

Response to “Splash dispersal of *Phyllosticta citricarpa* conidia from infected citrus fruit” by Perryman *et al.* (2014)

P.H. Fourie^{1,2}, G.C. Schutte¹, E. Carstens^{1,2}, G.J. Silva Junior³, M. Bellato Sposito⁴, V. Hattingh^{1,5}, T.G. Gottwald⁶, I. Paul⁷ and M.M. Dewdney⁸

¹Citrus Research International, PO Box 28, Nelspruit 1200, South Africa

²Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Stellenbosch 7602, South Africa

³Fundecitrus, Araraquara, Brazil

⁴University of Sao Paulo, Piracicaba, Brazil

⁵Department of Conservation Ecology and Entomology, Stellenbosch University, Private Bag X1, Stellenbosch 7602, South Africa

⁶U.S. Horticultural Research Laboratory, Agricultural Research Services, United States Department of Agriculture, Fort Pierce, FL, USA

⁷ExperiCo (Agri-Research Solutions), A Division of Agri R&D (Pty) Ltd, PO Box 4022, Ida's Valley, Stellenbosch, 7609, South Africa

⁸Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA

8 September 2015

The study of splash dispersal of *Phyllosticta citricarpa* from infected citrus fruit by Perryman *et al.* (2014) was commissioned by the European Food Safety Authority (EFSA; project EFSA-Q-2013-00011). Prior to its publication (Scientific Reports 4, Article number: 6568 doi:10.1038/srep06568), a report of this study (Perryman and West, 2014) was published as a supporting publication along with the “Scientific Opinion on the risk of *Phyllosticta citricarpa* (*Guignardia citricarpa*) for the EU territory with identification and evaluation of risk reduction options” (EFSA, 2014). The findings of this study were used by EFSA to demonstrate the hypothesised feasibility of longer-distance splash dispersal of *P. citricarpa* conidia from infected citrus fruit.

Perryman *et al.* (2014) concluded that “This study, using artificially inoculated fruit, demonstrates that conidia are able to be dispersed from discarded oranges at ground level to heights and distances that would allow deposition onto (susceptible) leaves of citrus trees growing in close proximity”. Whilst it can be argued that Perryman *et al.* (2014) demonstrated a theoretical possibility, the epidemiological probability of such dispersal leading to successful infection was not demonstrated and the inferences they draw from their study were not supported by the data. Moreover, their findings contradict multiple field studies with *P. citricarpa*, even under high-rainfall field conditions that should have supported splash dispersal from fruit if it was a likely prospect (Kiely, 1948; Wager, 1949; McOnie, 1965; Whiteside, 1967; Spósito *et al.*, 2007, 2008, 2011). Collectively, these studies showed that mature *P. citricarpa* conidia (also termed pycnidiospores) are embedded in a gelatinous / mucilaginous mass and emerge from pycnidia when wet. When the spore mass comes in contact with water, the mucilage dissolves and the spore suspension may then be washed down from lesions over short distances within the tree, leading to infection within a distance of less than 1 m from the source. Nowhere in literature is there any evidence of upward or long-distance horizontal splash-dispersal of conidia from infected citrus fruit to subsequently cause successful infection. In fact, Kiely (1948) concluded from the lack of airborne conidia and their predominant downward water dispersal that with regard to *P. citricarpa* infected fruit with pycnidia on the ground “their importance in providing water-borne inoculum is practically nil”. Water splash dispersal from lesions on fruit residing on

the ground would have to escape the still air “boundary layer”, which was demonstrated to severely limit dispersal for a number of pathogens (Aylor, 1978; Madelin and Madelin, 1994).

Perryman *et al.* (2014) demonstrated the mechanics of physical droplet dispersal but did not include sufficient biological data to complement it. Apart from splashes from orange fruit, this is not novel to existing scientific literature. Previous research (*inter alia* Fitt *et al.* 1982, 1989; Huber *et al.* 1996, 2006; Madden, 1992, 1997; McCartney *et al.*, 2006; Travadon *et al.*, 2007) showed that splash dispersal of fungal inoculum is essentially a short-range phenomenon. Perryman *et al.* (2014) likewise reported splash from droplet impact into spore suspensions or artificially inoculated fruit, with observation that some spores were transported short distances in the resultant splash. They subsequently infer biological significance under natural real-world conditions, but without any direct evidentiary support from the results of their study.

The laboratory study by Perryman *et al.* (2014) employed unrealistic methods and their findings therefore provide insufficient biological confirmation (*i.e.* that dispersal will lead to infection) to disprove prior scientific evidence of *P. citricarpa* conidium dispersal from studies conducted under orchard conditions (Kiely, 1948; Wager, 1949; McOnie, 1965; Whiteside, 1967; Spósito *et al.*, 2007, 2008, 2011). Specific aspects in the methodology and interpretation of the results are criticised below:

(1) Firstly, Perryman *et al.* (2014) used artificially inoculated oranges with lesions that were uncharacteristically large (*c.* 1-2 cm in diameter) and depressed, compared with naturally occurring CBS hard spot lesions that are typically <0.5 cm in diameter (Figure 1). Perryman *et al.* (2014) nonetheless considered that their artificial lesions were “typical ‘hard spot’ symptoms” and observed pycnidia after some 4–6 weeks. Several lesion-causing fungi typically occupy citrus fruit rinds (OEPP/EPPO, 2009), and Perryman *et al.* (2014) failed to confirm that their lesions were indeed caused by *P. citricarpa* (through back-isolation, morphological identification of conidia or PCR test). The lack of confirmation places some question as to the actual cause of these atypical lesions, especially since they used fruit that were “purchased commercially”. These fruit are mature, senescing and are resistant to natural *P. citricarpa* infection (Kotze, 1981; OEPP/EPPO, 2009).

(2) Perryman *et al.* (2014) did not quantify the number of pycnidia per lesion, but we assume pycnidia were atypically abundant as uncharacteristically high quantities of conidia were obtained following simulated release conditions. Only a few pycnidia are normally observed in hard spot lesions (Kotze, 1981; OEPP/EPPO, 2009; Figure 1). Lourenço *et al.* (2012) observed 1 to 31 pycnidia in freckle spot lesions, which generally contain more pycnidia than a hard spot lesion. The freckle spots exuded 0 to 109 875 spores *per lesion*. This is significantly less than the 31 250 to 462 500 spores *per mL* reported by Perryman *et al.* (2014).

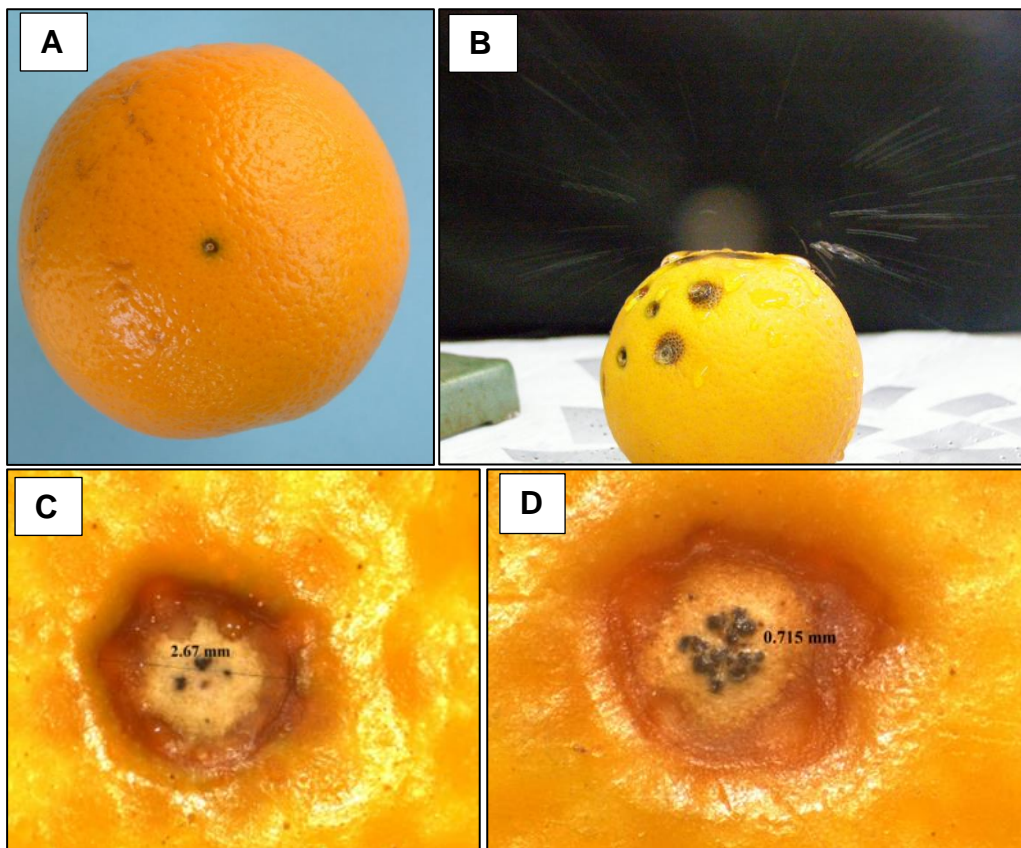


Figure 1. Typical Citrus Black Spot lesion caused by *Phyllosticta citricarpa* under natural field conditions (A) and lesions obtained from artificial inoculations of mature orange fruit [Fig.1c in Perryman *et al.* (2014)] (B). Average size of typical hard spot lesions are less than 3 mm (C) and larger pycnidia ‘nests’ can reach 0.7 mm, but are on average 0.16 mm in diameter (D).

(3) These uncharacteristically large and depressed lesions on artificially inoculated mature fruit, apparent abundance of pycnidia in lesions and high quantities of conidia obtained in these lesions following regular misting in the laboratory created an unrealistic and atypical situation. For example, this allowed for suspensions with unusually high spore loads to accumulate in the lesion depressions from which splash dispersal was subsequently simulated. Under natural conditions, CBS hard spot lesions do not resemble the symptoms illustrated by Perryman *et al.* (2014). Hard spots are considerably smaller and as droplets larger than 0.2 mL run off the citrus fruit surface, only water films are likely to occur on the rind.

(4) Perryman *et al.* (2014) used an excessively large droplet size to simulate rain splash. Raindrop sizes generally range from 0.5 to 4 mm, with size distribution quickly decreasing past diameters larger than 2-2.5 mm. The 5-mm droplets used by Perryman *et al.* (2014) are therefore in the largest and most infrequent droplet size range (Ulbrich, 1983; Villermaux and Bossa, 2009; McFarquhar, 2010), and therefore such large droplets striking a lesion would constitute a very rare event in nature. With these drops, Perryman *et al.* (2014) showed mostly short-distance droplet splash from oranges: 99.2% of all droplet sizes dispersed <50 cm from source, and splash heights reached a maximum of 41 cm with single 5-mm drops (<30 cm for 2.5-mm drops) and 61.7 cm in multiple splash experiments.

(5) Perryman *et al.* (2014) did not attempt to quantify the amount of spores that were dispersed from the artificially inoculated oranges, nor did they demonstrate the viability of these spores, but only stated that “[s]plash-droplets ... were observed to contain conidia,

whose numbers decreased with increasing height and distance”. Instead, Perryman *et al.* (2014) used dispersal data obtained from a spore suspension in a Petri dish to quantify spore dispersal, and invalidly inferred similar results in subsequent experiments of droplet splash from fruit.

(6) The experimental design of the Perryman *et al.* (2014) study was inadequate to demonstrate biological variation and therewith probability. For example, to simulate release of spores from an infected orange during a rain shower, the experiment was conducted on *two* artificially infected oranges, and spore release was enumerated twice only. For the frequency of conidia in splash droplets from a spore suspension in a Petri-dish, conidia were counted in *ten* droplets of each size range only and the means presented; this experiment was not repeated and therefore statistically questionable. To determine the presence of conidia in splashed droplets from artificially infected oranges, the experiment was conducted once from a 40-cm height and once from 11-m height in the rain-tower. In this experiment, the height and distance of droplet splash was quantified, but the spore concentration at source and number of conidia in the splashed droplets was not quantified (presence of conidia in droplets was *observed* only, but not measured in splash beyond 30 cm from source). In subsequent rain-tower experiments, the height and distance of droplet splash was quantified using water sensitive paper and videography, but conidia in the splashed droplets were evidently not quantified. Likewise, the amount of conidia in the splashed droplets was evidently not quantified in the experiments to simulate a rain shower event and the effect of wind on droplet dispersal. Therefore, Perryman *et al.* (2014) made a number of extrapolations in their conclusions that do not follow from the paucity of data and lack of replication, especially combined with simple physical splash dispersal experiments that did not quantify incorporation of conidia into droplets.

(7) Perryman *et al.* (2014) did not demonstrate any biological probability of long-distance splash dispersal of *P. citricarpa* conidia and failed to discuss their laboratory findings in context with the relevant scientific literature on this pathogen. Of particular concern, is Perryman *et al.* (2014)’s notion of aerosol dispersal of *P. citricarpa* conidia. They demonstrated that ballistic droplets with parabolic trajectories were relatively unaffected by wind, but that smaller splash droplets were affected by wind, and that the smallest droplets can become aerosolised to be dispersed much longer distances. Whilst these aerosolised droplets were evidently a minor proportion of splashed drops (Fig. 5b in their manuscript), the probability of these drops carrying conidia must be very small considering their finding that <1-mm droplets splashed from a spore suspension in a Petri-dish carried on average 1.7 spores. The aerosolised droplet spectrum is considerably smaller than 1 mm and should contain proportionally fewer conidia. Nonetheless, Perryman *et al.* (2014) stated for these aerosolised droplets that “[t]hese fine droplets, despite carrying an average of only one spore, are very numerous” and that “the pathogen can be dispersed at least 8 m and to heights of at least 75 cm”. Because they did not study or quantify the incorporation of conidia into fine droplets, this statement was not substantiated by their findings, and the biological possibility of this happening from infected fruit with typical CBS lesions was not demonstrated. The epidemiologically probability thereof is not supported by field studies (Kiely, 1948; Wager, 1949; McOnie, 1965; Whiteside, 1967; Spósito *et al.*, 2007, 2008, 2011).

To conclude, Perryman *et al.* (2014)’s findings are of little biological value as they did not attempt to demonstrate infection of a susceptible host subsequent to dispersal. The infectious potential of single spores randomly dispersed in small or aerosolised droplets should be considered in reference to conidium dispersal and subsequent inoculation in the orchard (Kiely, 1948; Wager, 1949; Whiteside, 1967; Spósito *et al.*, 2011), which cannot be regarded as a random single-spore inoculation event. The infectious potential of a single spore is also

very poor as *P. citricarpa* conidia typically have low viability and are short-lived (Kiely, 1948) and the likelihood of single-spore inoculation leading to infections is negligent, considering the strenuous infection conditions of at least 12 hours wetness at optimal conditions that are required (Noronha, 2002; Wang and Dewdney, 2014). Given these characteristics of the pathosystem, the inoculum potential of *P. citricarpa* conidia is clearly low and a relatively high inoculum density would be required to initiate infection (Baker, 1978). In *P. citricarpa* infection studies, researchers had to revert to inoculum concentrations of at least 1 000 spores per mL sprayed to the point of run-off to get a realistic prospect of infection (Truter, 2010; Aguiar *et al.*, 2012). Aerosol dispersal of *P. citricarpa* conidia leading to successful infection is biologically improbable given the low infective potential of conidia (Kiely, 1948; Truter, 2010; Aguiar *et al.*, 2012), the rapid evaporation of droplets <100 µm and detrimental effects of desiccation and solar radiation on the viability of small hyaline spores (Madelin and Madelin, 1994), such as *P. citricarpa* conidia.

Whilst the study by Perryman *et al.* (2014) can be recognised for its physical demonstration of dispersal of water *droplets* resulting from the impaction of very large drops onto citrus fruit (with artificially created, depressed lesions), it cannot be regarded as evidence of splash dispersal of *P. citricarpa* conidia, given the afore-mentioned shortcomings and the fact that this theoretical possibility was not biologically demonstrated under realistic conditions. In general, findings from a laboratory study cannot directly be extrapolated to field conditions. Despite these shortcomings, the Perryman *et al.* (2014) paper is nonetheless regarded by EFSA as “groundbreaking” and was used in support of assessing the real world probability of transfer of *P. citricarpa* from naturally infected citrus fruit pathway as “moderately likely” (EFSA, 2014, 2015). This assessment, as well as the conclusion of Perryman *et al.* (2014), is in clear contradiction of the available scientific evidence (Kiely, 1948; Wager, 1949; Whiteside, 1967; Spósito *et al.*, 2007, 2008, 2011) and other pest risk assessments (South African CBS PRA, 2000-2009; USDA APHIS, 2010).

References

- Aguiar RL, Scaloppi EMT, de Goes A and Spósito MB, 2012. Período de incubação de *Guignardia citricarpa* em diferentes estádios fenológicos de frutos de laranja ‘Valencia’. *Tropical Plant Pathology*, 37, 155–158.
- Aylor DE, 1978. Dispersal in Time and Space: Aerial Pathogens. Pages 159-180 in *Plant Disease an Advanced Treatise Volume II: How Disease Develops in Populations*. Ed. J.G. Horsfall and EB Cowling. Academic Press Inc., New York.
- Baker, R, 1978. Inoculum potential. Pages 137-157 in *Plant Disease an Advanced Treatise Volume II: How Disease Develops in Populations*. Ed. J.G. Horsfall and EB Cowling. Academic Press Inc., New York.
- EFSA PLH Panel (EFSA Panel on Plant Health), 2014. Scientific Opinion on the risk of *Phyllosticta citricarpa* (*Guignardia citricarpa*) for the EU territory with identification and evaluation of risk reduction options. *EFSA Journal*, 12(2), 3557, 243 pp. doi:10.2903/j.efsa.2014.3557
- EFSA PLH Panel (EFSA Panel on Plant Health), 2015. Statement on the comments by Hattingh *et al.* (2014) on the EFSA PLH Panel (2014) Scientific Opinion on Citrus Black Spot. *EFSA Journal*, 13(1), 3990, 26 pp., doi:10.2903/j.efsa.2015.3990
- Fitt BDL, Lysandrou M, Turner RH, 1982. Measurement of spore-carrying splash droplets using photographic film and an image-analysing computer. *Plant Pathology* 31, 19-24.
- Fitt BDL, McCartney HA and Walklate PJ, 1989. The role of rain in dispersal of pathogen inoculum.

Annual Review of Phytopathology, 27, 241–270.

Huber L, Fitt BDL, McCartney HA, 1996. The incorporation of pathogen spores into rain-splash droplets: a modeling approach. *Plant Pathology* 45, 506–17.

Huber L, Madden L, Fitt BDL, 2006. Environmental biophysics applied to the dispersal of fungal spores by rain-splash. In: *The Epidemiology of Plant Diseases*, 2nd edition, BM Cooke, D Gareth Jones and B Kaye (eds.), 417-444.

Kiely TB, 1948. Preliminary studies on *Guignardia citricarpa*, n. sp.: the ascigenous stage of *Phoma citricarpa* McAlp. and its relation to black spot of citrus. *Proceedings of the Linnean Society of New South Wales*, 68, 249–292.

Kotzé JM, 1981. Epidemiology and control of citrus black spot in South-Africa. *Plant Disease*, 65, 945–950.

Lourenço SA, Gasparoto MCG, Spósito MB, Amorim L, Gottwald TR, 2012. Infectious period of citrus black spot on sweet orange. In: *International Citrus Congress 2012, Valencia. Book of Abstracts*. p.261.

Madden LV, 1992. Rainfall and the dispersal of fungal spores. *Advances in Plant Pathology* 8, 39–79.

Madden LV, 1997. Effects of rain on splash dispersal of fungal pathogens. *Canadian Journal of Plant Pathology*, 19, 225-230

Madelin TM, Madelin MF, 1994. Biological analysis of fungi and associated moulds. Chapter 13 in: *Bioaerosols Handbook*, CM Wathes and CS Cox (eds.), Lewis Publishers Inc. CRC Press, Michigan.

McCartney HA, Fitt BDL, West JS, 2006. Dispersal of foliar plant pathogens: mechanisms, gradients and spatial patterns. In: *The Epidemiology of Plant Diseases*, 2nd edition, BM Cooke, D Gareth Jones and B Kaye (eds.), 159-192.

McFarquhar G, 2010. *Raindrop Size Distribution and Evolution*. Geophysical Monograph Series 191.

McOnie, KC, 1965. Source of infection for black spot of citrus. *S.A. Citrus Journal* June 5, 6, 9.

Noronha, MDA, 2002. Escala diagramatica para avaliacao da mancha preta em folhas de citros efeito da temperatura e da duracao do molhamento na prepenetracao de conidios de *Guignardia citricarpa* Kiely [*Phyllosticta citricarpa* (McAlp.) Van der Aa]. *Agronomy: Concentration of Plant Pathology Masters Dissertation*. Universidade de Sao Paulo.

OEPP/EPPO, 2009. Data sheets on quarantine organisms PM 7/17, *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* 39, 318-327.

Perryman SAM, Clark SJ, West JS, 2014. Splash dispersal of *Phyllosticta citricarpa* conidia from infected citrus fruit. *Scientific Reports* 4, 6568. doi:10.1038/srep06568.

Perryman SAM and West JS, 2014. Splash dispersal of *Phyllosticta citricarpa* pycnidiospores from infected citrus. *EFSA supporting publication 2014-EN-560*, 30 pp.

South African CBS PRA, 2000-2009. *Citrus Black Spot Consolidated Pest Risk Assessment Pertaining to the Export of Fresh Citrus Fruit from the Republic of South Africa to the European Union*. <http://www.citrusres.com/market-access>

Spósito MB, Amorim L, Ribeiro PJ, Bassanezi RB and Krainski ET, 2007. Spatial pattern of trees affected by black spot in citrus groves in Brazil. *Plant Disease*, 91, 36–40.

- Spósito MB, Amorim L, Bassanezi RB, Filho AB and Hau B, 2008. Spatial pattern of black spot incidence within citrus trees related to disease severity and pathogen dispersal. *Plant Pathology*, 57, 103–108.
- Spósito MB, Amorim L, Bassanezi RB, Yamamoto P, Felipe MR and Czermainski ABC, 2011. Relative importance of inoculum sources of *Guignardia citricarpa* on the citrus black spot epidemic in Brazil. *Crop Protection*, 30, 1546–1552.
- Travadon R, Bousset L, Saint-Jean S, Brun H and Sache I, 2007. Splash dispersal of *Leptosphaeria maculans* pycnidiospores and the spread of blackleg on oilseed rape. *Plant Pathology* 56, 595-603.
- Truter MP, 2010. Epidemiology of citrus black spot disease in South Africa and its impact on phytosanitary trade restrictions. PhD Thesis University of Pretoria, South Africa. 148 pp.
- Ulbrich CW, 1983. Natural Variations in the Analytical Form of the Raindrop Size Distribution. *J. Climate Appl. Meteor.*, 22, 1764–1775.
- USDA APHIS (United States Department of Agriculture Animal and Plant Health Inspection Service), 2010. Risk assessment of *Citrus* spp. fruit as a pathway for the introduction of *Guignardia citricarpa* Kiely, the organism that causes Citrus Black Spot disease. Center for Plant Health Science and Technology, Plant Epidemiology and Risk Analysis Laboratory, Raleigh, NC, USA.
- Villermaux E, Bossa B, 2009. Single-drop fragmentation distribution of raindrops. *Nature Physics*, 5 (9), 697–702.
- Wager VA, 1949. The occurrence of the black-spot fungus in the citrus areas of South Africa. *Farming in South Africa*, 24, 367–374.
- Wang N, Dewdney MM, 2014. Nutritional and environmental effects on germination and appressorium formation of *Guignardia citricarpa* conidia. *Phytopathology*, 102, S4.131
- Whiteside JO, 1967. Sources of inoculum of the black spot fungus, *Guignardia citricarpa*, in infected Rhodesian citrus orchards. *The Rhodesia, Zambia and Malawi Journal of Agricultural Research*, 5, 171–177.