Hort Innovation

Final Report

Joint Florida and Australian Citrus Black Spot Research Initiative

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Delivery partner:

The University of Queensland, Queensland Alliance for Agriculture and Food Innovation

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Joint Florida and Australian Citrus Black Spot Research Initiative – CT13021

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Content

Summary

The citrus black spot (CBS) disease, caused by the fungus *Phyllosticta* (syn. *Guignardia*) *citricarpa*, costs Australian growers ~$80M annually through export restrictions, fungicide applications and fruit damage. CBS has occurred in parts of Australia for over 100 years, but was only recently found in Florida. The need to manage CBS in Australia, and reduce the spread in Florida, provided an ideal opportunity for collaboration between the two countries. This collaboration was achieved through co-investment between the Citrus Research and Development Foundation (CRDF) in Florida, the University of Florida, the University of Queensland (UQ), and Hort Innovation.

Contemporary knowledge of CBS indicates that the disease primarily arises from the production of airborne ascospores that are formed in the citrus leaf litter in orchards. The ascospores then infect young susceptible fruit, eventually resulting in CBS symptoms when fruit mature. Based on this understanding of the disease, the project aimed to address three key areas:

- Inoculum (leaf litter) reduction in orchards.
- Sporulation patterns of the fungus.
- Sources of CBS resistance within Citrus germplasm.

Enhancing leaf litter degradation as a means of removing leaf litter before ascospores release was shown to be most effectively achieved by the application of an organic mulch layer covering the leaf litter. Reducing fungicide run-off is the next most effective approach, as it was found that simulated fungicide run-off from high volume spray application methods significantly reduced leaf litter degradation. Other leaf litter amendments were not highly beneficial.

Monitoring leaf litter in orchards found that at the beginning of the fruit season the majority of fungal fruiting bodies in leaf litter are pycnidia, which produce the water-splash dispersed conidia. The occurrence of pseudothecia, which produce ascospores, tended to peak in late December to early January. Quantitative PCR methods determined the relative proportions of DNA of the pathogen *P. citricarpa*, as well as the visually identical, but harmless endophyte, *P. capitalensis*. At the majority of sites and samples, *P. citricarpa* was highly dominant, and the infrequent occurrence of *P. capitalensis* is unlikely to be a significant source of confusion in studying sporulation patterns. Experiments to better understand the interactions between the pathogen and endophyte have suggested that the two species cannot readily occupy the same space, with co-inoculation studies on fruit showing pre-inoculation with the endophyte to reduce CBS development. Promoting *P. capitalensis* in orchards may be beneficial, however no significant differences in fungicide sensitivity between the two species could be found for exploitation to promote *P. capitalensis* over *P. citricarpa*.

Through the systematic inoculation of fruit of various *Citrus* accessions at the Bundaberg Research Facility, a small number of resistant accessions have been identified. Importantly, one of these accessions, the K15 pomelo, has several desirable horticultural traits that have previously led to its inclusion in the scion breeding program. This is in contrast to the sour orange, which has previously been considered as a source of CBS resistance for breeding purposes; however frequent inheritance of traits such as bitterness have limited progress with this approach. Identification of CBS resistance in an accession with good fruit quality in this project is a major step forward in breeding for resistance to CBS.

Keywords

citrus, pathology, black spot, *Phyllosticta*, *Guignardia*, *citricarpa*, breeding, fungi, endophyte, cultural control, biological control

Introduction

The fundamentals of the citrus black spot (CBS) disease cycle have been determined (Brodrick and Rabie, 1970; Kiely, 1948a, b; Kotze, 1981). Ascospores of *P. citricarpa* are ejected from fallen leaves on the orchard floor, and are considered the primary inoculum source. Spores infect young leaves and fruit, and then survive asymptomatically until latency breaks and symptoms develop, typically when fruit ripen. Fruit are most susceptible to infection during the first 20-24 weeks of development (Baldassari et al., 2006; Kotze, 1981; Wager, 1952). Pycnidia containing waterborne conidia are formed within black spot lesions, but are considered of lesser importance to the disease cycle. Considering that the most important inoculum source was always believed to be ascospores produced in the leaf litter, a more thorough understanding of the role the different spore types play in the disease cycle is needed to improve disease management strategies in Australia and Florida.

Previous studies have shown benefit from reducing ascospore production from leaf litter using grass mulching to cover the litter (Miles et al., 2008; Schutte and Kotze, 1997), or stimulation of leaf biodegradation with various chemical amendments (Bellotte et al., 2009; Mondal et al., 2007; Mondal and Timmer, 2003). However, these various approaches to leaf litter management can have limitations. Mulch application, for example, is relatively expensive and labour intensive. These limitations may be overcome to some extent using approaches that stimulate biodegradation of old leaves on the orchard floor, however the effectiveness of applied stimulants could be negatively impacted by fungicide run-off. For example, fungicides have been shown to directly inhibit microbial leaf litter decomposition (Rasmussen et al., 2012) and interfere with degradation by earthworms (Wright, 1977). This issue may be particularly relevant to Australian citrus production, where fungicide run-off to the orchard floor is significant due to spray applications volumes (up to 10,000 L/ha) which significantly exceed the theoretical canopy retention volume of mature citrus of 2,300 L/ha (Cunningham and Harden, 1998). It is therefore necessary to investigate if biodegradation can still be enhanced in orchards deploying these fungicide application methods.

Although removal of leaves under the trees has been considered, research in Brazil has shown that this does not give sufficient control of black spot (Spósito, et al, 2011). In addition, removal of leaves gives rise to increased soil erosion, increased water use, increased weed growth, reduction of natural suppression of soil-pupating insects as well as a reduction in soil carbon. The process of removal on a plantation scale requires specific equipment creating dust which has a negative impact on beneficial insects and increases the change of fruit inoculation due to disturbance of the infected leaves on the orchard floor which may release fungal spores.

Additional to enhancing CBS control by reducing the leaf litter inoculum source, monitoring of the leaf litter for spore production may assist in scheduling fungicide applications. However, a complicating factor is the occurrence of spores of both *P. citricarpa* and the harmless endophyte *P. capitalensis* (formally *G. mangiferae*). These two fungi are morphologically indistinguishable (Baayen et al., 2002), limiting accurate visual identification (Truter et al., 2007). This may hinder accurate fungicide forecasting based on ascospore monitoring. Also of interest is the direct interaction between the endophyte and pathogen. Any antagonism against the pathogen by the presence of the endophyte could be useful. Notable examples of such antagonism include suppression of fusarium wilt of banana by non-pathogenic forms of the causal fungus (Forsyth et al., 2006). Take-all of wheat has also been suppressed by non-pathogenic relatives of the causal fungus (Wong et al., 1996). One study from Brazil has shown reduced severity of CBS following inoculation of fruit with the endophtye (de Almeida, 2009). In terms of the practical application of any antagonism, commercially multiplied *P. capitalensis* could be applied. Alternatively, it may be possible to influence the *Phyllosticta* populations with certain orchard practices, such as fungicide applications. Evidence exists that the pathogen is sensitive to copper fungicide, whilst the endophyte is not (Hendricks and Roberts, 2012). Therefore, with further investigation it may be possible to choose fungicide programs that encourage the endophyte over the pathogen.

Superior to leaf litter management or spore monitoring would be the development of citrus varieties resistant to CBS. A major impediment to making progress in this area is a lack of clearly defined resistant accessions. The only *Citrus* thought to be resistant to CBS are Tahiti lime (*Citrus latifolia*) (Baldassari et al., 2008), and sour orange (*C. aurantium*) and its hybrids (Kotze, 1981). However, in the case of Tahiti lime it has been shown that leaves and fruit are "insensitive"; i.e. they can harbour infections of the pathogen, but these infections never produce CBS symptoms (Baldassari et al., 2008). Before any attempt can be made to pursue resistance to CBS, it is necessary to first characterise the disease response of a wide range of available *Citrus* accessions.

There is a clear need to improve the management of CBS in citrus orchards in Australia and Florida. Simultaneously there are a number of knowledge gaps that if addressed may lead to improved CBS control. Therefore, this project aims to address: i) inoculum (leaf litter) reduction in orchards; ii) sporulation patterns of the fungi associated with CBS; and iii) sources of CBS resistance within *Citrus* germplasm.

Methodology

The following methodologies were used to investigate the key project areas of: 1) leaf litter management to suppress inoculum production; 2) the seasonal dynamics of inoculum production by the two species of *Phyllosticta* in leaf litter, and 3) identification of resistant germplasm for future breeding projects.

Leaf litter management

In order to determine the effect of various leaf litter amendments on leaf litter degradation, and the influence of fungicide run-off from high-volume spraying on degradation by the various amendments, experiments were conducted in an orchard receiving minimal foliar fungicide applications. Green mature leaves were sampled randomly from the tree canopy and then distributed into poly-mesh bags. In the first season experiment, the bags of leaves were pinned to the ground under the trees in a randomized design, before being treated with either urea, calcium carbonate or a commercially available compost accelerator (Figure 1). In the second season an organic mulch was included as an additional treatment. Each treatment was prepared in duplicate, with one duplicate being subjected to simulated fungicide run-off from a typical CBS fungicide program. The experiment was performed in four replicates and included untreated controls. To determine the treatment effects on leaf degradation, a set of leaves were sampled every 2-3 weeks and assessed visually according to Mondal et al (2007), using a revised visual scale, and leaf litter dry weight data was collected.

Figure 1 - Leaves in mesh bags on the grove floor prior to treatment.

Seasonal dynamics of *Phyllosticta citricarpa* and *P. capitalensis*

Leaf litter monitoring

The seasonal dynamics of *Phyllosticta* in citrus orchards in Queensland, Australia, with a known history of CBS was determined. Every fortnight leaves were sampled from the ground in at least two orchards over three production seasons. Sampled leaves were inspected for fruiting bodies of *Phyllosticta* spp., the proportions of different fruiting bodies determined by microscopy, then waterborne conidia and airborne ascospores harvested separately from the leaf tissue, and quantified. Finally, DNA was extracted from the leaf tissue and spore suspensions, then quantitative PCR used to identify the relative proportions of the two *Phyllosticta* species (Hu et

al., 2014).

Pathogen endophyte antagonism in vitro

In order to determine if populations of the citrus endophyte *P. capitalensis* are antagonistic to the pathogenic *P. citricarpa* in orchards, *in vitro* competition assays were performed between the two fungi. Isolates of both fungi were first collected from fresh citrus tissue and or/an existing reference collection of isolates from Australia, and held by UQ. The cultures of the various isolates were then raised from single conidia to ensure purity, then identified as *P. citricarpa* or *P. capitalensis* and submitted to the Brisbane Plant Pathology Herbarium. Isolates of *P. citricarpa* and *P. capitalensis* were then inoculated onto the same Petri dish of potato dextrose agar (PDA) and incubated together. Any inhibition of growth *in vitro* was then observed.

Pathogen endophyte antagonism in planta

To investigate antagonism between the two fungi *in planta*, co-inoculation of fruit was performed in the field. Inoculum was prepared from characterised isolates of *P. citricarpa* and *P. capitalensis*. Two inoculations were performed per fruit at intervals of 48 hours or 14 days. At the first inoculation time, fruit were inoculated with a 10 mm-wide strip of sterile blotting paper soaked in a spore suspension and wrapped around the entire equator of the fruit, then covered with cling wrap to maintain humidity and aluminium foil to reduce field heat. The fruit were then incubated for ~48 hours before all coverings were removed from the fruit. At the second inoculation the procedure was repeated, but a 5 mm-wide strip was used instead of a 10 mm-wide strip to ensure that the second inoculation occurred within the same area of the first inoculation. Fruit were then left to mature before inspection for CBS symptoms. The first and second inoculations were various combinations of water controls and *Phyllosticta* isolates necessary to determine the existence and any antagonism between *P. citricarpa* and *P. capitalensis*.

Pathogen endophyte antagonism fungicide sensitivity

In order to determine any differences between the endophyte and pathogen in fungicide sensitivity, five representative isolates of each of the two fungi were grown on PDA amended with different concentrations of the major fungicides used in citrus production (copper, mancozeb, azoxystrobin and iprodione). Differences in fungicide sensitivity were determined based on colony growth rate.

Resistant germplasm

In order to identify germplasm with potential resistance to CBS, a fruit inoculation technique was developed and utilized on various citrus accessions located at the Bundaberg Research Facility, Queensland Department of Agriculture and Fisheries (Figure 2). Inoculum of *P. citricarpa* was prepared and applied to young citrus fruit. Fruit were then left to mature before being sampled and inspected for CBS. If symptoms were not present, fruit were incubated at 27˚C, high humidity and permanent light to break latency (Brodrick and Rabie, 1970). Suspect symptoms were inspected for pycnidia and conidia, then lesion tissue plated onto agar to attempt to recover *P. citricarpa*. Fruit were considered: 1) “susceptible” if typical lesions/pycnidia were observed and *P. citricarpa* recovered from lesion tissue; 2) “insensitive” if no lesions appeared, but *P. citricarpa* was recovered from the point of inoculation (Baldassari et al., 2008); or 3) “resistant” if no symptoms developed and no *P. citricarpa* could be recovered from the point of inoculation.

Figure 2 – Timothy Shuey undertaking fruit inoculations at the Bundaberg Research Facility

Outputs

Below is a summary of project outputs. Full details of experimental methods and results are provided in appendix 1.

Leaf litter management

The project has evaluated the ability of various ground-applied amendments (urea, dolomitic lime, compost accelerator, and sugar cane trash) to accelerate the decomposition of leaf litter in citrus orchards, with and without the presence of fungicide run-off. These evaluations found the sugar cane trash to be the most effective amendment for encouraging the decomposition of leaf litter. The next most important finding was the reduced decomposition of leaf litter in the presence of fungicide run-off. The remaining amendments were not highly effective.

Seasonal dynamics of *Phyllosticta citricarpa* and *P. capitalensis*

Leaf litter monitoring

Three production seasons of *Phyllosticta* spp. sporulation data has been collected from Qld citrus orchards, and identified a peak in ascospore producing pseudothecia production to occur in late December to early January. In contrast, conidia producing pycnidia were generally present in the leaf litter throughout the season, with a tendency to peak earlier in the season. The DNA analysis using quantitative PCR indicated that the pathogen was highly dominant over the endophyte.

Pathogen endophyte antagonism in vitro

The *in vitro* co-inoculation assays demonstrated a general inability of the *Phyllosticta* spp. isolates to inhabit the same space. Regardless of the *Phyllosticta* spp. combinations used, the mycelium of the two colonies always remained separated, even when the same isolate was paired on the same plate. This strongly suggests that *Phyllosticta* spp. cannot grow in close proximity, and could explain the antagonism reported between *P. citricarpa* and *P. capitalensis* in the past (de Almeida, 2009).

Pathogen endophyte antagonism in planta

The *in planta* co-inoculation studies found that pre-inoculation with *P. capitalensis* significantly reduced the amount of CBS that arises from subsequent inoculations with *P. citricarpa*, supporting previous findings by de Almeida (2009). Furthermore, the lack of CBS developing in control fruit inoculated only with *P. capitalensis* affirms that this species is not pathogenic to citrus.

Pathogen endophyte antagonism fungicide sensitivity

The comparison of fungicide sensitivity between *P. citricarpa* and *P. capitalensis* isolates failed to identify any significant differences in sensitivity between the two fungi for the commonly used fungicides azoxystrobin, copper, iprodione or mancozeb. This in contrast to previous studies which have shown significant differences in copper sensitivity (Hendricks and Roberts, 2012).

Resistant germplasm

A reliable technique for inoculating fruit in the field with *P. citricarpa*, and producing symptoms of CBS, was developed and used to screen the fruit of 50 *Citrus* accessions for their disease reaction. All the mandarin, sweet orange, lemon and papeda types that were tested were designated as susceptible, while the pomelo types were designated as resistant, and the lime types as resistant/insensitive. A small number of pomelo and *Poncirus* hybrids showed preliminary evidence of segregation for black spot resistance. Identifying resistance in pomelo is highly desirable from a breeding perspective due to the positive fruit quality traits associated with pomelo hybrids.

Industry conference presentations, posters, papers

Miles, A. K. 2017. Best orchard practices now for best biosecurity later. In: Advances in Disease Management Workshop, 2017 Citrus Technical Forum, 1-2 March Citrus Australia Limited, Mildura.

Miles, A. K., Tran, N., and Drenth, A. (2017). Citrus black spot - whether to worry about the weather. In: 2017 Citrus Technical Forum, 1-2 March Citrus Australia Limited, Mildura.

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Tran, N. T., Miles, A. K., Dietzgen, R. G., and Drenth, A. (2016). Citrus black spot: Sexual reproduction provides new insights into an old problem. Page 128. In: 13th International Citrus Congress International Society of Citriculture, Foz do Iguacu, Brazil.

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Industry publications/workshops

Miles, A. K., Tran, N., Shuey, T., and Drenth, A. 2016. Citrus black spot. In: Grower Workshop, 3 August, Spencer Ranch, Queensland.

Miles, A. K., Tran, N., Shuey, T. & Drenth, A. (2015). New international partnership takes on citrus black spot. Australian Citrus News March: 14-15.

Outcomes

Leaf litter management

This project has demonstrated that none of the commonly discussed alternatives to applying an organic mulch layer over leaf litter in orchards are capable of providing measurable increases in the rate of leaf litter degradation. This is in contrast to promising results that have been observed overseas, particularly in Florida. A possibly important difference between Florida and the Burnett region of Queensland is the generally higher and more consistent relative humidity observed in Florida. As moisture has been found to be a critical factor in leaf litter breakdown (Faber et al., 2001; Fidalski et al., 2010), the more humid environment in Florida may be more favourable to decomposition, and subsequently the enhancement of decomposition.

Importantly, the project has provided evidence that fungicide run-off can reduce the rate of leaf litter degradation. This is presumably due to declines in soil microflora and microfauna associated with fungicide build up in the leaf litter environment (Al-Assiuty et al., 2014; Seguin et al., 1983; Zhou et al., 2013). Reducing fungicide run-off is therefore likely to be more useful for encouraging leaf litter decomposition in Qld orchards than applying amendments such as urea.

Seasonal dynamics of *Phyllosticta citricarpa* and *P. capitalensis*

Leaf litter monitoring

The direct sampling of leaf litter in orchards has shown that it is possible to use this approach to identify the onset, peak, and decline of pseudothecia production throughout the growing season. The application of quantitative PCR has then shown the dominance of the pathogen *P. citricarpa* over the rare occurrence of the endophyte *P. capitalensis* in commercial orchards. This indicates that monitoring the pathogen via visual means (directly in leaf litter, or ascospores in a volumetric spore trap), is likely to give reliable results regarding pathogen progress throughout the season with little confusion from *P. capitalensis*.

Pathogen endophyte antagonism in vitro

The studies of the pathogen-endophyte interaction *in vitro* demonstrated most notably that the *Phyllosticta* spp. do not readily occupy the same space. This was observed even between fungal colonies of the same isolate. This most likely means that in nature whichever individual or species occupies a niche first, that individual or species will dominate that niche. The mechanisms responsible for this observation are currently unknown, but warrant investigation.

Pathogen endophyte antagonism in planta

Expanding on the results from the *in vitro* antagonism studies, *in planta* studies have shown that pre-inoculation of fruit with the endophyte *P. capitalensis* reduced development of CBS lesions from subsequent inoculation with the pathogen *P. citricarpa*. This suggests that *P. capitalensis* itself, or a derivative of inoculation with *P. capitalensis*, may have some value as an alternative to conventional chemical control of CBS.

Pathogen endophyte antagonism fungicide sensitivity

Project findings have not identified sufficient differences in fungicide sensitivity between *P. citricarpa* and *P. capitalensis* that could allow for revised fungicides schedules to promote naturally occurring populations of *P. capitalensis* over *P. citricarpa*.

Resistant germplasm

The systematic approach of screening fruit of various citrus accessions has allowed for the identification of a potential source of CBS resistance in a host background with desirable fruit quality traits, in particular the ‘K15’ pomelo. This is a major step forward for a breeding approach to CBS control, which has been hindered in past breeding attempts by relying on sour orange as a

source of CBS resistance. Furthermore, the project has identified preliminary evidence for the CBS resistance in 'K15' to be heritable, but this requires further confirmation.

Monitoring and evaluation

The collaborative research undertaken between Florida and Australia through project CT13021 has made significant progress towards minimising the impact of CBS on citrus producers. The project has taken a number of critical first steps towards long term, but high impact, approaches to dealing with CBS. Several of these steps have not before been possible without the availability of new technologies, or have been overlooked for the past 100 years as they were considered too 'long term' to invest in. These longer term, high impact, approaches include identifying sources of CBS resistance, such as the 'K15' pomelo, and creating new opportunities for breeding. In addition, gaining a better understanding of the seasonal dynamics of *Phyllosticta* spp. in orchards provides the foundation for developing inoculum production and/or infection models to facilitate pest monitoring approaches currently not possible for CBS.

Not to overlook nearer term goals, the project has investigated the potential of several easy to adopt approaches to decomposing leaf litter, as well as the potential for biological control using the endophyte *P. capitalensis*. Promoting leaf litter decomposition with compounds such as urea are commonly considered by growers to reduce leaf litter inoculum, but evidence for their efficacy under Qld conditions has been lacking. Alternatives to conventional chemical control for CBS are becoming increasingly desirable as agrichemical residues become more critical to consumers, but no effective options are currently available to growers. In this project it has not been possible to demonstrate any appreciable benefit from trying to enhance leaf litter degradation with easier options such as urea spraying, but instead the project has shown the need to limit fungicide runoff to avoid negative impacts on leaf litter decomposition. In terms of the potential biological control by *P. capitalensis*, the gains are most likely to be made by better understanding the mechanisms of CBS suppression afforded by *P. capitalensis*, and then look for ways to exploit those mechanisms. For example, determining if suppression of *P. citricarpa* is related to production by *P. capitalensis* of a metabolite may allow for synthesis of that metabolite.

Recommendations

Leaf litter management

- Development and evaluation of leaf litter management strategies to reduce the risk of infection of the fruit.
    - This project has shown the most effective leaf litter amendment to be organic mulch, however, this practice is expensive and time consuming regardless of its numerous additional production benefits.
    - Easily applicable amendments, such as urea or calcium carbonate, were found to be of limited value.
    - Reduction of fungicide run off to aid in leaf degradation while lowering cost of application.

Seasonal dynamics of *Phyllosticta*

- Further elucidate the timing of, and conditions for infection.
    - Past studies have tended to focus on the timing of sporulation of *Phyllosticta*, but the conditions for infection are poorly understood.
    - Understanding the conditions for infection may allow the prediction of infection risk without needing to undertake spore trapping, or more accurately predict infection based on infection conditions rather than sporulation conditions. Understanding infection risk will enable improved targeting of fungicide applicatons.

Resistant germplasm

- Confirm resistant accession and study the susceptibility of hybrid progeny to determine if resistance is a heritable trait.
    - As CBS resistance appears to be a rare trait in citrus, multiple rounds of testing are needed to confirm any potential resistance.
    - To have any long term value in breeding, the heritability of any CBS resistance will need to be determined.

Additional recommendations

- Undertake strategic genetic diversity studies in specific study groves to determine the relative contributions of clonal and sexual reproduction in CBS epidemics.
    - It is generally assumed that sexually-derived ascospores are the primary inoculum source in CBS epidemics, but this is a topic of debate in market access negotiations.
    - Specific genetic diversity studies could be undertaken to determine the occurrence of parental and hybrid genotypes in single CBS lesions occurring in fruit.

Refereed scientific publications

Journal articles

Miles, A.K., Wright, C., Tran, N.T., Shuey, T.A., Drenth, A., Dewdney, M., 2017. Does fungicide run-off from citrus delay leaf litter decomposition? Citrus Research and Technology 38, 52-61.

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Tran, N. T., Miles, A. K., Dietzgen, R. G., Mudge, S., Papacek, D., Chandra K. & Drenth, A. (2018). Timing of infection of citrus fruit by *Phyllosticta citricarpa* and the links with seasonal ascospore release. Plant Pathology (*in preparation*).

Miles, A. K., Smith, M. W., Tran, N. T., & Drenth, A. (2018). Expression of resistance in citrus fruit to *Phyllosticta citricarpa*, the cause of citrus black spot. Plant Disease (*in preparation*).

PhD Thesis

Tran, N. T. 2018. Biology and epidemiology of citrus black spot (*Phyllosticta citricarpa)* in Australia. PhD thesis, the University of Queensland.

Scientific conference presentations, posters, papers

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Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

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Appendices

Appendix 1: Joint Florida and Australia Citrus Black Spot Research Initiative

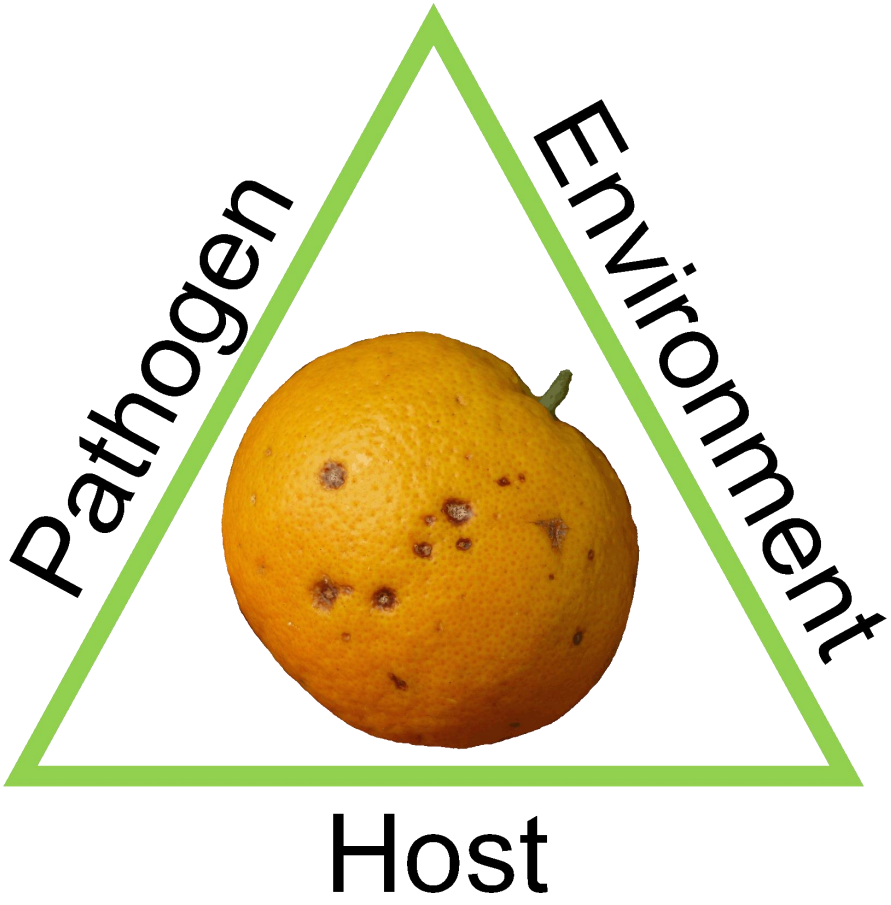
CT13021

Joint Florida and Australia Citrus Black Spot Research Initiative

Appendix to final report

Dr Andrew Miles, Nga Tran and Prof André Drenth

23 March 2018



Project Details

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Chapter 1

Leaf litter management

Does Fungicide Run-off From Citrus Delay Leaf Litter Decomposition?

Miles, A.K., Wright, C., Tran, N.T., Shuey, T.A., Drenth, A., Dewdney, M., (2017). Does fungicide run-off from citrus delay leaf litter decomposition? *Citrus Research and Technology* **38**, 52-61.

1.1 Abstract

Leaf litter is a major inoculum source for citrus diseases such citrus black spot caused by *Phyllosticta citricarpa*, and greasy spot caused by *Mycosphaerella citri*. In order to reduce this inoculum source, the efficacy of urea, dolomitic lime, a commercial compost accelerator, and an organic mulch, was assessed for enhanced leaf decomposition and reduction in sporocarps. However, due to the potential for run-off from high volume fungicide applications to disrupt leaf decomposition and microbial antagonism, the amendments were compared with and without simulated fungicide run-off. Mature green leaves of *Citrus sinensis* were removed from trees and placed inside mesh bags before being pinned to the orchard floor. The amendments were applied, and then simulated run-off from a typical citrus black spot fungicide program (copper, mancozeb, azoxystrobin) was applied. Leaf degradation was assessed every 2-3 weeks by visual ratings and dry weight. The results showed that the organic mulch was the most effective at enhancing decomposition, while there was significantly ($P < 0.05$) less decomposition in the presence of fungicide run-off. No direct effects on sporocarps could be observed due to insufficient infection.

1.2 Introduction

Citrus leaf litter in orchards is a source of inoculum for several fungal diseases including citrus black spot caused by *Phyllosticta* (*Guignardia*) *citricarpa* (McAlpine) van der Aa (McAlpine, 1899; Kiely, 1948) and greasy spot caused by *Mycosphaerella citri* (Whiteside) (Whiteside, 1970). Leaf litter is also an inoculum source for diseases in other tree crops; one particularly well studied example being apple scab caused by *Venturia inaequalis* (Cooke) G. Winter (Gadoury *et al.*, 1984). For all these examples, ascospores of the pathogen are released from leaf litter and are a source of airborne inoculum (Kiely, 1948; Whiteside, 1970; Gadoury *et al.*, 1984). For this

reason, various forms of leaf litter management have been investigated for their potential to reduce inoculum, and hence, reduce disease. Although removal of leaves under the trees has been considered research in Brazil has shown that this does not give sufficient control of black spot (Spósito, et al, 2011). In addition, removal of leaves gives rise to increased soil erosion, increased water use, increased weed growth, reduction of natural suppression of soil-pupating insects as well as a reduction in soil carbon. The process of removal on a plantation scale requires specific equipment creating dust which has a negative impact on beneficial insects and increases the change of fruit inoculation due to disturbance of the infected leaves on the orchard floor which may release fungal spores.

The application of grass mulch over the orchard floor has been demonstrated to reduce the incidence of citrus black spot in South Africa (Schutte and Kotze, 1997). Mechanical forms of leaf litter management such as shredding have been shown to be effective in reducing *V. inaequalis* inoculum and apple scab incidence in apple orchards (Holb, 2007; Sutton *et al.*, 2000). However, as applying mulch or shredding leaves can require additional equipment and labour costs, orchard operators often express interest in leaf litter management approaches that utilise existing equipment such as herbicide boom sprayers or fertiliser spreaders. Promising amendments complementing this desire include urea, $CaCO_3$ and dolomitic lime forms, and commercial compost accelerators (Rodrigues *et al.*, 2016).

In general, the aim of amendments such as urea, dolomitic lime, and compost accelerators is to promote microbial activity leading to increased leaf decomposition and/or antagonise the pathogens directly (Crosse *et al.*, 1968; Green *et al.*, 2006; Condron *et al.*, 1993; Bengtsson *et al.*, 2006). The application of these amendments to manage leaf litter inoculum sources has been evaluated in a number of studies in tree crops with promising results for reducing inoculum (Sutton *et al.*, 2000; Mondal and Timmer, 2003; Mondal *et al.*, 2007; Bellotte *et al.*, 2009; Spotts *et al.*, 1997), but in some cases significant improvements in disease control were not observed (von Diest *et al.*, 2016). However, it has been shown that the fungicides used to control diseases in apples, for example, can have negative impacts on non-target microbial populations in leaves and leaf litter (Walter *et al.*, 2007; Andrews and Kenerley, 1979). Rates of leaf decomposition can therefore be reduced as a result of these altered microbial communities (Duarte *et al.*, 2008; Rasmussen *et al.*, 2012). In citrus orchards, fungicides are routinely used for the control of diseases such as citrus black spot (CBS) and greasy spot. Consequently, attempts to stimulate leaf decomposition through enhanced microbial activity in response to amendments such as urea may be counteracted by fungicide run-off from trees.

In some citrus production areas in Australia and South Africa, fungicide run-off is significant due to the adoption of high fungicide application volumes (>7,000 L/ha) that exceed the theoretical canopy retention volume of mature citrus of 2,300 L/ha (Beattie *et al.*, 1989; Chapman *et al.*, 1981; Cunningham and Harden, 1998b; Cunningham and Harden, 1998a; van Zyl *et al.*, 2013; Fourie *et al.*, 2009). As fungicide programs for the control of citrus black spot, for example, typically incorporate monthly fungicide applications during the first 20-24 weeks of fruit development (Baldassari *et al.*, 2006; Kotze, 1981; Wager, 1952; Miles *et al.*, 2004; Agostini *et al.*, 2006; Schutte *et al.*, 2003), it is highly likely that citrus leaf litter under these circumstances is readily exposed to the fungicides being applied. Three of the most commonly used fungicides in citrus disease management are various copper-based formulations, dithiocarbamates and strobilurins (Schutte *et al.*, 2012; Miles *et al.*, 2004; Makowski *et al.*, 2014; Schutte *et al.*, 2003; Miles *et al.*, 2005). As these fungicides have efficacy against a wide range of fungal genera (Hewitt, 1998), off-target effects from run-off of these fungicides on microbial communities in leaf litter are a concern.

Leaf litter management in citrus orchards is considered a cultural practice which may improve the control of diseases such as citrus black spot. However, consideration needs to be given to the potential for other orchard practices, such as high volume fungicide application, to disrupt leaf litter management strategies. In this study we aim to investigate: 1) the efficacy of leaf litter amendments for enhancing leaf litter decomposition in citrus orchards in Queensland, Australia; 2) the efficacy of leaf litter amendments for directly reducing *Phyllosticta* sporocarp development in leaf litter; and 3) determine the impact of fungicide run-off on amendment efficacy and leaf litter decomposition. Addressing these aims will greatly assist citrus producers to determine the value of adopting leaf litter strategies for citrus disease control.

1.3 Materials and Methods

Experiment 1

In order to determine the effect of leaf litter amendments and fungicide run-off on leaf litter decomposition and sporocarp development, urea, calcium carbonate, and a commercially available compost accelerator, were applied to leaf litter and compared over time to untreated leaf litter. The effect on leaf decomposition of fungicide run-off from routine high-volume fungicide applications for CBS was also investigated by duplicating the application of urea,

calcium carbonate, the compost accelerator and the untreated control in the presence of simulated fungicide run-off.

Attached mature citrus leaves were harvested on the 10th December 2014 from sweet orange (*Citrus sinensis* (L.) Osbeck) trees in an orchard in Mundubbera, Queensland (-25.596598, 151.305108). The collected leaves were pooled, randomised through agitation, then 15 harvested leaves were arbitrarily assigned to each treatment in four replicates, and eight sampling times. The batches of 15 leaves were then laid out evenly in poly-mesh bags (35 mm x 23 mm) which were pinned to the ground under the canopy of trees in an orchard adopting a minimal fungicide regime (low frequency and volumes of application). One bag for each of the eight treatments was pinned under each tree. The bags evenly surrounded the trunk of each tree at a distance of 50 cm from the trunk, with the position of the treatments around the trunk determined using a random number generator. When applying the amendments, a 50 cm × 50 cm quadrat was placed around the bag to be treated, and the amendments applied to the entire area of the quadrat. Urea (46% N, Richgro, Australia) was applied at a rate of 23.3 kg/ha (20.81 lbs/acre) in a carrying volume of 467.5 L/ha (50 gal/acre). Dolomitic lime (14% Ca, 8% Mg, Richgro, Australia) was applied dry at a rate of 2,000 kg/ha (1,785 lbs/acre). The compost accelerator, Actizyme (proprietary enzymatic ingredients, Aware Environmental Products Pty Ltd, Australia), was applied at a rate of 40 kg/ha (35.69 lbs/acre) in a higher carrying volume of 2,600 L/ha (277 gal/acre) in order to best suspend the pelletised product. Control leaves were not treated. The amendments were applied immediately after placement of the mesh bags, and again 7 days later due to a period of stormy weather (~70 mm over 7 days).

Simulated amounts of fungicide run-off from typical fungicides used for controlling CBS (copper, mancozeb, and then azoxystrobin) were applied to the duplicated mesh bags during the experiment. A maximum, worst case, potential run-off amount of 7,700 L/ha (823 gal/acre) was used on the basis of the canopy retention volume for mature citrus being 2,300 L/ha (Cunningham and Harden, 1998b), and high-volume application rates of 10,000 L/ha. Therefore, the fungicides were applied directly to the ground in a carrying volume of 7,700 L/ha. Fungicide run-off was applied to the mesh bags using a knapsack sprayer, and the bags treated using the quadrat as previously mentioned. Simulated run-off applications of 0.675 g/L cuprous oxide (Red Copper WG, Melpat International Pty Ltd, Australia), 1.5 g/L mancozeb (Penncozeb 750 DF, NuFarm Ltd, Australia), and 0.1 mL/L azoxystrobin (Amistar 250 SC, Syngenta, Australia) were applied on the 18th December 2014, 22nd January 2015, and 4th February 2015, respectively.

The first seven samplings were conducted fortnightly, with the final sample collected at a 14 week interval when leaves were almost completely degraded. At each sampling time, four replicate sets of mesh bags were collected and visually rated for their state of decomposition and inspected for sporocarp development. Visual assessment of leaf litter decomposition was undertaken using the rating scale of Mondal *et al.* (2007) with a minor modification: 0 = dead, not decomposed, leaf firm; 1 = not decomposed, flexible, still intact; 2 = leaf slightly decomposed, no loss of lamina; 3 = moderately decomposed, some loss of lamina; 4 = moderately decomposed, considerable loss of lamina; 5 = highly decomposed, skeletonized leaves; and 6 = no recognisable leaf. A second rating scale that was customised for Queensland conditions was also used whereby: 0 = green, intact, flexible; 1 = brown, dry, curled; 2 = laminar loss commencing <25% area; 3 = moderate laminar loss 26-50% area; 4 = high laminar loss 51-75% area; 5 = fully decomposed/skeletonised >75% area; and 6 = no recognisable leaf. The estimated density of sporocarps was determined according to Mondal & Timmer (2003) whereby: 0 = none, 1 = 1 to 5%, 2 = 6 to 10%, 3 = 11 to 15%, 4 = 16 to 20%, 5 = 21 to 25%, 6 = 26 to 30%, 7 = 31 to 35%, 8 = 36 to 40%, 9 = 41 to 45%, and 10 = >50% leaf area covered with sporocarps. After visual assessments, the dry weight of leaf tissue in each mesh bag was determined after drying at 50˚C for 48 hours.

Experiment 2

In order to confirm the findings from experiment 1, a second experiment was conducted using largely the same methods as experiment 1 but with minor modifications. In addition to the four leaf amendments applied previously, sugar cane mulch (Rocky Point Mulching, Australia) was applied at the rate of 18 t/ha (8 t/ac). Attached mature citrus leaves were harvested on the 2nd December 2015, and leaves placed in mesh bags as described previously. The leaf amendments were applied once on the 17th December 2015. The simulated run-off applications of 0.675 g/L cuprous oxide, 1.5 g/L mancozeb, 0.1 mL/L azoxystrobin, and 1.5 g/L mancozeb were applied on the 17th December 2015, 7th January 2016, 9th February 2016, and 4th March 2016, respectively. Leaf samplings were conducted approximately every three weeks from week two to week 16, and leaf decomposition was assessed as previously described.

Statistical Analysis

In order to compare treatment effects in each experiment, the mean visual ratings of degradation for each bag were analysed using residual maximum likelihood (REML) which allows

the inclusion of smoothing splines in the model for investigating the presence of a non-linear response over time. The observed non-linear response in mean degradation over time was then modelled using an exponential curve.

Dry weight data were subjected to Analysis of Variance (ANOVA) with the fixed factors of amendment × fungicide run-off × time. Where the time factor was found to explain a large majority of the variance in the analysis, the area under the curve (AUC) was calculated for the dry weight values over time using the formula as previously described (Akinsanmi *et al.*, 2007; Campbell and Madden, 1990). The leaf litter decomposition constant (*k* value) was also determined using the formula as previously described (Yue *et al.*, 2016; Olson, 1963). The area under the curve and *k* value were then subjected to ANOVA with the fixed factors amendment × fungicide run-off.

Where a significant main effect or interaction was found ($p<0.05$), pairwise comparisons are made using Fisher's 95% least significant difference (LSD). All analyses were performed using GenStat for Windows (16th Edition, VSN International, Ltd., UK).

1.3 Results

The results of the REML analysis of the two visual assessment methods found the time effect to be significant ($P<0.05$), with decomposition ratings increasing as time increased, as expected. This increase was non-linear as decomposition rate slowed towards the end of the experiments (data not shown). The amendment factor was only found to be significant in experiment 2, whereby the mulch amendment was found to significantly ($P<0.05$) increase the decomposition ratings compared to the other amendments. The fungicide run-off factor was, however, found to be significant in both experiments for both rating scales, with the addition of fungicide run-off resulting in significantly ($P<0.05$) lower decomposition ratings (Table 1.3.1). Significant interactions were only observed in experiment 2, with the amendment × time interaction being significant ($P<0.05$). This interaction was explored by the fit of the data to an exponential model, with the model accounting for 90.5% and 82.7% of the variance for the Mondal and customised rating scales, respectively. Figure 1.3.1 shows the fitted exponential model to the customised rating scale data. Visual assessments of the prevalence of sporocarps in the leaf litter could not be meaningfully analysed due to low levels of leaf infection (data not shown).

Analysis of the leaf litter dry weight data by ANOVA for experiment 1 found the fungicide run-off and time factors to be significant for the dry weight data, and only the fungicide run-off factor to be significant for the area under curve data (Table 1.3.2). In both cases the addition of

simulated fungicide run-off resulted in significantly (P <0.05) higher mean dry weights and area under the curve of dry weight (Table 1.3.3). In experiment 2, the amendment, fungicide run-off and time factors were significant for the dry weight, and amendment and fungicide run-off factors were significant for the area under curve data (Table 1.3.2). No interactions between factors were significant. Within the amendments, mulch was the only amendment to significantly (P <0.05) reduce the mean dry weight and area under the curve compared to the control (Table 1.1.3). As in experiment 1, the addition of simulated fungicide run-off resulted in significantly higher mean dry weight and area under the curve. For both experiments, the time factor was observed to result in the leaf litter dry weight to decline with increasing time as expected (Table 1.1.3). The k values in both experiments were not significant (P<0.05) except for the amendment factor in experiment 2 (Table 1.3.2). In this case, the k value for the mulch amendment was significantly higher than all the other treatments (data not shown).

Table 1.3.1. Results of REML analysis of visual ratings of the effect of fungicide run-off on leaf litter decomposition ratings.[a]

	Mondal[b]	Customised[c]
Experiment 1		
No run-off	4.38 a	3.81 a
Run-off	4.30 b	3.70 b
P	0.032	0.034
95% LSD	0.07	0.100
Experiment 2		
No run-off	4.52 a	4.00 a
Run-off	4.35 b	3.79 b
P	<0.001	0.007
95% LSD	0.07	0.11

[a]Mean values followed by the same letter are not significantly different (P<0.05)
[b]Mean leaf litter decomposition rating based on Mondal *et al.* (2007) where: 0 = dead, not decomposed, leaf firm; 1 = not decomposed, flexible, still intact; 2 = leaf slightly decomposed, no loss of lamina; 3 = moderately decomposed, some loss of lamina; 4 = moderately decomposed, considerable loss of lamina; 5 = highly decomposed, skeletonized leaves; and 6 = no recognisable leaf.
[c]Mean leaf litter decomposition rating based on a customised scale where 0 = green, intact, flexible; 1 = brown, dry, curled; 2 = laminar loss commencing <25% area; 3 = moderate laminar loss 26-50% area; 4 = high laminar loss 51-75% area; 5 = fully decomposed/skeletonised >75% area; and 6 = no recognisable leaf.

Table 1.3.2. ANOVA of leaf dry weight, area under the curve of leaf dry weight (AUC), and decomposition rate constant (*k* value) from experiments 1 and 2.

Factor	**df**	**Mean square**			**F pr.**		
		Weight	AUC	*k* value	Weight	AUC	*k* value
Experiment 1							
Amendment (A)	3	1.3867	126.47	2.024	0.223	0.140	0.524
Time (T)	7	66.9029	-	-	<0.001	-	-
Run-off (R)	1	13.3271	832.83	5.802	<0.001	0.001	0.152
A × T	21	1.0365	-	-	0.350	-	-
A × R	3	0.1555	9.25	2.936	0.920	0.929	0.366
T × R	7	0.9912	-	-	0.396	-	-
A × T × R	21	0.2403	-	-	1.000	-	-
Experiment 2							
Amendment (A)	4	8.7171	176.880	41.284	<0.001	<0.001	<0.001
Time (T)	5	2.1319	-	-	0.013	-	-
Run-off (R)	1	102.7151	53.754	1.723	<0.001	0.003	0.524
A × T	20	0.2783	-	-	0.507	-	-
A × R	4	0.3563	6.870	4.104	0.392	0.272	0.429
T × R	5	0.4399	-	-	0.261	-	-
A × T × R	20	0.3212	-	-	0.515	-	-

Table 1.3.3. ANOVA results for the amendment, fungicide run-off and time factors for mean leaf litter dry weight, and area under the curve for leaf litter dry weight in the presence of various amendments and simulated fungicide run-off onto *Citrus sinensis* leaf litter in an orchard in Mundubbera, Queensland.[a]

	Experiment 1		Experiment 2	
Amendment	**Dry weight (g)**	**AUC**	**Dry weight (g)**	**AUC**
Control	3.2	58	2.7 a	31 a
Urea	3.1	55	2.5 a	29 a
CaCO3	3.4	60	2.7 a	31 a
Actizyme	3.4	64	2.5 a	29 a
Mulch	-	-	1.7 b	19 b
95% LSD	n.s.	n.s.	0.2	2
Fungicide run-off				
Yes	3.5 a	64 a	2.5 a	29 a
No	3.0 b	54 b	2.3 b	27 b
95% LSD	0.2	6	0.1	1
Time (weeks)				
2	5.4 a	-	5.4 a	-
4	4.3 b	-	-	-
5		-	2.9 b	-
6	4.0 b	-	-	-
7		-	2.2 c	-
8	3.8 b	-	-	-
10	2.6 c	-	1.6 d	-
12	2.6 c	-	-	-
13		-	1.1 f	-
14	2.4 c	-	-	-
16		-	1.3 e	-
28	0.7 d	-	-	-
95% LSD	0.5	-	0.3	-

[a]Means followed by the same letter are not significantly different (P<0.05); n.s. = not significant.

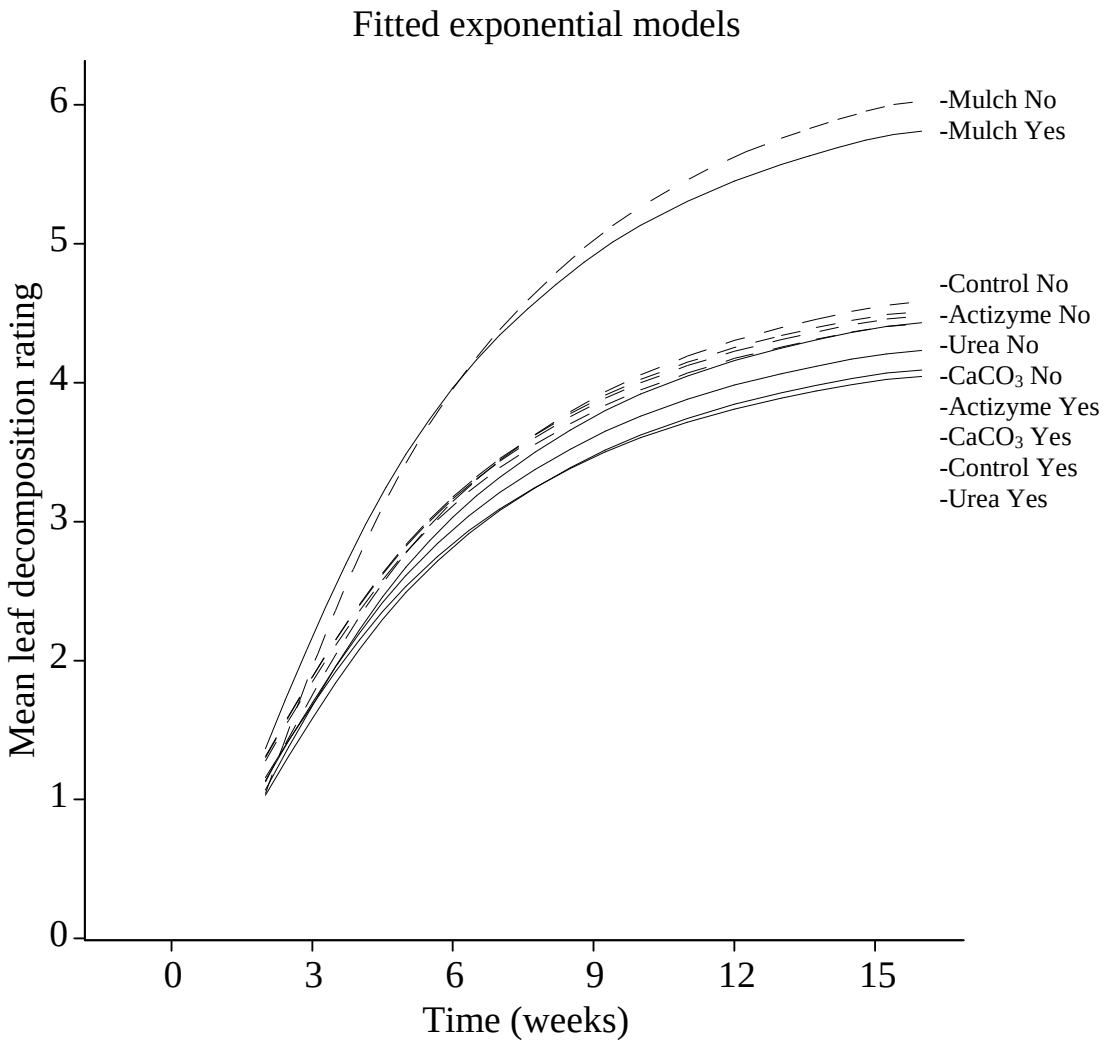

Figure 1.3.1. Exponential plots of mean leaf litter decomposition over time assessed using a customised rating scale from experiment 2, following the application of various amendments (mulch, urea, $CaCO_3$, actizyme, and an untreated control) in the presence (yes) and absence (no, dashed lines) of fungicide run-off. Mean leaf litter decomposition rating based on a customised scale where 0 = green, intact, flexible; 1 = brown, dry, curled; 2 = laminar loss commencing <25% area; 3 = moderate laminar loss 26-50% area; 4 = high laminar loss 51-75% area; 5 = fully decomposed/skeletonised >75% area; and 6 = no recognisable leaf.

1.4 Discussion

In this study we aimed to determine the efficacy of various leaf litter amendments for promoting leaf litter decomposition and/or *Phyllosticta* sporocarp development, as well as determine any effects of fungicide run-off from high volume fungicide applications. Our results have shown that significant (P<0.05) reductions in leaf litter decomposition were consistently observed in the presence of simulated fungicide run-off. The efficacy of the different leaf amendments was generally low, and/or inconsistent between seasons. However, the most effective amendment for significantly (P<0.05) increasing leaf decomposition was the sugar cane mulch amendment. While the cane mulch was only assessed in one season, the visual decomposition and dry weight measures were markedly more favourable for promoting decomposition than observed for the other amendments in either experiment. The ability to determine any direct effects on *Phyllosticta* sporocarps was limited due to low levels of leaf infection for assessment. Our findings strongly indicate that under Queensland conditions reducing fungicide run-off to leaf litter may be more beneficial to promoting leaf litter decomposition than applying urea, dolomitic lime or a compost accelerator.

Managing off-target pesticide losses is an important issue for horticulture. Off-target losses have been associated with negative impacts on plants, insects and fungi (de Jong *et al.*, 2008). In citrus specifically, studies have shown accumulation of several heavy metals in soil from agrichemical use including Cu and Mn which are key elements in Cu-based fungicides and mancozeb (Kelepertzis, 2014; Fan *et al.*, 2011; Hewitt, 1998). Accumulation of these elements/fungicides in horticultural soils has been associated with declines in soil microflora and microfauna (Zhou *et al.*, 2013; Al-Assiuty *et al.*, 2014; Seguin *et al.*, 1983). However, to our knowledge our study is the first to show a measurable reduction in leaf litter decomposition in a citrus orchard associated with the application of simulated fungicide run-off, probably resulting from disruptions to soil microflora and microfauna. Of concern is that measurable differences in decomposition were observed in our study within the 16-28 week lifespan of our experiments, whereas most citrus trees in the Queensland region are currently 13 years old or greater (Hancock, 2014). The level of soil exposure to fungicide run-off after 13 years in orchards using high volume application methods is likely to be significant. Further investigation of the long term impacts of fungicide run-off on leaf litter decomposition and associated aspects of soil biology are warranted.

The efficacy of urea, dolomitic lime and Actizyme was generally low and inconsistent in our study. This is consistent with findings from Florida that show a reduction of *M. citri* inoculum with urea and dolomitic lime applications, but generally not significant increases in leaf litter decomposition (Mondal *et al.*, 2007; Mondal and Timmer, 2003). However, in Brazil increases in leaf litter decomposition were observed with similar amendments (Bellotte *et al.*, 2009). A likely reason for

differences among the studies are climatic differences between study regions. In particular, rainfall and/or humidity are likely to be important. For example, additional irrigation of citrus leaf litter in Florida was one of the most effective treatments for promoting decomposition (Mondal *et al.*, 2007). The increased decomposition in the sugar cane mulch amendment in our study was also probably the result of higher moisture provided by the organic mulch (Faber *et al.*, 2001; Fidalski *et al.*, 2010). Interestingly, other relevant differences between study regions are evident when comparing the two visual decomposition rating scales in our study. While our results from using the two rating scales did not differ substantially, the rating scale from Florida suggests a different sequence of decomposition than that of the customised scale we developed specifically for our Queensland study site (Mondal *et al.*, 2007). The most notable difference being that leaves in Queensland turn brown and curl very early in decomposition, while leaves in Florida remain flexible. This would suggest a drier overall climatic situation in Queensland relative to Florida, and therefore differences in decomposition.

Our study has provided evidence that fungicide run-off from high volume fungicide spraying for the control of citrus black spot may be contributing to the preservation of leaf litter which itself is an important inoculum source of the causal fungus. Furthermore, our study has shown that leaf litter amendments such as urea are not likely to be of significant benefit to Queensland citrus growers managing citrus black spot. Instead, it is recommended that growers aim to reduce fungicide run-off using lower volume application equipment, and/or consider organic mulching, to more effectively promote leaf litter decomposition.

1.5 Acknowledgments

The authors gratefully acknowledge Gavin Ford for the use of his orchard, and Dan Papacek and the team at Bugs For Bugs for their assistance and use of their facilities. We are grateful for the financial support provided by the University of Florida through the Citrus Research and Development Foundation and Horticulture Innovation Australia

Chapter 2

Seasonal dynamics of *Phyllosticta citricarpa* and *P. capitalensis*

2.1 Leaf litter monitoring

Introduction

Citrus in Australia, and many other parts of the world, are hosts of two *Phyllosticta* species: the pathogenic *P. citricarpa*; and the endophytic *P. capitalensis* (Miles *et al.*, 2013; Baayen *et al.*, 2002). The two species are morphologically indistinct, apart from some *in vitro* colony characteristics such as growth rate and pigment production (Glienke-Blanco *et al.*, 2002). Both species are capable of producing three fructification and spore types, namely: spermogonia which produce dumbbell-shaped spermatia; pycnidia which produce waterborne conidia, and; pseudothecia which produce the airborne ascospores (Baayen *et al.*, 2002; Sutton and Waterson, 1966; Baayen *et al.*, 2003). Recent studies (Tran *et al.*, 2017; Wang *et al.*, 2016) have demonstrated that pseudothecia and the airborne ascospores of *P. citricarpa* are the result of sexual reproduction between isolates of complementary mating types of *P. citricarpa*, which can be facilitated by spermatia. These studies have confirmed the heterothallic nature of *P. citricarpa*, while *P. capitalensis* is homothallic and an individual isolate readily produces psedothecia (Baayen *et al.*, 2002). The waterborne conidia on the other hand are clonally produced by a single mating type. Of the three spore types, only the ascospores and conidia have been shown to cause disease, suggesting the function of spermatia is purely as a sex gamete (Kiely, 1948). The two species also produce these structures in citrus leaf litter, suggesting similar life cycles. The indisinct morphology, and similar lifestyles of the two fungi therefore present the opportunity for significant confusion if visually monitoring for ascospore development directly on leaves, or by aerial spore trapping, whereby it is impossible to know if they are spores of the pathogen or the endophyte (Truter *et al.*, 2007). This may render these monitoring approaches to disease management, such as for fungicide scheduling, erroneous if the seasonal sporulation dynamics of the two fungi are significantly different. For example, if the endophyte tends to produce ascospores before the pathogen, a fungicide may be mistakenly scheduled based on the harmless endophyte, instead of the damaging pathogen. Alternatively, if there are no significant differences in seasonal dynamics between the two fungi, fungicide scheduling based on net ascospore production would be acceptable. Considering this potential source of confusion, and that the occurrence of both the species co-inhabiting citrus has been known for several years, it is surprising that more detailed investigations have not been undertaken.

A key limitation to studying the seasonal dynamics of the two *Phyllosticta* spp. has been the lack of a method to readily identify and quantify the two species in environmental samples such as leaf litter. Recently, CT13021 project collaborators developed a quantitative polymerase chain reaction (qPCR) assay for achieving this very goal (Hu *et al.*, 2014). It is therefore now possible to determine the seasonal dynamics of both *P. citricarpa* and *P. capitalensis* in commercial citrus orchards. By combining this modern approach with classic techniques, it is possible to determine the relative abundance of each species within the leaf litter directly, but also in spores harvested from this same leaf litter. The waterborne condia can be extracted by soaking leaf litter in water, while ascospores can be stimulated to eject from the pseudothecia by briefly soaking leaf tissue and then suspending the tissue over water (Korf *et al.*, 2001; McOnie, 1967). Leaf tissue and spore suspensions produced by applying these methods to samples collected over time, can then be subjected to the qPCR assay in order to determine which fungus is producing what spores, when. The findings of this type of study can improve our understanding of the two fungi, identify key points in time when inoculum may be more readily available, as well as assess the potential for the presence of the endophyte to misinform disease management decisions.

The overall aim of this study was determine the seasonal dynamics of *P. citricarpa* and *P. capitalensis* in commercial citrus orchards in Queensland, Australia. The specific questions were: 1) when are pycnidia and pseudothecia present in leaf litter during the season; 2) when are conidia and ascospores produced from leaf litter during the season; and 3) what is the relative abundance of *P. citricarpa* and *P. capitalensis*? The implications of the findings for our understanding of the lifecycles of the two fungi, and for disease control are discussed.

Methods

Leaf litter sampling, microscopy and spore extraction. In order to study the seasonal dynamics of the *Phyllostica* spp. in commercial citrus orchards, leaf litter was routinely sampled from the orchard floor during the main fruit production season from flowering in October, until April. The main study orchard was a block of *C. sinensis* c.v. Valencia planted in 1959 located near the township of Mundubbera (-25.611816, 151.261043), with a known history of CBS epidemics. This orchard was sampled during the 2013-14, 2014-15, 2015-16 and 2016-17 production seasons. In each season except 2016-17 additional groves were also sampled. These groves were: in 2013-14 a block of *C. reticulata* c.v. Imperial planted in 1976 (-25.627877, 151.506941); in 2014-15 a block of *C. sinensis* c.v. Washington Navel planted in 1978 (-25.615044, 151.474306) and a block of *C. sinensis* c.v. Arnold Blood planted in 2006 (-25.598926, 151.189857); and 2015-16 a block of *C. limon* c.v. Eureka planted in 1999 (-25.632486, 151.243258).

At each sampling date, 600 leaves were sampled from the orchard floor from October to April. The 600 leaves were systematically sampled using a strategy whereby samples of 25 leaves were collected from each of six trees, for each of the four cardinal points (N,S,E,W), resulting in the 600 leaves being taken from a total of 24 different trees. The leaves were collected from within a 50 cm^2 area just inside the drip line of each tree. The sampled leaves were each inspected under a dissecting microscope for signs of fructifications typical of *Phyllosticta* (Sutton and Waterson, 1966). The leaves without fructifications were separated, the number recorded, then they were discarded. The portions of the leaves with fructifications were excised, then wet mounts were made of at least 30 fructifications (or two fructifications from each leaf portion) from each replicate and examined microscopically to identify the different fructifications present on the leaf tissue.

Spore extraction was conducted on leaves collected in all years except 2016-17. For spore extraction the samples of leaves with fruiting bodies from a single tree for each cardinal position were pooled to give six replicate batches of leaves. In 2013-14, leaves were pooled from two trees for each cardinal position to give three replicated batches of leaves. Spore extraction from leaves began by first removing surface contaminants on the leaf tissue by washing thoroughly with tap water using a kitchen sieve to prevent loss of the leaf tissue. To stimulate ascospore ejection, the washed leaf tissue was then placed into a beaker containing 40 mL of sterile distilled water (SDW) at 40˚C, partially submerged in a water bath at 40˚C for 5 mins (Truter *et al.*, 2004). The leaf tissue was then removed and immediately placed fructification side down on a 6 mm plastic mesh covering a 90 mm Petri dish containing 20 mL of SDW + 0.02% Tween 20 (Sigma-Aldrich, Australia). The leaves were left suspended over the Petri dishes at room temperature overnight to allow any ascospores to be liberated into the water from any mature pseudothecia present. The bottom of the Petri dishes were swept lightly with a paint brush to dislodge any ascospores from the bottom of the dish. The water in the Petri dishes was transferred into individual 40 mL centrifuge tubes, and the tubes set aside. The leaf tissue was then transferred to beakers containing 40 mL of sterile distilled water (SDW) + 0.02% Tween 20, and incubated at room temperature for 30 mins to allow any conidia to be liberated from any mature pycnidia. The contents of the beakers were transferred to 40 mL centrifuge tubes via a 150 micron, then a 38 micron sieve, to remove debris from the spore suspension. All the spore suspensions were then centrifuged at 3,500 rpm for 30 minutes to pelletise the spores. All but approximately 1-2 mL of the supernatant was carefully pipetted off and discarded, and the spores resuspended using a pipette before being transferred to 2 mL microfuge tubes. To wash the spores, the tubes were centrifuged for 5 mins at 5,000 rpm to pelletise the spores, the supernatant removed, then the spores resuspended in 1 mL of SDW before pelletising the spores a second and third time before returning the volume of the spore suspension to 100 µL. The quantity of conidia and ascospores was determined using a

hemocytometer. The leaf tissue from which spores were collected was dried for 72 hrs in an oven at 50˚C, the dry weight recorded, then temporarily stored in a paper envelope. The paper envelopes and spore suspensions were stored at -20˚C until DNA extractions were performed.

DNA extraction and qPCR. In order to purify total DNA from the leaf tissue, an 8 mm diameter biopsy punch was used to take discs of leaf tissue from samples processed above. Typically, two discs were collected from each of 5 leaves, and these 10 discs (approximately 30-40 mg dry weight) were placed in a 2 mL conical screw-cap tube with a 5 mm diameter steel ball. Tubes were frozen at -80°C overnight in a TissueLyser adapter (Qiagen), and then immediately ground to a powder using the TissueLyser (2 x 30 seconds at 24Hz) without letting the samples thaw. DNA was extracted from this ground tissue using the PowerSoil DNA Isolation Kit (MoBio Laboratories).

Primer and probes for qPCR were as described (Hu *et al.*, 2014), except that the probes include an internal quencher in addition to the 3' quencher and 5' label (GCITSP1: 5' -6-FAM-AGCCGCCCG-ZEN-ACCTACCTTCA-Iowa black FQ -3'; and GMITSP1: 5' HEX-CGCTACAAC-ZEN-GCCGAAATGACCTTCT-Iowa black FQ -3'). Both of these were PrimeTime probes synthesised by Integrated DNA Technologies. The qPCR reactions were performed in a total volume of 10 µl using the QuantiTect Multiplex PCR Kit (Qiagen), 400 nM forward and reverse primers, 200 nM probe and 2 µl template DNA, and were run in a Rotor-Gene 6000 thermal cycler (Qiagen) with fluorescence measured in the green and yellow channels (for *P. citricarpa* and *P. capitalensis*, respectively). Separate reactions were performed with each set of primers and probe rather than duplex reactions, due to template concentration-dependent inhibition effects (Hu *et al.*, 2014). A standard curve consisting of serial 10-fold dilutions from 2.5 ng to 2.5 fg genomic DNA per assay was included in every qPCR run. These standard curves used genomic DNA from *P. citricarpa* isolate BRIP 53711 and *P. capitalensis* isolate BRIP 54242, both extracted using a Wizard Genomic DNA Extraction Kit (Promega).

Prior to deploying the qPCR for general *Phyllosticta* population studies, the assay was applied to DNA from a population of 24 Australian *P. capitalensis* isolates. This was done to ensure that the genotypic diversity detected in a previous study of *Phyllosticta* (Miles *et al.*, 2013) did not result in isolates falling outside of the detection specificity of the qPCR primers. To further validate the assay, template DNA extracted from leaf litter in Florida, and previously characterised with the qPCR assay oringally developed in Florida, was obtained and subjected to the assay by the CT13021 project team to ensure reproducibility of results. Following these validation steps, the qPCR assay was used to determine the relative abundance of *P. citricarpa* and *P. capitalensis* DNA in leaf litter samples collected from the previously described study sites. When leaf litter samples showed more than 0.1% *P. capitalensis,* and the corresponding spore extraction samples had more than 10^3 spores total, the

qPCR assay was applied to the spore sample to determine the relative abundance of *P. citricarpa* and *P. capitalensis* spores. To extract DNA from spore suspensions, the suspensions were incubated at -80°C overnight, then transferred directly into a 65°C waterbath for 10 minutes, and then vortexed for 3 minutes in the presence of 0.5 mm diameter glass beads. Following vortexing, the tubes were centrifuged for 1 minute and the supernatant was used for qPCR.

To compare the ability of the two *Phyllosticta* species to reproduce (sporulate) from leaf litter, the fecundity (F) of each species was determined using the formula:

$$F^t = (g_{sporeDNA} / g_{sporeleaf}) / (g_{leafDNA} / g_{extractedleaf})$$

Where: ^{t} is the specific sampling time; $g_{sporeDNA}$ is the mass of the specific *Phyllostica* DNA present in the spore suspension; $g_{sporeleaf}$ is the dry mass of leaf tissue from which the spore suspension was prepared; $g_{leafDNA}$ is the mass of specific *Phyllostica* DNA present in the leaf tissue; and $g_{extractedleaf}$ is the dry mass of leaf tissue from which DNA was extracted. F values were determined for both *Phyllosticta* species for the 39 sampling times where more than 0.1% *P. capitalensis* was detected in the leaf litter sample, and the corresponding spore extraction samples had more than 10^3 spores total.

Statistical analysis. In order to determine if the proportions of leaves with fructifications were significantly influenced by time or cardinal position, the data were analysed by Analysis of Variance (ANOVA) using Genstat for Windows (16th Edition, VSN International Ltd., UK). To show the seasonal dynamics of sporulation in the leaf litter during the period of fruit susceptibility, the relative proportions of the different fructifications, and the associated spore yields, were plotted against time with the standard error determined for each time point. The relationship between the proportions of pycnidia and pseudothecia to the spore yields of conidia and ascospores, respectively, was explored by regression analysis in Genstat.

Where the qPCR data permitted, statistical comparisons of the relative populations of the two *Phyllosticta* species were made. A two sample t-test was used to determine if the fecundity of the two species is significantly different.

Results

Leaf litter sampling, microscopy and spore extraction. Analysis of the proportions of leaves with fructifications showed that in most years, in most sites, the sampling time, cardinal position (Table 2.2.1) and sampling time × cardinal position interaction were significant (P<0.05). However, no single sampling time, cardinal position, or sampling time × cardinal position was identified as being

consistently more or less likely to have a significantly different proportion of leaves over all years in trial sites.

Observations of the seasonal dynamics of *Phyllosticta* spp. in leaf litter from the long term study site of 'Valencia' orange showed the production of spermogonia to be variable throughout the study period each year, with no well defined peak at any point in time (Fig. 2.2.1 b-d). The production of pycnidia was similar, but with a clear peak in production at the beginning of the season in all seasons except 2015-16. In 2015-16, the production of pycnidia fluctuated throughout the season (Fig. 2.2.1 e-h). For pseudothecia, the relative abundance tended to peak in late December or early January in all years except 2016-17 (Fig. 2.2.1 i-l). In 2016-17 the abundance of pseudothecia (and spermogonia and pycnidia) was low relative to the other years, with a small increase in pseudothecia in late April.

Observations of the seasonal dynamics of *Phyllosticta* spp. in leaf litter from the other study sites 'Imperial' mandarin, 'Arnold Blood' orange, 'Washington' navel orange and 'Eureka' lemon showed similar trends to the long term 'Valencia' orange study site. In particular the spermagonia (Fig. 2.2.2 a-d) and pseudothecia (Fig. 2.2.2 i-l); the latter also showing peaks in pseudothecia production in late December (Fig. 2.2.2. I,j) similar to that observed at the 'Valencia' orange site. The production of pycnidia did not show the same tendancy for a clear peak in pycnidia early in the season as was observed for the 'Valencia' orange, but rather the relative abundance of pycnidia fluctuated over the season (Fig. 2.2.2. e-h).

Spore harvesting showed that the yields of conidia generally were related to the relative abundance of pycnidia (Fig. 2.2.1 and 2.2.2). This was similar for ascospores and pseudothecia, but to a less obvious extent. Regression analysis of the combined data for all sites and seasons found that for both spore and fructification types, the relationship between spore and fructification was signiticant (P<0.001). In both cases, an exponential curve accounted for the most variance, but with adjusted R^2 values of only 21.3 and 23.1 for conidia/pycnidia and ascospores/pseudothecia, respectively.

Table 2.2.1. Proportion of leaves under trees with fructifications.[A]

	2016-17	2015-16		2014-15			2013-14	
	Valencia	**Valencia**	**Lemon**	**Valencia**	**Blood**	**Navel**	**Valencia**	**Imperial**
Cardinal								
North	22 b	30	45 b	23 a	6 a	23	24 a	27
South	22 b	30	37 a	29 b	9 b	20	30 b	29
East	17 a	31	42 b	24 a	8 ab	20	31 b	28
West	18 a	33	37 a	22 a	9 b	22	26 a	22
LSD	3	3	5	4	2	5	4	6
P-value	<0.001	NS	<0.001	0.012	0.048	NS	0.002	NS
Time[B]								
1		15 bc						
40	21 def	3 a						
50			33 ab	15 abc	6 a	21 (4) ab		
60	9 ab	12 b	26 a					
70	14 bc	48 g	43 cde	14 ab	10 ab	34 (12) d	17 a	44 (19) d
80	7 a	57 h		12 a	8 a	15 (2) a	32 b	33 (11) c
90		44 fg	51 ef	18 abcd	7 a	17 (3) a		
100	15 bc	56 h	49 def				26 b	33 (11) c
110	18 cde			37 f	6 a	21 (4) a	39 c	27 (7) bc
120		47 g		23 cde	9 a		40 c	21 (5) ab
130	22 efg	39 f	42 cd			19 (4) a		
140	18 cde	49 g	36 bc	37 f	5 a	21 (5) abc	24 b	14 (2) a
150					7 a		26 b	31 (10) c
160		28 e	36 bc	27 e	13 b	18 (3) a	25 b	13 (2) a
170	25 fg	28 e		21 bcde		18 (3) a		
180	18 cde	18 bc	37 bc	41 f	9 a	30 (9) bd	25 b	15 (2) a
190			51 f	26 e	8 a	21 (5) ab	26 b	33 (11) c
200	53 h	16 bc		25 de	9 a			
210		25 de				22 (5) abc		
230	27 g	20 cd						
240		26 de						
LSD	5	6	7	7	4	8 (1)	6	9 (1)
P-value	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001

[A]Where needed values were subjected to an arcsince angular transformation to normalise the data. Values in parentheses are back-transformed means. NS = not significant (*P*>0.05)

[B]Time in days after 1st of September in each year.

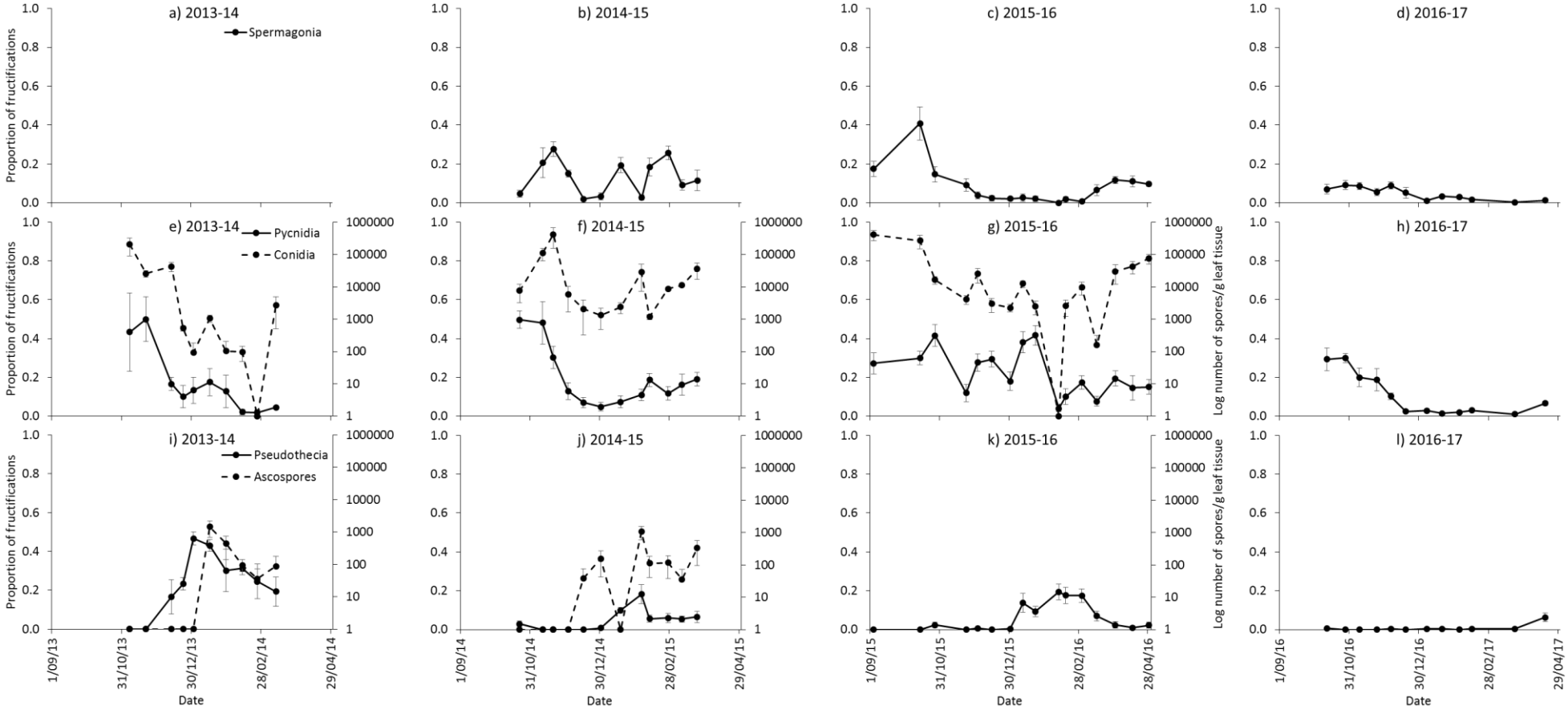

Figure 2.2.1. The seasonal dynamics of the production of spermagonia (a-d), pycnidia and conidia (e-g), and pseudothecia and ascospores (i-k) by *Phyllosticta* spp. during the period of fruit susceptibility for the seasons 2013-14 (a,e,i), 2014-15 (b,f,j), 2015-16 (c,g,k), and spermogonia, pycnidia and pseudothecia only for 2016-17 (d,h,l) in 'Valencia' orange located in Mundubbera, Qld. Error bars indicate Standard Error.

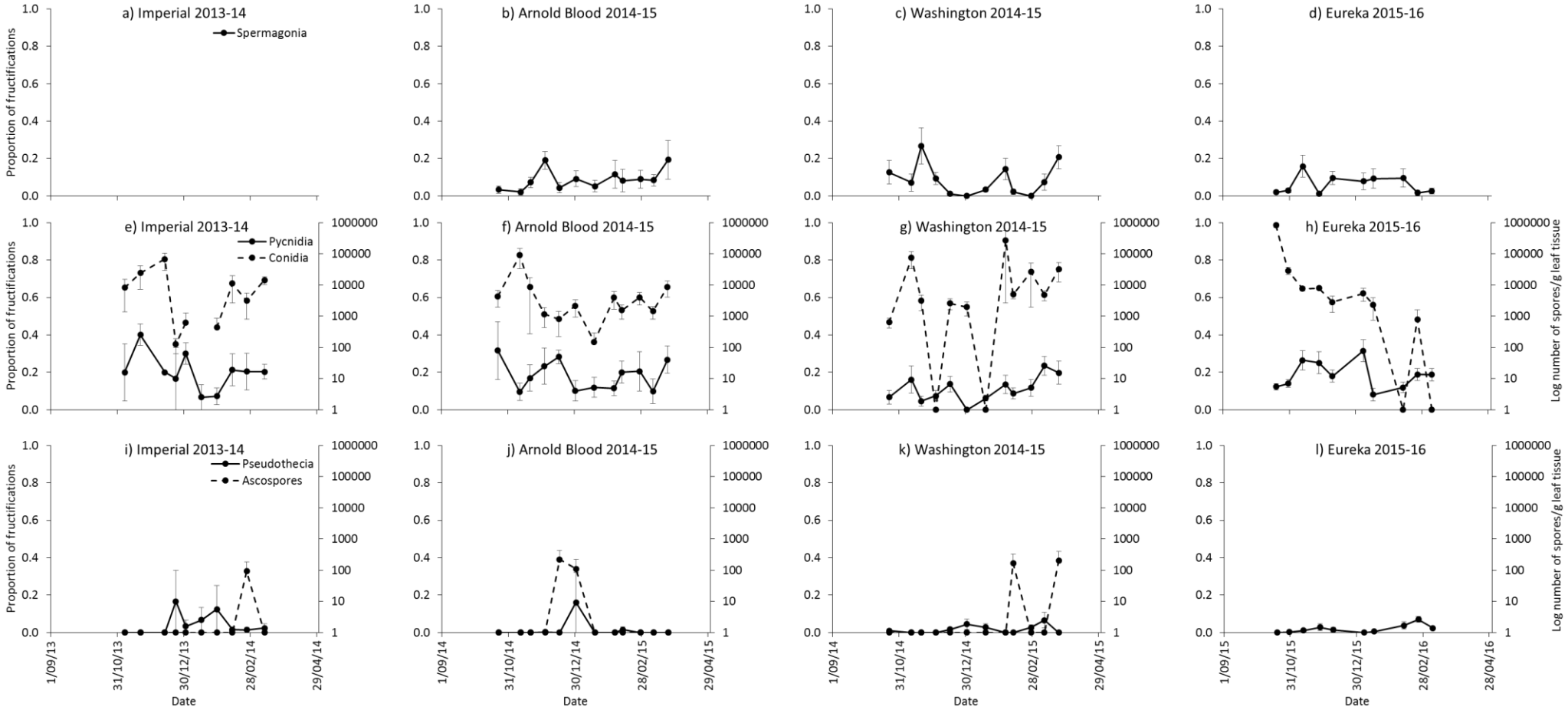

Figure 2.2.2. The seasonal dynamics of the production of spermagonia (a-d), pycnidia and conidia (e-h), and pseudothecia and ascospores (i-l) by *Phyllosticta* spp. during the period of fruit susceptibility for 'Imperial' mandarin in the season 2013-14 (a,e,i), 'Arnold blood' orange (b,f,j) and 'Washington' navel (c,g,k) in 2014-15, and 'Eureka' lemon in 2015-16 located in the Central Burnett, Qld. Error bars indicate Standard Error.

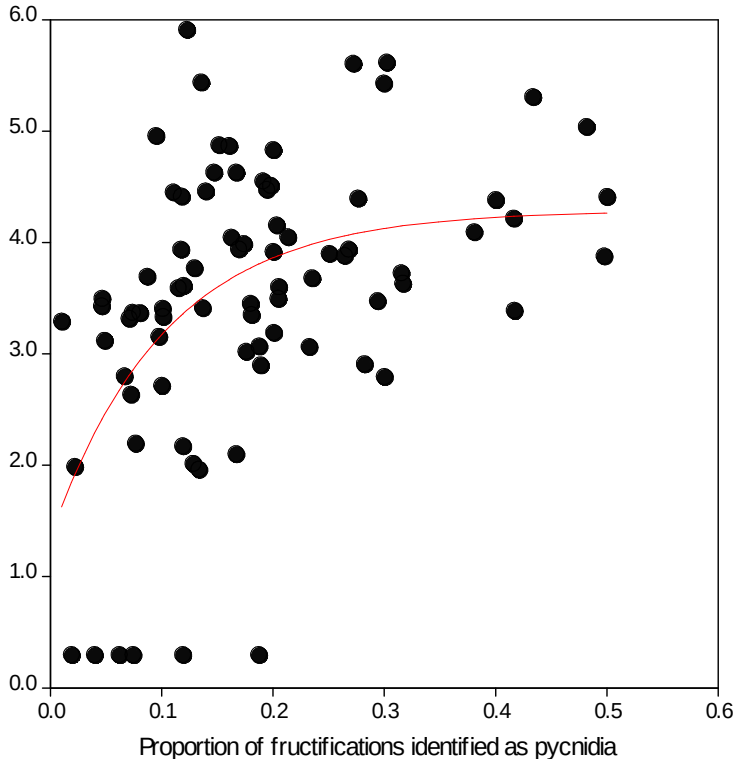

Figure 2.2.3 Exponentrial relationship between pycnidia abundance and conidia recovery where y = $4.288 - 2.93(0.000064^{x})$; adjusted $R^2 = 21.3$

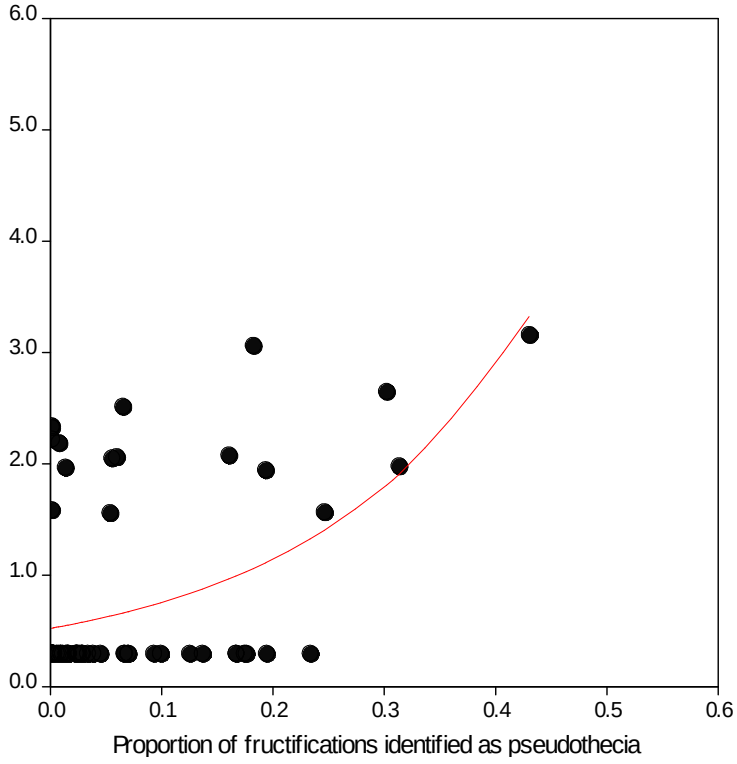

Figure 2.2.4 Exponentrial relationship between pseudothecia abundance and ascospore recovery where y = $0.74 + 0.35(166^{x})$; adjusted $R^2 = 23.1$

Species identification and quantification. The successful application of the qPCR assay to DNA from the Australian population of *P. capitalensis* isolates indicates the assay is likely to detect the *P. capitalensis* genotypes present in Australia. Furthermore, the application of the assay to characterised template DNA from leaf litter from Florida, and production of results consistent with those from Florida, further validate the use of the assay on Australian samples.

The relative abundance of *P. citricarpa* and *P. capitalensis* varied across the different study sites. The long term Valencia study site consistently showed the lowest levels of *P. capitalensis* in all seasons (Figure 2.2.5.a, b, c), with the highest level being 2.4% of total *Phyllosticta* DNA, but most samples being less than 0.1% *P. capitalensis*. The other sites all showed higher levels of *P. capitalensis* compared to the Valencia site in all seasons. These included: Imperial, with up to 68% *P. capitalensis* in a specific sample (Figure 2.2.5.d); Washington, with up to 60% *P. capitalensis* (Figure 2.2.5.e); Arnold Blood, with almost 100% *P. capitalensis* in one sample (Figure 2.2.5.f); and Eureka, with up to 7.9% *P. capitalensis* (Figure 2.2.5.g). Despite these occasionally high levels of *P. capitalensis*, however, the majority of leaf litter samples had much lower levels compared to *P. citricarpa*.

In several cases, the proportion of *P. capitalensis* was often highly variable between replicate samples at a particular timepoint and site. In extreme cases, replicate leaf litter samples could range from almost 100% *P. capitalensis* to almost 100% *P. citricarpa* (Figure 2.2.5.d,e,f). These variable results between replicates were confirmed in multiple independent assays done on different days. Due to this high variation, and overall very low occurrence of *P. capitalensis* in most samples, the raw data for each replicate sample are presented in lieu of a meaningful statisitical analysis.

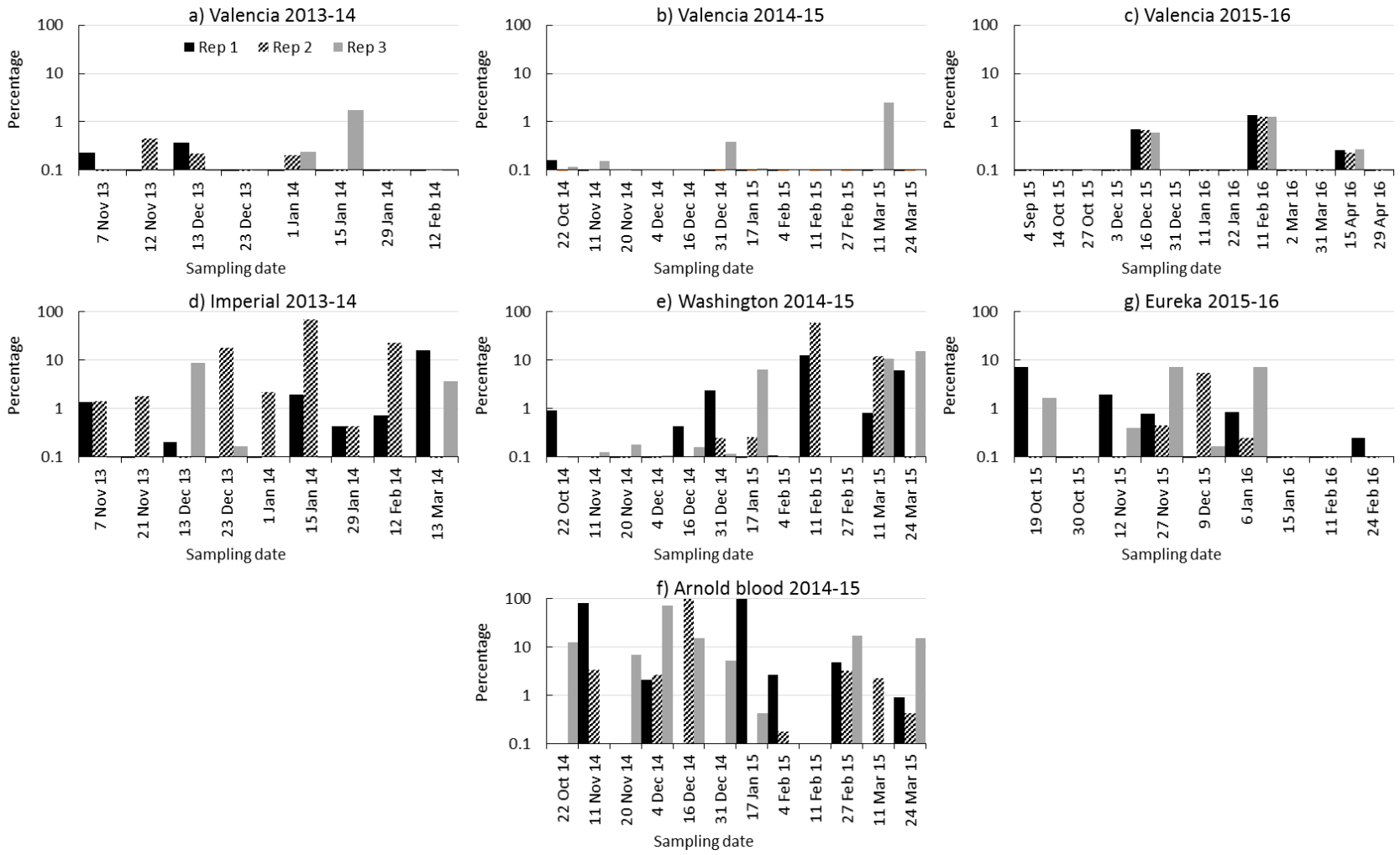

Figure 2.2.5. The proportions of *Phyllsticta capitalensis* DNA in the total *Phyllosticta* DNA present in leaf litter sampled from commercial production blocks near the townships of Mundubbera (Valencia, Eureka, Arnold Blood) and Gayndah (Imperial, Washington) from the 2013-14 to 2015-16 periods when fruit are susceptibility to citrus black spot caused by *Phyllostcita citricarpa*.

In the application of the qPCR assay to the spore samples to determine the relative abundance of *P. citricarpa* and *P. capitalensis* spores, the recovery of ascospores from the leaf litter was generally extremely low (with the vast majority of samples yielding below the detectable limit of the hemocytometer). Therefore, in all cases the spore analysis was performed on conidia suspensions. In many cases these water-extracted conidia suspensions also contained numerous spermatia, although these were not quantified in this study. In total, we assayed 39 spore suspensions by qPCR. As with the leaf litter samples, it was possible to amplify *P. citricarpa* DNA from every spore suspension tested, but only about half of the suspensions (21/39) contained *P. capitalensis* DNA based on this assay (Fig. 2.2.6). Only one spore sample had *P. capitalensis* levels approaching those of *P. citricarpa* (47% and 53%, respectively), and the corresponding leaf litter had more *P. capitalensis* (60%) than *P. citricarpa.* High levels of *P. capitalensis* in leaf litter did not always translate into high levels of *P. capitalensis* in spore suspensions, however. For example, a conidia suspension from a leaf litter sample with 80.6% *P.capitalensis* contained only 7.8% *P. capitalensis*.

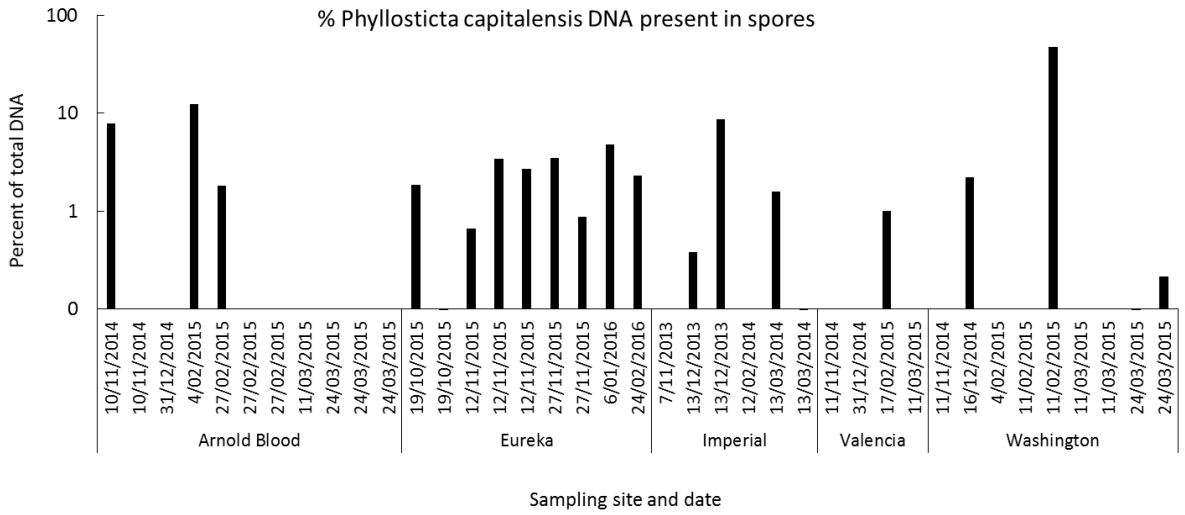

Figure 2.2.6. The percentage of *Phyllosticta capitalensis* DNA present in the total *Phyllosticta* DNA amplified from spore suspensions extracted from citrus leaf litter.

As indicated in the examples above, the association between levels of the two species in leaf litter and the corresponding spore suspensions was poor, and in most cases (83%) the percentage of *P. capitalensis* in spore suspensions was less than that in leaf litter. While this implies that the pathogenic *P. citricarpa* generally produces more conidia (or conidia plus spermatia) in leaf litter than the endophytic *P. capitalensis*, application of the t-test to the fecundity values (Fig. 2.2.7) did not reveal a

statistically significant difference between the fecundity of the two species (*P*=0.14). Nevertheless the mean fecundity of *P. citricarpa* (0.89) was higher trendwise than *P. capitalensis* (0.14).

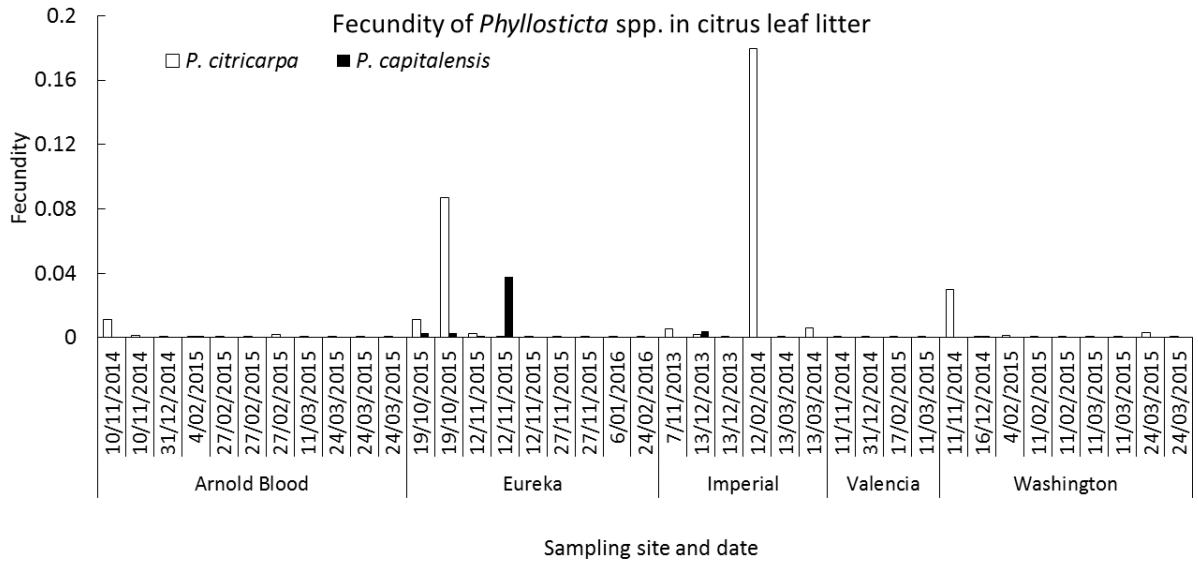

Figure 2.2.7. The fecundity of *Phyllosticta citricarpa* and *P. capitalensis* in citrus leaf litter, as determined by the quanities of DNA of each species in leaf tissue and recovered spores.

Discussion

In this study we aimed to determine the seasonal dynamics of *P. citricarpa* and *P. capitalensis* in commercial citrus orchards in Queensland, Australia. Our major finding is that the pathogen *P. citricarpa* was far more abundant in Queensland orchards than the endophyte *P. capitalensis*. *P. citricarpa* was detected in every sample from every timepoint at every site, whereas *P. capitalensis* was only detected in a proportion of samples. For example, across all sites in the 2014-15 season we were only able to detect *P. capitalensis* in 69.6 % of leaf litter DNA samples. Across all sites and seasons, *P. capitalensis* was detected in 83% of leaf litter samples. *P. citricarpa* was also dominant in the harvested conidia. Conidia, and pycnidia, were also found to be the most abundant spore and fructification type in leaf litter. This was particularly obvious at the start of the season in most cases. Ascospores and pseudothecia, despite being generally accepted as the main inoculum source in CBS epidemics (Fourie *et al.*, 2013; Kiely, 1948; Kotze, 1981), were relatively less abundant than the conidia and pycnidia. Furthermore, detection of ascospores and pseudothecia generally occurred with a defined peak in abundance around late December to early January (mid summer). Assuming ascospores are the primary inoculum source during epidemics, this would suggest the majority of infection in the field should coincide with this peak in ascospores and pseudothecia. Importantly, the

overall dominance of *P. citricarpa* in orchards suggests the presence of *P. capitalensis* is unlikely to be a significant source of confusion in commercial orchards.

The dominance of *P. citricarpa* over *P. capitalensis* may be due to a number of factors, the first being simply the possibility of *P. citricarpa* being present at the study sites longer than *P. capitalensis*. However, definitive evidence for the time of introduction into individual sites is lacking. Assuming similar introduction times, an obvious advantage for *P. citricarpa* is the ability to sporulate from lesions on fruit and twigs, for which there is no evidence for *P. capitalensis*. *P. capitalensis* is only known to sporulate in leaf litter, and trendwise at least, with a lower mean fecundity than *P. citricarpa* in our samples. Indeed, we have previously shown that *P. citricarpa* produces significantly more conidia and spermatia in culture than *P. capitalensis* (Tran *et al.* in preparation). In contrast, the homothallic nature of *P. capitalensis* would presumably suggest an ability to more readily produce pseudothecia and ascospores in leaf litter than *P. citricarpa*, which requires a compatible mating type. However, the rare recovery of ascospores in our study precluded comparison of ascospore fecundity, and in the few cases were the qPCR assay could be applied to ascospores *P. citricarpa* was again highly dominant (data not shown). It is also of note that evidence exists for *P. capitalensis* being suppressive of *P. citricarpa* (de Almeida, 2009), which is further explored in section 2.3. Another possible explanation are differences in fungicide sensitivity between the two species, as has been observed for copper (Hendricks *et al.*, 2013), which is further explored in section 2.4. The general notion that *P. citricarpa* can displace *P. capitalensis* will be best observed by our collaborators in Florida, where *P. capitalensis* has been well established prior to the recent introduction of *P. citricarpa* in 2010 (Schubert *et al.*, 2012).

Our study is not the first attempt to determine the seasonal dynamics of *Phyllosticta* in citrus leaf litter in Qld, however it is the first to include evidence for the identity of the observed fructifications. In both our study and previous work (Wyatt *et al.*, 2008), direct leaf litter examinations were made, and both studies show an initial peak in pyncidia early in the season, followed by relatively consistent production of pycnidia, while pseudothecia production occurred in more isolated peaks later in the season. The most notable difference between the 2005-06 data (Wyatt *et al.*, 2008) and our study is the start of the peak in pseudothecia production occurred slightly earlier (late November to Mid December) in 2005-06, compared with late December to early January in our study. This difference is most likely to differences in prevailing weather conditions, which have been shown to influence the maturation of pseudothecia (Fourie *et al.*, 2013). Importantly, our evidence for *P. capitalensis* being relatively less common than *P. citricarpa* assists to validate the 2005-06 data as being relevant to *P. citricarpa*. Furthermore, the combined findings suggest that peak inoculum availability, and potentially infection, typically occur in December. The actual timing of infection has

been investigated in PhD studies supplementary to CT13021 to better understand the relationship between pseudothecia production and CBS infection in the field.

A possible explanation for the initial peak in pycnidia production maybe the early peak in mature leaf abscission that occurs in early spring, followed by a consistent, low level of abscission (Erickson and Brannaman, 1960). During the peak of leaf abscission in spring, the environmental conditions (e.g. rainfall, temperature, wetting/drying cycles) needed for pseudothecia production are unlikely to have occurred, but such specific conditions are not necessary for pycnidia and spermogonia development to take place (Kiely, 1948; Fourie *et al.*, 2013). Hence, formation of pycnidia and spermogonia is more likely, more often. Pseudothecia formation is reported to be associated with wetting and drying cycles driven by rainfall (Kiely, 1948; Kotze, 1981; McOnie, 1964b), but the reasons why these cycles are needed are rarely speculated. Considering recent findings demonstrating the requirement of compatible mating types in order to produce pseudothecia (Tran *et al.*, 2017), and that conidia and spermatia are capable of fertilising complementary mating types, it would seem likely that the wetting part of the cycle creates an opportunity for conidia and spermatia to be liberated and disseminated to make contact with the compatible mating type. Furthermore, the findings of section 3.3 have shown that mycelium of *P. citricarpa* colonies will not readily occupy the same space, likely precluding mycelium contact between mating types from being able to facilitate mating. For these reasons it is likely that our observations of defined peaks in pseudothecia production are the result of mating opportunities being tightly confined to the onset of specific weather conditions (wetting and drying).

This investigation of the seasonal dynamics of *Phyllostica* spp. in citrus in Qld supports the potential for forecasting disease management options such as fungicides or leaf litter management on the basis of visual assessments (spore trapping, leaf litter etc). Volumetric spore trapping has the benefit of collecting ascospores once mature and airborne, however the time taken to check the trap manually means infection may have taken place prior to reporting of their presence. Direct leaf assessment has the potential advantage of detecting and report the development of pseudothecia prior to sporulation events, allowing time to take protective action in the field. However, of note is that the direct leaf litter monitoring in this study was used to advise the commencement of a fungicide treatment in an experiment conducted under project CT13020 *Increasing market access, profitability and sustainability through integrated approaches to fungal disease control*, but commencing the fungicide regime after the first detection of pseudothecia did not effectively control CBS. Further work is needed to better understand the relationship between inoculm sources and CBS development in the field.

Acknowledgements

The authors gratefully acknowledge the invaluable assistance of Mal Wallis (Citricare). We also thank the trial site collaborators Mark Trott, Greg Parr and Carl Robinson. We are grateful for the financial support provided by the University of Florida through the Citrus Research and Development Foundation and Horticulture Innovation Australia.

2.2 Pathogen endophyte antagonism *in vitro* and *in planta*

Introduction

At present in Australia, *P. citricarpa* and *P. capitalensis* are the only *Phyllosticta* species that have been found associated with *Citrus* (Miles *et al.*, 2013). *P. citricarpa* has the highest economic impact due to its wide distribution and ability to infect almost all commercial citrus cultivars. *P. capitalensis* is a widespread endophyte and morphologically similar to *P. citricarpa* (Baayen *et al.*, 2002; Kiely, 1948; McOnie, 1964a; Wager, 1952). *P. capitalensis* has been well-documented to co-exist in the same tissues of citrus trees as *P. citricarpa* and exists under similar ecological conditions for growth and reproduction (Baayen *et al.*, 2002; Baldassari *et al.*, 2008; Bonants *et al.*, 2003). The nature of the co-existence of the two species and their interactions, if any, is unclear. The interaction between endophytes and plant pathogens within a host plant can be antagonistic, neutral or facilitative (Busby *et al.*, 2016). Antagonism of endophytes towards plant pathogens has been reported and used to develop biological control agents in some pathosystem (Busby *et al.*, 2016). For example, the endophyte *Trichoderma hamatum* isolated from wheat plants was found to significantly reduce colony growth, spore germination and disease severity of wheat tan spot caused by *Pyrenophora tritici-repentis* (Larran *et al.*, 2016). In the case of citrus black spot, one study reported that pre-infection of citrus fruit with *P. capitalensis* reduced the severity of hard spot lesions (de Almeida, 2009). The apparent antagonism, or any interaction between *P. citricarpa* and *P. capitalensis*, if confirmed, may be useful and provide an avenue to use the endophyte in disease management. Understanding the interaction between the endophyte and the pathogen may also improve our knowledge of the citrus black spot pathosystem.

In this study, we aimed to identify any interaction between the endophyte *P. capitalensis*, and the citrus black spot pathogen, *P. citricarpa*. The specific questions we sought to address were: (i) does one species impact on the growth of the other *in vitro* and; ii) does the endophyte impact on the development of the disease *in planta*? The possible mechanisms of the interactions between the species are discussed.

Methods

Fungal isolates In total, 27 isolates of *P. citricarpa* and 33 isolates of *Phyllosticta* endophytes recovered from *Citrus* in Australia were used. All the *P. citricarpa* isolates, and 4 isolates of the endophytic population were previously identified as *P. citricarpa* and *P. capitalensis*, respectively, by sequence analysis of the internal transcribed spacer region and partial translation elongation factor 1-α gene (Miles *et al.*, 2013). The other 29 endophytic isolates were identified based on colony morphology,

and spore types produced in culture, and the presence of a yellow halo around colonies grown on Oatmeal Agar (Baayen *et al.*, 2002; Baldassari *et al.*, 2008). After identification all isolates were cultured on half-strength potato dextrose agar (1/2 PDA) (BD Difco, BD Australia) at room temperature for 2 weeks prior to commencement of the experiments.

***In vitro* co-cultivation assays** Following a preliminary screening of isolates, each of four *P. citricarpa* isolates (BRIP 27889, 52614, 53720, and 54241) was co-cultivated with four *P. capitalensis* isolates (BRIP 53721, 53722, BRIP 61605c and 54242) in all possible combinations including with themselves. Two mycelial plugs (5 mm diameter) obtained from two week old cultures were placed on petri plates of ½PDA at a distance of 5 cm from each other. There were three replicate petri plates per combination. As the pathogen grows more slowly than the endophytes, they were plated 3 days earlier than the endophyte to ensure adequate opportunity for colonies of *P. citricarpa* to establish. The plates were then incubated at 25˚C with 12h/12h light/dark regime. Development of any inhibition zone was visually assessed and colony growth areas were measured at 14 days after co-cultivation using NIS-Elements Basic Research Imaging Software (Nikon Instruments Inc., Japan). Inhibition of colony growth relative to the controls (same isolate paired with itself) was then determined using the formula: $\frac{A - B}{A}$, where A = mean growth areas of two colonies of the same isolate paired with itself, and B = growth area of a single colony of the same isolate as A when paired with a different isolate. Data of colony growth inhibition of each isolate was analysed separately using GenStat for Windows (16th Edition, VSN International Ltd., UK). The data were subjected to one way ANOVA. Pairwise comparisons of means were based on least significant difference (P<0.05).

In order to test if mycelial strips provide a particularly large and uniform zone of interaction between the resulting two colonies of *P. citricarpa* and *P. capitalensis*, an alternative method was used with some modifications of the mycelial plug methods. In particular, instead of using plugs, strips of mycelium (3 cm × 2 mm) were cut from one-month-old colonies and then placed parallel to one another at a distance of 3 cm. The same set of isolates as the mycelial plugs assays was paired in all possible combinations, including themselves. In addition, plates with only one mycelial strip were included as a control to determine if inhibition in colony growth is due to competition for resources between the two colonies when they are paired. Colony growth inhibition was calculated as described forin the mycelial plug assay sectiondescribed previously. Data of colony growth inhibition of each isolate was analysed separately using GenStat. The data were subjected to one way ANOVA. Pairwise comparisons of means were based on least significant difference (P<0.05).

***In planta* co-inoculation** In order to identify any interactions between *P. citricarpa* and *P. capitalensis* on citrus fruit, a co-inoculation experiment was conducted on fruit from three mandarin hybrid accessions (OOCO18, O1CO49 and Daisy) at the orchard arboretum of the Bundaberg Research Facility, Qld.

Fruit were inoculated using the blotting paper strip technique (Miles *et al.*, 2016) described previously with two inoculations performed per fruit at an interval of 14 days. In the first inoculation, a 10 mm-wide strip was used while a 5 mm-wide strip was used in the second inoculation to ensure that the second inoculation occurred within the same area of the first inoculation. Treatments included fruit inoculated first with one of the endophytic *P. capitalensis* BRIP 53722, BRIP 54242 or BRIP 61605c, which were found to be most promising in inhibiting colony growth of the *P. citricarpa* ex-epitype isolate BRIP 52614, and most consistent across the four *P. citricarpa* isolates tested in the *in vitro* assays. Fourteen days later, the fruit pre-inoculated with the endophytes were inoculated with *P. citricarpa* BIRP 52614. Fruit that were inoculated with sterile water followed by sterile water, or one of the endophytic isolates followed by sterile water at the 14 days interval served as negative controls. Positive controls included fruit inoculated with sterile water, then *P. citricarpa* BRIP 52614 at 14 days later. Conidia suspensions were prepared and adjusted to 5×10^4 conidia/mL. There were 10 replicate fruit (3 to 4 fruit each of the three hybrid host accessions) per treatment. After inoculation, fruit were left to mature on the tree for as long as possible and covered with a mesh bag prior to harvest to reduce the risk of losing fruit. After harvest, fruit were incubated at constant light, 27^{o}C and 80% relative humidity for three weeks before assessment of disease.

Disease incidence was defined as the proportion of fruit with black spot symptoms. Disease severity was defined as the number of lesions per infected fruit. In the case of disease severity, severity of each black spot symptom type was assessed separately. For discrete lesion types (i.e. hard spot and freckle spot), disease severity was assessed by counting individual lesions on each fruit. For spreading lesion types (i.e. virulent spot), disease severity was assessed by estimating percentage of diseased surface areas (% virulent) which was then converted to an equivalent number of 3-mm-in-diameter lesions (no. lesions), which is the common size of the discrete lesions, using the formula:

$$No.lesions = \frac{\%\ virulent\ \times surface\ area\ of\ fruit}{area\ of\ a\ 3-mm-dia\ lesion}$$

Where: Surface area of fruit = $4 \times \pi \times R^2$ (mm^2), with R being mean radius of 20 randomly selected fruit of the citrus varieties used; area of a 3-mm diameter lesion = $\pi \times (1.5)^2 = 7.065$ mm^2. Overall disease severity was then calculated by adding all together values for all the three symptom types.

Data analyses. Disease incidence and severity for were analysed separately using GenStat . A Generalized Linear Model with a binomial distribution and logit link was applied to the binomial

incidence data. Disease severity data were $\log_{10}$ transformed to stablise variance before subjected to unbalanced ANOVA to account for the small, but variable number of missing fruit resulting from decay (mould) during post-harvest incubation. To test the relationships between means, pairwise comparisons were performed for all significant analyses (P<0.05) using Fisher's least significant difference test.

Results

Zones of mycelium inhibition were consistently observed for all combinations in both mycelial plug and strip assays. Generally, some inhibition in colony growth of all four studied *P. citricarpa* isolates was observed when paired with *P. capitalensis* as indicated by the positive growth inhibition values (Fig. 2.2.1). However, such growth inhibition was not statistically different (P>0.05) compared with the control of the same *P. citricarpa* isolates paired with themselves. In some cases, growth inhibition values of *P. citricarpa* by certain endophytic isolates were negative (e.g. *P. citricarpa* isolate BRIP 53720 by *P. capitalensis* isolates BRIP 53721 and 54242, Fig. 2.2.1.C). Across all four *P. citricarpa* isolates, *P. capitalensis* isolates BRIP 53722 and 54242 had the highest and most consistent colony growth inhibition against *P. citricarpa*, especially the ex-expitype BRIP 52614 isolate (Fig. 2.2.1.A).

Assessment of colony growth of the endophye in the presence of *P. citricarpa* showed negative growth inhibition values in most cases although the inhibition values were not statistically different (P>0.05) between combinations of different species and same isolate (Fig. 2.2.2).

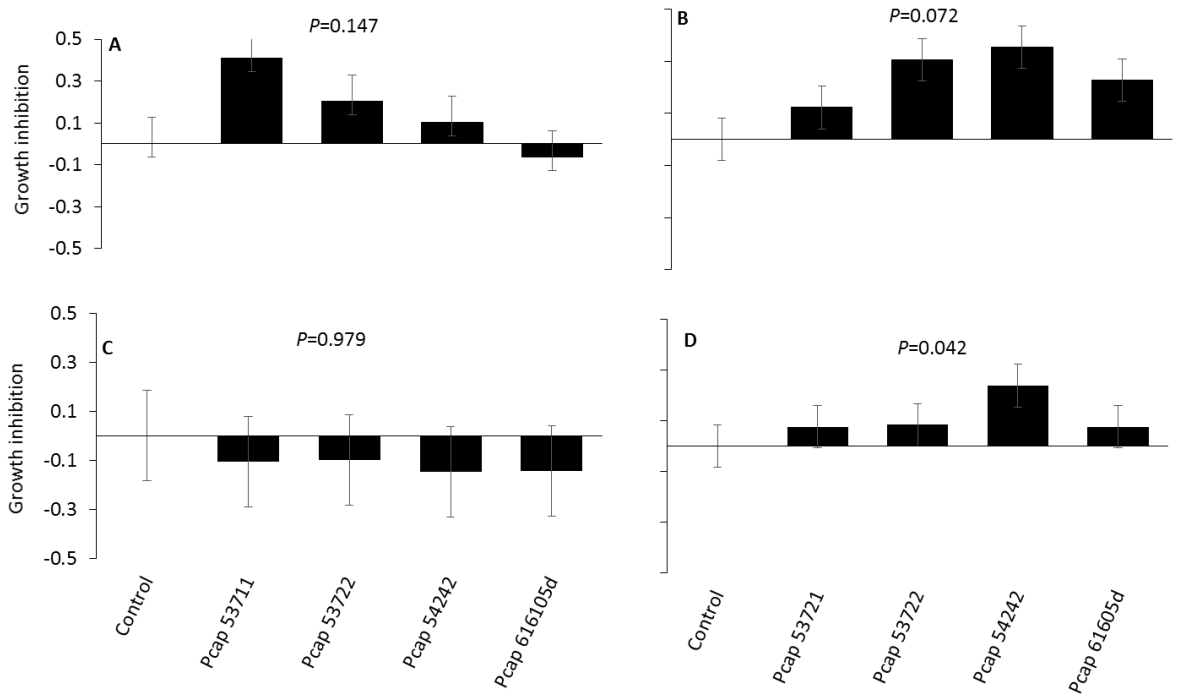

Figure 2.2.1. Colony growth inhibition of *P. citricarpa* isolates 27889 (A), 52614 (B), 53720 (C) and 54241 (D) by *P. capitalensis* employing mycelial plug co-cultivation method. Where Control = the *P. citricarpa* isolate being tested paired with itself, Pcap = *P.* capitalensis. Error bars indicate Standard Error.

The revised method using mycelial strips showed a similar result to the mycelial plug assay that mycelium-free zones were observed with all combinations of isolates. However, no significant difference in colony growth between the same isolates paired with themselves and paired with isolates of the other species. As expected, the controls where one colony grown by itself had significantly less inhibition values than any other combination (data not shown).

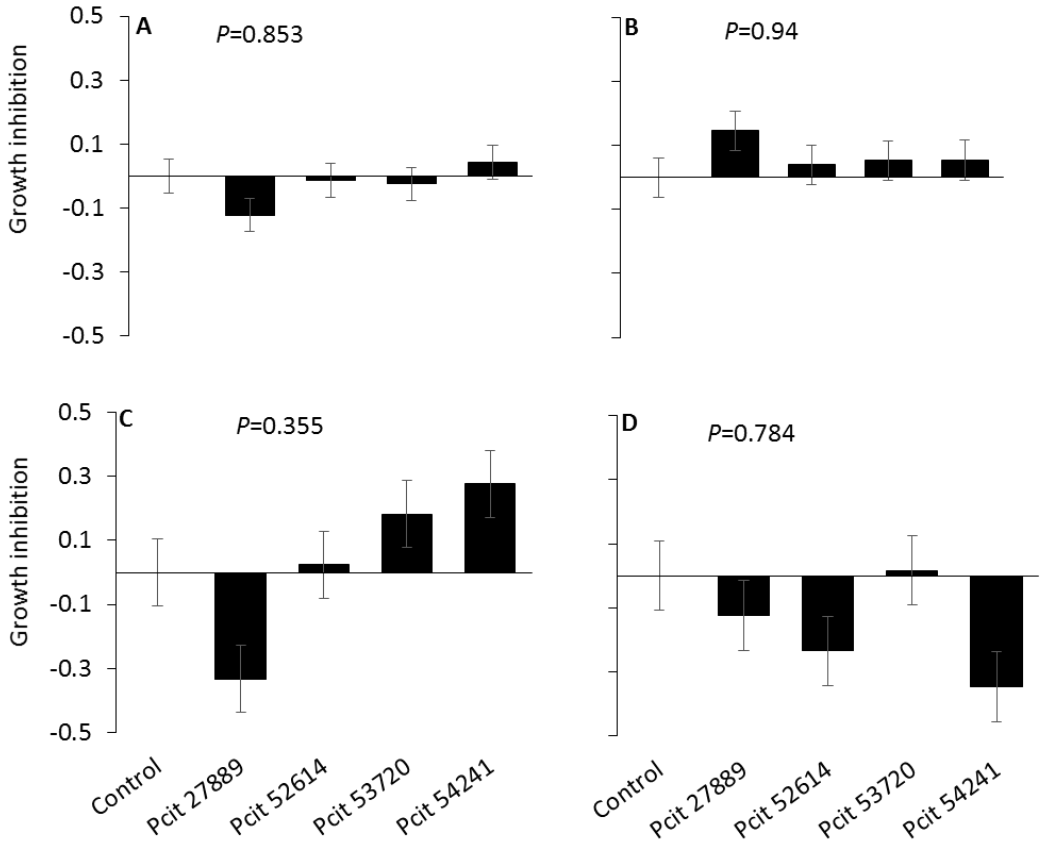

Figure 2.2.2. Colony growth inhibition of *P. capitalensis* isolates (A) 53721, (B) 53722, (C) *5*4242, (D) 61605d, by *P. citricarpa*. Where Control = the *P. capitalensis* being tested paired with themselves, Pcit = *P. citricapra*. Error bars indicate Standard Error.

Co-inoculation of citrus fruit with *P. capitalensis* and *P. citricarpa* at a 14 days interval showed a significant reduction (*P*<0.001) in disease incidence of citrus black spot (Fig. 2.2.3.A). As expected, control treatments of water and the endophytes did not result in any citrus black spot symptoms. Fruit pre-inoculated with water, then *P. citricarpa* BRIP 52614 had 100% disease incidence which was significantly higher (*P*<0.001) than that for fruit pre-inoculated with *P. capitalensis* BRIP 52722, BRIP 54242 or BRIP 61605d (Fig. 2.2.3A). Disease severity of the fruit pre-inoculated with water then *P. citricarpa* did not differ signficantly from fruit pre-inoculated with *P. capitalensis* BRIP 54242 then *P. citricarpa*, but did from other treatments (Fig. 2.2.3.B).

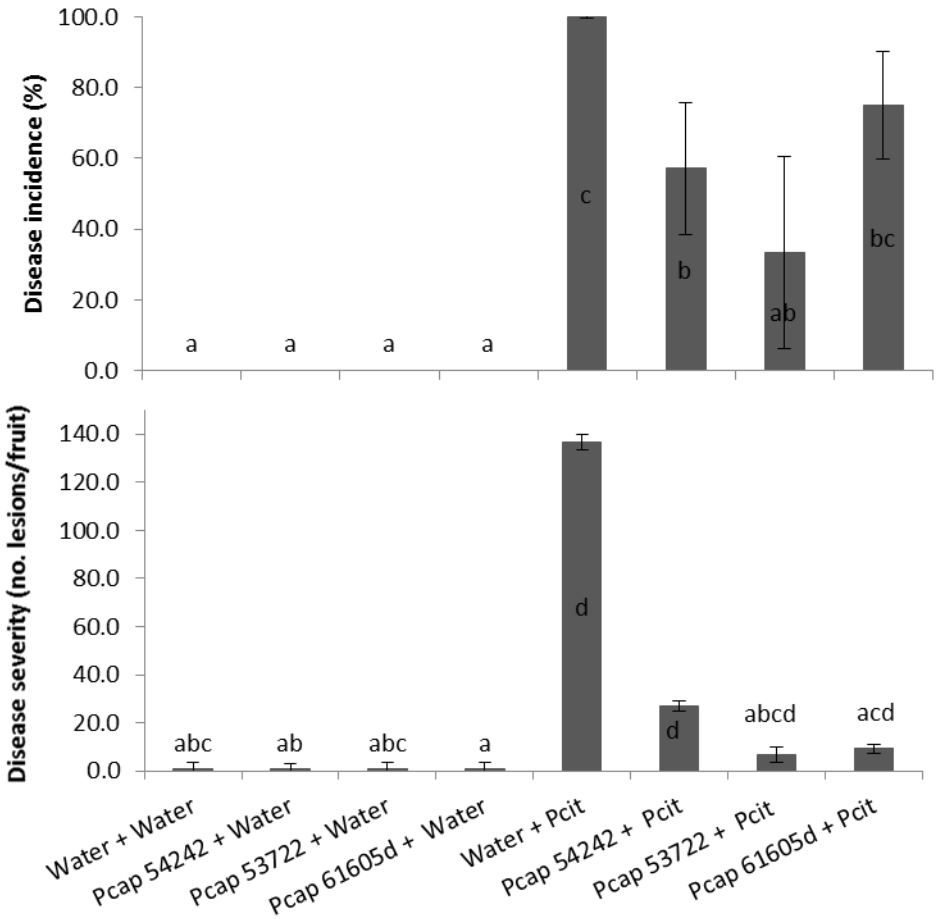

Figure 2.2.3. Disease incidence (top) and severity (bottom) of citrus black spot for fruit of co-inoculation experiment in the 2015-16 season. Each treatment label in the X axis indicates the first inoculation followed by second inoculation at 14 days later. Pcit = *P. citricarpa*, Pcap = *P. capitalensis*. Error bars indicate Standard Error.

Discussion

To investigate the interactions between the endophytic species and the citrus black spot pathogen, *P. citricarpa*, we performed *in vitro* and *in planta* co-inoculation assays. Our results showed no significant reduction of colony growth from the *in vitro* assays. However, disease incidence was significantly less when fruit co-inoculated with the pathogen and the endophyte at a 14 day interval compared with fruit inoculated with only the pathogen, indicating the potential of the endophytes to suppress expression of citrus black spot. This supports the finding by de Almeida (2009) that inoculating 'Pêra-Rio' orange fruit with *P. capitalensis* 48 hours prior to inoculation of *P. citricarpa* showed significant reduction in disease severity of black spot, which needs to be confirmed to be deployed in citrus black spot management.

The underlying mechanisms by which endophytes affecting host plant diseases are unclear. The common documented mechanisms include direct antagonism by which endophytes antagonise pathogens via hyperparasitism, competition or antibiosis resulting in decreasing disease severity, and indirect antagonism by which the presence of endophyte helps induce host resistance to the pathogen (Busby *et al.*, 2016). The research for potential biocontrol of endophytes often involves *in vitro* assays (e.g. co-cultivation) to preliminarily screen for potential antagonistic endophytes, *in vivo* glasshouse and field tests (Busby *et al.*, 2016). However, in some cases, results from these assays can be discrepant. For instance, Martín et al. (2015) reported strong inhibition of the Dutch elm disease pathogen, *Ophiostoma novo-ulmi*, via antibiosis in *in vitro* competition assays with the endophytes *Monographella nivalis* and *Alternaria tenuissima*, however, this interaction was not repeatable in the field experiments. In contrast, de Capdeville et al. (2002) found combination of alternative control agents including yeast antagonists induced resistance of "Red Delicious" apple fruit against postharvest blue mold caused by *Penicillium expansum* rather than directly inhibiteding the pathogen. In the case of citrus black spot, if any of the endophyte-induced suppression of citrus black spot in the field can be confirmed, inducing host resistance to plant disease may be the case for this endophyte as no direct antagonism was observed in our *in vitro* dual culture assays.

Acknowledgements

We thank Mark Trott and Malcolm Smith and team at the Bundaberg Research Facility for the use of their orchards.

2.3 Pathogen endophyte antagonism fungicide sensitivity

Introduction

In terms of the practical application if any antagonism is confirmed in this study, the direct application of commercially multiplied *P. capitalensis* as a biological control agent could be investigated in a subsequent project to CT13021, however it is not the aim of this project to develop and commercialise a biocontrol agent against *P.citricarpa*. Alternatively, project CT13021 will first investigate if populations of the endophyte might be encouraged/discouraged by certain orchard practices. Routine fungicide applications are the most likely practice to impact upon fungal populations, therefore the impact of different fungicides on the pathogen and endophyte should be investigated. Evidence exists for such differences between the pathogen and endophyte, with the pathogen being sensitive to copper fungicide, whilst the endophyte was unaffected by the fungicide (Hendricks and Roberts 2012). Similar to the ability of fungicide applications to significantly alter fungal populations in orchards when individuals within the population become insensitive/resistant to a particular fungicide, leading to significant disease outbreaks, it may be possible that any significant differences in fungicide sensitivity between *P. citricarpa* and *P. capitalensis* could be positively exploited.

Methods

In order to compare the sensitivity of mycelia growth of *P. citricarpa* and *P. capitalensis* to commercially used fungicides *in vitro*, the colony growth of 5 isolates of *P. citricarpa* (BRIP 52614, 53714, 53717, 53720, 54241) and 5 isolates of *P. capitalensis* (BRIP 53710, 53721, 53722, 54242, 61605a) were compared on ½ PDA amended with various rates of the fungicides copper ($CuSO_4$), mancozeb (Dithane Rainshield), azoxystrobin (Amistar 250), and iprodione (Rovral Aquaflo). The PDA was amended to final concentrations of 0.01, 0.1, 10 and 1000 ppm of each fungicide. PDA without any fungicide was included as a control. PDA plates were inoculated in the centre with 3 mm diameter plugs of mycelium from 2-week-old colonies of the isolates. All plates were incubated in the dark at 25°C. The mean colony diameter was determined after 3 weeks incubation by taking the average diameter across two axes of the colony. Growth inhibition was expressed as a proportion of the colony diameter relative to the control. Curves of the $\log_{10}$ concentration versus percent growth inhibition were generated and tested for fit to various models (simple linear, exponential, Gompertz, and logistic curves). The concentration to inhibit growth by 50% (EC_{50}) was then determined.

Results and discussion

The $\log_{10}$ concentration versus percent growth inhibition curves for each fungicide and *Phyllosticta* spp. could be successfully fit (R^2 >70) to various standard curves, with the only exception being

azoxystrobin. For the remaining fungicides, the responses to copper were best described by the exponential curve, iprodione by the Gompertz, and mancozeb by the Gompertz for *P. capitalensis* and logistic for *P. citricarpa* (Table 2.3.1). The calculated EC_{50} values varied by factors of 10 to 1000 between the different fungicides, but not within the different *Phyllosticta* spp. (Table 2.3.1), providing limited evidence for any exploitable differences in fungicide sensitivity between the two species. However, trendwise the EC_{50} values for *P. capitalensis* were higher than for *P. citricarpa*, consistent with previous work that has shown *P. capitalensis* maybe significantly less sensitive to copper than *P. citricarpa* (Hendricks *et al.*, 2013). The growth response for azoxystrobin was atypical, with the growth inhibition for both fungi being highly variable and on average not exceeding 50%. Past studies with similar isolates of *P. citricarpa* have estimated an EC_{50} value of 0.155 mg/kg for azoxystrobin. While the azoxystrobin response curves were atypical, these curves were not greatly different between the two *Phyllosticta* spp..

From the perspective of promoting *P. capitalensis*, over *P. citricarpa*, in commercial orchards by exploiting differences in fungicide sensitivity, the differences in sensitivity shown in Table 2.3.1 are unlikely to be sufficient to promote observable change. The differences in EC_{50} between fungicide sensitive and resistance isolates that leads to a failure of a fungicide may provide some insight into the differences required to promote observable change. For example, EC_{50} values of fungicide sensitive versus resistant isolates have been found to be 0.20-0.60 mg/kg and 280 mg/kg for *Alternaria alternata* on tangelo (Solel *et al.*, 1996), <1 mg/kg and 61.89-109.21 mg/kg in a second study on *A. alternata* on tangelo (Erklc *et al.*, 1999), and <5 mg/kg and >100 mg/kg for *A. alternata* on pistachio (Avenot *et al.*, 2008). These examples suggest that differences in EC_{50} values of around 100-fold confer a difference likely to result a significant change in population.

Table 2.3.1. *In vitro* growth inhibition responses of *Phyllosticta* spp. to commonly used fungicides.

Fungicide	**Fungus**	**Standard curve**	**R^2**	**EC_{50} (mg/kg)**
Azoxystrobin	*P. capitalensis*	Exponential	9.7	-
	P. citricarpa	Exponential	28.1	-
Copper	*P. capitalensis*	Exponential	90.2	918.9
	P. citricarpa	Exponential	75.8	524.2
Iprodione	*P. capitalensis*	Gompertz	87.4	4.4
	P. citricarpa	Gompertz	72.9	1.9
Mancozeb	*P. capitalensis*	Gompertz	77.9	24.7
	P. citricarpa	Logistic	76.5	20.1

Chapter 3

Resistant germplasm

Expression of resistance in citrus fruit to *Phyllosticta citricarpa*, the cause of citrus black spot

In preparation for *Plant Disease*

3.1 Abstract

Citrus black spot is an important fungal disease of *Citrus* caused by the fungus *Phyllosticta* (syn. *Guignardia*) *citricarpa* (McAlpine) van der Aa. The disease primarily causes blemishes on fruit in tropical and subtropical production areas, with control of black spot relying on the application of fungicides. Cultivars resistant to black spot would be highly beneficial to producers. Since the occurrence and expression of resistance is poorly understood, a method was developed for inoculating fruit in the field that reliably produced symptoms of black spot consistent with natural field infection. The inoculation method was used to screen 50 *Citrus* accessions and characterise their expression of black spot symptoms. Control fruit were inoculated with water or the endophyte *P. capitalensis* Henn. Our results showed that all the mandarin, sweet orange, lemon and papeda types were susceptible; the pomelo types expressed resistance; while the lime and sour orange types were designated as resistant/insensitive. When screening hybrid progeny from crosses using pomelo (*C. maxima* (Burm.) Merr.) or *Poncirus trifoliata* (L.) Raf as a parent, preliminary evidence of segregation for black spot resistance was identified. The implications of the results are discussed.

Keywords: breeding, pathology, Citrus, *Guignardia citricarpa*

3.2 Introduction

Citrus black spot, caused by the fungus *Phyllosticta* (syn. *Guignardia*) *citricarpa* (McAlpine) van der Aa (Kiely, 1948; McAlpine, 1899), is an important disease of citrus in most humid tropical and subtropical production areas of the world including parts of Australia, Asia, South America, Africa, and most recently North America (Kiely, 1948; Korf *et al.*, 2001; Kotze, 1981; McOnie, 1964c; Schubert *et al.*, 2012; Wager, 1952). Black spot is characterised by a range of symptoms on fruit including: hard spot (Fig. 3.2.1a); freckle spot (Fig. 3.2.1b); virulent spot (Fig. 3.2.1c); speckled blotch (Fig. 3.2.1d) and; false melanose/cracked spot-like (Fig. 3.2.1e) (de Goes *et al.*, 2000; Kiely, 1948; Kotze, 2000). Severe symptom expression on fruit can be associated with premature fruit abscission, and symptoms similar

to hard spot can sometimes be observed on leaves and twigs. Pycnidia of *P. citricarpa* often develop within lesions, for example within hard spot (Fig. 3.2.1f). The pycnidia contain asexual conidia which are water splash dispersed over short distances, and may contribute to black spot epidemics in *Citrus* varieties with overlapping crops (Kiely, 1948; Sposito *et al.*, 2008). Aerial dispersal over longer distances is reported to occur via sexually produced ascospores released from pseudothecia produced in orchard leaf litter (Kiely, 1948; Tran *et al.*, 2017; Wang *et al.*, 2016). Fruit are considered most susceptible to infection for the first 20-24 weeks after fruit set, with symptoms typically expressing when latent infections break dormancy as fruit near maturity (Baldassari *et al.*, 2006; Kiely, 1948; Kotze, 1981; Wager, 1952). Control of black spot in orchards relies almost entirely on protectant fungicide applications during this 20-24 week period of fruit susceptibility (Kiely, 1948; Kotze, 1981; Miles *et al.*, 2004; Schutte *et al.*, 2003; Silva Junior *et al.*, 2016), with low implementation of cultural practices such as mulching and pruning (Schutte and Kotze, 1997; Loest, 1968; Miles *et al.*, 2008). Genetic solutions via scion resistance have been largely ignored, with only one preliminary and short-lived attempt to breed for resistance to this disease (Anonymous, 1974).

Genetic resistance to black spot is apparently rare, with only sour orange (*C. ×aurantium* L.) and its hybrids, and Tahitian lime (*C. ×latifolia* Yu. Tanaka) considered resistant (Baldassari *et al.*, 2008; Kotze, 1981). Even then, the reported resistance of sour orange and its hybrids is seemingly based on general field observations rather than specific experimentation. Furthermore, use of the term 'resistant' for sour orange and Tahitian lime has recently been superseded by the term 'insensitive', due to reports of symptomless fruit of sour orange and Tahitian lime hosting asymptomatic infections of *P. citricarpa* (Baldassari *et al.*, 2008; Kotze, 1981; Wickert *et al.*, 2009). As a consequence, in this study we attempt to better define the fruit reaction of *Citrus* accessions to *P. citricarpa* by using three categories of response: i) 'susceptible', whereby visible black spot symptoms occur; ii) 'insensitive', where fruit are asymptomatic, but *P. citricarpa* infection and colonisation can be confirmed; and iii) 'resistant', where fruit are asymptomatic, and *P. citricarpa* infection and colonisation cannot be confirmed.

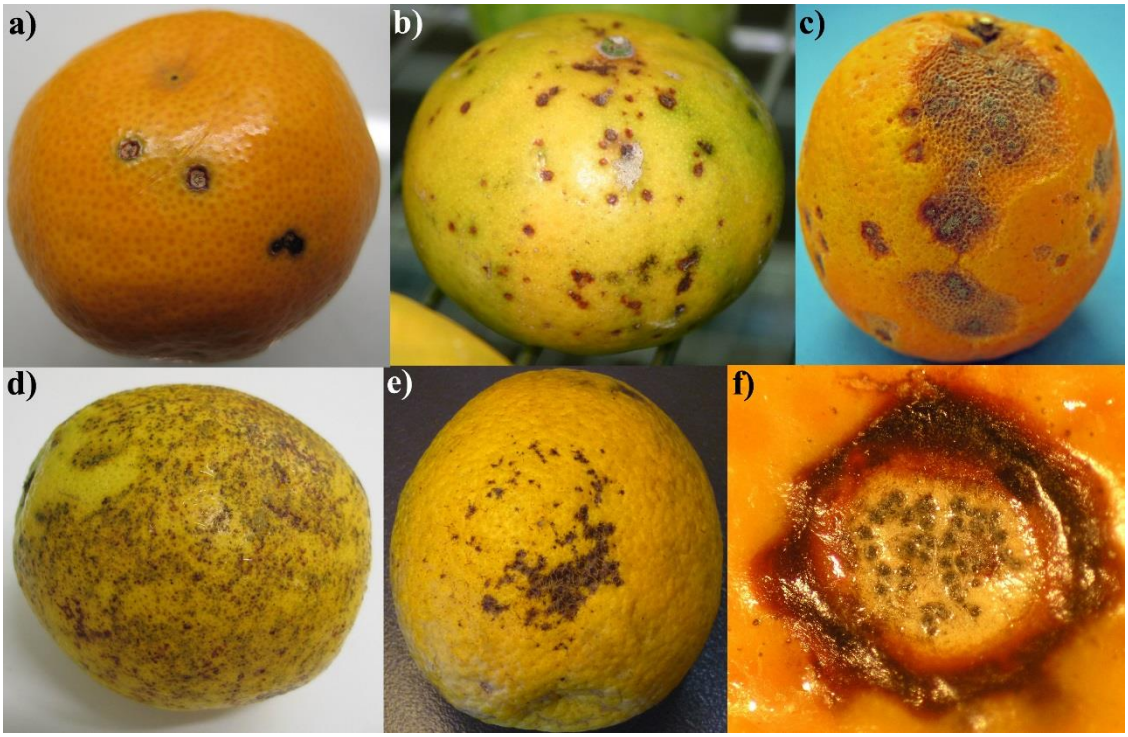

Figure 3.2.1. Symptoms of citrus black spot caused by *Phyllosticta citricarpa* on fruit resulting from natural infection including a) hard spot, b) freckle spot, c) virulent spot, d) speckled blotch, e) false melanose/cracked spot-like, and f) a hard spot lesion containing pycnidia.

Understanding the disease expression in fruit also requires an appreciation of the taxonomic status of the *Citrus* accessions being tested. The long history of cultivation, frequent occurrence of apomixes, and ease with which interspecific hybrids can be generated, have resulted in wide genetic diversity combined with sufficient levels of genetic stability. Consequently, many apomictic interspecific hybrids have mistakenly acquired species status including important commercial types such as grapefruit (*C. paradisi* Macfad.), lemon (*C. limon* (L.) Osbeck), lime (*C. aurantiifolia* (Christm.) Swingle *C. ×latifolia*), sour orange (*C. ×aurantium*), and sweet orange (*C. sinensis* (L.) Osbeck). An abundance of recent molecular studies (Carbonell-Caballero *et al.*, 2015; Curk *et al.*, 2015; Wu *et al.*, 2014) have confirmed the original views of Scora (1975) and findings of Barrett and Rhodes (1976) that cultivated *Citrus* are highly heterozygous interspecific admixtures of just a few basic taxa, now considered to be *C. reticulata* Blanco; *C. maxima* (Burm.) Merr.; *C. medica* L.; and *C. micrantha* Wester. This has important implications in developing genetic solutions to black spot, because commercial *Citrus* types can potentially be re-constituted from black spot resistant accessions of their base taxa, retaining only resistant hybrids. For example, the pomelo (*C. maxima*) is a progenitor of sweet orange and grapefruit (Barrett and Rhodes, 1976) which are susceptible to black spot, as well as of sour orange which is insensitive. Consequently, in attempting to breed black spot-resistant cultivars it may be more efficient to find progenitor accessions with black spot resistance rather than use the highly

heterozygous sour orange as a source of resistance. Furthermore, sour orange is undesirable as a parent due its pronounced bitterness in both carpellary membranes and albedo (Hodgson, 1967).

In the case of the pathogen, the literature needs to be interpreted with care, as there has been confusion regarding the various *Phyllosticta* spp. associated with *Citrus* and other plants. At present, the main pathogenic species associated with *Citrus* are *P. citricarpa* causing black spot; *P. citriasiana* Wulandari (Wulandari *et al.*, 2009) and *P. citrimaxima* Wikee, Crous, K.D. Hyde & McKenzie (Wikee *et al.*, 2013b) associated with tan spot of pomelo (*C. maxima*); and *P. citrichinaensis* Wang, Hyde and Li associated with symptoms on leaves and fruit of pomelo, sweet orange, and mandarin (*C. reticulata*) (Wang *et al.*, 2012). The latter three species have so far only been reported from specific regions of Asia. Another potentially pathogenic species *P. paracitricarpa* Guarnaccia & Crous has recently been described, but pathogenicity has so far only been inferred from detached mature fruit inoculations (Guarnaccia *et al.*, 2017). The non-pathogenic species of *Phyllosticta* have also confused the interpretation of the *P. citricarpa* host status among *Citrus*. Initially, *P. capitalensis* Henn. was the designated anomorph of *Guignardia mangiferae* Roy (Baayen *et al.*, 2002) however, subsequent revision determined *G. mangiferae* and *P. capitalensis* to be separate taxa (Glienke *et al.*, 2011). At present, *P. capitalensis* is considered the main endophytic *Phyllosticta* spp. associated with *Citrus* in most production areas, while *G. mangiferae* is restricted to mango (*Mangifera indica* L.). This suggests that past references to *P. citricarpa* having an extensive host range outside of *Citrus* (Kiely, 1948) were most likely referring to reports of *P. capitalensis* occurring in a wide range of woody plants (Wikee *et al.*, 2013a; Baayen *et al.*, 2002; McOnie, 1964a). In the specific case of Australia, only the pathogen *P. citricarpa* and endophyte *P. capitalensis* have been reported from *Citrus* (Miles *et al.*, 2013).

Considering the complexities detailed above, it is perhaps not surprising that little attention has been given to the potential of breeding for resistance to black spot, nor was breeding for resistance considered likely to succeed (Calavan, 1960). Efforts have been further hindered by focussing on the use of sour orange as a source of resistance, which while apparently resulting in hybrids free of black spot symptoms (Anonymous, 1974), is also likely to transmit many undesirable traits such as bitterness (Matsumoto, 1995). Identifying sources of resistance combined with better internal quality characteristics would therefore greatly improve progress using conventional breeding. Alternatively, identifying a segregating population would assist in studies of the mechanisms and/or genetics of resistance, to support marker assisted breeding to develop black spot resistant cultivars.

Host resistance to black spot would be highly beneficial to citrus producers as it would break the reliance on chemical control, eliminate direct losses of fruit due to cosmetic downgrading and fruit drop, while at the same time overcome trade restrictions on fruit from production areas where black spot is present. However, for this to occur an important first step is identifying resistant phenotypes

using a well characterised source of the pathogen, and a reliable field inoculation method. Therefore, the overall objective of this study was to develop a simple, reliable, field inoculation method to be able to characterise the black spot phenotype of fruit of a wide range of *Citrus* accessions. The specific hypotheses we sought to address include: i) can reliable expression of black spot on fruit be achieved using inoculation in the field; ii) can fruit resistance to black spot be accurately identified among a range of different *Citrus* accessions; and iii) is there segregation of disease expression among hybrid progeny between resistant and susceptible accessions? Reliable identification and genetic characterisation of resistance in different genetic backgrounds opens up new potential for breeding for resistance to black spot.

3.3 Materials and Methods

In order to address the aims of this study, field inoculations were conducted in the *Citrus* arboretum located at the Department of Agriculture & Fisheries, Bundaberg Research Station, Queensland, Australia. This arboretum was ideal for this study due to the wide variety of *Citrus* accessions available; citrus black spot being endemic to, but not severe in, the Bundaberg area; and that fungicides are not routinely applied in the arboretum. The general experimental approach was to inoculate fruit with *P. citricarpa*, along with *P. capitalensis* and water as negative controls. Fruit of known susceptible accessions (such as sweet orange and mandarin) were included as susceptible controls each year, while suspected resistant accessions (sour orange, sour orange hybrids) were included as resistant/insensitive controls each year. The accessions included in the study were selected based on relevant reports in the literature, an absence of citrus black spot from field observations, and/or the importance of the accessions to scion breeding. Up to 20 fruit were inoculated on each accession, but the final number that were inoculated was highly dependent of the number of fruit having set on each accession, and the number of those fruit that were free of rind blemishes such as wind rub that may disrupt the successful infection and development of symptoms. This was particularly relevant to young hybrid plants that in several cases were producing fruit for the first time.

Fungal isolates and inoculum preparation Fully characterised isolates of the ex-expitype accession BRIP 52614 of *P. citricarpa*, and BRIP 54242 of *P. capitalensis* (Miles *et al.*, 2013) were retrieved from the Queensland Plant Pathology Herbarium (BRIP). Two different methods were used in the production of inoculum. For inoculations made in the 2013-14 fruit production season, inoculum was prepared largely by the methods of Baldassari *et al.* (2009). Mature leaves of *C. reticulata* c.v. 'Imperial' were harvested from trees free of any fungicides. A 10 mm diameter borer was used to extract discs from the leaves. The leaf discs were then autoclaved and immediately placed abaxial side

down into partially solidified water agar plates. Once the leaf discs and agar were fully cooled, a small block of mycelium from 2-week-old colonies of the *Phyllosticta* spp. on ½ strength potato dextrose agar (PDA, BD Difco, BD Australia) were placed adjacent to the leaf discs. The plates were incubated at 25˚C under a 12 hour cycle of black light and white light for 14 days. Pycnidia readily formed on the surface of the leaf discs after 14 days. A different inoculum production procedure was used in subsequent seasons. *Phyllosticta* colonies were established on ½ PDA and grown for 14 days. The colonies were then flooded with sterile distilled water and left to stand for 30 minutes to induce spore release and the resulting conidia (and ascospores in the case of *P. capitalensis*) suspensions were decanted into a centrifuge tube and adjusted to 5×10^4 spores/mL using a hemocytometer.

Field inoculation Inoculation of fruit in the field was undertaken on fruit ranging from 8 to 12 weeks old on field grown trees free of any fungicides. Two different methods of inoculation were used. In the 2013-14 fruit production season, fruit were lightly misted with distilled water, then inoculated by placing a single leaf disc colonised by *P. citricarpa* pycnidia-side-down on the surface of the fruit. A strip of cotton wool surgical dressing saturated in sterile distilled water was then wrapped around the fruit to hold the leaf disc against the fruit and maintain moisture. For an initial batch of fruit, the cotton wool and fruit was covered with a latex finger cot, then wrapped in aluminium foil to minimise field heat. The foil and finger cot was held in place by a clothes peg around the fruit stem. The fruit were incubated as such for 7 days. For a later batch of fruit, domestic plastic cling wrap was used instead of the finger cot, and the incubation time reduced to 48 hrs. Control fruit were inoculated with water.

In the 2014-15 and 2015-16 fruit production seasons, the inoculation procedure was modified. After misting the fruit with water, a 5 mm-wide strip of sterile blotting paper soaked in the spore suspension was wrapped around the entire equator of the fruit. The blotting paper was then covered with a strip of domestic cling wrap to maintain high moisture conditions. Finally, the entire fruit was wrapped in aluminium foil to minimise field heat. After 48 hours the foil, cling wrap and blotting paper were removed from the fruit. Control fruit were inoculated with water or *P. capitalensis*.

Incubation and disease evaluation After inoculation, the fruit were left on the trees for as long as possible to reach maximum fruit maturity. Fruit nearing maturity were surrounded with a mesh bag to prevent losses due to abscission. When fruit either abscised or had reached full maturity they were taken to the laboratory for inspection. Fruit were inspected under a dissecting microscope for any symptoms of black spot or signs of *Phyllosticta* spp, namely pycnidia and conidia. Where no symptoms were visible, the fruit were incubated at 27˚C, 80% relative humidity and permanent light for as long as possible (up to 127 days) to maximise black spot symptom expression (Brodrick and Rabie, 1970).

When symptoms of black spot were evident, the presence of symptoms and/or pycnidia and conidia of *Phyllosticta* was noted. After visual inspection and light microscopy, fruit were surface disinfested by swabbing with 70% ethanol, then symptomatic tissue was plated onto ½ PDA to attempt to recover any *Phyllosticta* colonies. Fruit that failed to produce black spot symptoms during incubation were tested for the presence of asymptomatic infections by plating tissue from the fruit equator onto ½ PDA just prior to decaying from other postharvest conditions such as mould. Colonies of *Phyllosticta* arising from the plated tissue were then subcultured onto plates of Oatmeal Agar (OA, BD Difco, BD Australia) and incubated as previously described for 7 days. Colonies were identified as either *P. citricarpa* or *P. capitalensis* on the basis of the production of yellow pigment on OA around the colony margin, or lack of pigment, respectively (Baayen *et al.*, 2002; Baldassari *et al.*, 2007; Guarnaccia *et al.*, 2017). Fruit were considered 'susceptible' where visible black spot symptoms developed, 'insensitive' if no visible black spot developed but *P. citricarpa* could be recovered from the inoculation point, and 'resistant' where no symptoms developed and *P. citricarpa* could not be recovered. Details of the inoculated *Citrus* accessions are provided in Table 3.4.1.

3.4 Results

The inoculation method utilising the spore-soaked blotting paper strip applied around the equator of fruit reliably achieved the production of typical citrus black spot symptoms in the field that were reproducible over multiple seasons and various susceptible accessions. These symptoms included hard spot (Fig. 3.4.2a), freckle spot and virulent spot (Fig. 3.4.2b,d), and false melanose/cracked spot-like (Fig. 3.4.2c). The lesions were observed forming around the equator of fruit, corresponding with the point of inoculation (Fig. 3.4.2a-d). The colonised leaf disc inoculation method used in the 2013-14 fruit production season, while producing black spot symptoms in fruit of susceptible accessions included as positive controls, resulted in overly severe symptoms of virulent spot not commonly observed under natural conditions (Fig.3.4. 2e). This included the development of an atypical pycnidial crust in some cases (Fig. 3.4.2f). The method also resulted in excessive growth of superficial mould fungi on the inoculation constituents and potentially anoxic conditions after the 7 days of incubation on the tree. For the second batch of fruit in 2013-14 the incubation time was reduced to minimise mould and anoxia, but black spot symptoms were often still expressed as very severe virulent spot. In no cases did inoculation with water or *P. capitalensis* result in black spot symptoms or the recovery of *P. citricarpa*. However, *P. capitalensis* was commonly recovered from both water and *P. capitalensis* inoculations, suggesting a background level of naturally occurring *P. capitalensis* in the germplasm collection.

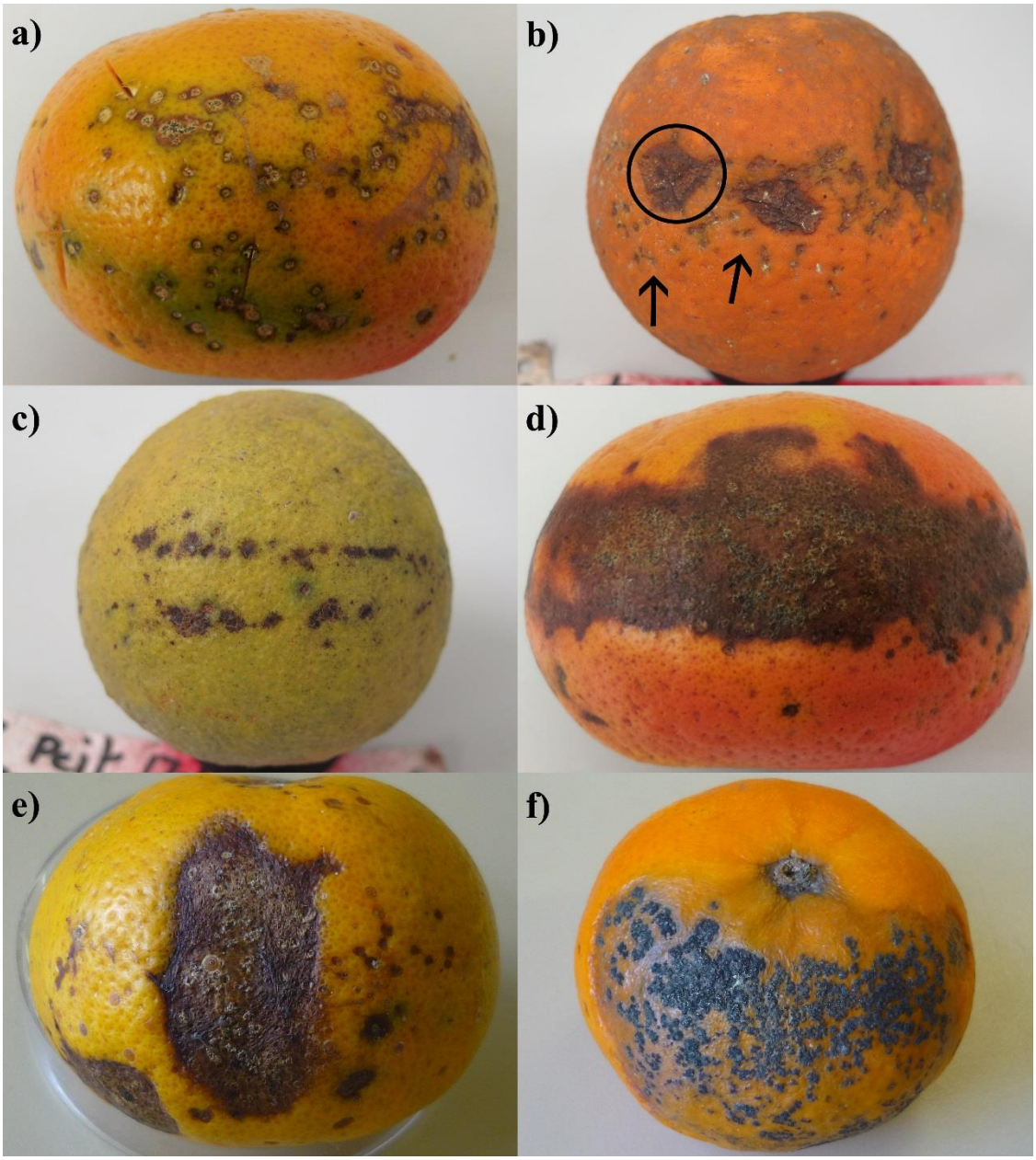

Figure 3.4.2. Symptoms on *Citrus* fruit resulting from inoculation using conidia of *Phyllosticta citricarpa* and the equatorial blotting paper strip method, resulting in symptoms of a) hard spot, b) freckle (e.g. arrows) and virulent spot (e.g. circle), c) false melanose/cracked spot-like, and d) virulent spot containing pycnidia. Symptoms resulting from inoculation using the colonised leaf disk method resulting in severe symptoms of e) virulent spot including the production of an extensive f) pycnidial crust.

The production of pycnidia in association with black spot symptoms was very consistent for the major *Citrus* 'types', but pycnidia production was greatly reduced among the hybrids expressing visible black spot symptoms (Table 3.4.1). It was also rare that pycnidia were observed without conidia also being present. In almost all cases where black spot symptoms were expressed for a particular accession, *P. citricarpa* could be recovered from other symptomatic tissue of that accession, with the hybrid *P. trifoliata* F9W1 being the only exception, suggesting an overall very high recovery rate of *P. citricarpa*. Regardless of this, the black spot symptoms expressing on F9W1 still produced pycnidia and conidia consistent with *Phyllosticta*.

Using the inoculation method to screen fruit of the various accessions for their disease response, it was possible to identify resistance to citrus black spot in a small number of accessions (Table 3.4.1). Most notable of these were the two accessions of *C. maxima*, while the observation of resistance in the lime and sour orange types was anticipated. For the remaining *Citrus* types (mandarin, sweet orange, lemon and lime, pomelo, sour orange, papeda) that were inoculated, the majority of fruit were found to be susceptible, in particular the mandarin and sweet orange types. These include susceptible control accessions such as 'Imperial' and 'Washington', for example, which are well known to be susceptible to black spot in the field (Kiely, 1948; Miles *et al.*, 2004). Within the lemon and lime types, the representative 'Limoneira' lemon was susceptible as expected.

Preliminary evidence was found for segregation of resistance to citrus black spot among hybrids using either pomelo, *Poncirus trifoliata* (L.) Raf or finger lime as a parent (Table 1). Although fruit numbers for inoculating were typically low due to the young age of these hybrid trees, susceptibility was confirmed in the majority of cases, with a small number of hybrids (such as the K15 7-2-16 and the *P. trifoliata* F8W10) appearing consistently resistant in replicate fruit and replicate seasons.

Table 3.4.1. Details and results of inoculation studies to determine the citrus black spot (CBS) phenotype of various *Citrus* accessions.[A]

Accession	Year	No. Fruit[B]	CBS symptoms[C]	Pycnidia[D]	Conidia[D]	*Phyllosticta* recovered[E]	Pigment on OA[F]	CBS fruit phenotype[G]
MANDARIN types								
00C018	2015-16	10	+	+	+	+	+	Susceptible
	2014-15	10	+	+	+	+	+	
	2013-14	4	+	+	+	+	+	
01C011		1	+	+	+	+	+	Susceptible
02C036		1	+	+	+	+	+	Susceptible
03C066		2	+	+	+	+	+	Susceptible
05C014		1	+	+	+	+	+	Susceptible
05C028		1	+	+	+	+	+	Susceptible
07C004		4	+	+	+	+	+	Susceptible
08C010		2	+	+	+	+	+	Susceptible
'Imperial'		1	+	+	+	+	+	Susceptible
SWEET ORANGE types								
'Washington'	2015-16	6	+	+	-	+	+	Susceptible
'Salustiana'	2014-15	10	+	+	+	+	+	Susceptible
LEMON and LIME types								
'Limoneira'	2013-14	1	+	-	n/a	+	+	Susceptible
Tahitian lime	2015-16	4	-	-	n/a	-	n/a	**Resistant (insensitive)**
West Indian lime		1	-	-	n/a	-	n/a	**Resistant**
Finger lime		8	-	-	n/a	+	-	**Resistant**
POMELO types								
'Shatian You' T5	2015-16	10	-	-	n/a	-	n/a	**Resistant**
'K15'		13	-	-	n/a	+	-	**Resistant**
	2014-15	14	-	-	n/a	-	n/a	
	2013-14	4	-	-	n/a	-	n/a	
SOUR ORANGE types								
Sour orange (CO55)	2015-16	22	-	-	n/a	+	-	**Resistant (insensitive)**
PAPEDA types								

Accession	Year	No. Fruit[B]	CBS symptoms[C]	Pycnidia[D]	Conidia[D]	*Phyllosticta* recovered[E]	Pigment on OA[F]	CBS fruit phenotype[G]
Papeda (D19)	2014-15	2	+	+	+	+	+	Susceptible
HYBRIDS								
'Chinotto' 11Q003	2014-15	15	-	-	n/a	+	+	**Insensitive**
C. ichangensis F15E30	2015-16	2	+	+	+	+	+	Susceptible
C. ichangensis F15T28		2	+	+	-	+	+	Susceptible
C. ichangensis F15W37		4	+	+	+	+	+	Susceptible
Finger lime 09Q002	2015-16	2	-	-	n/a	-	n/a	**Resistant**
'Gou Tou Cheng' (D2)	2014-15	13	+	+	+	+	+	Susceptible
	2013-14	11	+	-	n/a	+	+	
'Gou Tou Cheng' (D3)	2014-15	13	+	+	+	+	+	Susceptible
	2013-14	7	+	-	n/a	+	+	
'K15' 7-1-2	2014-15	1	+	-	n/a	+	+	Susceptible
'K15' 7-1-19		2	+	+	+	+	+	Susceptible
'K15' 7-1-23		1	+	+	+	+	+	Susceptible
'K15' 7-2-5		1	-	-	n/a	+	+	**Insensitive**
'K15' 7-2-6		1	+	-	n/a	+	+	Susceptible
'K15' 7-2-16	2015-16	3	-	-	n/a	+	-	**Resistant**
	2014-15	1	-	-	n/a	+	-	
'K15' 7-4-19		2	+	+	+	+	+	Susceptible
'K15' 7-4-99		1	+	+	+	+	n/a	Susceptible
'K15' 7-4-125	2015-16	4	+	-	n/a	+	+	Susceptible
	2014-15	1	+	-	n/a	+	-	
'K15' 7-5-11		1	+	+	+	+	+	Susceptible
'K15' 7-5-55		1	+	+	+	+	+	Susceptible
'K15' 7-5.5-5		1	+	+	+	+	+	Susceptible
'K15' 7-5.5-18		1	+	+	-	+	+	Susceptible
'K15' 7-6-14		1	+	-	n/a	+	+	Susceptible
'K15' 7-7-16		1	+	-	n/a	+	+	Susceptible
'K15' 15Q028	2015-16	2	-	-	n/a	+	-	**Resistant**
'K15' 14Q056		4	-	-	n/a	+	+	**Insensitive**
P. trifoliata C196		2	-	-	n/a	-	n/a	**Resistant**
	2014-15	1	-	-	n/a	-	n/a	
P. trifoliata F7E16	2015-16	9	+	-	n/a	+	+	Susceptible

Accession	Year	No. Fruit[B]	CBS symptoms[C]	Pycnidia[D]	Conidia[D]	*Phyllosticta* recovered[E]	Pigment on OA[F]	CBS fruit phenotype[G]
	2014-15	1	+	-	n/a	-	n/a	
P. trifoliata F8W10	2015-16	7	-	-	n/a	-	n/a	**Resistant**
	2014-15	1	-	-	n/a	-	n/a	
P. trifoliata C237		1	+	-	n/a	+	+	Susceptible
P. trifoliata F9W1		1	+	+	+	-	n/a	Susceptible
P. trifoliata C222		1	+	-	n/a	+	+	Susceptible
P. trifoliata F8E9		1	+	+	+	+	+	Susceptible
Water inoculations								
00C018	2015-16	10	-	-	n/a	-	n/a	n/a
	2014-15	4	-	-	n/a	-	n/a	n/a
'Imperial'	2013-14	1	-	-	n/a	-	n/a	n/a
'Salustiana'	2014-15	3	-	-	n/a	+	-	n/a
'Gou tou cheng' (D3)		4	-	-	n/a	+	-	n/a
	2013-14	3	-	-	n/a	+	-	n/a
'Gou tou cheng' (D2)		3	-	-	n/a	+	-	n/a
***P. capitalensis* inoculations**								
00C018	2015-16	10	-	-	n/a	-	n/a	n/a
	2014-15	3	-	-	n/a	-	n/a	n/a
'Salustiana'		4	-	-	n/a	+	-	n/a
'Gou tou cheng' (D3)		4	-	-	n/a	+	-	n/a

[A]Rows without a specified year denote the same year as the row above; "+" denotes a positive result; "-" denotes a negative result; "n/a" denotes non-applicable.

[B]Number of fruit collected at fruit maturity.

[C]Symptoms included hard spot, freckle spot, virulent spot, false melanose / cracked spot-like, and speckled blotch.

[D]Presence of pycnidia and/or conidia consistent with those of *Phyllosticta* spp. as determined by light microscopy.

[E]Colonies of *Phyllosticta* spp. recovered onto ½ strength potato dextrose agar.

[F]Colonies of *P. citricarpa* produce a yellow pigment surrounding the colony when grown on Oatmeal Agar (OA), while *P. capitalensis* does not.

[G]Phenotypes designated as susceptible (CBS symptoms and *P. citricarpa* recovered), insensitive (no CBS symptoms and *P. citricarpa* recovered); and resistant (no CBS symptoms and no *P. citricarpa* recovered). Phenotype in parentheses denotes the phenotype reported by Baldassari *et al.* (2008) and Wickert *et al.* (2009).

3.5 Discussion

In this study, we aimed to develop a simple and reliable field inoculation technique and use it to characterise the susceptibility, insensitivity or resistance to black spot in fruit of a wide range of accessions of *Citrus* and relatives. As anticipated, almost all accessions were found to be susceptible to black spot which is consistent with previous reports (Baldassari *et al.*, 2008; Kotze, 1981). We have demonstrated that reliable expression of black spot on fruit in the field following inoculation can be achieved. Using this method, we identified a small number of accessions that expressed fruit resistance to black spot. Sour orange and Tahiti lime fruit gave resistant responses which corresponds with previous reports of resistance and/or insensitivity of these accessions (Baldassari *et al.*, 2008; Kotze, 1981). The resistant response of the pomelo (*C. maxima*) accessions 'K15' and 'Shatian Yu' is of particular interest, with these fruit never producing any symptoms of black spot, nor could *P. citricarpa* be recovered from the inoculated tissue. To our knowledge, this is the first evidence that these particular varieties of *C. maxima* are resistant to *P. citricarpa*. Also of note is the resistant response of the finger lime (*C. australasica* F.Muell.). Importantly, our study provides evidence for segregation in black spot expression in hybrid progeny using *C. maxima*, *P. trifoliata* or *C. australasica* as a parent, based on the occurrence of a small number of seemingly resistant hybrids with 'K15'and *P. trifoliata* parentage, and the apparent resistant phenotype of the *C. australasica* hybrid 09Q002. In these examples, the apparently resistant parents 'K15' and *C. australasica* have been hybridised with susceptible accessions that themselves are complex hybrids involving susceptible 'Ellendale', 'Imperial' and 'Murcott' mandarins. This finding suggests that resistance to black spot is a heritable trait that can be exploited in future breeding programs.

The potential resistance of *C. maxima* to *P. citricarpa* may have been previously overlooked due to confusion between black spot symptoms and tan spot symptoms on pomelo, prior to the recent discovery of the novel fungi *P. citriasiana*, *P. citrichinaensis,* and *P. citrimaxima* associated with tan spot (Wang *et al.*, 2012; Wikee *et al.*, 2013b; Wulandari *et al.*, 2009). Reports of black spot on pomelo have been made previously (Truter, 2010), and while it was not possible to confirm the reports, it may be that symptoms of tan spot caused were confused with those of black spot. In the case of Australia, neither black spot or tan spot have been observed on pomelo, nor has *P. citriasiana*, *P. citrichinaensis,* or *P. citrimaxima* been identified among Australian accessions of *Phyllosticta* (Miles *et al.*, 2013). It is also possible that *C. maxima* accessions vary in their resistance to black spot, and that we have selected two that are seemingly resistant. A wider range of *C. maxima* germplasm needs to be tested in order to confirm whether there is variation in the black spot reaction between pomelo cultivars. It would also be beneficial to undertake companion inoculation studies with *P. citriasiana*, *P. citrichinaensis,* or *P. citrimaxima* to confirm the pathogenicity of these species to pomelo, and act as

a positive control for *P. citricarpa* inoculations. However, as these species do not occur in Australia it was not possible to undertake inoculations using these species in this study. Nevertheless the *C. maxima* fruit inoculated in our study were well within the age when *Citrus* fruit are considered susceptible to *P. citricarpa (REF)*; fruit were repeatedly inoculated over multiple seasons; and fruit remained free of black spot and *P. citricarpa* while known susceptible accessions readily produced black spot symptoms.

Resistance to *P. citricarpa* in pomelo is highly useful when aiming to breed for both fruit quality and resistance to black spot. The two pomelo accessions we have used in this study were previously selected from a collection of more than 30 named and seedling pomelos evaluated at Bundaberg Research Station between 1998 and 2010 based on fruit eating quality, rind thickness, shape, and skin texture. Furthermore, pomelo is an ancestral taxa of sweet orange and grapefruit, and of most modern mandarin and lemon cultivars (Curk *et al.*, 2015), and has thus played a critical role in the domestication of *Citrus*. Having identified two pomelo accessions with desirable commercial traits such as eating quality, as well as the apparent heritability of resistance to black spot, creates a significant new opportunity in breeding. It may now be possible to reconstitute key commercial cultivars using resistant pomelo accessions as the base taxa. This approach may be far more efficient and effective than the use of sour orange accessions in previous resistance breeding attempts (Anonymous, 1974) that were probably unsuccessful due to traits such as bitterness associated with sour orange (Hodgson, 1967; Matsumoto, 1995). To have identified black spot resistance in pomelo accessions with commercially desirable traits augers well for the introgression of disease resistance into new cultivars of sweet oranges and mandarins.

From the perspective of investigating the mechanisms of resistance to *P. citricarpa*, the pomelo is of interest as the evidence so far suggests it is resistant, rather than insensitive, while at the same time being susceptible to tan spot associated with *P. citriasiana*, *P. citrichinaensis,* and *P. citrimaxima* (Wang *et al.*, 2012; Wikee *et al.*, 2013b; Wulandari *et al.*, 2009). This provides researchers with a model system for investigating the comparative infection processes of the various *Phyllosticta* spp.; the possible mechanisms underpinning the differential susceptibility of pomelo to the various *Phyllosticta* spp.; as well the opportunity to further investigate the heritability of resistance from hybridisation. Such future studies would benefit from the equatorial strip inoculation method developed in this study. This method was fast to implement (~100 fruit per hour, with two field operators), and resulted in typical black spot symptom expression from a controlled inoculum dose, at a known location on the fruit surface. By contrast, we found the colonised leaf disc approach to be relatively more time consuming; resulting in particularly severe symptom expression on some accessions; delivering a potentially variable inoculum dose; and more difficult identification of the

inoculation point when undertaking isolations to recover the *Phyllosticta* spp. after the long latent period of up to 8 months. However, the production of conidia using the leaf disc method may be useful for producing spore suspensions for the equatorial strip method from isolates of *P. citricarpa* that do not readily sporulate on agar. Also of note is the recent demonstration of equivalent pathogenicity of *P. citricarpa* conidia and ascospores (Tran *et al.*, 2018), supporting the use of these simple conidia-based inoculation methods. As well as our study providing an opportunity to develop and compare methods for characterising the susceptibility of fruit from different accessions, it has also demonstrated the need for both systematic testing of fruit for resistance, as well an understanding of the host taxonomy. For example, the Gou Tou Cheng is sometimes referred to as a sour orange (Liao *et al.*, 2013), and perhaps assumed to be resistant to black spot. However, the Gou Tou Cheng accessions included in our study were confirmed to be susceptible to black spot, most likely due to Gou Tou Cheng being a hybrid of sour orange (Lee and Keremane, 2013; Zhang *et al.*, 1988). In contrast, the true sour orange (CO55) included in our study was found to be resistant to black spot.

The other notable accession found to be resistant in this study was the finger lime. This finding is supported by lack of black spot symptoms on finger lime fruit observed during a survey of an arboretum located in a region of coastal New South Wales subject to severe black spot pressure (Donovan *et al.*, 2009). In addition, no black spot has been observed on finger lime after fifteen official black spot inspections for export certification by the Queensland Government, Department of Agriculture and Fisheries. Such surveys and inspections have been required on the assumption that all *Citrus* are susceptible to black spot. This requirement only recently applied to finger lime subsequent to it being reunited with the genus *Citrus* (Mabberley, 1998), after originally being assigned to the former genus *Microcitrus* (Swingle, 1915).

While this study makes a contribution towards renewing interest in breeding for resistance to black spot, rapid progress in breeding for black spot resistance remains hindered by relying on fruit for characterising the disease status of each new hybrid. This is in contrast to the significant progress that has been made in breeding for resistance to the brown spot disease caused by *Alternaria alternata* (Fr.) Keissl. (Pegg, 1966), which has been possible due to the large scale screening of seedlings based on the rapid production of disease symptoms on leaves instead of fruit (Miles *et al.*, 2015). The next major step forward in breeding for black spot resistance is most likely to come from the development of a leaf-based screening, or marker assisted, approach to screening hybrid *Citrus* populations. To ensure such a leaf-based assay is reliable, the relationship of leaf susceptibility to fruit susceptibility needs to be further explored. The recent demonstration of reliable foliar symptom development in 'Troyer' citrange (*Citrus sinensis* × *Poncirus trifoliata*) (Tran *et al.*, 2018) provides new opportunities for comparing foliar to fruit susceptibility. It would be also beneficial to have consistent

definitions of leaf susceptibility for *P. citricarpa*. Leaf symptoms tend to be rare, even in susceptible cultivars like 'Valencia' sweet orange, but ascospore and conidial inocula are formed routinely in the leaf litter from infections prior to leaf senescence (Kotze, 1981; Sposito *et al.*, 2008). If no symptoms are present in the field, but spores are formed in the leaf litter, is the accession defined as susceptible or insensitive? Whichever conclusion the community of black spot researchers ultimately arrives at, it is imperative that the definitions are consistently used or it will be very difficult to compare data. This question is more than academic, as it has been reported by Baldassari et al. (2008) and Wickert et al. (2009) that *P. citricarpa* ascospores were formed in sour orange and Tahiti lime leaves. The fruit of these accessions generated in this report were found to be resistant (no symptoms and *P. citricarpa* was not isolated from the fruit), but spore production data were not gathered from the leaves in this study. Some preliminary data have been gathered in a separate experiment to develop an *in vitro* assay to evaluate leaf susceptibility. Results have been inconsistent with 'Chinotto', shown to be insensitive (no symptoms but *P. citricarpa* isolated) to fruit infection here. The number of pycnidia/leaf area on inoculated 'Chinotto' leaves was significantly lower than found on those from the highly susceptible 'Valencia' in one assay but in a subsequent assay were not significantly different (*data not shown*). Another question is whether leaf infection matters since the infections are not causing an economic loss. In terms of inoculum production and pathogen movement, the answer is yes, but the level of importance is still unclear (Sposito *et al.*, 2008; Tran *et al.*, 2017).

Resistance of *Citrus* to *P. citricarpa* remains poorly understood even after more than a century since black spot was first described. Past confusion over host and pathogen taxonomy, and the subsequent inability to routinely screen germplasm, have been likely contributors to the tardy progress of research. However, new opportunities have arisen from this study including: a simple and reliable inoculation method to characterise germplasm; strong evidence for resistance to *P. citricarpa* in pomelo and some other accessions; and preliminary evidence for resistance to black spot being a heritable trait. In addition, a better understanding of the genetic make-up and pedigree of all major citrus types and varieties and its original progenitors enables us to recreate these varieties while at the same time ensuring that we introgress black spot resistance. There is little doubt that introgression of resistance to black spot in commercially desirable *Citrus* cultivars would greatly benefit citrus producers and consumers worldwide.

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