

EXTERNAL SCIENTIFIC REPORT

Splash dispersal of *Phyllosticta citricarpa* conidia from infected citrus fruit¹

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ABSTRACT

Simulated rain splash experiments using a rain tower and wind tunnel determined the potential for dispersal of *Phyllosticta citricarpa* (synonym *Guignardia citricarpa*) conidia (pycnidiospores) from infected oranges. High Power microscopy demonstrated presence of conidia in splash droplets. In still air, the highest splash droplets were produced by the largest (5 mm) incident drops, reaching mean maximum height of 41.9 cm as opposed to 35.5 cm with 3.5 mm drops and 28.8 cm with 2.5 mm drops. The largest splashes (2-3 mm diameter) were recorded up to 20 cm high. Larger drops contain more spores (4-5.5 mm splashes averaged 308 spores), but get splashed <30 cm. Most (80-90%) splashes were <1 mm diameter but carry far fewer spores per droplet. The 0-0.99 and the 1-1.99 mm droplets which splash furthest in still air (up to 70 cm) contained an average of 1 and 21 spores respectively. In multiple splash experiments, splashes combined, rebounded and were forced higher, up to 72.2 cm (mean 64.3cm), compared to the single splash experiments. In experiments combining wind speed with rain-splash, progressively higher wind speeds carried an increasing proportion of splashes downwind and these splashes travelled increasingly further downwind – up to 8 metres in the case of the highest wind speed (7m/sec). At wind speeds up to 4m/s, all splash droplets described an arc that was skewed due to the wind but at 7m/s a small proportion of droplets (<1mm) were dispersed higher than originally splashed (up to 73.2cm) suggesting they remain aerosolised rather than behaving as ballistic droplets. These experiments showed that spores were dispersed from the infected oranges when carried in splashes of water. Laboratory experiments showed that infected oranges misted to simulate light rainfall continued to exude spores for at least an hour. Infected oranges are a potential source of spores to be dispersed by rainfall events.

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KEY WORDS

rain-splash, splash dispersal, oranges, citrus black-spot, spores, plant pathogen, quarantine pest

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SUMMARY

Simulated rain splash experiments were conducted to determine the potential for dispersal of *P. citricarpa* conidia (pycnidiospores) from infected oranges in a rain tower and wind tunnel at Rothamsted Research. Single incident drops (2.5, 3.5 and 5 mm diameter) or multiple drops (5mm) were dropped onto infected oranges from a height of 11 meters which enable them to reach terminal velocity. Experiments were conducted either in still air or in wind. The pattern of splash droplets dispersed from oranges was determined using water sensitive paper. The presences of spores in splashes were assessed using transparent melinex tape and observation by high power microscopy.

In single incident drop experiments in still air, the highest splash droplets were produced by the 5 mm initial drops and were on average, 41.9 cm high as opposed to 35.5 cm with 3.5 mm drops and 28.8 cm with 2.5 mm drops. This mean maximum height of splash was reached 20 cm away from the target orange. 90% of splashes on vertical strips were <1mm diameter. 32% of these smallest splashes fall below 10 cm and 56% fall below 20 cm high. The largest splashes (2-3 mm diameter) did not splash very high – all below 20 cm. 81% of splashes on horizontal strips were <2 mm. 76% of these had fallen within 10 cm of the orange. These smallest splashes also splash the furthest away – sometimes reaching up to 70 cm from the orange in still air. These more numerous smaller splashes carry far fewer spores. The 0-0.99 and the 1-1.99mm droplets which splash furthest have only an average of 1 and 21 spores present respectively. Larger drops contain more spores – as expected, but do not get splashed so far away. The largest splashes (4-5.5 mm) averaged 308 spores per droplet.

In single incident drop experiments combined with wind speed, progressively higher wind speeds carry an increasing proportion of ballistic splashes downwind instead of upwind and, under higher wind speeds, splashes travel increasingly further downwind – up to 8 metres downwind in the case of the highest wind speed (7m/sec). The lowest wind speed, (1m/sec) produced a pattern of splashes virtually identical to that in still air. The 7m/sec wind speed carried splashes up and away from the main splash area reaching heights up to 75 cm (and could have been higher) due to fine droplets (<1mm) becoming aerosolised.

In multiple drop experiment splashes combined and rebounded and were forced higher than occurred in single drop experiments, up to 60cm, compared to a maximum height of 47 cm for the single splash experiments.

Laboratory experiments showed infected oranges, misted to simulate light rainfall, continue to exude spores from pycnidia for at least an hour. Spores were shown to be dispersed from the infected oranges carried in splashes of water. These experiments showed that infected oranges can provide a source of spores with the potential to be dispersed by rainfall events.

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BACKGROUND AS PROVIDED BY EFSA

Following a request from the European Commission (EFSA-Q-2012-00992), a working group of the Panel on Plant Health has been established to prepare a pest risk assessment for the EU territory, with identification and evaluation of risk reduction options, of *Guignardia citricarpa* Kiely. The adoption of this scientific opinion is scheduled for July 2013. *P. citricarpa* is a pathogen of citrus (orange, lemon etc.), causing the disease, citrus black spot. The pathogen is absent from Europe but weather conditions in some areas of Southern Europe, where susceptible citrus trees are grown, have been shown having a potential for infection by this disease (EFSA Panel on Plant Health (PLH), 2008).

There is potential for dispersal of asexually produced spores (conidia or conidia) to citrus trees by upward rainsplash from infected citrus fruit imported from areas where *P. citricarpa* is currently present (EFSA Panel on Plant Health (PLH), 2008). *P. citricarpa* conidia are produced in pycnidia and are surrounded by a mucilage (van der Aa, 1973), which, as with other pycnidiospore-producing fungi, prevents their dispersal by wind and is an indicator of spores adapted for splash-dispersal (Fitt et al., 1989). The importance of *P. citricarpa* conidia in the epidemics of citrus black spot and their splash dispersal have been discussed by several authors and studied in field experiments (e.g. Whiteside, 1967; Kotzé, 1981; Spósito et al., 2011), however no experiments have been conducted under experimental controlled conditions to determine precisely the potential distances and direction of the *P. citricarpa* conidia splashed upwards from citrus fruit by rain drops.

In still air, splash-dispersed pathogens are usually dispersed up to a height of 50 cm above the inoculum source or up to a distance of 1 m from the host, with the number of conidia deposited on the plant surfaces decreasing steeply with increasing height or distance from the source (Fitt et al., 1989). However the dispersal of conidia depends on a number of factors, such as the size and velocity of the incident drop, the size of conidia and the occurrence of air currents. Rain tower experiments have shown that in still air one splash may disperse many thousands of conidia, but most are carried in the largest droplets (diameter >1 mm) and very few in droplets <100 µm in diameter (Fitt et al., 1989). Moreover, the largest drops spread the conidia over shorter distances when compared with the smaller drops. Generally, raindrops that reach the ground are 0.2-5 mm in diameter, since smaller drops evaporate rapidly unless relative humidity is near to 100 %, and larger drops break up when they fall at speeds approaching their terminal velocities. Nevertheless, rain with many large drops will be most effective in dispersal of these pathogens (Fitt et al., 1989). In the presence of wind, inoculum carried in small splash droplets may also become airborne as an aerosol of fine spray (Fitt et al., 1989). The significance of wind in the dispersal of pathogens removed from the inoculum source in splash droplets becomes greater as the size of the inoculum particles becomes smaller. Drops formed on the leaves due to fog, dew, mist or overhead irrigation may also cause drip splash of inoculum under canopies. This may be as important as direct rain-splash. These drip drops may be larger than 5 mm diameter because they fall only short distances and are less likely to break up compared to raindrops. Thus, they may have sufficient impact force for the dispersal of conidia in splash droplets (Fitt et al., 1989). In Mediterranean countries, the distance above ground of low hanging citrus fruit and foliage can vary from 0 to 80 cm, depending on the cultivation techniques. Therefore, the small-sized *P. citricarpa* conidia have the potential, from fruit/peel discarded underneath citrus trees, to be dispersed by direct rain-splash or drip-splash dispersal mechanisms.

The rain tower of Rothamsted Research (UK) is a unique facility in Europe to perform splash dispersal studies for plant pathogens, which, combined with a wind tunnel, allows to investigate splash heights and concentrations of spores per droplet with different combinations of wind and rain (Fitt et al., 1986).

TERMS OF REFERENCE AS PROVIDED BY EFSA

To reduce the uncertainty on the assessment of the probability of entry of *P. citricarpa* into the European Union, preparatory work is needed to collect experimental data, produced under controlled conditions, on the splash dispersal of *P. citricarpa* pycnidiospores from infected citrus fruit, particularly on the distance and direction of the fungal spores splashed upwards from citrus fruit by rain drops. These data can be used to improve the assessment of the probability of transfer to a suitable host plant from the entry pathway of the imported citrus fruit, and therefore to reduce the uncertainty on the overall probability of entry of this plant pathogen. Although some field work has been conducted, experimental data, produced under controlled conditions, are not available in scientific literature on splash dispersal of *P. citricarpa*, but the methodology to conduct such experiments is very well developed and has been already tested for spores of many other phytopathogenic fungi.

Therefore, to address these needs, the objective of this contract is to conduct an experiment under controlled conditions on the splash dispersal of the pycnidiospores of *P. citricarpa* from infected citrus fruit. The task of the contractor will be to investigate the splash dispersal characteristics of pycnidiospores of *P. citricarpa* from the surfaces of artificially infected oranges, using a rain-tower facility. The contractor should analyse data, perform statistical analysis and submit a preliminary report to EFSA ahead of attending a meeting at the EFSA with the working group and/or the Scientific Panel on Plant Health. The contractor should also write an external/final scientific report and a research paper on the results of the experiment to be submitted by the Contractor to a refereed journal, to be agreed with EFSA

This contract was awarded by EFSA to:

Professor Jon S West, Rothamsted Research, Harpenden, Herts., AL5 2JQ, UK

Contract title: Data collection to support DG SANCO mandate on pest risk assessment of *Guignardia citricarpa* Kiely: splash dispersal of *G. citricarpa* conidia from infected citrus

Contract: NP/EFSA/PLH/2013/01- CT1

INTRODUCTION AND OBJECTIVES

Phyllosticta citricarpa (McAlpine) Van der Aa, which was previously named *Guignardia citricarpa* Kiely, is a pathogen of citrus plants such as orange and lemon, causing the disease citrus black spot. The pathogen is absent from Europe but the suitability of weather conditions for it to complete its life cycle in southern parts of Europe where susceptible citrus trees are grown, including commercial citrus plantations, is debated (Paul et al., 2005; EFSA, 2008; Vicent and García-Jiménez, 2008; Yonow et al., 2013; Fourie et al., 2013). There is potential for dispersal of asexually produced spores (conidia or pycnidiospores) by rain splash from infected fruit. Wind dispersed ascospores are produced from infected leaf debris but are not known to be produced on infected fruit. However, there is potential for the disease to enter and establish in Europe if rain-splashed conidia could initiate infections of susceptible citrus trees, leading with time to the development of the sexual stage on leaf litter releasing wind-dispersed spores. To provide information to the EFSA Panel on Plant Health (PHL), as part of their risk assessment for the EU territory, this study investigated the splash dispersal characteristics of conidia of *P. citricarpa* from the surfaces of artificially infected oranges, using a purpose-built rain tower facility at Rothamsted, Research, UK.

Objectives were to use established methods to collect experimental data, produced under replicated conditions, on the splash dispersal of *P. citricarpa* conidia from infected citrus fruit, particularly on the distance of the fungal spores splashed upwards and from the citrus fruits by rain drops. Splash dispersal (trajectory of splashed droplets and concentrations of spores per droplet) of various pathogens such as *Septoria nodorum*, *Rhynchosporium secalis* and *Pyrenopeziz brassicae* has been investigated previously at Rothamsted using the rain-tower and combined wind-tunnel facility (Fitt et al. 1986). The approach used in the present study was to simulate rain splash events using distilled water drops falling from various heights onto infected oranges in still air initially or at later stages, combined with a wind current allowing the combination of wind and rain to be investigated. Splashed droplets were collected after individual and multiple incident rain drops, of known diameters. The horizontal and vertical location of deposited droplets was assessed and their frequency and trajectories determined. A sub-set of splashed droplets was assessed for numbers of conidia present and data analysis was carried out to determine effects of rain splash on *P. citricarpa* spore dispersal from rain splash events.

MATERIALS AND METHODS

1. Fungal cultures and inoculum production

Two isolates of *P. citricarpa* designated as IVIA-GC072 (GenBank Accession No. KF709953), and IVIA-GC 092 (sequence submitted to GenBank) (A. Vicent, Centro di Protezione Vegetale y Biotecnologia, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada 46113, Valencia, Spain), obtained from sweet orange fruit from South Africa, were used in the study. The isolates were received as small agar cubes in 1.5ml Eppendorf tubes. These were each sub-cultured at Rothamsted onto ten PDA (potato dextrose agar) agar plates and half kept at 20°C incubator under UV light. Half were kept in dark incubator at 18°C. After several days, dark masses of mycelium were observed, colonising the plates (Fig. 1a). Some of these were used to produce a spore suspension to inoculate the oranges for use in the experiments below. When masses of spores were observed on mycelium, plates were flooded with sterile distilled water in a flow cabinet and rubbed/agitated with a sterile L-shaped glass rod and the resulting suspension poured into a tube. A small amount was placed on a glass cavity slide and observed microscopically to ensure presence of spores (Fig. 1b).

Rothamsted holds a permit from the UK plant health authorities to keep fungal cultures for research (FERA UK plant health permit amended 101941/201284/1) and a risk assessment was made for the experiments described in this report. All experiments were done within a single building in contained conditions with all surfaces disinfected with 70% alcohol and waste materials autoclaved. No air was vented directly to outdoors. Additionally, no citrus trees are known to be grown outside near Rothamsted Research.

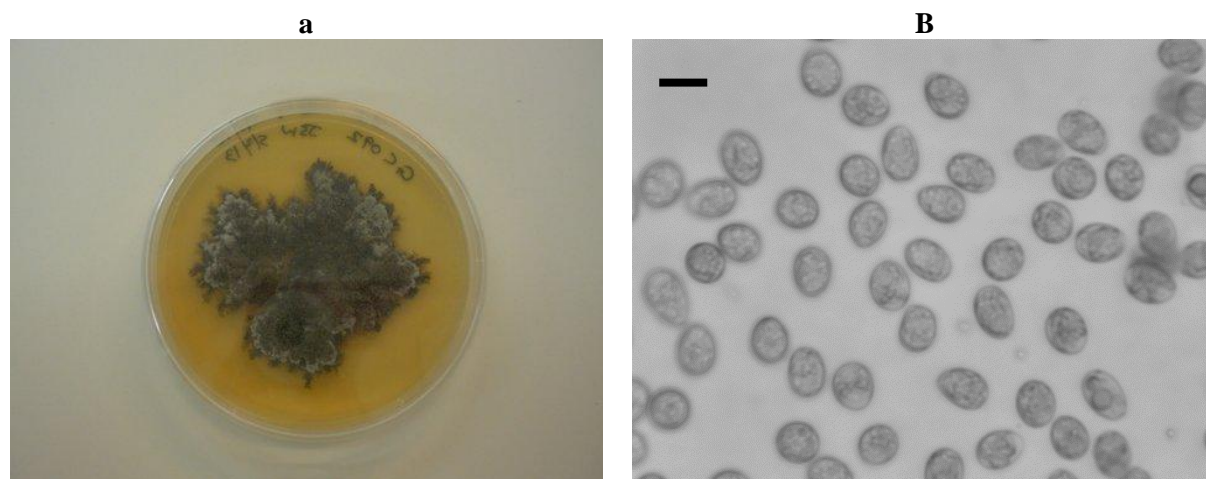


Figure 1: a. *Phyllosticta citricarpa* colony on PDA agar plate; b. *P. citricarpa* spores in aqueous suspension (bar represents 10 µm)

1.1. Orange inoculation

The experiments required fruit with lesions with pycnidia. Mature fruits of sweet orange (*Citrus sinensis* Osbeck), cultivar Navel Late (from Spain and S. Africa) were purchased commercially. Fruits were washed and surface disinfected using 70% ethanol. Oranges were inoculated in batches with one or other of the isolates, they were never used mixed. Three methods were used to inoculate the fruits with single isolates of *P. citricarpa* in a sterile flow cabinet: A). A suspension of spores (in sterile distilled water) was injected, 100ul at a time, into a dozen different locations on the top surface of each orange, using a hypodermic needle. It was carefully inserted into the albedo of the orange (the white pith area just below the peel) (Fig. 2a). B). additionally, some fruit were inoculated with mycelia. Growing edges of a fungal colony were collected from the margin using a fine scalpel. A small incision was made in a dozen parts of the upper surface of the oranges. The mycelia were inserted into the incisions being careful to ensure the material reached the white albedo beneath the peel (Fig. 2c). C). an alternative method sprayed the oranges with a spore / mycelia suspension.

The inoculated oranges were incubated in sterile plastic boxes at 20°C under a lighting rig providing 12 hour photoperiod (Fig. 2b). After a few weeks, signs of infection were observed, with development of lesions and subsequently pycnidia after some 4-6 weeks (Figs. 2d-f). Some oranges (approx. 20%) developed *Penicillium* and were discarded. Eight batches of oranges were inoculated over several months to ensure a continuous supply of infected oranges for use in experiments. Oranges were misted with sterile distilled water and a drop of film collected and observed for spore production by microscopy. Statistical analysis of experimental data found no significant trend caused by either the source of the oranges (from Spain or South Africa) nor the two isolates used in the study.

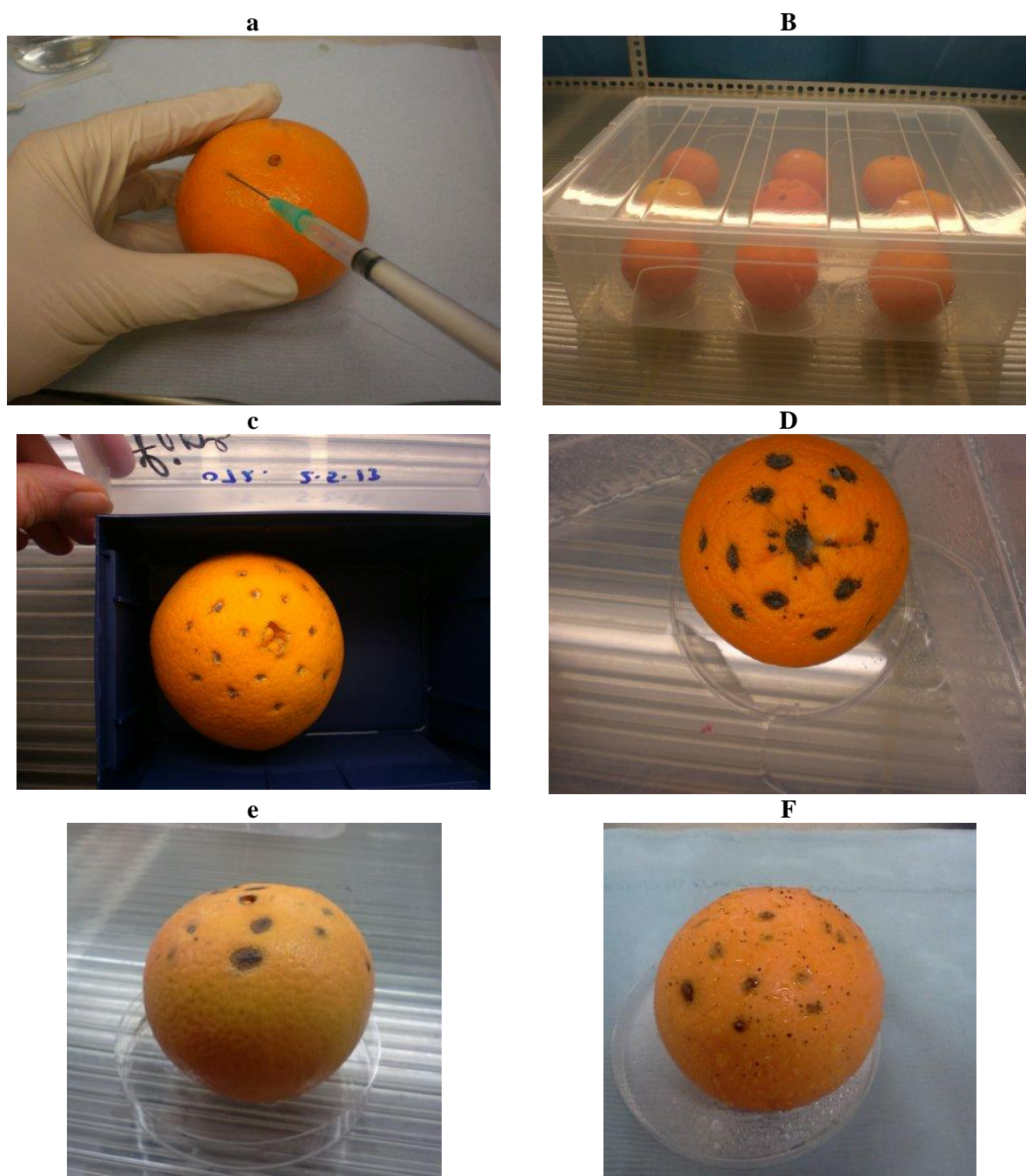


Figure 2: a. Inoculation of oranges with *Phyllosticta citricarpa* spores in suspension; b. Incubation of oranges in sterile transparent boxes; c. Orange impregnated with chunks of mycelium; d. Infection resulting from mycelium; e. Infection resulting from needle inoculation; f. Orange infected by injection and sprayed with a spore suspension.

2. Laboratory experiment

2.1. Release of spores from surface film on infected oranges in flow cabinet (simulated duration of spore production during rain shower)

An infected orange with distinct citrus black spot disease lesions was misted with sterile distilled water three times in the minutes preceding the experiment in a flow cabinet (Fig. 3). This was to encourage spores to ooze from the disease lesions. The film of water rapidly coagulated into large drops of water on the orange surface, most of which then runs off the orange. However, drops of water remain in the hollows associated with lesions. After set periods of time up to one hour, water was drawn off the orange surface and placed on slides for microscopic observation to determine presence and numbers of *P. citricarpa* spores. The orange was repeatedly misted throughout, as in simulation of a continuous light rain shower. The aim of this was to determine how long spores were produced for during a rain shower and was repeated three times, twice with isolate GS072 and once with isolate GC092.



Figure 3: Infected orange after misting with sterile distilled water in flow cabinet

2.2. Frequency of conidia in splashed droplets

2.2.1. Spore suspension and melinex tape

This experiment was done to determine numbers of spores that were able to be carried in splashes of different sizes. A spore suspension was produced by flooding a fungal colony (GC072) with sterile distilled water. The surface was then scraped and the content poured off. Observation showed few spores in suspension so the black fruiting bodies were crushed with a sterile glass rod and this effectively released more spores into suspension, which was filtered through sterile muslin. The resulting spore concentration was found to be 70,000 per ml using a haemocytometer.

On the platform at the base of the rain tower, strips of melinex tape were placed horizontally around a small glass Petri dish containing 1 mm layer of the above spore suspension. Fifty drops of sterile

distilled water were released in the rain tower on to the dish containing the spore suspension and drop splashes caught on the surrounding melinex strips (Fig. 4). These were then carefully lifted and left in a sterile flow cabinet to dry. The number of spores in ten drops each of various size categories was recorded using microscopy.

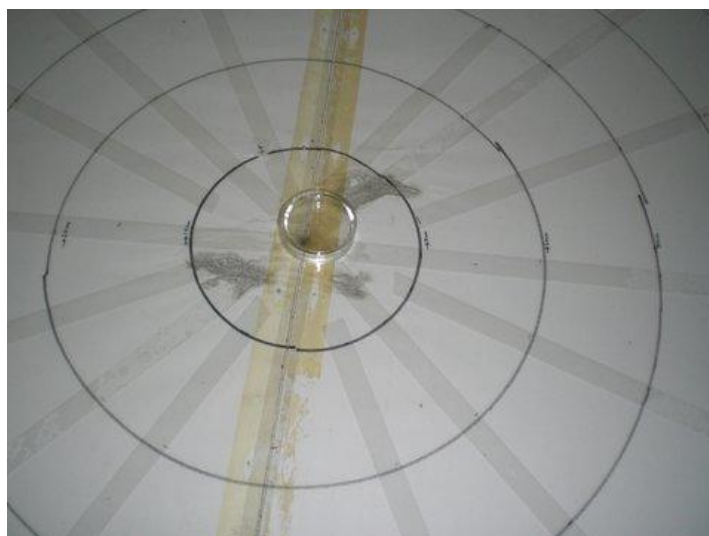


Figure 4: Rain splash experiment in Rothamsted Rain Tower; transparent melinex strips placed horizontally at various distances from target *Phyllosticta citricarpa* spore suspension

2.2.2. Infected oranges and slides / melinex tape

2.2.2.1. In flow cabinet

This experiment was done to determine whether spores were carried within the splashes from an infected orange. In a sterile flow hood, an infected orange (isolate GS092) was misted with sterile distilled water. After 10 minutes 0.1 ml of the liquid-film on the surface of the infected orange was drawn-up using a hypodermic syringe and placed on a slide. High power microscopic observation showed numerous spores of *P. citricarpa* were present and thus able to be potentially splashed off the orange. A series of slides were placed around the orange inside the flow cabinet at 5 cm and 10 cm distances. A 1 ml syringe filled with distilled water was held in place 40 cm above the orange. Water was forced down out of the syringe onto the orange to produce splashes. The slides were assessed under high power microscope for presence of spores and these were counted.

2.2.2.2. In rain tower

This experiment was repeated in the rain tower using sections of melinex tape as droplet collection surfaces radiating out from an infected orange (Fig. 5). (To confirm the tape was an appropriate surface to use, a spore suspension of differing size drops (concentration 50,000 per ml) was dripped onto melinex strips and measured whilst wet. They were left to dry in a flow hood to determine firstly whether the position of the dried drop was still visible and secondly whether spores could be observed and counted accurately once dried out). The presence of spores in the liquid-film on the surface of a

sterile water misted infected orange (isolate GS092) was checked by placing a drop onto a microscope slide at the start of the experiment by observing under a light microscope. This confirmed that there were indeed *P. citricarpa* spores present. Incident drops (one hundred, 5 mm size) were dropped on to an infected orange in a rain tower experiment. Sections of tape were observed under high power microscopy to determine presence of spores in splashes dispersed from the infected orange.



Figure 5: Rain splash experiment on infected orange using melinex tape to determine dispersal of *P. citricarpa* spores in splashes: view from top of rain tower showing infected orange and experimenter setting up tapes to catch splashed droplets

3. Rain tower experiments

Rain-splash experiments were conducted in the Rothamsted Rain Tower (Fitt et al., 1986). This is an 11 m tower 1.2 m X 1.2 m wide the top of which is open and has a framework for attaching syringes for creating drops, the height of drop allows the drops to reach terminal velocity. The bottom is also open and is at the leading end of a wind tunnel. The base is enclosed with transparent Perspex doors. At the base there is a flat platform on which a target (bulls-eye ring) was overlain with 10 cm increasing circles, up to a maximum of 70 cm from the central point (Figs. 6). Vertical rods were placed at various locations on this platform on which tapes can be placed to catch vertical drops and splashes.

3.1. Effect of raindrops on splash height, distance and trajectory in still air - Infected oranges and water sensitive paper (wsp); drop sizes 5 mm, 3.5 mm and 2.5 mm using single incident drops

An orange, with pycnidia visible in infection lesions, was placed in the target-centre and was misted with sterile distilled water to encourage a film (including individual drops) of water on the surface in which spores were suspended. Individual drops of water (of pre-determined diameters (5 mm, 3.5 mm and 2.5 mm)) were dropped onto the orange (25 drops per experiment) from 1 ml syringe (5 mm drops) or 1ml syringe plus two varying hypodermic needles (for 2.5 and 3.5 mm drops). The resulting splashed droplets, from the oranges were collected on water sensitive paper strips (70 mm x 20 mm)

and (50 cm x 20 mm) which were placed at differing heights and distances from the orange either sides of the narrow column of falling simulated rain (a) horizontally at increasing distance from the target (5, 10, 20, 30, 40, 50, 60, 70 cm) and (b) vertically around the target orange, at different heights (10, 20, 30, 40, 50 cm high) at different distances from the target (10, 20, 30, 40, 50 cm) (Fig. 3.). Melinex strips were also placed on the platform below the infected orange in the case of the 3.5 m incident drop to confirm presence of spores in the splashed droplets carried away from the infected orange. These were observed under high power microscope.

The optimal heights and distance were predetermined by initially using uninfected oranges. Digital and video photography was used to record the pattern of splash from the oranges. The average concentration of spores in the film around the misted orange was assessed by microscopy using a haemocytometer slide at the start of a rain simulation event.



Figure 6: Rain splash experiment at the base of the 11 m Rothamsted Rain Tower; water sensitive paper strips placed vertically and horizontally at various distances and heights from target orange

3.2. Simulated rain shower event – multiple splashes

This experiment involved releasing a 15 second timed shower on to a collection of infected oranges (isolate GS 072 in one repeat, GS092 in the other repeat) placed on a wire mesh cage at the base of the rain tower. The mesh cage platform was surrounded by paper tissue to soak up any drops that had either missed the oranges or would be secondarily splashed from the solid platform to interfere with those splashes directly from the oranges. Water sensitive strips were placed vertically on rods on clamp stands radiating out from 10 cm to 70 cm away from the infected oranges. The rods were also raised to the same base-height as the oranges. The oranges were misted to induce spore production and then a rain shower was simulated for 15 seconds (Fig. 7) and is estimated to have comprised approximately 2000 incident drops.



Figure 7: Multiple rain splash experiment in Rothamsted Rain Tower; batch of infected oranges on metal cage and surrounded by tissue to soak up excess splashes *P. citricarpa* spore suspension

3.3. Combined rain tower / wind tunnel experiments

This experiment investigated the effect of different wind speeds on the splash pattern resulting from 5 mm drops onto infected oranges. It was conducted using the rain tower and integral wind tunnel which is shut at both ends and includes a filtration system on the circulating air. The wind speeds investigated were 1, 2, 4 and 7 m/sec. The oranges were either inoculated with isolate GS072 or GS092. Instead of being placed in a radial layout as in (A) the clamp stands containing vertical series of water sensitive paper strips were placed in a slightly offset linear pattern up-wind and down-wind of the infected orange at the base of the rain tower. The reason to offset collection positions was to avoid shielding subsequent positions. The stands were placed at different distances up to a maximum of 8 metres downwind from the orange (Fig 8). Speeds 1 and 2 m/sec were repeated twice and speeds 4 and 7m/sec were repeated three times.



Figure 8: Rain splash and wind effects experiment in the combined Rothamsted Rain Tower-Wind Tunnel facility; rods with water sensitive paper strips placed downwind from the infected oranges up to a maximum distance of 8m

RESULTS

4. Laboratory experiment

4.1. Release of spores form surface film on infected oranges in flow cabinet (simulated duration of spore production during rain shower)

This experiment tested for how long fresh spores were released from an infected orange into the water-film on the surface of the orange (Table 1). Infected oranges released spores into the water film on an infected orange for up to 1 hour (Fig. 9). During this hour the average concentration of spores in the liquid was estimated to be 475,000 per ml.

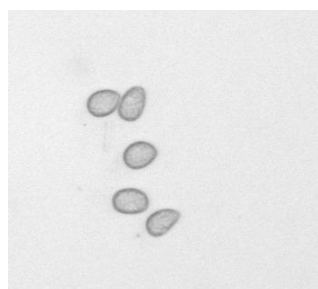


Figure 9: *P. citricarpa* spores present in water film/drops on infected orange in a simulated rain shower

Table 1: Results of test to determine the release of spores from an infected orange

Time lapse (minutes after misting or rinsing)	Numbers / presence of spores		
	Number of spores per ml (cm ³) in film of water on surface after frequent misting	Number of spores per ml (cm ³) in film of water on surface after frequent rinsing	In run-off below orange
1	125,000	300,000	yes
5	625,000		yes
10	750,000	280,000	yes
20	125,000	300,000	yes
30	375,000	48,000	yes
40		25,000	
50		1,000	
60	200,000	3,000	yes

4.2. Frequency of conidia in splashed droplets

4.2.1. Spore suspension and melinex tape

The melinex tape was effective in collecting maintaining the size of the splashed droplets (Fig. 10) and, once dried out, the diameter of the drops was easily measured by HP microscope. The size of the drops varied from 0.1 mm to 5.5 mm in diameter. The smaller drops were more numerous. Ten droplets of each size category were observed under HP microscope and numbers of spores counted. The mean number of spores by droplet size is shown, Fig. 11.



Figure 10: Splash collection at base of rain tower on melinex strips

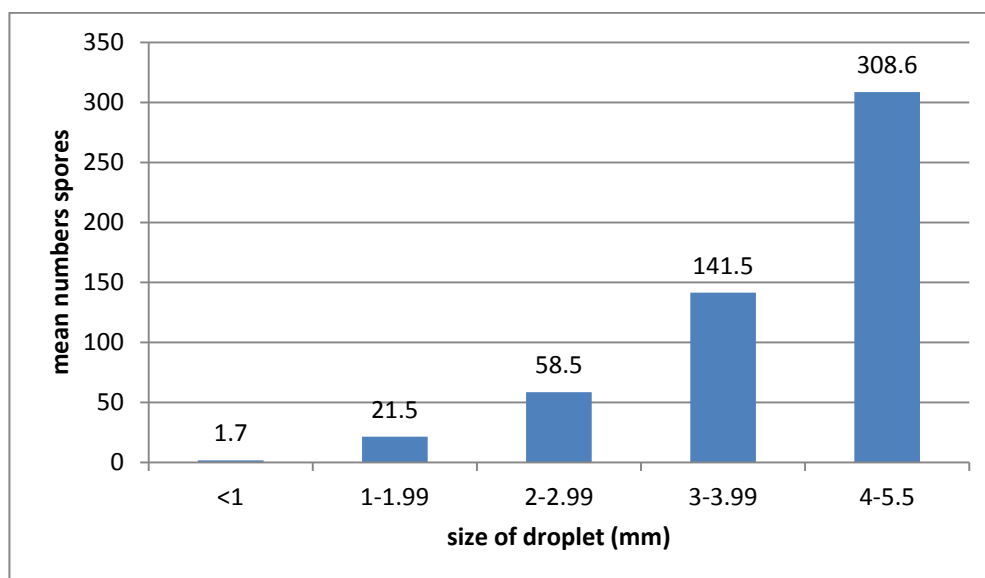


Figure 11: Mean numbers of spores per droplet size (10 droplets counted per size)

The maximum number of spores was 473 in a droplet measuring 5.1 mm. On average, there were 308 spores in the largest droplet size category. The smallest droplets, which were the most numerous, only contained 0-4 spores, with an average of 1.7. As expected, larger drops were found to contain more spores than small droplets. *P. citricarpa* spores were clearly visible with in the droplets (Fig. 12).

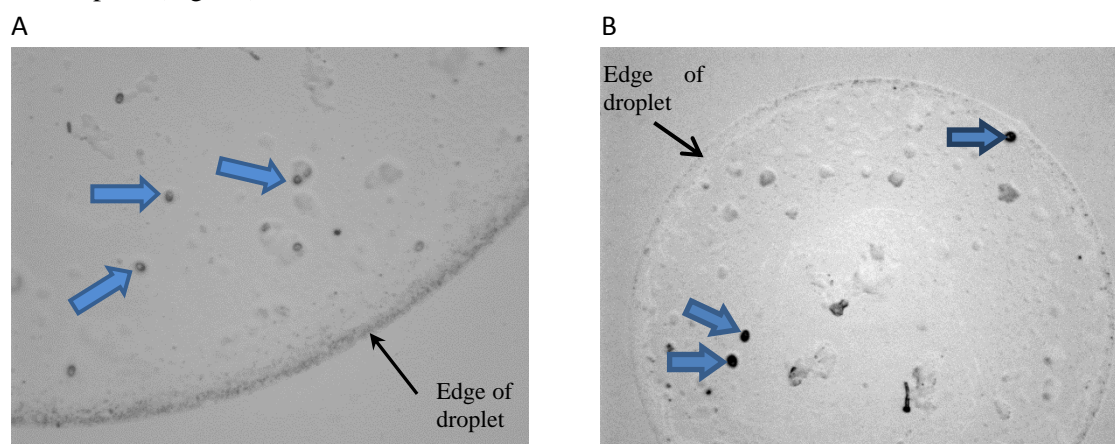


Figure 12: Spores (some indicated by blue bold arrows) in splashed droplets (edge indicated by fine arrow) observed under High Power microscopy a. Spores in a large droplet and b. Three spores in a 1 mm droplet

4.2.2. Infected oranges and slides / melinex tape

4.2.2.1. In flow cabinet

The infected orange had been misted with sterile water and checked to confirm that spores were present in the film/beads of water on the surface. Observation of some of the slides showed that spores

were present in the splashes which came from the infected orange – present in 5 cm distance slides. The splashes were 5-6 mm in diameter. This confirmed that splashes from infected misted oranges could produce spores that were then transported away from oranges by rain drops. It showed that spores were produced from the lesions and came into the water and were able to be splashed away from the orange. However, not all splashes were found to contain spores as drops hitting un-infected parts of the orange did not pick-up spores. The number of spores in droplets varied from 10-125 spores (5 mm diameter) (Table 2).

Table 2: Presence of *P. citricarpa* spores within splashes ejected from an infected orange in four different compass directions

Direction from orange	Presence/Absence of spores
	5 cm from orange
North West	51 spores in one splash 18 in one splash 10 in one splash Two splashes with no spores
North East	30 spores in one splash Two splashes with no spores
South East	No splashes
South West	125 spores in one splash One splash with no spores

4.2.2.2. In rain tower

The repeat experiment in the rain tower confirmed that spores were present in splashes dispersed from the infected orange. Microscopic observation at high power showed there to be numerous spores in the splashed droplets. Photography captured the pattern of splash trajectory and dispersal of drops from the infected orange (Fig. 13).

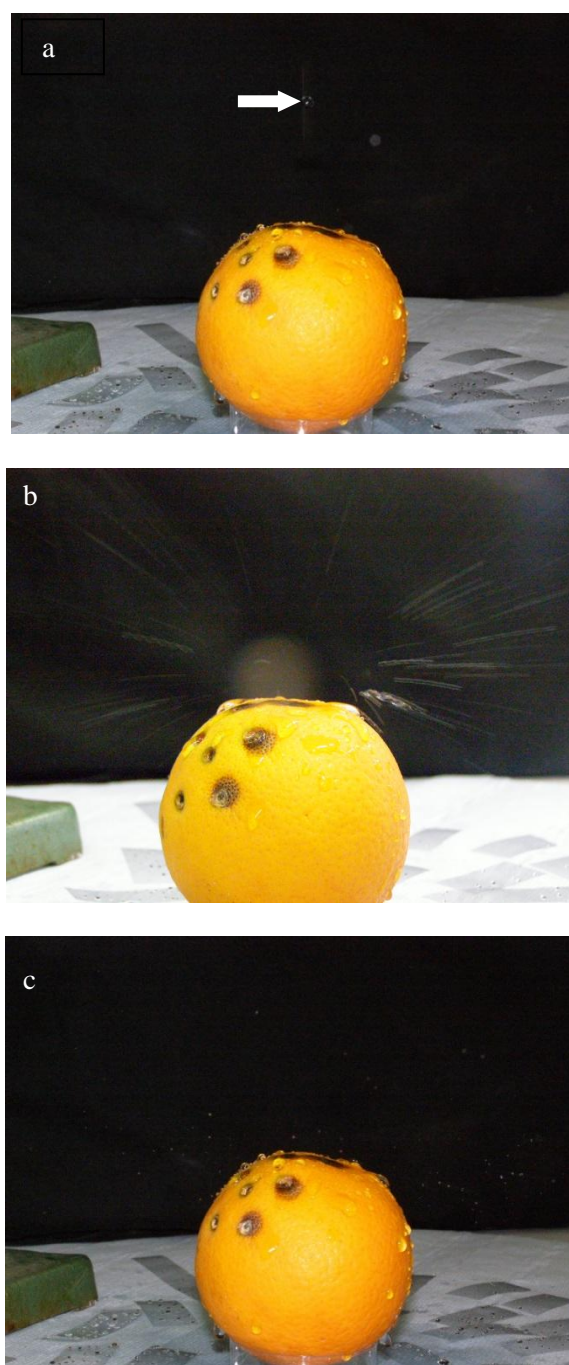


Figure 13: Splash emanating from infected orange; a. drop (arrowed) falling mid-flight above orange; b splash event (long exposure to show splash trajectories); c splash droplets mid-flight (flash, short-term exposure photo)

5. Rain tower Experiments

5.1. Effect of raindrops on splash height, distance and trajectory in still air - Infected oranges and water sensitive paper (wsp); drop sizes 5 mm, 3.5 mm and 2.5 mm using single incident drops

Observations confirmed the rain tower experiments worked well and splash deposits were clearly seen on the water sensitive paper (Fig. 14).

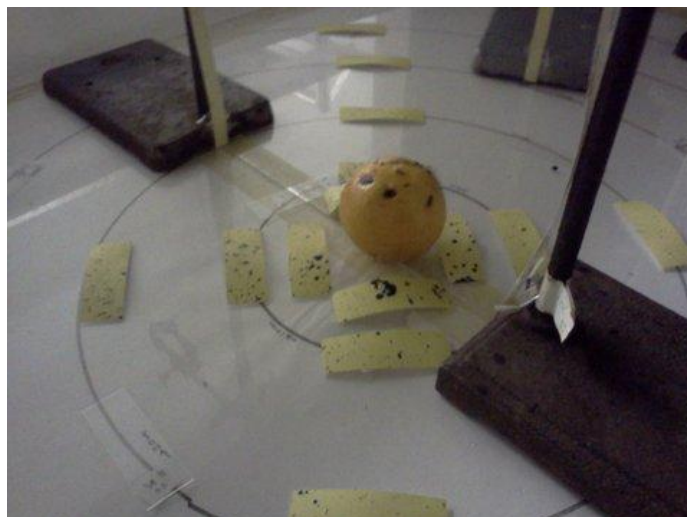


Figure 14: Splashes on water sensitive paper strips at increasing distances from infected orange on platform at base of Rothamsted Rain Tower

5.1.1. Splash trajectories in still air, produced using single incident drops

5.1.1.1. Results from vertical strips

The larger incident drop size resulted in a higher splash dispersal from the infected orange (Fig. 15). The maximum recorded vertical height of the splashes varied with distance from the orange being at their highest when 20 cm from the orange – at which point the splash reached 47.4 cm high (Fig. 16). The height of the splash increased suddenly and splash trajectories declined towards 50 cm horizontal distance from the orange with few travelling further.

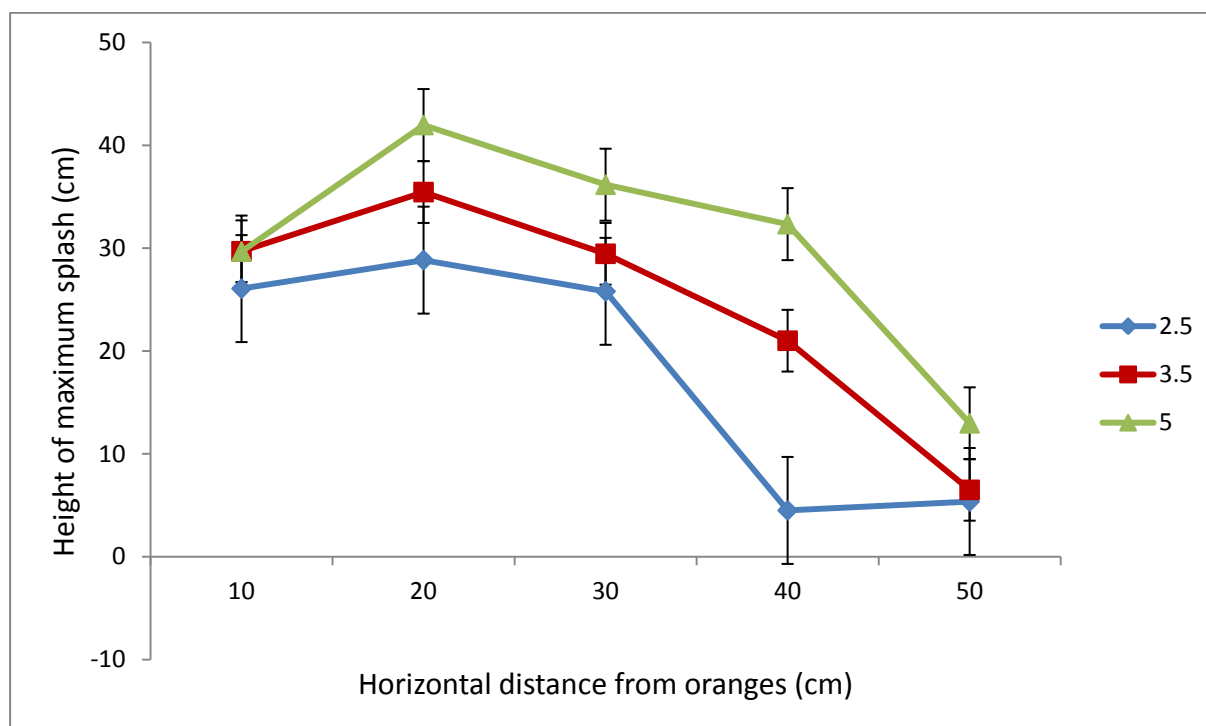


Figure 15: Maximum vertical splash height with horizontal distance from infected orange in still air (mean of three repeats)



Figure 16: Splash pattern on vertical strips of water sensitive paper; the splashes are more dense at the base of the strips (left hand side of figure) and less frequent at the top. The height of the maximum splash is marked by an arrow.

The variation and frequency of different size splash was significant; most splashes (90%) are less than 1 mm in diameter, 9.4% of splashes are 1-2 mm and only 0.5 % of the splashes are over 2 mm (Fig.17).

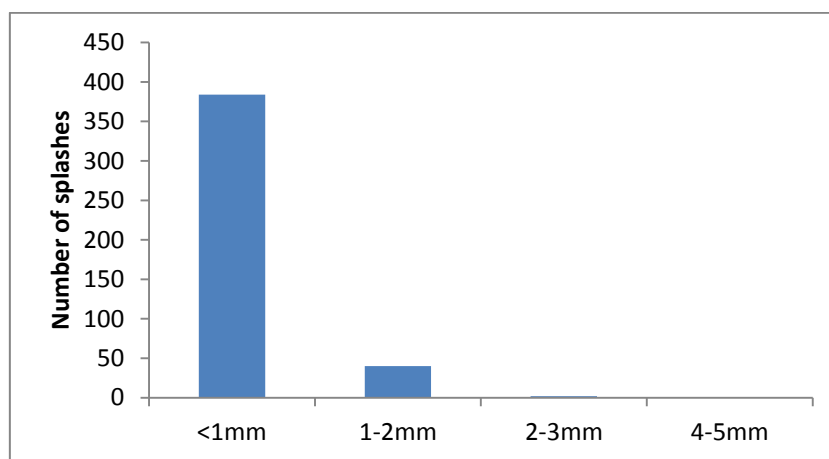


Figure 17: The frequency of splash droplets of different size categories

The numbers of splash droplets of different sizes reduced with distance as they radiated out from the orange (Fig. 18); most splashes (32%) were less than 1mm in diameter and fell within 10 cm horizontal distance of the orange. 24% splashes were less than 1 mm and hit a maximum height at 20 cm from the orange. The largest splash landing on the vertical strips was 2-3 mm in diameter and fell on the strip 20 cm from the orange at a height of 10-20 cm. The number of spores collected decrease with increasing height.

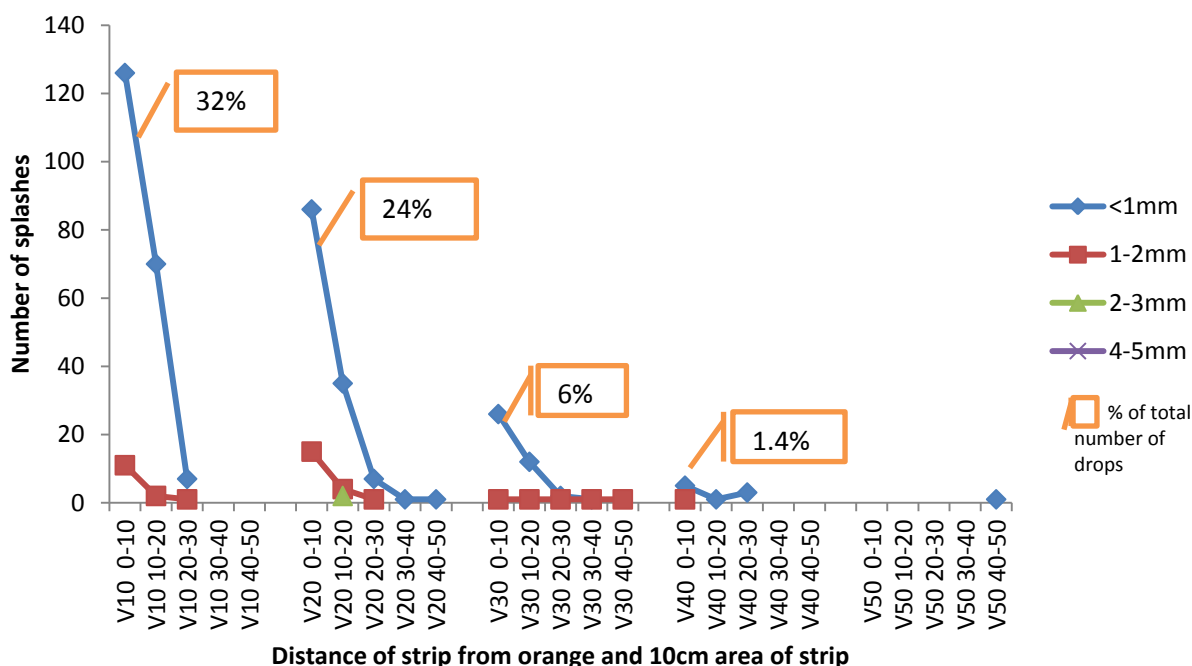


Figure 18: Numbers of splash droplets of different sizes falling on vertical strips with distance from infected orange and relative percentages of the total numbers of droplets (5 mm incident drop). Data were also collected using 2.5 mm incident drops, producing a similar pattern (data not shown). Results from horizontal strips:

The smallest splashes, under 1 mm in diameter, were by far the most frequent (81%) size of splash (Table 3). Most (76%) of splashes fell within 10cm of the orange and 90% fell within 20 cm (Fig. 19-21). The smallest splashes as well as being the most numerous occasionally splashed the furthest away – only splashes less than 2mm travelled further than 50 cm from the orange. The largest splashes (4-5 mm) did not travel so far and all fell within 30 cm of the orange, half of these (52.3%) were within 5 cm of the orange (96.8% within 10 cm of the orange). Although most splash droplets were <1 mm, the ones that travelled furthest in still air were 1-2 mm in diameter.



Figure 19: Splash pattern on horizontal strips of water sensitive paper; the splashes are clearly more dense on the strips nearer the orange (left hand side of figure) and less dense further away from the orange (note: wsp strips shown rearranged to be closer together than in the experiment).

Table 3: Frequency of splash droplets of different sizes at increasing distances from an infected orange. Means of four compass directions (NW, NE, SW, SE)

Distance (cm) from orange	Droplet frequency				Total	%
	<1mm	1-2mm	2-3mm	4-5mm		
5	313.3	31.7	11.25	8.25	364.5	35.9
10	323	63.25	10	7	403.3	39.7
20	128.75	18	1.25	0.25	148.3	14.6
30	55	12	1.25	0.25	68.5	6.8
40	3.5	12.5	0.5		16.5	1.6
50	2	3.75			5.8	0.6
60	1	5			6.0	0.6
70	1	1			2.0	0.2
Total	827.6	147.2	24.3	15.8		
%	81.6	14.5	2.4	1.6		

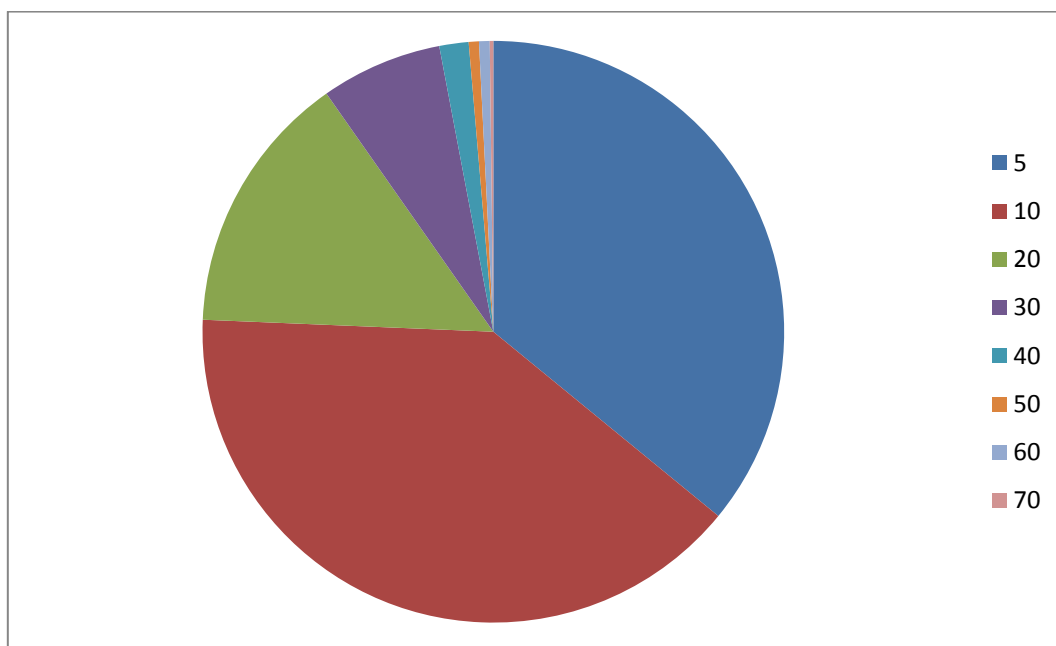


Figure 20: Frequency of total splashes horizontally away from the orange (mean of four values)

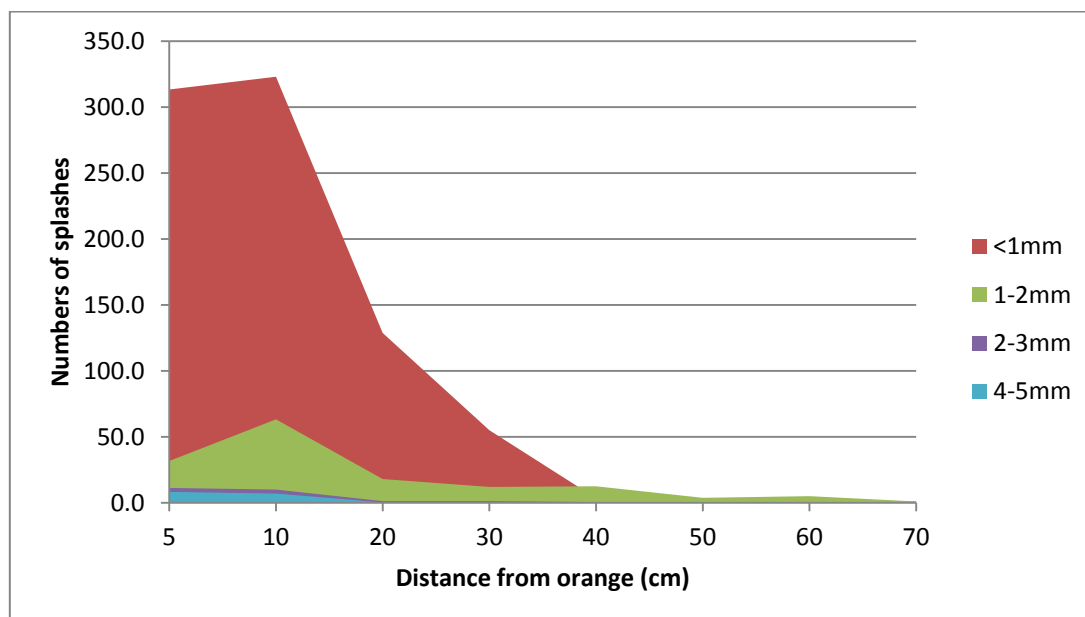


Figure 21: Distribution of various sized splashes horizontally away from the orange (mean of four values)

6. Simulated rain shower event – multiple splashes

The maximum height of splash droplets in the simulated rain shower was higher than found with individual incident drops (Fig. 22), suggesting that splashes combined / rebounded and resulted in erratic splashes which went much higher than in the single drop experiments; 70+cm high from the infected oranges. This is thought to occur due to combination of adjacent splashes, which alters the splash trajectory to force some of the splashed droplets to go higher than from a single splash event.

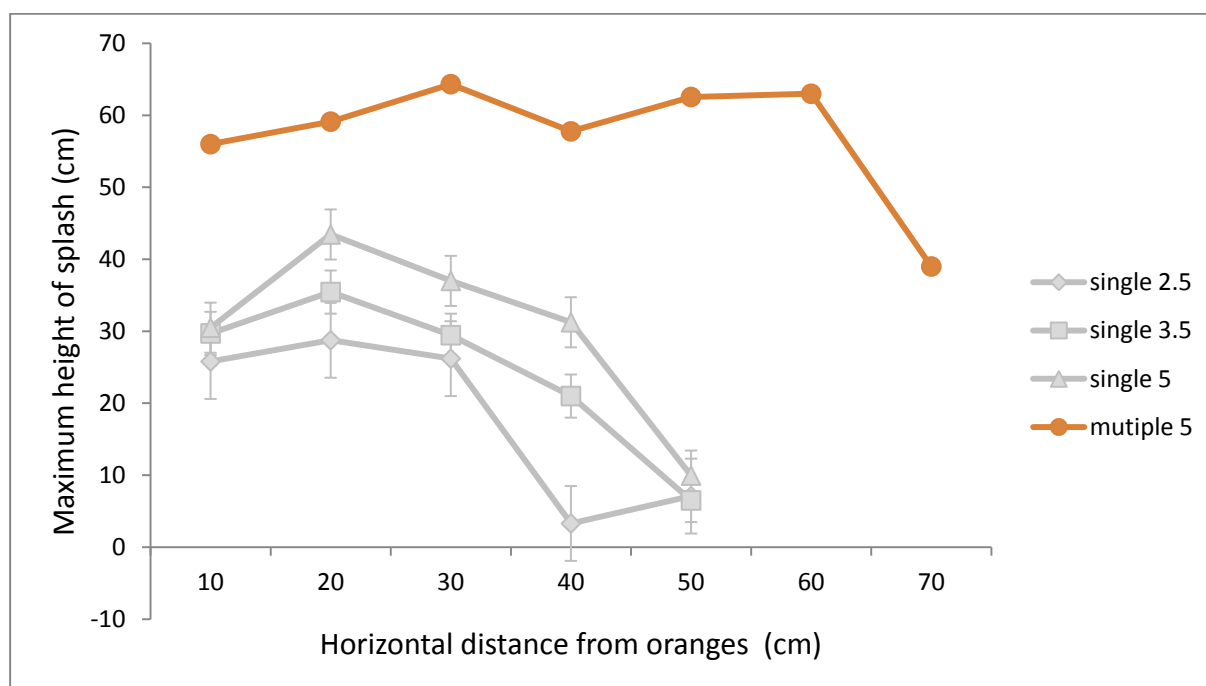


Figure 22: Maximum vertical splash height with increasing horizontal distance from infected orange in still air in a rain shower (*mean of two repeats*)

6.1. Combined rain tower / wind tunnel experiments

Higher wind speeds dispersed the splashes from the oranges further downwind than in still air or in low wind speeds (Figs. 23-25). Some ‘ballistic splashes’ went upwind, especially at the lower wind speeds. The 1 m wind speed produced a similar pattern of splash trajectories to that of still air. The 4m/sec wind speed carried splashes to 2 metres downwind and the 7 m/sec wind speed carried some splashes up to 8metres away (Figs. 23 and 24). Numbers of splashes dispersing downwind increased with wind speed as increasing numbers were influenced by the wind to be blown downwind rather than being dispersed more radially (Fig. 23). At the highest wind-speed (7 m/s), a component of the smallest splash droplets (<1 mm in diameter) appear to become aerosolised and become entrained into the airflow, staying at their original splash height or even dispersing higher, up to a mean of 73.2 cm 8 metres distance downwind (Fig. 24). This occurred in three separate repeat runs at this wind speed.

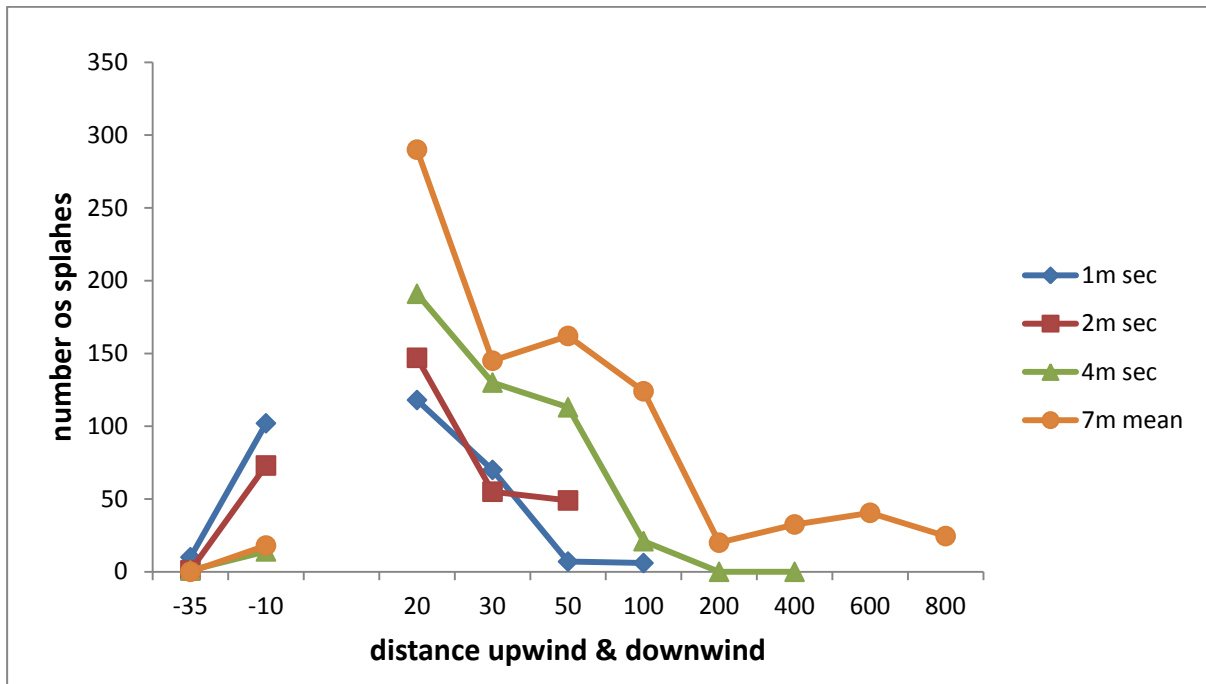


Figure 23: Frequency of splashes at differing wind speeds with distance upwind and downwind

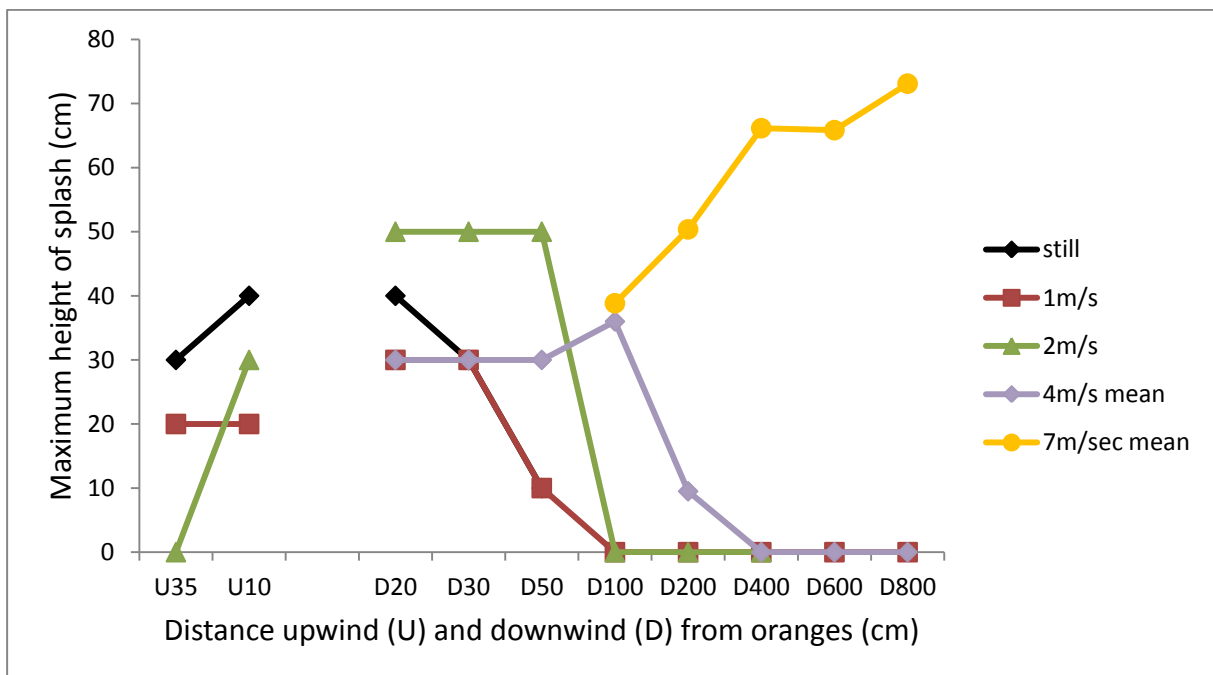


Figure 24: Maximum height of splashes in wind speed experiments (mean of 2 runs at 1 and 2 m/s or 3 runs, in the case of 4 m/s and 7 m/s wind speeds) at different distances from the source (NB horizontal distance not shown to scale)

The proportion of droplets travelling to different distances up and down wind at various heights is shown in Figure 25. It suggests that ballistic drops are dispersed up to 2 m at wind speed of 7 m/sec, but an increase in number of droplets reaching 60 cm height at over 2 m away suggests some droplets at this wind-speed were aerosolised and air borne droplets were recorded 8 m away.

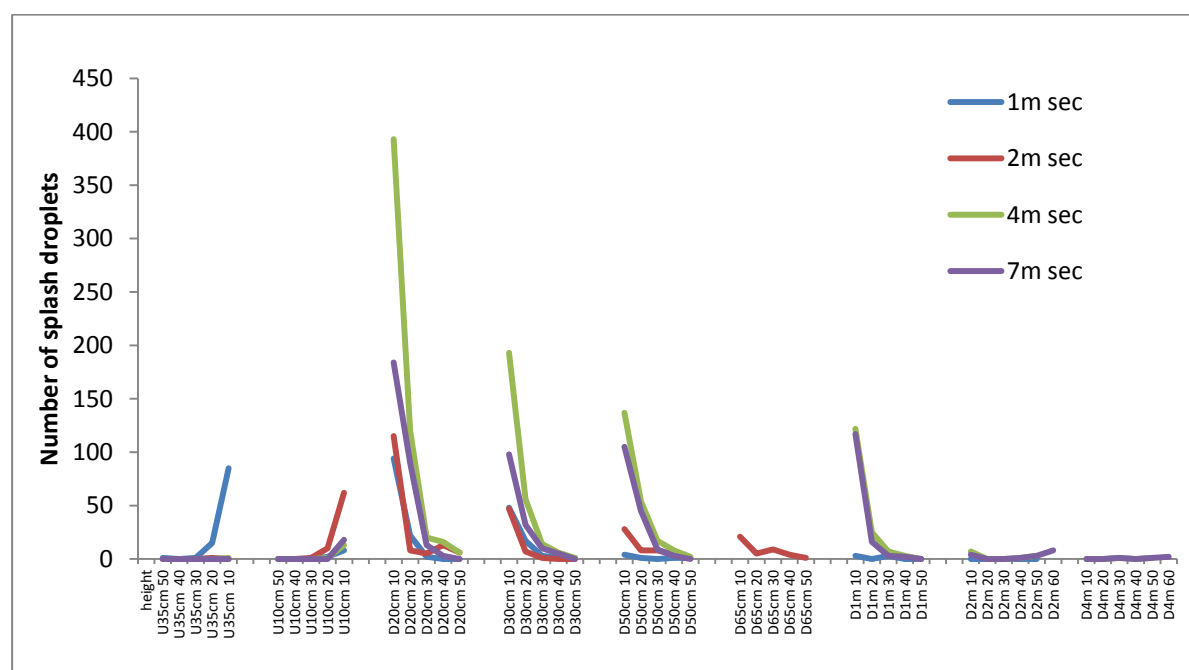


Figure 25: Effect of wind on rain splashes: Height sections split

7. Estimation of numbers of spores dispersed from an infected orange: Example calculation

These data can be used to provide a one-off estimation of numbers of *P. citricarpa* spores potentially spread by rain fall splash. It must be emphasised that this is just an example. An estimation of the number of spores dispersed by one drop is as follows (Table 4);

Table 4: An estimation of the number of spores dispersed by one drop

5cm incident drop	<1mm	1-2mm	2-3mm	3-5mm	total
Numbers of splashes	384	40	2	1	
Number of splashes containing spores	96	30	1.98	1	
Number spores per splash size	2	22	55	148	
Total number spores	192	660	108.9	148	1108.9

1109 spores in 5 degrees (5 vertical rods in rain tower) x 72 = 79840 in full 360 degrees radiating out from an infected orange. If the estimate of total number of spores splash dispersed by 25 x 5mm rain drop is 79840, then the estimate of total number of spores splash-dispersed by one 5 mm rain drop is approximately 3193. Only 25% splashes of 1mm or under contained spores – however, these may have the potential to become air borne.

The average concentration of spores in water film on surface of infected orange (Fig. 26) were therefore 475,000 spores per ml. In one 5mm drop experiment, all splashes combined are estimated to disperse a total of 3,900 spores per one rain drop. Hence approx. 1/120th of spores in 1 ml of water film on orange surface are dispersed by a single raindrop.

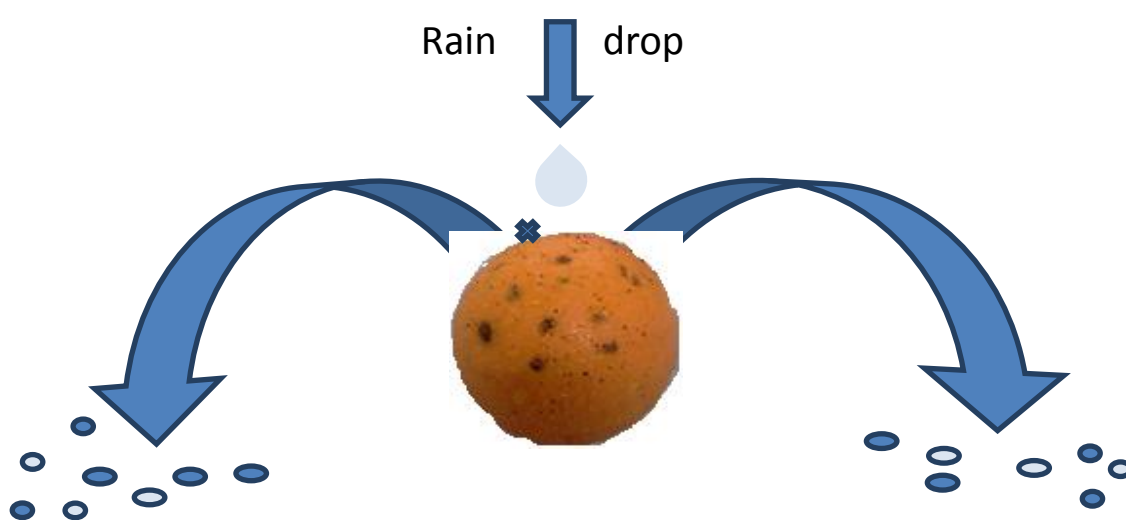


Figure 26: Diagrammatic representation of dispersal of spores from infected orange subjected to rain splash. Bold X: Average concentration of spores in water film on surface of infected orange = 475,000 spores per ml; Dark ovals: All splashes combined are estimated to disperse a total of 3,900 spores per one rain drop; Pale blue ovals: Some splashes did not have spores; Hence approx. 1/120th of spores in 1 ml of water film on orange surface are dispersed by a single raindrop

CONCLUSIONS

Simulated rain splash experiments using a rain tower and wind tunnel were used here to determine the potential for dispersal of *P. citricarpa* conidia (pycnidiospores) from infected oranges. Infected oranges were able to exude spores for over an hour, which means that water on the surface of the infected orange, which occurs as a film over infected positions and as droplets on healthy peel, will contain a suspension of spores throughout a rain event and these spores will be available to be splash-dispersed. It is known that rain and irrigation splashes remove spores in water films from plant surfaces by incorporating them into splash droplets, most of which travel only a few centimetres and some over 1 m (Fitt et al. 1989). High Power microscopy in this study demonstrated presence of

conidia of *P. citricarpa* in splash droplets. The surface texture, angle of orientation, flexibility and plasticity of a surface is known to affect the characteristics of splashes from the surface. To our knowledge, splash dispersal of spores from oranges has not been studied previously. In still air, splash droplets were splashed highest by the largest (5 mm) incident drops, reaching on average 41.9 cm high as opposed to 35.5 cm with 3.5 mm drops and 28.8 cm with 2.5 mm drops. Maximum heights were found between 20 and 30 cm horizontal distance. Larger splashed droplets were found to contain the most spores (4-5.5 mm splash droplets averaged 308 spores), but get splashed <30 cm, while most (80-90%) splashes were <1 mm diameter but carried on average only one spore. The droplets that were splashed the greatest horizontal distance in still air were 1-1.99 mm in diameter, which reached 70 cm and contained an average of 21 spores. Results of this study therefore fit well with previous studies on other splash-dispersed pathogens, e.g. Yang et al (1990) demonstrate splash dispersal of spores of *Colletotrichum acutatum* to distances up to 80 cm from plastic sheeting, 60 cm from soil and 50 cm from straw, while MacDonald and McCartney (1987) modelled theoretical maxima of ballistically splashed droplets 1 mm in diameter as 75 cm height and 120 cm horizontal distance in still air. Large incident rain drops are known to remove more spores and to splash them further due to their increased kinetic energy compared to small rain drops (McCartney and Fitt, 1986; Fitt et al 1988; Yang et al. 1991).

In multiple splash experiments, in which a 15 second simulated shower of rain fell onto infected oranges, maximum splash heights in still air reached 60 cm, compared to 47.4 cm with single splash experiments. This is likely to be due to a vastly increased number of splash events i.e. greater technical replication since the multiple splash experiment was estimated to have comprised about 2000 splash events, compared to 25 individual incident drops per run with single splashes. However, it was also observed in the multiple splash experiments that adjacent splashes occasionally combined together and the altered trajectory appeared to be forced higher than was initially the case.

In addition to ballistically splashed droplets, which describe parabolic trajectories and are relatively unaffected by wind, smaller splash droplets that are also produced, are affected by wind and particularly for the smallest droplets, can become aerosolised and able to be dispersed much longer distances. In experiments combining wind speed with rain-splash, progressively higher wind speeds carried an increasing proportion of splashes that would have travelled upwind but were turned downwind and generally splashes travelled increasingly further downwind. At wind speeds up to 4 m/s, splash droplets described an arc that was skewed due to the wind but travelled less than 4 m horizontal distance downwind. However, at wind speeds of 7 m/s, splash droplets were found to disperse at least 8 m (they were recorded still within the airflow at a horizontal distance from the source of 8 m, which was the maximum distance that could be tested within the wind tunnel) and a small proportion of droplets (<1 mm) were found to dispersed higher than originally splashed (up to 75 cm) suggesting that they remain aerosolised and were affected by turbulence rather than behaving as ballistic droplets.

This study demonstrates that conidia of *P. citricarpa* are able to be dispersed from infected oranges in splashes of rain and particularly when combined with moderate wind-speeds (7 m/s equates to 25.2 Km/h), which is not unusual, the pathogen can be dispersed at least 8 m and to heights of at least 75 cm. Rain events are often combined with strong winds and although modelling of splash dispersal during rain events is complex due to secondary splash, loss of spores due to wash-out and depletion of the spore source over time, diffusion models (Yang et al 1991) and random jump models (Pielaat and van den Bosch, 1998) have been used. Studies of dispersal from citrus trees to the nearest newly infected tree in Florida suggest that rain-splash dispersed pathogens can travel up to 3.5 km, most probably in a tropical storm event (Gottwald et al. 2002).

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