technical and diagnostic support.

## **7 EXTENSION / VOORLIGTING**

By Hannes Bester, M.C. Prestorius, Wayne Mommsen, Keith Lesar, Catherine Savage, David Groenewald, Andrew Mbedzi and Melton Mulaudzi

### **7.1 VOORLIGTINGOORSIG**

#### **7.1.1 Die 2020 Seisoen**

Die 2020 seisoen het in groot onsekerheid afgeskop a.g.v die Covid pandemie. Dit was vroeg duidelik dat die kwaliteit van die vrugte besonder goed is, pryse in die markte was goed, maar die logistiek in veral die hawens was 'n groot uitdaging op daardie stadium. Daar is egter steeds met optimisme na die seisoen uitgesien, aangesien alles daarop gedui het dat die vraag na sitrus wêreldwyd sterk sou bly a.g.v die vraag na Vitamien C.

Wisselvallige klimaatstoestande het sporadies probleme in sekere areas geskep. Koueskade op veral sagtesitrus het in die tweede kwartaal voorgekom gedurende 'n onverwagse koue front oor groot dele van die land. Aan die produksiekant was daar oor die algemeen 'n besondere goeie vrugset. Australiese wolluis en blaaspootjie skep egter toenemend probleme.

Die pakseisoen het vroeg ten einde geloop en rekordvolumes van goeie gehalte vrugte is uitgevoer en teen goeie pryse verkoop. Daarby was die wisselkoers ook baie gunstig vir uitvoere. As gevolg van die Covid pandemie is groot ontwrigtings in die hawens en ander komponente van die logistieke ketting ervaar. Producente was vroeg reeds bekommerd oor die nadelige effekte wat Covid op oes, verpakking en verskeping sou veroorsaak, met die gevolg dat almal hul oes so gou moontlik wou verpak en verskeep. Gevolglik was daar vroeg reeds groot volumes vrugte wat in verskeie markte aangekom het, maar a.g.v die sterk vraag na Vitamien C het vrugte relatief vinnig verkoop. Die sterk mark het meeste potensiële eise a.g.v gehalteprobleme self geabsorbeer en min eise is aan producente deurgegee. Die pandemie, wat aanvanklik 'n donker wolk oor die uitvoerseisoen gegooi het, het uiteindelik in ons guns gewerk a.g.v die groter vraag na Vitamien C.

Die seisoen het nuwe eise ook ten opsigte van kommunikasie gestel en nuwe maniere van tegnologie oordrag moes beproef word. Virtuele vergaderings, kongresse en werksinkels moes vinnig ingestel word as noodmaatreëls om inligting oor te dra en te kommunikeer. In sekere gevalle hou dit baie voordele in vir toekomstige gebruik, maar daar is ook gevalle waar dit beslis nie die mees gewenste wyse van kommunikasie is nie. Fisiese byeenkomste vir bv. CRI werksinkels en die simposium bly die mees gesogte keuse omdat

daar 'n groot premie op interaksie tussen al die verskillende rolspelers geplaas word, aanbiedinge in meer detail gedoen kan word en 'n wyer reeks onderwerpe gedek kan word.

Diensverskaffers en ander rolspelers moes groot uitdagings te bowe kom om sitrus-uitvoere suksesvol te maak werk. Papier- en kartonvervaardigers, paletvervaardigers en hawefasiliteite kan uitgesonder word wat, ten spyte van moeilike omstandighede, die wa suksesvol deur die drif kon sleep. Selfs ook pakhuse moes verskeie aanpassings maak om die oes betyds verpak te kry. Die afgelope seisoen het weereens geillustreer dat ten spyte van massiewe uitdagings, die Sitrus bedryf nogsteeds volhoubaar kan funksioneer.

Die vooruitsigte op die 2021 seisoen vanuit 'n produksie-oogpunt lyk belowend. Die skattings vir die seisoen dui op rekord volumes vir die derde agtereenvolgende jaar, met 163 miljoen kartonne in die vooruitsig. Die markvooruitsigte op hierdie vroeë stadium is moeilik om te voorspel, maar dit wil voorkom of die dringende vraag na Vitamien C as gevolg van Covid effens getaan het, soos weerspieël in die laer pryse vir sitrus wat op hierdie stadium in Europa heers. Die neerdrukkende effek van Covid op die wêreld-ekonomie bring ook onsekerheid oor die markvooruitsigte vir 2021.

'n Bron tot kommer is die ernstige tekort aan skeepshouers in die suidelike halfmond. As gevolg van hierdie tekort is die verwagting dat logistieke koste beduidend hoër as verlede jaar gaan wees. Samesprekings met verskepingmaatskappye is reeds aan die gang om maatreëls in plek te sit om logistieke uitdagings aan te spreek.

## 7.2 CRI-PTF

### 7.2.1 Akkreditasie van kartonvervaardigers

Na verskeie samesprekings met Sappi en die Sappi Technology Centre se bestuur het hulle besluit dat die toets van die geakkrediteerde kartonvervaardigers se kartonne net soos in die verlede, gedurende 2020/2021 sal voortgaan. Alle kostes sal weer deur Sappi gedra word.

Ongelukkig moes die geskeduleerde kartontoetse gedurende vlak-5 van die inperking gestaak word. Die kartonvervaardigers is versoek om die lewering van kartonne vir toetse volgens die skedule tot verdere kennisgewing te staak. Ander maatreëls is getref om die kwaliteit van kartonne te monitor. Die kartonvervaardigers is versoek om volledige inligting met betrekking tot die papier, asook die basiese massa van die "Liners" en die "Fluting" vir die volle reeks sitruskartonne vir die CRI-PTF te stuur. Daar is toe op 'n *ad hoc* basis kartonne getrek en die basiese massa van die papier is bepaal en toetse is ook gedoen om te verseker dat "Virgin Liners" gebruik word. Na die beëindiging van vlak-5 inperking is 'n nuwe skedule opgestel en die kartontoetse het weer voortgegaan. As gevolg van gereelde krag-onderbrekings kan die kondisionering van kartonne nie gedoen kon word nie en daar is toe besluit dat daar gedurende beurtkrag "edge crush" toetse op 100 x 50mm monsters van die kartonne gedoen sal word.

Ongelukkig is daar gedurende 2020 seisoen heelwat probleme ondervind met sekere kartonvervaardigers wat nie by die skedule gehou het nie en die inligting oor die papierkombinasies was ook foutief. Dit is met die betrokke kartonvervaardigers opgeneem en hulle is versoek om streng by reëls te bly.

### 7.2.2 Klagtes oor kartonne

Gedurende die jaar is daar enkele gevalle gerapporteer van kartonne wat inmekaar gesak het. Al die gevalle is ondersoek en die probleme was hoofsaaklik deur die volgende faktore veroorsaak:

- Uiters rowwe hantering: Fotos het baie duidelik getoon dat kartonne en palette erg deur vurkhysers beskadig is.
- Palettiserings protokolle wat nie gevolg is nie: Byvoorbeeld, die aantal "securing sheets" en plastiese bande was nie voldoende volgens die protokolle nie. In sommige gevalle was die hoekstukke tot onder op die vloer geneem en nie bo-op die palette geplaas nie.

- In een geval het die basiese massa van die papier afgewyk van wat op die betrokke kartonvervaardiger se vraelys aangedui is.
- 'n Geval het voorgekom waar kartonne gebruik is wat nie deur een van die geakkrediteerde kartonvervaardigers vervaardig is nie, en dus nie aan die minimum vereistes voldoen het nie.
- Paletvragte wat tydens padvervoer vanaf pakhuis na die hawens nie na behore gestablisier en vasgemaak is nie.

**Die volgende aksies is geneem:**

- Met die hulp van die logistieke personeel van die CGA is daar 'n dringende beroep op alle hawens gedoen om die probleem met rowwe hantering aan te spreek.
- 'n Snykant artikel waarin produsente versoek is om by die palettiserings protokolle te hou, slegs geakkrediteerde kartonvervaardigers se kartonne te gebruik en seker te maak dat paletvragte tydens padvervoer na behore vasgemaak en gestablisier word, is opgestel en uitgestuur.

### 7.2.3 **Palette**

Die groot verskeidenheid uitvoerpalette wat tans gebruik word is kommerwekkend. Ondersoeke het getoon dat daar tans van agt verskillende ontwerpe gebruik gemaak word:

- 'n Koper in Italië wat aandring op 'n spesifieke ontwerp.
- Heelwat pakhuis in die Oos Kaap wat 'n sogenaamde "10 Slat" palet gebruik.
- Lidl palette. Sover vasgestel kan word, is daar 4 verskillende Lidl palet ontwerpe. Wat baie interessant is, is dat die oorgrote meerderheid van die pakhuis gesê het dat wanneer hulle vir Lidl pak, hulle die 1 210 x 1 010 mm CRI palet gebruik en dat hulle geen probleme ondervind het nie.
- Vrugte wat vir Bama gepak word moet op een van die Euro-palette gepak word. Daar bestaan verskillende Euro-palet ontwerpe. Sover vasgestel kon word is dit verpligtend om te gebruik.
- Summer Citrus se palet vir uitvoere na die VSA.
- 'n Pakhuis in die Oos Kaap wat sy eie ontwerp gebruik.
- Die CRI 1 210 x 1 010 mm palet. Ongeveer 80% van SA se sitrus word op die CRI palet uitgevoer en terugvoering is baie positief.

Die volle reeks uitvoerkartonne word tydens evaluasie op modelle van die nuwe palet-ontwerpe geplaas om te kyk hoe die ventilasie-gate van die kartonne oplyn met die openinge tussen die bo-dek planke en of die hoeke van die kartonne op bo-dek planke rus. In sekere gevalle is daar 'n geringe verbetering in die oplyn van die ventilasie-gate (beslis nie die geval met Euro palette nie) maar in al die gevalle staan die hoeke van die kartonne nie op bo-dek planke nie en dit is 'n groot probleem. Die groot verskeidenheid palette sal so spoedig moontlik met die uitvoerorganisasies en ander rolspelers opgeneem moet word.

Die skatting vir die 2021 seisoen staan tans op 162 miljoen kartonne. Dit plaas veral baie druk op die paletvervaardigers. Op versoek van die paletvervaardigers is daar 'n omsendskrywe aan alle produsente/pakhuis gerig met die versoek om hulle palette so vroeg moontlik te bestel.

### 7.2.4 **Everest Packaging**

Bogenoemde instansie het die CRI-PTF genader vir akkreditasie. Gedurende die 2020 seisoen het hulle die vereiste prosedures gevolg en aan die einde van 2020 'n baie volledige verslag ingedien.

Opsommend: 'n Totaal van 344 699 kartonne (teleskopiese- sowel as oop vertoon-kartonne) is gepak en na 20 verskillende lande uitgevoer. Dit sluit ook koue-sterilisasie markte in. Die verslag sluit ook briewe van

betrokke pakhuis, 'n toonaangewende uitvoerorganisasie en SANAS geakkrediteerde laboratorium toetsresultate in. Alle terugvoering was positief en Everest Packaging is geakkrediteer.

#### 7.2.5 **CRI-Packaging Material Specifications and Palletisation Protocols for the 2021 Citrus Export Season.**

Bogenoemde dokument is gefinaliseer en in Januarie 2021 aan alle produsente/pakhuis en al die ander rolspelers gestuur.

#### 7.2.6 **Disruptive Packaging**

'n Verteenwoordiger van bogenoemde Australiese maatskappy in Suid Afrika het die CRI-PTF gekontak rakende 'n "Barrier Coated" waterdigte karton wat deur die sitrusprodusente in Australië gebruik word. Op CRI-PTF se versoek is van die kartonne na SA gestuur. Met ontvangs is gevind dat dit "Extruded Corrugated Plastic Containers" is. Daar is gedurende die afgelope paar jaar verskeie toetse op soortgelyke plastiese houers gedoen en om verskeie redes is hierdie plastiese houers hoegenaamd nie vir SA Sitrus aanvaarbaar nie. Dit is so aan "Disruptive Packaging" oorgedra.

#### 7.2.7 **Navorsing- en ontwikkelingswerk op meer koste-effektiewe kartonne**

Navorsing- en ontwikkelingswerk op meer koste-effektiewe kartonne, wat steeds aan die minimum spesifikasies voldoen, is baie belangrik. Gedurende die 2020 seisoen is daar proewe gedoen met A15C kartonne waarvan die basiese massa van "Liners" en "Fluting" ligter is. Gedurende die vorige drie seisoene is A15C kartonne met 175g/m<sup>2</sup> "Liners" en 165g/m<sup>2</sup> "Ultraflute" vervaardig. Gedurende die 2020 seisoen is proewe met 'n nuut ontwikkelde 170g/m<sup>2</sup> "Kraftpride Liners" en 150g/m<sup>2</sup> "UltraflutePlus" gedoen. Op die A15C dubbelwand binnestukke is die middel "liners" ook vervang met 150g/m<sup>2</sup> "UltraflutePlus". Die belangrikste fisiese eienskappe van die nuut ontwikkelde "UltraflutePlus" is dat die stapelsterkte van kartonne tussen 20 en 22% hoër is. Dit is moontlik gemaak deur 'n hoër persentasie "virgin" materiaal, asook die optimalisering van verskeie operasionele prosesse. Die proewe was suksesvol en A15C kartonne wat met bogenoemde ligter papier vervaardig sal word, sal in 2021 geïmplementeer word. Gebaseer op die lysprys van "liners" en die "fluting" ten tye van die proewe is die besparing in die omgewing van R0,70 per karton.

#### 7.2.8 **Fruit Technical Services and Support Ltd**

Bogenoemde doen evaluasies vir sommige SA uitvoerorganisasies en kartonvervaardigers. Na 'n verduideliking oor hoe CRI funksioneer het hulle ingewillig om CRI op hoogte te hou met alle evaluasies rakende verpakkings-aangeleenthede waarby hulle betrokke is. Gedurende die 2020 seisoen is twee baie volledige en waardevolle verslae van hulle ontvang. Hierdie insette wat nou deur alle betrokke instansies gelewer word, is 'n wen-wen situasie vir almal.

#### 7.2.9 **Versoeke van Morrisons en Lidl**

Verskeie uitvoerorganisasies het CRI-PTF in kennis gestel dat vrugte wat voortaan vir Morrisons in oop vertoonkartonne gepak word, sonder "securing sheets" (SS) gepalettiseer moet word. Wanneer palette oorsee afgepak word, is die verwydering van die SS 'n probleem en bowendien gebruik Peru nie SS op hulle oop vertoonkartonne nie. Die hele aangeleentheid is deeglik ondersoek en daar is toe gevind dat Peru beslis ook SS gebruik. Morrisons is in kennis gestel dat oop vertoonkartonne baie beslis nie sonder SS gepalettiseer kan word nie. Dit is ook aan hulle duidelik gemaak dat indien hulle daarop gaan aandring dat die gebruik van SS gestaak moet word, hulle dit op skrif moet sit dat hulle volle verantwoordelikheid vir enige verliese sal vat. Gelukkig het hulle aanvaar dat oop vertoonkartonne met SS gepalettiseer moet word.

Lidl het 'n versoek gerig het dat alle A15C kartonne se binnestukke ten volle gedruk moet word. Die rede vir die versoek is dat alle teleskopiese kartonne wat hulle van regoor die wêreld ontvang, se buitestukke verwyder word, en wannere hierdie binnestukke met vol drukwerk daarop op die rakke in hulle winkels vertoon word, moet alle binnestukke dieselfde lyk. Dit gaan eenvoudig geweldige logistieke probleme vir die

kartonvervaardigers en ook al die sitruspakhuis veroorsaak. Bo en behalwe dit gaan dit die prys van die binnestukke verhoog en die stapelsterkte van die kartonne gaan nadelig beïnvloed word. Daar is begin met verskeie aksies om al die probleme aan hulle uit te wys en daar gaan 'n dringende beroep op alle produsente in suidelike Afrika, die uitvoerorganisasies en ander rolspelers gedoen word om saam te staan en Lidl te oorreed om hierdie "belaglike" versoek te laat vaar.

#### **7.2.10 Versoek vanuit Japan vir 'n 18Kg teleskopiese karton vir Suurlemoene**

Een van die groot uitvoerorganisasies het 'n versoek uit Japan gekry om suurlemoene in 'n 18 kg teleskopiese karton te verpak. Dit is aan die organisasie uitgewys dat die meeste tellings in A15C teleskopiese kartonne se bruto massa tussen 17,0 en 17,4 kg wissel. Nadat die inligting aan die kopers in Japan deurgegee is, het hulle laat weet dat die bestaande A15C kartonne aanvaarbaar is.

#### **7.2.11 CRI Na-oes Werkwinkels 2021**

Die CRI Na-oes werkwinkels vir 2021 kon as gevolg van die Covid19 pandemie nie soos in die verlede "lewendig" in die verskillende streke regoor die land aangebied word nie. Dit is vervang met 'n webinar aanbieding van drie sessies. Die aantal onderwerpe moes aansienlik verminder word en die aanbiedinge van maksimum 15 minute elk van die mees belangrikste onderwerpe is aangebied. 'n Aanbieding oor verpakking is gedoen. Ten spyte van die feit dat dit 'n eerste webinar aanbieding wat deur CRI aangebied is, het alles goed afgeloop. Die terugvoer was goed, hoewel 'n opname toon dat deelname na raming slegs helfte van die fisiese werkwinkels se bywoning verteenwoordig. Meer as 60% van die persone wat aan die meningspeiling deelgeneem het, het aangetoon dat hulle die lewendige werkwinkels verkies.

### **7.3 POSTHARVEST**

#### **7.3.1 COVID-19**

The greatest challenge of the 2020 season was undoubtedly the impact of Covid-19. The national lockdown coincided with the start of the citrus packing season and many packhouses made an effort to complete packing ahead of the peak of infections, starting earlier and faster than normal. Fruit entered the markets early but on average was very well received. Covid-19 seems to have in fact been a blessing in disguise since the markets were hungry for fruit and the increased demand of fruit high in vitamin C meant that the 2020 season had one of the lowest claim rates to date. Several areas reported poor colour and high blemishes but the majority of markets took the fruit without complaint. Oranges and grapefruit demand was particularly high, however there were some problems concerning the soft citrus, primarily due to an oversupply which was exacerbated by shipping delays resulting in an overlap of cultivars from different regions, but also partly due to an overall lower demand for soft citrus.

Despite high fears at the start of the season, very few packhouses had Covid-19 cases and there have been no long term shutdowns. Mask wearing in the packhouse environment is not pleasant but all packhouses have implemented Covid-19 mitigation protocols which seem to be working.

One of the greatest challenges at the start of the 2020 season was the sudden influx of sanitation products for hands and surfaces. The obvious concern was residue transfer to the fruit but the overall efficacy of the products came into question too. With time, most packhouses did away with the new wonder products and treatments, such as misting tunnels, and went back to the tried and tested basics. The initial fear that sanitation products would be unavailable also ceased as the season progressed smoothly and panic buying desisted.

During the lockdown and the 2020 season when uncertainty of the severity of the pandemic was high, unfortunately no physical packhouse visits were able to be conducted, however the industry has embraced technology and communication was not hampered between CRI and the packhouses. Several Cutting Edges were published early in the season to aid packhouses in Covid-19 mitigation, as well as collaboration with Citrus Academy to publish checklists and protocol documents. On the whole, packhouses handled the season very well and have carried on with business as usual with some packhouses even taking the opportunity to make improvements such as increased automation to reduce the labour force.

### 7.3.2 **Decay concerns**

Overall claims were low and quality high during the 2020 season. Very few decay reports came through. The main decay concern was storage mould (saprophyte mould) which is a grey–white fluffy growth on the stem end of citrus. It is most commonly seen on lemons but has been reported on other citrus types. CRI is collecting evidence and patterns such as that chem-free and organic fruit are worse hit. Typically storage mould was seen only on fruit arriving in far markets such as Canada but claims are also coming from closer markets such as Europe.

Frost damage occurred on Navels and Valencias due to the very cold weather, with the Groblersdal area most severely affected. Despite this, the affected fruit that were exported and still sold well.

### 7.3.3 **Fungicide Concerns**

Control of sour rot and the loss of adequate fungicides remains a top concern for packers and exporters. The most worrying aspect is the change to the EU's MRL of Propiconazole from between 5 and 9 mg/kg to 0.01 mg/kg. The change will only be enforced by September 2021 however multiple reports from both local and international packhouses gave figures of Propiconazole residue lingering on the line well past the date of usage stop and after cleaning. CRI issued Cutting Edge No. 314 with a detailed cleaning regime and advised packhouses to be cautious of using Propiconazole during the 2021 season, in order to give enough time to thoroughly clean the line and check the residue levels before the cut-off date.

With the loss of Propiconazole for one of our main export markets, the concern has been for adequate control of sour rot decay. With no replacement fungicide, packhouses are advised to be extremely strict with their sanitation practices, both in the orchard and in the packhouse, and to use GRAS or green chemicals that offer some activity against sour rot spores. These practices are vital as the problem may be compounded by the heavy rain seen in the Northern parts of the country at the beginning of the year. Brown rot has also been highlighted as a potential concern for this season. CRI published Cutting Edge No. 317 in light of these concerns.

Overall, packhouses are concerned over the general lowering of MRLs, both for wholesale markets, as well as by supermarkets. They are having to make sacrifices and adjustments that are not scientifically backed or sustainable. They have expressed a need for alternatives and strategies for lower residues while still maintaining quality.

## 7.4 **EXTENSION - KEY EVENTS**

### 7.4.1 **CRI Symposium**

As gevolg van die wêreldwye COVID-19 pandemie en gepaardgaande beheermaatreëls het dit onmoontlik geword om die Sitrusnavorsings simposium gedurende Augustus aan te bied, soos beplan. Na deeglike oorweging is besluit om die simposium uit te stel tot Augustus 2021. Gelukkig was al die hoofborge te vinde daarvoor dat die simposium tot 2021 uitgestel word, maar voel dat hulle die voortdurende interaksie met die bedryf wil behou en is dankbaar dat dit wel in 2021 sal plaasvind.

Daar is vir elke onderwerp wat by die simposium aangebied sou word, 'n twee-bladsy opsomming opgestel en aan die bedryf uitgestuur aan die begin van September.

### 7.4.2 **CRI Biosekuriteit/Voorligting vergroening-opleiding**

CRI Biosekuriteit en Voorligtings personeel het Sitrusvergroeningsiekte simptome en vektor moniterings opleiding aan DALRRD personeel in die Suid-Kaap aangebied. Opleiding is in samewerking met produsente en chemiese verteenwoordigers op verskeie plase en kultivars gedoen. Boorde met vergroening is geïdentifiseer en tipiese blaar- en vrugsimptome is aan studente uitgewys, asook nie-tipiese blaarsimptome. Moniterings riglyne en korrekte uithang en afhaal van geel 'ACP sticky traps' is ook gedemonstreer. Opleiding

is opgevolg met praktiese identifikasie en geel valletjie uithang en afhaal prosesse. Teen die einde van die week kon studente 80% akkurate visuele vergroeningssimptome identifiseer. Opvolg opleiding is wel nodig in die toekoms

#### **7.4.3 IPM en Scouting opleiding met LAC Nelspruit**

Laeveld Agrochem in Nelspruit het nuwe ontwikkelinge in hulle tegniese afdeling wat Scouting bied vir hulle kliënte. Dit is belangrik vir CRI om diensverskaffers in die bedryf tegniese ondersteuning te gee, wat sal verseker dat die regte beginsels toegepas word en goeie samewerking in die bedryf verseker.

#### **7.4.4 CRI Werkswinkels**

Die jaarlikse CRI Geïntegreerde Plaagbestuur & Siektebestuur werksinkels is gedurende Augustus virtueel aangebied. Sprekers het hul aanbiedinge vooraf opgeneem, waarna dit op die CRI-website gelaa is vanwaar produsente dit kon aflaai en deurwerk. Gedurende die eerste week van September is lewendige vrae- en antwoord sessies virtueel aangebied, waartydens produsente kon inskakel vir vrae en insette. Hoewel die terugvoer oor hierdie manier van tegnologie oordrag as 'n noodmaatreël goed was, is meeste wel van mening dat dit nie 'n plaasvervanger vir die normale werksinkels kan wees nie. Onderwerpe kan nie so wyd en deeglik gedek word soos by normale werksinkels nie, en die gebrek aan interaksie tussen sprekers, produsente en borge laat 'n groot leemte.

#### **7.4.5 CRI Postharvest Workshops 2021**

The planning meeting for the 2021 CRI Postharvest Workshops was forced to be held online as several participants were unable to travel. Topics were discussed and an agenda laid out with the intention to hold live workshops as in the past. A survey conducted amongst previous workshop attendees indicated that the majority would prefer live meetings. However, with increases in infections, and stricter lockdown measures, the difficult decision to move the workshops into a one day webinar was taken.

Die CRI Na-oes werksinkels vir 2021 is met 'n webinar van drie sessies vervang. Die aantal onderwerpe moes aansienlik verminder word en aanbiedinge van maksimum 15 minute elk van die mees belangrikste onderwerpe is aangebied. Ten spyte van die feit dat dit 'n eerste webinar was wat deur CRI aangebied is, het dit goed afgeleef. Die terugvoer was goed, hoewel 'n opname toon dat deelname na raming slegs die helfte van die fisiese werksinkels se bywoning verteenwoordig. Meer as 60% van die persone wat wel aan die peiling deelgeneem het, het aangetoon dat hulle die fisiese werksinkels verkies.

#### **7.4.6 Packaging Working Group meeting**

The Packaging Working Group, co-ordinated and chaired by Dawid Groenewald, held their annual meeting during November at the Sappi Technology Centre in Pretoria. The meeting was well attended by all the carton- and paper manufacturers, with excellent co-operation and interaction. It was a very difficult season, but they were complimented by the chairman for their outstanding performance during these challenging times.

#### **7.4.7 Exporters Technical Panel and Cooling Working Group meeting**

The ETP meeting and Cooling Working Group meeting was combined into one meeting this year. Despite the pandemic, the meeting was very well attended with very good input and discussions by the attendees. The venue must be complimented for their excellent arrangements, especially to ensure that the meeting fully complied to Covid regulations.

#### **7.4.8 Integrated Pest Management Course**

By default, strict market requirements for MRL's and phytosanitary measures require more intensive management of citrus pests and more growers are introducing biological control options in their pest control programmes. This has brought about a need for training on integrated pest management (IPM). CRI presented

an IPM course for new farm managers in Letsitele. This was one of the first IPM information sessions CRI has presented in quite a few years and was very well received. There have been requests for this course in more growing regions and we expect to present more courses in future.

## 7.5 **PRODUKSIESTREKE**

### 7.5.1 **Suidelike Produksiestreek**

Die pakseisoen in die suidelike produksiestreke het suksesvol ten einde geloop en word algemeen as 'n baie suksesvolle seisoen beskou. Oeste van baie goeie gehalte is vir 2020 in die suidelike gebiede gerapporteer, met goeie uitpakpersentasies. Uitvoere na die VSA het rekordhoogtes bereik, so ook totale uitvoere wat rekordhoogtes bereik het.

Pakhuse moes enorme uitdagings t.o.v Covid-19 trotseer, maar kon uiteindelik die oes suksesvol verpak. As gevolg van die goeie aanvraag na sitrus was die mark sterk en vrugte het vinnig verkoop. Min eise a.g.v vrugkwaliteit en bederf is uit die markte ontvang. Meeste van die probleme wat normaalweg in eise sou eindig, is a.g.v die sterk vraag na sitrus deur die mark self geabsorbeer.

Verskeie plaë is toenemend besig om problematies te word in meeste produksiegebiede in die suide. Veral blaaspootjie en witluis skep probleme, asook Australiese wolluis wat in getalle toeneem. Die beskikbaarheid van chemiese produkte om siektes en plaë effektief te beheer, sonder om MRL probleme te veroorsaak, raak ook meer van 'n probleem.

Vrugset vir die 2021 seisoen was beter a.g.v koel weer vroeg in die blom- en vrugset periode. Die toename in oes in die suide is hoofsaaklik a.g.v nuwe aanplantings wat in drag kom en jong aanplantings wat groter oeste oplewer. As gevolg van die koel tot koue weer is vruggrootheid oor die algemeen effens kleiner in feitlik al die produksiestreke in die suide. Verskeie gevalle is opgemerk waar suurlemoene se blommetjies nie vrugbeginsels vorm nie, en dan afspeen. Algemeen wil dit voorkom of suurlemoene in die Suid- en Wes-Kaap 'n laer oes gaan lewer in 2021.

Ernstige watertekort a.g.v die droogte staar die Gamtoos-rivier vallei in die gesig, en dit het nou kritiese afmetings aangeneem. Daar is groot onsekerheid oor wat die finale uitvoer-volume gaan wees.

Valskodlingmot-getalle was effens hoër as normaal vroeg in die 2021 seisoen in dele van die Oos-Kaap. Dit wil voorkom of die oes-seisoen so 10 tot 14 dae later as verlede seisoen gaan wees.

### 7.5.2 **Sentrale Produksiestreek**

Covid-19 het 'n groot uitdaging aan produsente gestel t.o.v implimentering van Covid maatreëls asook vervoer reëlins van plukkers en pakkers na plase en pakhuse. CRI se nasionale plan het dit moontlik gemaak dat personeel kon werk gedurende hierdie moeilike tydperk. Alhoewel beweging beperk was, is daar steeds aan produsent behoeftes aandag geskenk. Tegnologie is gebruik om vergaderings te fasiliteer en beplanning te doen asook om produsente en buite organisasies van hulp te wees.

Vroeë reën in die lente het die blom oor die algemeen gesinchroniseer en 'n meer egalige en goeie blom is geset. Uitpak persentasies by meeste van die kultivars was vêr bo 80%. Meeste produsente/pakhuse het tot drie weke vroeër as normaal hul verpakking gefinaliseer. Uitstekende uitpakte is behaal en oor die algemeen is daar min probleme uit die markte gerapporteer. Uitstekende pryse is ontvang vir vroeë suurlemoen kultivars.

Scout en moniterings opleiding is aangebied in die Nelspruit gebied. Witluis en VKM beheermaatreëls is ondersoek by sekere plase wat na Korea uitvoer; uit die ondersoek blyk dit dat produsente wel die protokolle streng nagevolg het. Witluis en bladspringer skade op mandaryne en sekere lemoen kultivars het toegeneem sowel as die populasie getalle wat toegeneem het in sekere boorde. Die beheer daarvan gedurende die somer maande was 'n groot uitdaging. Rooidopluis populasies begin op sekere plase toeneem; opskerping van monitering en beheer is nodig. Vrugtevlug en valskodlingmot druk was laer as vorige seisoene. Blaaspootjie

druk was laag tydens lente periode maar witluis en blaaspootjie getalle het wel gedurende Desember begin toeneem.

Die verwagte oesskattings vir die 2021 seisoen lyk belowend en die drag in meeste boorde mag dieselfde tot effens hoër wees as die vorige jaar. Die algemene vrug grootte verspreiding op meeste kultivars blyk kleiner te wees weens die grootte van die oeslading maar die laat Januarie/Februarie reëns kan moontlik 'n noemenswaardige verbetering tot gevolg hê. Bogemiddelde reënval vanaf einde Januarie tot vroeg Maart het damvlakke dramaties laat styg. Die Kwêna dam wat Nelspruit, Malelane en Komatipoort areas voed was begin Januarie slegs 23%, maar het wel later die 100% perk bereik.

Vrugkleur is in meeste gevalle vroeër en dit wil voorkom of 'n meer egalige kleur gedurende die 2021 seisoen voorkom. Die stikstofvlakke is laer in baie Laeveldse boorde, waarskynlik weens die hoër reënval en minder hael wat gedurende die seisoen voorgekom het. Die interne kwaliteit tot dusver is baie goed, behalwe indien laat reëns dalk gaan voortduur. Midnight op growweskil in die Nelspruit area mag dalk nie die standaard haal weens die baie reën nie.

Vrugtevlug en valskoddingmot-getalle is effens hoër as normaalweg, maar Bactrocera getalle is uitermatig hoog. Produsente het daadwerklik ingespring met bespuitings om getalle onder beheer te bring. Groenstinkbesies het sporadies in Groblersdal/Marble Hall, Ohrigstad/ Burgersfort en Nelspruit produksie areas voorgekom. Skade is tans beperk tot enkele boorde, hoofsaaklik mandaryne. Witluis getalle het teen einde Desember en begin Januarie begin kop uitsteek in enkele boorde. Die insidensie was wel laer as vorige seisoene maar die boorde met probleme was wel erg geaffekteer. Rooimyt voorkoms is wel laer maar rooidopluis in sekere boorde blyk steeds 'n probleem te wees. Bladspringer skade en getalle begin toeneem in meeste boorde en dringende aandag aan beheermaatreëls is nodig om skade tot die minimum te beperk.

### 7.5.3 Noordelike Produksiestreek

The Level 5 lockdown initiated on 26 March by the South African government forced all enterprises to seriously adjust their operations and CRI was no different. Most individuals and companies grasped the use of technology using online meetings and conferences where needed. Positives included shorter and more focussed meetings, reduction in travel costs and ultimately the health and safety of all.

A well devised action plan was drafted by CRI which ensured staff in Research and Extension could continue to work and travel to see growers and packhouses, which was limited to essential on-site visits. Between April and June, technical support was provided to farms and packhouses, packaging trials were conducted in Letsitele and training on IPM and scouting took place.

In die Noorde was daar heelwat minder druk op die oes en verpakking van sitrusvrugte. As gevolg van droogte het produsente in Letsitele nog boorde uitgehaal, of afgesaag vir oorwerking. Die oeslading op draende bome was ook matig gewees en vir hierdie redes was daar heelwat minder vrugte om te pak. Ooral in die Noorde was daar suurlemoenblomme wat nie reg ontwikkel het nie. Hierdie ongewone simptome kan beskryf word as 'n blom sonder enige vrugbeginsel. Daar is ongelukkig nie baie literatuur hieroor nie en hopelik is dit seisoengebode.

Nuwe aanplantings van sagtesitrus in die Capricorn gebied neem toe, hoofsaaklik deur nuwe sitrus produsente en voorligting oor sitrusproduksie word meer en meer aangevra.

Snoei demonstrasies was by verskeie maatskappye in die Letsitele gebied afgelê. Die aanvraag het gekom van groot maatskappye om verskillende snoeipraktyke te bespreek en fisies in die boord te snoei sodat die plaasbestuurders kan sien wat verwag word van verskillende snoei metodes.

As ons kyk na die laaste gedeelte van die seisoen kan produsente tevrede wees met min skade a.g.v peste en plaeg op vrugte, en vrugkleur was hierdie seisoen minder van 'n probleem op Valencias, alhoewel "re-greening" op die laat valencias het nog steeds impak gehad op uitpakte. Hier en daar was laat blaaspootjieskade op vrugte waargeneem.

Na die goeie voorkoms van blom van die komende 2021 seisoen was produsente baie opgewonde oor die vroeë reën wat geval het einde September. Die uitkoms was meer 'n matige vrugset in Oktober in die Noorde. By sekere variëteite het vrugte heelwat afgespeen, veral Pomelos. Oor die algemeen is produsente tevrede met die drag op die bome. Boorde wat verlede seisoen uit produksie gehaal is, is weer terug in produksie.

CRI Voorligting het saam met Biosekuriteit afdeling die inisiatief op die been gebring om monitoring van ACP in die Noordelike gebied te doen. Daar word nog steeds klem geplaas op produsente om hulle eie monitoring te doen.

In October CRI presented an IPM course for farm Managers in Letsitele. This was one of the first IPM information sessions CRI has presented in quite a few years and was very well received. There have been requests for this course in more growing regions and we expect to present more courses in future. Strict market requirements for MRL's and phytosanitary measures require more intensive management of citrus pests and more growers are introducing biological control options in their pest control programmes. This has brought about a need for training on integrated pest management (IPM).

Witluisbeheer op die vroeë variëteite was bietjie van 'n uitdaging wat die bestuur van MRL'e aan betref, asook die korter tydperk vir parasiete en predatore om te werk. IPM bestuur word al hoe belangriker. Blaaspootjie getalle in Hoedspruit en Letsitele was nogals hoog gewees gedurende November en vroeg Desember. Dit was interessant dat die voorkoms van blaaspootjie nie noodwendig gekoppel was aan blaaspootjie-skade op die vrugte nie.

Die vooruitsig was dat meer vrugte gedurende 2021 gepak sal word as die vorige seisoen, al was die drag laer in baie gevalle. Nuwe aanplantings en boorde wat weer in produksie gekom het na die droogte het bygedra tot 'n verhoging in die aantal kartonne. Die sitrus blomperiode het omtrent twee weke vroeër as normaal gebeur. Die suurlemoen blom was baie uitgerek in Hoedspruit as gevolg van die baie reën wat in April 2020 geval het. Daarom is die eerste suurlemoene al in Desember ge-oes. Die reënval in Jan/Feb het baie vertraging op die suurlemoen-oeste gehad en die voorkoms van oleo was ook problematies.

Valskodlingmot lokvalvangste in Letsitele was hoër gewees in die begin van 2021 in vergelyking met verlede jaar die tyd. Terugvoer was dat larwes vroeg ook opgespoor is in die Turkey Valencias. Maar dit is nog steeds 'n bekommernis vir produsente dat hulle minder VKM in boorde identifiseer as wat by die pakhuis reflekteer. Voorligting het die Biosekuriteit-span ondersteun met bietjie bewusmaking oor die belangrikheid van monitoring vir ACP. CRI het ekstra lokvalle uitgehang en het ook in Hoedspruit begin moniteer. Verder het produsente in Hoedspruit en Letsitele hand opgesteek om te begin moniteer en lokvalle sal maandeliks deur CRI gelees word.

**Opsomming van aktiwiteite vir periode April – Junie 2020**

Datum	Studiegroep/Aktiwiteit	Onderwerpe/Aksies	Betrokkes/ Sprekers
9 Apr 20	Extension Meeting	Video Conference	Wayne Mommsen MC Pretorius Liezl Van der Linde Catherine Savage Hannes Bester Dawid Groenewald
9 Apr 20	Extension Meeting	Video Conference	Wayne Mommsen MC Pretorius Liezl Van der Linde Catherine Savage Hannes Bester Dawid Groenewald

16 Apr 20	Plaas besoek en advies	De Wagendrift Boerdery – groen vrugte en suurvrot beheer in boorde	MC Pretorius Pieter Nel
23 Apr 20	Indulini: Gustav Bell	Vruggehalte	Hannes Bester
24 Apr 20	Farm visit and advice	Letaba Estates Hail damage and severe Sour rot	Wayne Mommsen Harry Grove Johan Botma Johan Van Zyl Billy Van Staden
30 Apr 20	Video Conference	Indigo Farms IPM planning discussions	Wayne Mommsen Andrew Cooper Sakkie Bruwer Adriaan Serfontein
5 May 20	Farm Visit	Letsitele: CBS Fruit collections for DALRRD Training	Wayne Mommsen Harry Grove
8 May 20	Farm Visit	Gubitz Yanetsi: Pruning demonstration and advice	Wayne Mommsen Farm Manager-Renier
15 May 20	Farm Visit	Mahela Trial Block: Mandarin Cultivar Evaluation before harvest	Wayne Mommsen Johan Gubitz Paul Gubitz Henk Van Rooyen
18-20 May 20	Packhouse and farm visits	Investigate Packhouse Monitoring for Mealybug Hoedspruit, Malelane and Letsitele	Wayne Mommsen MC Pretorius
25 May 20	Augrabies: Augrabies Bdy Kakamas: Zwartbooisberg	Snoeidemonstrasie en oesmanipulasie	Hannes Bester
26 May 20	Sappi Technology Centre	Beplanningsvergadering om weer te begin met toets van kartonne	Donald Nonyane Dawid Groenewald
26 May 20	Marchand: Krismar Bdy JH Retief Bdy Augrabies Kwekery Loveren Bdy	Snoei, oesmanipulasie en algemene praktyke	Hannes Bester
27 May 20	Kakamas: Karsten Bdy Augrabies: Groenheuwel Bdy	Snoei en oesmanipulasie	Hannes Bester
2 Jun 20	Ohrigstad Studiegroep Tegniese vergadering	Mahela Smutsfield raadsaal: IPM en bemestings beplanning	MC Pretorius Eddie Vorster Carl Fourie Desi Fourie Lezel Beetge Dewald Kamffer Dick Marinus Neetling Karlien Grobler Albert Winterbach

4 Jun 20	Studiegroep Technical meeting	Mahela Boardroom: IPM planning meeting	Wayne Mommsen Eddie Vorster Carl Fourie Desi Fourie Lezel Beetge Gerhard Vorster Dewald Kamffer Kobus Van Heerden
9 Jun 20	IPM and Scout Training Nelspruit	LAC Technical Division	Wayne Mommsen MC Pretorius
9 Jun 20	IPM and Scout Training Nelspruit	LAC Technical Division	Wayne Mommsen MC Pretorius
11 Jun 20	Farm Visit Letsitele	FCM identification and packhouse grading	Wayne Mommsen
15 Jun 20	Farm Visit Letsitele	Fruit Burn Investigation	Wayne Mommsen
16 Jun 20	Video Conference	CIS - soilborne	MC Pretorius Paul Fourie Jan v Niekerk Elaine Basson
17 Jun 20	Houers Koöperatief, Letsitele	Beplanning en vervaardiging van 150g/m <sup>2</sup> Ultraflute Plus eksperimentele kartonne	Wimpie Mostert Frikkie van Wyk - Houers Dawid Groenewald
18 Jun 20	Houers Koöperatief, Letsitele	Vervaardiging van bogenoemde kartonne en trek van monsters vir laboratorium toetse	Wimpie Mostert Frikkie van Wyk – Houers Dawid Groenewald
19 Jun 20	Sappi Technology Centre	Laai monsters af en bespreek die toetse wat gedoen moet word.	Donald Nonyane Dawid Groenewald
24 Jun 20	Video Conference	Biosekuriteits vergadering – opleiding en opnames en beplanning vir uithang van geel valletjies.	MC Pretorius Elma Carstens Wayne Kirkman

**Opsomming van aktiwiteite vir periode Julie – September 2020**

Datum	Studiegroep/Aktiwiteit	Onderwerpe/Aksies	Betrokkes/ Sprekers
6/7 Jul 20	Houers en DNB Packers in Letsitele	Pak van A15C eksperimentele kartonne	Jacques Nel Frikkie van Wyk Dawid Groenewald
15 Jul 20	Sappi Technology Centre	Toets van “A11D” kartonne	Laboratorium personeel Dawid Groenewald
15-17 Jul 20	Noordlike Grens Plaasbesoeke	Biosecurity – ACP lokvalle uitsit vir monitering	Wayne Kirkman Xolani Sibiyi Wayne Mommsen
17 Jul 20	Nelspruit	Grobbersdal produsente – Midnight boord besoeke	MC Pretorius James Warrington
22 Jul 20	Nelspruit	Nadorcott probleem boord besoek	MC Pretorius Chris Kellerman

			Johan Joubert
22 Jul 20	Swellendam: vergadering en boordbesoeke: Sarel Neethling & Cornelis van Tonder	Vergroening	Hannes Bester Hanno Maree Rachel Bester
23 Jul 20	Swellendam: boordbesoeke: Jacques Franken Piet van Deventer	Vergroening	Hannes Bester Hanno Maree Rachel Bester
26 Jul 20	Voorligting – Zoom vergadering	IPM werkswinkel beplanning	Hannes Bester MC Pretorius Wayne Mommsen Catherine Savage Liezl v d Linde Dawid Groenewald
27 Jul 20	Farm Track Zoom vergadering: Johan De Swardt Johan Botha Suné Joubert	GPS tegnologie vir vrugteverbouing	Hannes Bester
28 Jul 20	Hoedspruit Pakhuisbesoek en Produksie bespreking	“Automation” in die pakhuis en Cultivars	Wayne Mommsen Gert Bezuidenhout Coenie Scheepers
29 Jul 20	CFB	Snoei van saadbronne	Hannes Bester Paul Fourie Jacolene Meyer Johané Nieman
4 Aug 20	CRI Manco meeting	Agenda	Vaughan Hattingh Hannes Bester MC Pretorius Wayne Mommsen Tim Grout Christine Stoppel-Grove Jon Pinker Sean Moore Paul Cronje Jan van Niekerk
6 Aug 20	Patensie: Rikus Du Preez	Boordbesoeke: Snoei Wortelvrot Oesmanipulasie	Hannes Bester
12 Aug 20	Voorligting – Zoom vergadering	CRI App vergadering	Hannes Bester MC Pretorius Wayne Mommsen Catherine Savage Liezl v d Linde Dawid Groenewald
14 Aug 20	Polokwane Plaasbesoek	Probleemboorde met Phytophthora - aanbevelings	Wayne Mommsen Jan De Klerk Manie Beytell
20 Aug 20	Barberton	Probleem boordbesoeke	MC Pretorius Tian Schutte Schalk Schoeman

20 Aug 20	Pretoria: Jan Botha	Nuwe aanplanting	Hannes Bester
21 Aug 20	Letsitele: Boordebesoeke: Johan Gubitz Kallie Erasmus Paul Gubitz	Snoei en oesmanipulasie	Hannes Bester
24 Aug 20	Letsitele Plaasbesoeke: Groep 91 Henk v Rooyen	Snoei en oesmanipulasie	Hannes Bester Wayne Mommsen
25 Aug 20	CRI Board meeting	Agenda	Hannes Bester Vaughan Hattingh Tim Grout Jon Pinker
	Letsitele boordbesoeke: Letaba Landgoed	Snoei en oesmanipulasie	Hannes Bester Wayne Mommsen
26 Aug 20	Letsitele boordbesoeke: Mahela Bdy Laeveld Sitrus	Snoei en oesmanipulasie	Hannes Bester Wayne Mommsen
27/28 Aug 20	Houers en Gubitz pakhuis in Letsitele	Pak van eksperimentele A15C kartonne.	Nicolaas Gubitz Frikkie van Wyk Dawid Groenewald
27 Aug 20	Ohrigstad	Produsent besoeke – probleem boorde en beplanning voor seisoen: Smit le Roux, Cobus Beetge, Mahela	MC Pretorius Johan Joubert
31 Aug 20	Stellenbosch: Live MS Teams sessions	CRI IPM & DM MS- Teams Q&A sessions	Hannes Bester Sean Moore Tim Grout Wayne Kirkman Aruna Manrakhan Jan van Niekerk Providence Moyo
	FarmProf: Jan van der Merwe Johannes Cilliers	Blaarsap-analises	Hannes Bester Pieter Raath
1 Sept 20	Stellenbosch: Live MS Teams sessions	CRI IPM & DM MS- Teams Q&A sessions	Hannes Bester Sean Moore Tim Grout Wayne Kirkman Aruna Manrakhan Jan van Niekerk Providence Moyo
	MS Teams vergadering	CRI en CGA App	Hannes Bester Liezl vd Linde Paul Hardman Portia
2 Sept 20	Stellenbosch: Live MS Teams sessions	CRI IPM & DM MS- Teams Q&A sessions	Hannes Bester Sean Moore Tim Grout Wayne Kirkman Aruna Manrakhan Jan van Niekerk Providence Moyo

2 Sept 20	Sappi Technology Centre	Toets van kartonne en bepaling van basiese massa van papier	Laboratorium personeel Dawid Groenewald
3 Sept 20	Letsitele Scout Opleiding	Opleiding van nuwe scouts, Alesia Bdy.	Wayne Mommsen
31 Aug – 4 Sept 20	Suid Kaap - Swellendam	DALRRD vergroenings opleiding	MC Pretorius Wayne Kirkman Hano Maree
4 Sept 20	Tzaneen - Fasiliteite besoek	Insect Science Nuwe Laboratorium besoek	Wayne Mommsen Martin Van Niekerk Johan Visage (LAC)
10 Sept 20	Tom Burke/Baltimore boordbesoeke	Probleemboorde, suurlemoene met steriele blom/geen drag	Wayne Mommsen Rudolph Kruger Braam Du Plessis Fanie Schoeman
14 Sept 20	Laeveld Agrochem: Ernst De Beer	Uitruil van tegniese inligting	Hannes Bester
16 Sept 20	Letsitele Plaasbesoek	Snoei demonstrasies	Wayne Mommsen Fanie Schoeman Renier Ouwenkamp Albie Gotze Jaco Lindeque
22 Sept 20	Nelspruit – Online	Siza Environmental Audit Training Webinar	Catherine Savage
22 Sept 20	WorldFocusAgri: At van Schalkwyk Willem Venter	Nuwe grondkundige produkte	Hannes Bester
23 Sept 20	Nelspruit	Voorligting jaarbeplanning en begrotings vergadering	Hannes Bester MC Pretorius Wayne Mommsen Catherine Savage Liezl vd Linde Dawid Groenewald
28 Sept 20	Pretoria: Dawid Groenewald	Een-tot-een gesprekke	Hannes Bester Dawid Groenewald
29 Sept 20	Nelspruit: MC Pretorius	Een-tot-een gesprekke	Hannes Bester MC Pretorius
	Chris Kellerman	SASCCON aangeleenthede	Hannes Bester
30 Sept 20	Nelspruit: Liezl vd Linde Catherine Savage	Een-tot-een gesprekke	Hannes Bester Liezl vd Linde Catherine Savage
	James Warrington	Oesmanipulasie	Hannes Bester

**Opsomming van aktiwiteite vir periode Oktober – Desember 2020**

Datum	Studiegroep/Aktiwiteit	Onderwerpe/Aksies	Betrokkenes/Sprekers
1 Okt 20	World Focus Agri: Willem Venter	Bemestingsprodukte	Hannes Bester
2 Okt 20	SIZA meeting	Understanding the services provided	Catherine Savage

5 Okt 20	IPM Research Committee meeting	Vetting of research projects	Tim Grout Hannes Bester Sean Moore
6 Okt 20	Disease Management Research Committee meeting	Vetting of research projects	Tim Grout Hannes Bester Jan van Niekerk Paul Fourie
7 Okt 20	Cultivar Evaluation Committee meeting	Vetting of evaluation trials	Tim Grout Hannes Bester Paul Cronje Johan Joubert Werner Swiegers
7 Okt 20	Onderberg	Produsent besoeke: GFC + Radley	MC Pretorius
8 Okt 20	Moorland Kwekery: Rian Moor Ri-Ellen Kemp	Boomstandaarde	Hannes Bester
9 Okt 20	CFQM Research Committee meeting	Vetting of research projects	Tim Grout Hannes Bester Paul Cronje Pieter Raath Tarl Berry
13 Okt 20	Besoek Letsitele Produsente	"Soil Classification" Profielgate	Wayne Mommsen Pieter Raath Vivian White
14 Okt 20	Letsitele Junction	IPM Kursus	Wayne Mommsen Sean Moore
15 Okt 20	Letsitele Junction	Studiegroep: FMS werkswinkel	Wayne Mommsen Sean Moore
15 Okt 20	Winterton: CSR	Symposium meeting	Hannes Bester
	Winterton: Leon vd Merwe	Vestiging van nuwe sitrus-aanplanting	Hannes Bester
19 Okt 20	FMS Working Group meeting	Veranderinge aan die FMS vir 2021	Vaughan Hattingh Hannes Bester Sean Moore Paul Cronje Tarl Berry Elma Carstens
22 Okt 20	CRM meeting	Introduction and understanding CRM for CRI	Eugene Beetge Hannes Bester Liezl vd Linde Catherine Savage Elaine Basson Jon Pinker Tshidi Ramabu
26 Okt 20	CRM meeting	Follow up on finer details of CRM	Eugene Beetge Hannes Bester Liezl vd Linde Catherine Savage
28 Okt 20	CMF meeting	Agenda	Hannes Bester
5 Nov 20	Nelspruit Landbou Unie - jaarvergadering	CRI verteenwoordig by jaarlikse SA Landbou Unie – Nelspruit tak. Komati Croc Valley	MC Pretorius Johan Joubert

5 Nov 20	Global Citrus Congress Live	Online congress of the WCO	Catherine Savage
6 Nov 22	Innovation Hub, Pta.	Vergadering met Everest Packaging	Brian Seggar, Deon Zwanepoel      Dawid Groenewald
9 Nov 202	Plaasbesoek      Uitdraai Boerdersy Polokwane	Ondersoek, Phytophthora wortel vrot, suurlemoene	Wayne Mommsen Jan De Klerk Manie Bytell
10 Nov 20	Pretoria: Packaging Working Group meeting	Agenda	Dawid Groenewald Hannes Bester Tarl Berry
10 Nov 20	Plaas/Kwekery besoeke	Tarlton/Brits	Wayne Mommsen
11 Nov 20	CRI Na-oes Werkswinkels Beplanningsvergadering	Agenda	Hannes Bester Dawid Groenewald Catherine Savage Wayne Mommsen Wilma Du Plooy Tarl Berry Liezl vd Linde Jan van Niekerk
11 Nov 20	Postharvest workshop presentation planning	Postharvest practices session	Catherine Savage Wilma Du Plooy Jan van Niekerk Liezl vd Linde
11 Nov 20	Letsitele Junction	Splat inligting Sessie RB XSIT/Wenkem	Wayne Mommsen Sean Thackery Rob Elfick Richard Schulze
12 Nov 20	DALRRD Citrus Coordinating meeting	Agenda – post season feedback	Catherine Savage
17 Nov 20	Letsitele Junction	Studiegroep	Wayne Mommsen
18 Nov 20	CRI Board meeting	Agenda	Vaughan Hattingh Hannes Bester Tim Grout Jon Pinker
19 Nov 20	Schoemanskloof: Moontlike Viroied probleem ondersoek	Ent oordraagbare siekte span na boord vergesel – Nadorcot viroied probleem	MC Pretorius Glynnis Cook Kobus Breytenbach
17 – 19 Nov 20	Postharvest Symposium Webinar	3 sessions of the online version of the New Zealand Postharvest Symposium cancelled for 2020	Catherine Savage
23 + 24 Nov 20	Ohrigstad en Burgersfort	Produsent boord besoeke: Probleem boorde en nuwe kwekery boom probleem	MC Pretorius Johan Joubert
24 Nov 20	Jan van Niekerk	Navorsing en voorligting aangeleenthede	Hannes Bester Jan van Niekerk
	Ashanti: Exporters Technical Panel meeting	Agenda	Hannes Bester Dawid Groenewald

			Vaughan Hattingh Paul Cronje Wilma du Plooy Tarl Berry
	Vaughan Hattingh	Een-tot-een vergadering	Vaughan Hattingh Hannes Bester
	Teunis Vahrmeijer	Bemestings- aanbevelings van Agritechnovation	Hannes Bester
24 Nov 20	Hoedspruit	Studiegroep	Wayne Mommsen
25 Nov 20	ICA: Wouter Schreuder Kobus Serfontein	CRI Na-oes webinar Borgskap	Hannes Bester
	Out of the Blue: Arina Vermeulen	Na-oes webinar	Hannes Bester
	FarmTrack: Suné Joubert Johan De Swardt Johan Botha	GPS tegnologie in landbou	Hannes Bester
	Pieter Raath	Bemestings-handleiding en bemestings-kursus	Hannes Bester Pieter Raath
26 Nov 20	Nelspruit area	Produsent besoeke: Komati, Friedenheim en Karino Pakhuis	MC Pretorius James Warrington
26 Nov 20	Burgersfort CRI Entomoloëë	Witluis inseksie Onder net en buite net.	Wayne Mommsen Sean Moore Evans Mauda Martin Hill
30 Nov en 1 Des 20	Houers, Letsitele	Vervaardiging van addisionele A15C kartonne met 150UF+	Wimpie Mostert, Frikkie van Wyk Dawid Groenewald
2 Des 20	Sappi Technology Centre	Toets van A15C 150UF+ kartonne en samesprekings oor 2021 laboratorium toetse	Sappi bestuur. Dawid Groenewald
2 Des 20	Tom Burke Boordbesoek	Witluis bestuur op Vroeë suurlemoene	Wayne Mommsen
2 – 3 Des 20	Pongola produsente besoek Laeveld + Scout opleiding Nelspruit en onderberg	Pongola produsente vergesel na Onderberg, plase en pakhuis asook Karino Kooperasie. Ondersteun Wayne Mommsen met scout opleiding	MC Pretorius Chris Kellerman Wayne Mommsen
3 Des 20	Nelspruit gebied	Scout Opleiding	Wayne Mommsen MC Pretorius Xolani Sibiyi
8 Des 20	Pontdrift CRI Entomologie	Witluis monsterneming vir identifikasie	Wayne Mommsen Evans Mauda
9 Des 20	CGA-CRI App meeting	Integration and design of a CGA-CRI app	Hannes Bester Liezl vd Linde Catherine Savage Paul Hardman

10 Des 20	CGA-CRI app and CRM system meeting	Meeting with Eugene Beetge	Hannes Bester Liezl vd Linde Catherine Savage Paul Hardman Eugene Beetge
14 Des 20	Onderberg	Komati Kwekery besoek	MC Pretorius
14 Des 20	Eiland Konferensiesaal Letsitele Produsente en bemesting konsultante	Bespreking oor Bemesting App.	Pieter Raath Vivian White Wayne Mommsen
15 Des 20	Hoedspruit	Produsente Besoek	Wayne Mommsen
17 en 18 Des 20	Groblersdal	Produsent besoeke	MC Pretorius

**Opsomming van aktiwiteite vir periode Januarie – Maart 2021**

Datum	Studiegroep/Aktiwiteit	Onderwerpe/Aksies	Betrokkenes/Sprekers
11 Jan 21	Onderberg/ Burgersfort	Vroeë suurlemoen oes versoek vanaf die streke – (Inligting versamel: VH en EC aan DALRRD)	MC Pretorius Chris Kellerman James Warrington Andrew Cooper - Indigo
13 Jan 21	Nelspruit	Fruitspec – Deon Pelser oesskattinge en vruggrootte	MC Pretorius
14 Jan 21	Nelspruit	NSSA meeting – symposium planning	MC Pretorius NSSA executive body
14 Jan 21	Citrosol Web training	Na-oes siektebeheer	Hannes Bester Catherine Savage
14 Jan 21	Orchard Visits Burgerfort	Nadorcotte orchards with high Mealybug infestation	Wayne Mommsen
14 Jan 21	Planning meeting Re: Mealybug and Korea Market	CRI, PPECB, DALRRD	Elma Carstens Vaughan Hatting Sean Moore Wayne Mommsen
15 Jan 21	Voorligting beplanning	Agenda	Hannes Bester MC Pretorius Catherine Savage Wayne Mommsen Dawid Groenewald Liezl vd Linde
18 Jan 21	Nelspruit	HLB – safe systems workshop	MC Pretorius Solomon Gebeyehu Vaughan Hatting Paul Fourie Elma Carstens Wayne Kirkman Wayne Mommsen Aruna Manrakhan
21 Jan 21	Korea VC	Agenda	Hannes Bester MC Pretorius Wayne Mommsen Catherine Savage
	Citrosol Webinar	Na-oes siektebeheer	Hannes Bester

25 Jan 21	Nelspruit	AFFL maatskappy: CBS produk + watersuiwering in pakhuis. werkswinkel	MC Pretorius Wilma du Plooy Providence Moyo
26 Jan 21	CRI Postharvest webinar	Agenda	Hannes Bester MC Pretorius Catherine Savage Wayne Mommsen Dawid Groenewald Liezl vd Linde
27 Jan 21	Noord-Kaap: Orania	Nuwe sitrus-aanplanting	Hannes Bester Phillip Dempsey
27 Jan 21	Groblersdal	Produsent besoeke + witluis opname vir SM. Schoonbee, Rosle, A Creyhton, Hertzog broers	MC Pretorius
28 Jan 21	Hartswater: Oase kwekery	Boomgehalte	Hannes Bester Phillip Dempsey
1 Feb 21	Sappi Tech Centre	2021 Akkreditasie toetse	Sappi Bestuur Dawid Groenewald
1 Feb 21	CSR Zoom meeting	Simposium	Hannes Bester
3 Feb 21	Orchard Visits Tzaneen	Soft citrus Alternaria control	Wayne Mommsen
4 Feb 21	Citrosol webinar	Na-oes siektebeheer	Hannes Bester Catherine Savage
8 Feb 21	PE: Vaughan Hattingh	CRI 4Y Plan	Vaughan Hattingh Hannes Bester
8 Feb 21	Onderberg	Komati Fruit besoek + Paul Cronje navorsings persele en bespreking	MC Pretorius Paul Cronje Marinus Neetling Karlien Grobler 5 Plaasbestuurders en 3 Pakhuisbestuurders
9 Feb 21	Nelspruit	CBS meeting – Benlate sprays, spray progammes	MC Pretorius Elma Carstens Jan v Niekerk Providence Moyo
10-11 Feb 21	XSIT workshop	Agenda	Vaughan Hattingh Hannes Bester Sean Moore Tim Grout Wayne Kirkman
16 – 17 Feb 21	Sunday's River – Packhouse visits	San Miguel (2 Packhouses)	Catherine Savage
17 Feb 21	Sondagsrivier: Pieter Nortje	Bederf op suurlemoene	Hannes Bester Catherine Savage Wilma Du Plooy
18 Feb 21	Sondagsrivier: SRCC André Combrink Dewalt Viviers	Jongboom-bestuur Boordbesoeke	Hannes Bester
18 Feb 21	Sunday's River – Packhouse visits	SRCC (4 Packhouses)	Catherine Savage
18 Feb 21	In Field demonstration	Mahela Soil and leaf sampling	Wayne Mommsen

19 Feb 21	Relining Africa: Ewen Niske	Besproeiings-pype herstel tegnologie	Hannes Bester
22 Feb 21	Nelspruit	Performance appraisals	Hannes Bester Liezl vd Linde Wayne Mommsen
23 Feb 21	Nelspruit: Extension meeting	Agenda	Hannes Bester MC Pretorius Catherine Savage Wayne Mommsen Dawid Groenewald Liezl vd Linde
		Performance appraisals	Hannes Bester Dawid Groenewald
24 Feb 21	Nelspruit: Citrus Sustainability Forum meeting	Agenda	Hannes Bester Catherine Savage
		Performance appraisal	Hannes Bester Catherine Savage MC Pretorius
25 Feb 21	Pretoria: WeSpeak Eugene Beetge	IT Tegnologie ondersteuning	Hannes Bester
25 Feb 21	Farmers day	Sitrus: Local Studygroup start-up	Wayne Mommsen
26 Feb 21	Hectorspruit	Turfbult packhouse visit	Catherine Savage
26 Feb 21	Komatipoort	Karlien Grobler visit	Catherine Savage
26 Feb 21	Nelspruit	BASF – NSSA symposium meeting	MC Pretorius
2 Mrt 21	Hoedspruit	Biosecurity: Placement of ACP traps	Wayne Mommsen Evans Mauda Wayne Kirkman Leanie Serfontein
	Letsitele Studiegroep	FCM en Biosecurity	Wayne Mommsen Wayne Kirkman
8 Mrt 21	CSR Zoom meeting	Simposium	Hannes Bester
9 Mrt 21	Innovation Hub	HB Fuller. “Global Adhesives”	Lulu da Silva Dawid Groenewald
9 Mrt 21	VC Pre-season Meeting	PPECB and DALRRD	Wayne Mommsen Catherine Savage
10 Mrt 21	Patensie	Phillip Dempsey	Hannes Bester
10 Mrt 21	Nelspruit	HLB- Steering com meeting	MC Pretorius Paul Fourie Solomon Gebeyehu Aruna Manrakhan Wayne Kirkman Vaughan Hatting
10 Mrt 21	Letsitele Vallei	Vrugbrand Onderzoek	Wayne Mommsen Sean Thackeray
11 Mrt 21	Nelspruit	Bayer meeting – Produkte en nuwe Bayer plaas konsultasie	MC Pretorius Tim Grout Jan v Vuuren Dirk Uys
12 Mrt 21	Nelspruit	FMC teams – CRI/ FMC tegniiese vergadering	MC Pretorius Sean Moore

			Tim Grout Wayne Kirkman Wayne Mommsen
15 – 16 Mrt 21	Ohrigstad	Mahela: Tegniiese produksie vergadering; Produsent besoeke – Tommy Landman	MC Pretorius Eddie Vorster Kobus Beetge
16 Mrt 21	Nelspruit	Croc Valley Packhouse visit	Catherine Savage
16 Mrt 21	PE: Sean Moore	Navorsing update	Hannes Bester
17 Mrt 21	CMF Zoom meeting	Agenda	Hannes Bester
19 Mrt 21	Laeveld Agrochem: Ernst De Beer	FCM en IPM update	Hannes Bester
20 Mrt 21	Nelspruit	ADAMA – meeting produkreeks	MC Pretorius
23 Mrt 21	Tulbach: Montpellier Wine Cellars	Nuwe sitrus-aanplanting	Hannes Bester Phillip Dempsey
24 Mrt 21	Lynnwood Bridge	Vergadering met New Era Packaging	New Era Bestuur Dawid Groenewald
24 Mrt 21	Stellenbosch: afspraak met 24 navorsers, Hortgro, Agrimotion	Jacques Fouché Paul Cronje Coenraad Fraenkel Hugh Campbell Pieter Raadt	Hannes Bester
24 – 25 Mrt 21	Hoedspruit	Studiegroep vergadering – HLB aanbieding en produsent besoeke	MC Pretorius Johan Joubert Wayne Mommsen
25 Mrt 21	Stellenbosch	Jan v Niekerk	Hannes Bester
30 Mrt 21	CGA Roadshow webinar	Agenda	Hannes Bester Catherine Savage
31 Mrt 21	Nelspruit	Wenkem: tegniiese vergadering	MC Pretorius
31 Mrt 21	Nelspruit	CRM Training	Catherine Savage Liezl vd Linde
31 Mrt 21	Evergreen Mini Mark, Pretoria	Vergadering met Rosslyn Pallets	Johan du Toit Dawid Groenewald

## 7.6 Other means of Technology Transfers

### 7.6.1 CRI website by Tim G Grout

Numbers of unique visitors and numbers of visits declined in 2020/1 compared to 2019/20 (Table 7.6.1). CRI are in the process of redesigning our current website from DRUPAL into WORDPRESS. The Wordpress platform allows for more design elements to be incorporated onto the site. We would like to modernize our existing site to include an events page featuring all CRI's upcoming events as well as an online shop for all CRI's products.

**Table 7.6.1.** Visits and page requests on [www.cri.co.za](http://www.cri.co.za) since April 2020.

Month	Unique visitors	Number of visits	Pages	Hits	Bandwidth
<b>Total 2019/20</b>	39 997	79 085	249 408	1 399 434	42.0 GB
Apr 2020	2 728	6 905	18 705	107 675	3.11 GB

May 2020	3 161	7 159	19 344	105 596	3.12 GB
Jun 2020	2 592	6 030	23 551	88 040	2.34 GB
Jul 2020	2 639	5 959	16 957	80 079	2.18 GB
Aug 2020	2 827	6 007	16 317	93 386	2.59 GB
Sep 2020	2 835	6 711	19 153	90 445	2.48 GB
Oct 2020	3 110	7 369	18 549	91 064	2.42 GB
Nov 2020	2 855	7081	15 973	85 157	2.39 GB
Dec 2020	2 937	6 889	14 033	69 632	1.70 GB
Jan 2021	3 658	8 542	19 821	114 936	2.93 GB
Feb 2021	3 830	7 875	21 092	106 165	2.87 GB
Mar 2021	4 007	8 363	20 787	132 995	3.72 GB
<b>Total 2020/1</b>	<b>37 179</b>	<b>84 890</b>	<b>224 282</b>	<b>1 165 170</b>	<b>31.9 GB</b>

#### 7.6.2 CRInet by Tim G Grout

Yahoo Groups shut down all their groups on 15 December 2020. CRInet was therefore also shut down. Latterly, CRInet had mostly been used for dissemination of information from CRI or CGA and that will continue via email. Over almost 20 years, CRInet had been a successful means of transferring technical citrus information to the industry, academia and other role players.

#### 7.6.3 X2 CRM System

CRI has launched a cloud-based CRM database to be used as a single point of contact to manage contacts and clients throughout CRI. Existing data has been kept in dispersed spreadsheets throughout CRI making it difficult to have a single consolidated view or effectively and professionally manage the CRI contact data.

#### 7.6.4 CRI Cutting Edge

**Table 7.6.2** Cutting Edge issues during 2020-21.

No.	Title	Month	Author/s
294	Consumer Assurance Update	Apr	Paul Hardman
295	Consumer Assurance and Dithiocarbamate (Mancozeb) Update	May	Paul Hardman
296	HLB now in Kenya: Preparations to mitigate its impact on the southern African citrus industry	May	Solomon Gebeyehu, Tim Grout, Paul Fourie and Vaughan Hattingh
297	Complaints and Losses as a result of Packaging	May	Dawid Groenewald
298	Mealybug and South Korea	May	Vaughan Hattingh, Elma Carstens and Sean Moore
299	COVID-19: Sanitation precautions	Jun	Catherine Savage and Wilma du Plooy
300	Warning: Incidence of frost and freeze damage of citrus fruits in various production areas	Jun	Paul Cronje, Hannes Bester and Johane Niemann
301	Container cooling – Best practice guidelines	Jun	Tarl Berry and Paul Cronje

302	Consumer assurance update	Jul	Paul Hardman
303	Identification of citrus pests	Aug	Sean Moore and Tammy Marsberg
304	Updated Citrus Black Spot Spray Programmes 2020 – 2021	Oct	Providence Moyo, Jan van Niekerk, Elma Carstens and Paul Fourie
305	How to retrieve data from CRI-PhytRisk	Oct	Providence Moyo and Paul Fourie
306	Fruit thinning strategies for 2021 season	Oct	Paul Cronje, MC Pretorius and Wayne Mommsen
307	Consumer assurance update	Nov	Paul Hardman
308	Changes to the Citrus FCM Risk Management System (FMS) for the 2021 Export Season	Dec	Elma Carstens, Vaughan Hattingh, Keziah Naidoo, Sean Moore and Paul Cronje
309	Alert: Important Citrus Black Spot Control Principles for orchards potentially exposed to early rain-related CBS infection risk prior to commencing with fruit protection sprays	Dec	Elma Carstens, Vaughan Hattingh, Providence Moyo, Jan van Niekerk and MC Pretorius
310	Corrective treatments for FCM within the citrus FCM risk management system (FMS)	Jan	Sean Moore, Vaughan Hattingh and Elma Carstens
311	Certified nurseries in the Southern African Citrus Improvement Scheme, tree standards and regulated movement of citrus propagation material	Feb	Paul Fourie, Thys du Toit and Michael Nell
312	Update on Propiconazole EU MRL	Feb	Paul Hardman
313	Advisory notice: Orchards potentially exposed to Citrus Black Spot infection during heavy rains experienced in January and February 2021	Feb	Providence Moyo, Jan van Niekerk, Elma Carstens, MC Pretorius, Wayne Mommsen, Paul Fourie, Tankiso Mpholo and Vaughan Hattingh
314	Removing propiconazole residue from citrus packing lines	Mar	Catherine Savage, Wilma du Plooy and Paul Hardman
315	Laboratory testing to exclude mealybug species of phytosanitary importance on fruit destined for the South Korean export market	Mar	E. Basson, G. Cook, A. Manrakhan, H.J. Maree, J. van Niekerk, E. Carstens, S. Moore, V. Hattingh, N Africander, W. Pieterse, M. Arendse, K. Naidoo and T. Mpholo
316	Identification of mealybug species on citrus	Mar	Sean Moore, Vaughan Hattingh, Elma Carstens, Aruna Manrakhan and Glynnis Cook

317	Sour rot and Brown rot warnings	Mar	Catherine Savage, Wilma du Plooy, MC Pretorius and Jan van Niekerk
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## 8 PUBLICATIONS IN 2020-21

### 8.1 Refereed Publications (or ISI ranked journals)

- Acheampong, M.A., M.P. Hill, S.D. Moore and C.A. Coombes. 2020. UV sensitivity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates under investigation as potential biological control agents in South African citrus orchards. *Fungal Biology* 124: 304-310
- Albertyn, S., S.D. Moore, T. Marsberg, C.A. Coombes and M.P. Hill. 2021. The influence of citrus orchard age on the ecology of entomopathogenic fungi and nematodes. *Biocontrol Science and Technology* 31(1): 80-96.
- Berry, T.M., T. Defraeye, W. Wu, M.G. Sibiyi, J. North, P.J.R. Cronje. 2021. Cooling of ambient-loaded citrus in refrigerated containers: What impacts do packaging and loading temperature have? *Biosystems Engineering* 201: 11-22.
- Bester R, Cook G and Maree HJ .2021. Citrus Tristeza Virus Genotype Detection Using High Throughput Sequencing. *Viruses* 13. doi:10.3390/v13020168.
- Bester R, Karaan M, Cook G and Maree HJ. 2021. First report of citrus virus A in citrus in South Africa. *Journal of Citrus Pathology* 8. doi:https://escholarship.org/uc/item/5gr6p8zh.
- Coetzee, B., E. Carstens, M. Dewdney, P.H. Fourie and A.E. Bester-van der Merwe. 2021. Extending the knowledge of *Phyllosticta citricarpa* population structure in USA with re-sequencing and genome wide analysis. *Physiological and Molecular Plant Pathology* 113: 101591.
- Cook, G., Breytenbach, J.H.J., Steyn, C., de Bruyn, R., van Vuuren, S.P., Burger, J.T., Maree, H.J. 2021. Grapefruit Field Trial Evaluation of Citrus Tristeza Virus T68-Strain Sources. *Plant Dis.* 105(2):361-367.
- Cook, G., B. Coetzee, R. Bester, J.H.J. Breytenbach, C. Steyn, R. de Bruyn, J.T. Burger and H.J. Maree. 2020. Citrus Tristeza Virus isolates of the same genotype differ in stem pitting severity in grapefruit. *Plant Disease* 104: 2362-2368.
- Cook, G., Steyn, C., Breytenbach, J. H. J., de Bruyn, R., and Fourie, P. H. 2020. No detection of seed transmission of citrus tatter leaf virus in 'Meyer' lemon. *Journal of Plant Diseases and Protection* 127: 895-898.
- Grout, T.G. and Stoltz, K.C. 2020. Carbon Dioxide Fumigation to Shorten Cold Disinfestation Treatments for *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in Citrus Fruit. *J. Econ. Entomol.* 113 (1): 144-151.
- Grout, T.G. and P.R. Stephen. 2021. Are Yeast Autolysate Attractants for *Ceratitidis* Species (Diptera: Tephritidae) in South Africa More Attractive and Palatable Than a Currently Used Protein Attractant? *J. Econ. Entomol.* 114(2): 1005-1008.
- Hattingh, V., S. Moore, W. Kirkman, M. Goddard, S. Thackeray, M. Peyper, G. Sharp, P. Cronjé and K. Pringle. 2020. An Improved Systems Approach as a Phytosanitary Measure for *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in Export Citrus Fruit From South Africa. *J. Econ. Entomol.* 113(2): 700-711.
- Makumbe, L., Moropa, T. P., Manrakhan, A. and Weldon, C. W. 2020. Effect of sex, age and morphological traits on tethered flight of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) at different temperatures. *Physiological Entomology* 45. DOI: 10.1111/phen.12323
- Manrakhan A, T. Grout, T. Grove, J.H. Daneel, P. Stephen, C. Weldon, M. De Meyer, E. Carstens and V. Hattingh. 2020. A survey of *Ceratitidis quinaria* (Bezzi) (Diptera: Tephritidae) in citrus production areas in South Africa. *African Entomology* 28: 35-43.
- Moore, S.D. 2021. Biological Control of a Phytosanitary Pest (*Thaumatotibia leucotreta*): A Case Study. *Int. J. Environ. Res. Public Health* 18, 1198.
- Moyo, P.; Fourie, P.H.; Masikane, S.L.; de Oliveira Fialho, R.; Mamba, L.C.; du Plooy, W.; Hattingh, V. 2020. The Effects of Postharvest Treatments and Sunlight Exposure on the Reproductive Capability and Viability of *Phyllosticta citricarpa* in Citrus Black Spot Fruit Lesions. *Plants* 9(12):1813.

- Moyo P., Du Raan S., Fourie P.H. 2020. Models for predicting pseudothecium maturity and ascospore release of *Phyllosticta* spp. in South African citrus orchards. *S Afr J Sci.* 116(11/12), Art. #7955, 10 pages.
- Pieterse, W., Manrakhan, A., Terblanche, J.S. and Addison, P. 2020. Comparative demography of *Bactrocera dorsalis* (Hendel) and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) on deciduous fruit. *Bulletin of Entomological Research* 110, 185—194.
- Weldon, C. W., Mnguni, S., Demares, F., DuRand, E., Malod, K., Manrakhan, A. and Nicolson, S. W. (2019). Adult diet of a tephritid fruit fly does not compensate for impact of a poor larval diet on stress resistance. *Journal of Experimental Biology* 222: jeb192534. DOI: 10.1242/jeb.192534
- White, V.G., A.G. Hardie and P.J. Raath. 2020. Relationships between commonly-used South African and international soil phosphorus extraction tests on pristine and cultivated soils. *SA J. Plant and Soil* 37(4): 265-272.