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Final Report

Increasing market access, profitability and sustainability through integrated approaches to fungal disease control

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Citrus (R&D Levy)

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Chapter 1
Citrus black spot

1.1 Introduction

Citrus black spot (CBS), caused by *Phyllosticta* (*Guignardia*) *citricarpa*, is an important fungal disease of most commercial citrus cultivars. The disease has been present in Australia since at least 1895 when it was first discovered in the Sydney area of New South Wales (Benson, 1895), and has since spread to parts of coastal New South Wales, Queensland, and the Northern Territory (Miles *et al.*, 2013). CBS is absent from the inland, winter rainfall areas of Australia, including the Riverland (South Australia), Sunraysia (NSW and Victoria border), and Riverina (Southern NSW) (The Commission of the European Communities, 1998; Barkley, 1988; Broadbent, 1995; Wall, 1989). Globally, CBS occurs in parts of Africa, Asia, North America and South America (Kotze, 1981; Kiely, 1948; Wager, 1952; Calavan, 1960; McOnie, 1964; Korf *et al.*, 2001; Schubert *et al.*, 2012).

Symptoms of CBS arise from infection of fruit during the first 20-24 weeks of fruit development, after which time fruit become resistant (Baldassari *et al.*, 2006; Kotze, 1981; Wager, 1952). Infection occurs via aerially dispersed ascospores of the fungus liberated from pseudothecia in leaf litter on the orchard floor, as well as via water dispersed conidia produced from pycnidia in lesions on twigs and diseased fruit hanging in the canopy (Kiely, 1948; Kotze, 1963; Sposito *et al.*, 2011). Symptoms of CBS on fruit generally appear as fruit mature on the tree, or after harvest (Kiely, 1948). A range of symptoms of CBS can occur on fruit (Fig. 1.1.1), the most distinctive symptom being ‘hard spot’. Hard spot is described as a red-brown spot that develops into a red-black rimmed depressed lesion with a light grey or brown centre that may contain pycnidia of *P. citricarpa* (Kiely, 1948; McOnie, 1964). Other symptoms include ‘freckle spot’, ‘virulent spot’, ‘speckled blotch’ and ‘cracked spot’ (Kiely, 1948; de Goes *et al.*, 2000). The expression of CBS symptoms postharvest can be maximised by the incubation of fruit for 3 weeks at 27°C, 80% relative humidity, and permanent light (Brodrick and Rabie, 1970). However, in some seasons and/or locations CBS symptoms may be severe enough to induce premature fruit drop and reduce yield prior to harvest (Kiely, 1948).

Management of CBS primarily relies on the preharvest application of fungicides, with the level of control generally offering economic returns to growers. While this is generally suitable for the domestic market, management of the disease to export standards would benefit from the incorporation of effective postharvest fungicides. However, previous studies have shown limitations for both the existing preharvest and postharvest fungicides (see sections

1.2 In *vitro sensitivity to postharvest* fungicides and *1.3 Field evaluation of fungicides* for detailed discussion). The aim of this chapter is to improve our understandind of the response of *P. citricarpa* to existing postharvest fungicides, as well as evaluate alternative options for field control of the fungus.

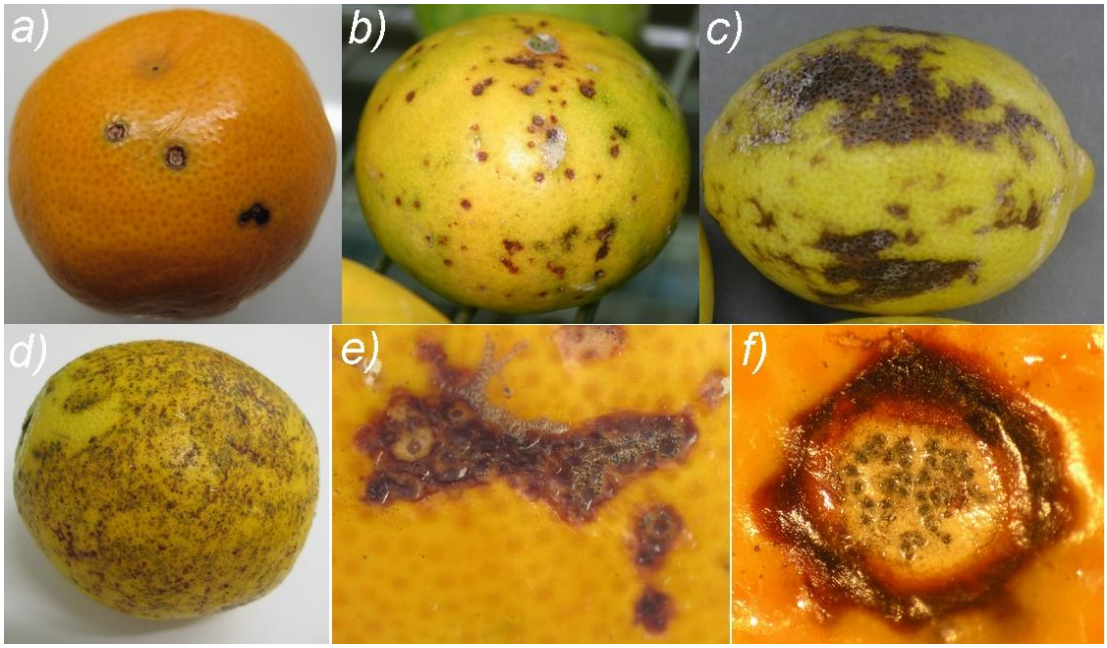

Figure 1.1.1. The various symptoms of citrus black spot (*Phyllosticta citricarpa*): a) hard spot, b) freckle spot, c) virulent spot, d) speckled blotch, e) virulent spot with pycnidia, and f) pycnidia contained within a hard spot lesion.

1.2 *In vitro* sensitivity to postharvest fungicides

Introduction

The management of CBS could be greatly improved by the development of a systems approach that combines field control with a postharvest treatment. As postharvest fungicide treatment is already a standard practice, improving this approach for CBS is a logical starting point. However, past efforts to develop postharvest fungicide treatments for CBS only sometimes resulted in significant reductions in CBS (Agostini et al. 2004; Agostini et al. 2006; Korf 1998; Korf et al. 2001; Wyatt et al. 2008). A possible explanation is that the approach used to evaluated postharvest fungicides has been to arbitrarily dip fruit with available fungicides, without thorough consideration of how these products may or may not be effective. For example, blue/green moulds are controlled by postharvest fungicides. However, infections arise from spores of the mould fungi that exist on the fruit surface (Brown and Eckert 2000). These spores are readily in contact with the fungicides when fruit are dipped postharvest. On the other hand, at harvest the fungus causing CBS exists just below the fruit surface, between the fruit cuticle (a wax layer on the outside of the fruit) and the top layer of epidermal cells (McOnie 1967). It is therefore possible that the cuticle layer is interfering with the fungicide contacting the fungus reliably. It may therefore be the case that inconsistent control of CBS is because of poor contact between the fungicide and fungus.

In order to improve the effectiveness of post harvest treatments a rigorous approach is needed to answer the following three questions: 1) are the fungicides directly effective against the fungus; 2) if so, why do the fungicides only work sometimes; and 3) can the impediments to efficacy be overcome (e.g. by addition of adjuvants / penetrants)? Answering these questions is critical to developing an effective postharvest treatment. If the cuticle is demonstrated to be interfering with the

fungicide, specific processes to overcome, or pass through, the cuticle could be developed in collaboration with a fungicide or adjuvant manufacturer.

In this experiment we aim to answer the first question (Are the fungicides directly effective against the fungus?) by determining the *in vitro* sensistivity of the fungus to the registered fungicides guazatine and imazalil. A reliable postharvest fungicide treatment would greatly assist in accessing the $67.5M USA market for fruit from CBS areas.

Methods

In order to confirm that *P. citricarpa* is sensitive to the widely-used postharvest citrus fungicides guazatine and imazalil, the effective concentration to inhibit growth by 50% (EC_{50}) of the fungus was determined *in vitro*. Five isolate of *P. citricarpa* (BRIP 52614, 53714, 53717, 53720, 54241) were grown in triplicate on ½ strength potato dextrose agar (PDA) adjusted to 0.01, 0.1, 10 and 1000 ppm of active ingredient of both fungicides. PDA without fungicide was used as a control. The amended agar was inoculated in the centre with a 3 mm diameter plug of mycelium from 2-week-old colonies of the isolates. The plates were incubated at ambient conditions (~25°C and 12 hr cycle of natural light and darkness). Colony growth of three replicate colonies was measured after 7 days as the total colony area using image analysis software (NS Elements, Nikon). Growth inhibition was expressed as a proportion of the colony area relative to the growth on the control plates. 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) using GenStat 16th Edition (VSN International, UK). The EC_{50} was then determined.

Results

The growth response curve to guazatine best fit a logistic model (adjusted $R^2 = 53.6$), with the EC_{50} determined to be 0.32 ppm (Fig. 1.2.1). The response curve for imazalil best fit the exponential model (adjusted $R^2 = 56.4$), with the EC_{50} determined to be 0.03 ppm; indicating *P. citricarpa* to be approximately 10 times more sensitive to imazalil than guazatine.

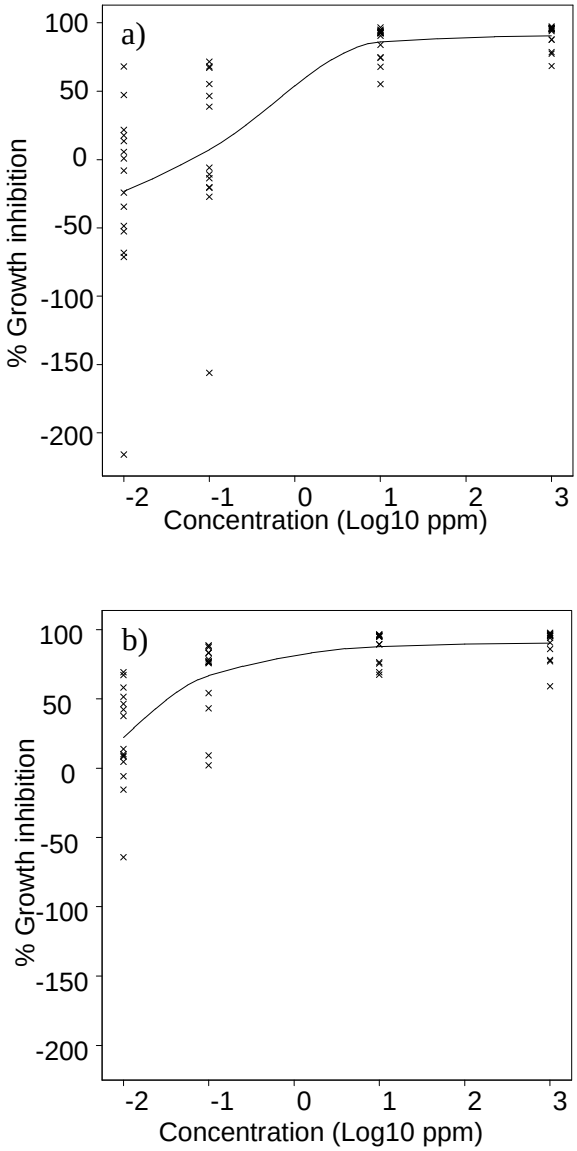

Figure 1.2.1. Growth response curves for a) guazatine and b) imazalil

Discussion

The results of this study have shown the EC_{50} values for guazatine and imazalil to be approximately 0.32 ppm and 0.03 ppm, respectively. These results suggest that of the two fungicides, *P. citricarpa* is more sensitive to imazalil. For means of comparison to fungicides with known efficacy against CBS in the field; studies with azoxystrobin (Amistar) have reported EC_{50} values for *P citricarpa* in the range of 0.155 ppm (Miles and Drenth, 2013), down to 0.027 ppm (Hincapie *et al.*, 2014); though the results of Miles and Drenth (2013) are more comparable, being based on similar protocols and populations of the fungus. It is possible to infer from this comparison that the fungicides azoxystrobin and imazalil, and to a lesser extent guazatine, show similar toxicity to *P. citricarpa* when there is adequate contact between the fungicide and the fungus undergoing vegetative growth. During the postharvest development of CBS the fungus

is growing vegetatively, as opposed to spore germination during infection in the field. These results support the hypothesis that a lack of efficacy of postharvest fungicide application is possibly due to poor contact between the fungicide and fungus.

This experiment aimed to answer the question whether postharvest fungicides are directly effective against *P. citricarpa*. The results of this experiment suggest that the existing postharvest fungicides, imazalil in particular, are directly effective. Progress towards a postharvest fungicide solution to CBS may therefore be more likely to be made by addressing the need for more direct contact between the fungicide and fungus in fruit.

1.3 Field evaluation of fungicides

Introduction

Reducing yield losses to CBS relies mainly on the application of protective fungicides during the first 20-24 weeks of fruit development (Kiely, 1948; Kotze, 1981; Schutte *et al.*, 2003; Miles *et al.*, 2004; Silva Junior *et al.*, 2016). The most common fungicides used for CBS control include copper-based formulations, dithiocarbamates such as mancozeb, benzimidazoles such as benomyl, and strobilurins such as azoxystrobin (Agostini *et al.*, 2006; Blackford, 1941; Kellerman and Kotze, 1977; Rodriguez and Mazza Gaiad, 1996; Schutte *et al.*, 1997; Schutte *et al.*, 2003; Tsai, 1981). While this suite of fungicides collectively offer good control of CBS in most circumstances, each has its limitations. For example, copper-based fungicides can be prone to rind stippling (Schutte *et al.*, 1997). Dithiocarbamates are within the priority 1 group for review by the Australian Pesticides and Veterinary Medicines Authority (APVMA), largely due to the metabolite ethylene thiourea (ETU) and its potential disruption of thyroid function (Vettorazzi *et al.*, 1995). Furthermore, dithiocarbamate residue limits vary extensively between countries, resulting in challenges for fruit exporters. The benzimidazole fungicide benomyl was reported to be mutagenic to bacteria, embryotoxic and teratogenic in rats, and possibly linked to anophthalmia in developing foetuses (Cummings *et al.*, 1992; Hewitt *et al.*, 2005; Seiler, 1972; Zeman *et al.*, 1986), therefore the APVMA completely prohibited the supply and use of benomyl in Australia in 2006. At present the main limitation with the strobilurin fungicides is their elevated resistance risk due to their highly specific mode of action, only requiring mutation within the target cytochrome *b* protein (Bartlett *et al.*, 2002; Stammler *et al.*, 2013). Due to the presence of an intron within the cytochrome *b* of *P. citricarpa* a lower mutation risk is expected (Stammler *et al.*, 2013), however, the resistance risk of other fungi in citrus orchards should also be considered. For example, anti-resistance strategies for strobilurin use are still needed as strobilurins are also used to control ‘Emperor’ brown spot (Miles *et al.*, 2005).

In order to overcome the limitations of the fungicides currently used for controlling CBS, it is necessary to expand the range of active ingredients with efficacy against the pathogen. As there are excess of 100 fungicide active ingredients used in crop protection (Hewitt, 1998), a short list of potential options was developed under previous project CT07012 (Miles and Drenth, 2013) around three main criteria: 1) high efficacy potential based on existing studies; 2) active ingredients outside of the resistance activity groups already used in citrus; and 3) have favourable residue profiles for domestic and export markets. One fungicide group with potential to fit these

criteria are the succinate dehydrogenase inhibitor (SDHI) fungicides. The SDHI group of fungicides target fungal respiration through inhibition of the ubiquinone-binding sites in the mitochondrial complex II (Avenot and Michailides, 2010). However, previous evaluation of various SDHI fungicides for CBS control did not look promising for commercial use at the evauated fungicide rates (Miles and Drenth, 2013). Furthermore, like the strobilurin fungicides, the specific mode of action of the SDHI group increases the risk of resistance development. This risk can be mitigated by also investigating the efficacy of a range of multisite activity fungicides against CBS. Multisite activity fungicides disrupt cell function across a range of processes, therefore resistance is unlikely to develop from any single point mutation such as for strobilurins or SDHI fungicides. For example, the pthalimide fungicide, captan, and quinone fungicide, Compound 4, disrupt general enzyme function (Hewitt, 1998). Also of interest are the emerging range of commercially available microbial products, as well as the increased use of sanitisers. Biological products are particularly desirable for their reduced residue exposure to consumers (Thomidis *et al.*, 2015; Lima *et al.*, 2011). The *Bacillus subtilis* product Serenade®, for example, has in tank mixes with other fungicides shown some potential efficacy against a range of citrus diseases including CBS (Quadrado *et al.*, 2016; Highland and Timmer, 2004). Several modes of action have been reported for these types of biological products including: resource/niche competition; direct inhibition through production of antimicrobial compounds and/or low molecular weight compounds (e.g. enzymes); and induction of systemic acquired resistance in the host plant which in turn reduces susceptibility of the host to the pathogen (Cawoy *et al.*, 2011). The sanitiser Compound 10 has also shown some promise for efficacy against CBS in combination sprays with reduced rates of conventional fungicides (Schutte, 2008). Compound 10 has potential to work in two ways, one being the disruption of fungal cell wall permeability, and the other being surfactant-like behaviour (Juergensen *et al.*, 2000) improving the coverage and efficacy of the fungicide it was applied with.

Independent field evaluations of alternative fungicides for the control of CBS in Australia have not been undertaken since 2003 (Miles *et al.*, 2004). While this previous work resulted in the registration of azoxystrobin for CBS control in Australia, the loss of benomyl has meant the number of options remains restricted. The aim of this study was to evaluate the efficacy of alternative fungicides for the control of CBS in Australia, following on directly from work commenced under previous project CT07012 (Miles and Drenth, 2013).

Methods

Treatment application, fruit incubation and disease assessment

In order to determine the efficacy of various fungicides against CBS (Table 1.3.1), a series of field experiments were conducted in the Central Burnett region of Queensland, Australia. Various fungicide treatment applications were made during the first 20-24 weeks of fruit growth when fruit are susceptible to *P. citricarpa* (Wager, 1952; Baldassari *et al.*, 2006; Kotze, 1981). All treatments were applied to four individual replicate trees in a randomised complete block design within commercial orchards. Treatments were applied using a custom built hand lance sprayer with dual D4 hollow cone nozzles, operating at 50 psi delivered by a 6.0 horsepower Subaru Robin EX17 gas engine-driven pressure pump (Subaru, Japan). All experiments included an untreated control, and a standard (mancozeb) as a positive control.

At commercial maturity, approximately 50 fruit were harvested from each row-side of each data tree for a total of 100 fruit per tree, and the total weight of the fruit from each data was measured to determine the average fruit weight.

To ensure all CBS symptoms were fully expressed in fruit prior to assessment, fruit from each experiment were incubated for 3 weeks at 27°C, 80% relative humidity, and permanent light to break the latency of all *P. citricarpa* infections (Brodrick and Rabie, 1970). After incubation CBS symptoms on each fruit were quantified by inspecting each fruit by eye and light microscopy. Hard spot was characterised as red to black-rimmed depressed lesions with a light grey or brown centre containing pycnidia. Freckle spot was characterised as slightly depressed, orange to brick red spots. Virulent spot was characterised as a coalescence of freckle spots, and speckled blotch as areas of many minute black spots on the fruit surface. In the case of hard spot and freckle spot, the numbers of lesions of each type on each fruit were counted. For virulent CBS lesions and speckled blotch, estimates of the percentage of the fruit surface area affected were made. Disease incidence was defined as the proportion of fruit with one or more lesions. Disease severity was defined as the number of lesions per fruit. To be able to analyse the effect of the various treatments on the combined severity of all the observed forms of CBS, the estimates of fruit surface area affected by virulent spot were converted to an equivalent number of lesions; assuming 1% of surface area was equivalent to 10 spots of 3 mm diameter. In all trials fruit were also observed for signs of phytotoxicity or other abnormal blemishes. If observed, the incidence of fruit with blemish was recorded and analysed as for disease incidence.

Table 1.3.1. Product names, active ingredients, chemical group, and standard rates used in CBS chemical control experiments carried out in Queensland.

Product name	Active ingredient	Group (FRAC code)	Standard rate of product	Standard rate of active ingredient
Amistar 250 SC	25% azoxystrobin	Quinone outside inhibitors (C3)	0.40 mL/L	0.100 mL/L
Chief Aquaflo	50% iprodione	Dicarboximide (E3)	1.00 mL/L	0.500 mL/L
Compound 3	n/a	Microbial (F6)	2.00 g/L	-
Compound 4	70% a.i.	Quinone (multi-site)	0.70 g/L	0.500 g/L
Compound 6	50% a.i.	Succinate dehydrogenase inhibitors (C2)	0.30 g/L	0.150 g/L
Penncozeb 750DF	75% mancozeb	Dithiocarbamate (multi-site)	2.00 g/L	1.500 g/L
Red copper WG	50% cuprous oxide	Inorganic (multi-site)	1.35 g/L	0.675 g/L
Compound 7	40% a.i.	Anilino-pyrimidines (D1)	1.00 g/L	0.400 mL/L
Compound 10	12% a.i.	Sanitiser (not specified)	1.00 mL/L	0.120 mL/L

Table 1.3.2. Schedule of fungicide treatments applied in experiment 1.3.1.

	Application date									
Treatment[a]	**25/10/13**	**6/11/13**	**18/11/13**	**4/12/13**	**17/12/13**	**2/1/14**	**17/1/14**	**3/2/14**	**14/2/14**	**25/2/14**
Control	-	-	-	-	-	-	-	-	-	-
Mz standard	Mz	-	Mz	-	Mz	-	Mz	-	Mz	-
Full program	Cu	-	Mz	-	Azo	-	Mz	-	Comp 6	-
Comp 6 2×	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	-
Comp 6	Comp 6	-	Comp 6	-	Comp 6	-	Comp 6	-	Comp 6	-
Comp 6 0.5×	Comp 6 0.5×	-	Comp 6 0.5×	-	Comp 6 0.5×	-	Comp 6 0.5×	-	Comp 6 0.5×	-
Comp 6 late	-	-	-	-	-	-	-	-	Comp 6	-
Mz 0.5×	Mz 0.5×	-	Mz 0.5×	-	Mz 0.5×	-	Mz 0.5×	-	Mz 0.5×	-
Mz 0.5×	Mz 0.5×	-	Mz 0.5×	-	Mz 0.5×	-	Mz 0.5×	-	Mz 0.5×	-
+ Comp 10	+ Comp 10		+ Comp 10		+ Comp 10		+ Comp 10		+ Comp 10	
Comp 10	Comp 10	-	Comp 10	-	Comp 10	-	Comp 10	-	Comp 10	-
Comp 4 2×	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	-
Mz late	-	-	-	-	Mz	-	Mz	-	Mz	-
Mz 0.5× (14 d)	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×
Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×	Mz 0.5×
+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3	+ Comp 3
Comp 3	Comp 3	Comp 3	Comp 3	Comp 3	Comp 3	Comp 3	Comp 3	Comp 3	Comp 3	Comp 3

[a]Mz = mancozeb, Cu = cuprous oxide, Azo = azoxystrobin, Comp 6 = Compound 6, Comp 10 = Compound 10, Comp 4 = Compound 4, values followed by "×" refer to a multiple of the standard rate according to table 1.3.1.

Experiment 1.3.1

Field experiment 1.3.1 was established in a high disease pressure area near Gayndah, Qld (-25.627877, 151.506941), during the 2013-14 production season. The trial comprised of 'Imperial' mandarin trees on 'Cleopatra' mandarin rootstock, planted in 1976 at an 8 m × 4 m spacing. Treatment applications were made to run-off at 12.5 L per tree. The treatment schedule in experiment 1.3.1 is shown in Table 1.3.2. The trial was harvested on the 16th April 2014.

Experiment 1.3.2

Field experiment 1.3.2 was established in a high disease pressure area near Mundubbera, Qld (-25.598926, 151.189857), during the 2014-15 production season. The trial comprised of 'Arnold' blood orange (*C. sinensis*) trees on 'Troyer' rootstock (*C. sinensis* × *Poncirus trifoliata*), planted in 2006 at a 6.5 m × 3 m spacing. Treatment applications were made to run-off at 10 L per tree. The treatment schedule is shown in Table 1.3.3. The trial was harvested on the 18th June 2015.

Table 1.3.3. Schedule of fungicide treatments applied in experiment 1.3.2.

	Application date				
Treatment[a]	**5/10/14**	**5/11/14**	**16/12/14**	**13/1/15**	**17/2/15**
Control	-	-	-	-	-
Mz standard	Mz	Mz	Mz	Mz	Mz
Comp 4 2×	Comp 4 2×	Comp 4 2×	Comp 4 2×	Comp 4 2×	Comp 4 2×
Comp 4	Comp 4	Comp 4	Comp 4	Comp 4	Comp 4
Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×
Comp 4 use pattern	Mz	Comp 4	Comp 4	Mz	Comp 4
Comp 7 2×	Comp 7 2×	Comp 7 2×	Comp 7 2×	Comp 7 2×	Comp 7 2×
Comp 7	Comp 7	Comp 7	Comp 7	Comp 7	Comp 7
Comp 7 0.5×	Comp 7 0.5×	Comp 7 0.5×	Comp 7 0.5×	Comp 7 0.5×	Comp 7 0.5×
Ipr	Ipr	Ipr	Ipr	Ipr	Ipr

[a]Mz = mancozeb, Comp 4 = Compound 4, Comp 7 = Compound 7, Ipr = iprodione, values followed by "×" refer to a multiple of the standard rate according to table 1.3.1.

Experiment 1.3.3

Field experiment 1.3.3 was established in a high disease pressure area near Mundubbera, Qld (-25.611744, 151.262775, during the 2015-16 production season. The trial consisted of 'Imperial' mandarin (*C. reticulata*) trees on 'Benton' citrange rootstock (*P. trifoliata* × *C. sinensis*), planted in 2009 at a 7.3 m × 2.7 m spacing. Experiment 1.3.3 was a smaller experiment conducted in excess of project CT13020 requirements, consisting of only three treatments: 1) unsprayed control; 2) mancozeb at the standard rate; and 3) Compound 4 at 0.25 g/L. Treatment applications were made to run-off at 10 L per tree. Treatments were applied on the 18th December 2015, 20th January, 18th February and 16th of March 2016. The trial was harvested on the 4th May 2016.

Statistical analysis

The mean disease incidence and severity was determined for each data tree. The mean values was then subjected to analysis of variance (ANOVA) in GenStat 16th Edition (VSN International, UK). Arcsine angular transformation was applied to incidence data, and arcsine angular, $\log_{10}$, square root or third root transformations applied to severity data, where required to normalise the data. Fruit presentation data were also analysed by ANOVA. In order to account for any effects of fruit size, mean fruit weight was included as a covariate.

Results

Experiment 1.3.1

The incidence and severity of total CBS in the untreated control was observed to be 58% and 4.78 equivalent lesions per fruit, respectively (Table 1.3.4). The most common symptom types were hard spot and freckle spot, with the least common symptom being virulent spot. The incidence and severity of CBS was generally highest or equal highest in the untreated control, whilst the incidence and severity of CBS was generally lowest or equal lowest in fruit treated with the industry standard fungicide, mancozeb. The treatments found to result in an equivalent incidence and severity of CBS to mancozeb, across all symptom types, included the highest rate of Compound 6, nearly all the treatments incorporating mancozeb (at the standard or half rate), and the Compound 4 treatment. The late Compound 6 and mancozeb treatments, and the Compound 3 treatment were equivalent to the untreated control.

Regarding the set of treatments investigating possible synergism between mancozeb and Comp 10, no evidence for synergy was found. By several measures, Comp 10 alone was not significantly different to the control, but the total incidence of CBS was significantly lower in the Comp 10 alone treatment than the control. Most interestingly, reducing the rate of mancozeb by half had no significant effect on efficacy compared to the standard rate of mancozeb. Similarly, the use of Compound 3 with reduced rates of mancozeb showed no additional value in controlling CBS. Compound 3 on its own also had no significant effect on CBS.

In experiment 1.3.1 there were no signs of abnormal fruit blemishes or insect pests to report.

Experiment 1.3.2

The incidence and severity of CBS in the untreated control was observed to be 54% and 14.2 equivalent lesions per fruit, respectively (Table 1.3.5). The most common symptom type was freckle spot. A small amount of virulent spot was observed, however the severity of virulent spot, and level of hard spot, were too low to be meangingfully analysed separately. However, these values were included in the total CBS analysis. The only fungicides shown to significantly reduce the incidence and severity of CBS compared with the control were the mancozeb standard and Compound 4. While Compound 4 showed excellent CBS control, the Compound 4 treatments were associated with significant phytotoxicity (Fig. 1.3.2). The incidence of phytotoxicity reduced with the rate of Compound 4 applied, but even when Compound 4 was alternated with mancozeb, phytoxicity was still observed in this experiment. Compound 7 and iprodione were not significantly different to the control.

Figure 1.3.2. Symptoms of phytotoxicity on 'Arnold' blood orange associated with applications of Compound 4 in experiment 1.3.2. Photo: Nga Tran.

Experiment 1.3.3

In experiment 1.3.3 the incidence and severity of CBS was the highest observed in the untreated controls of the three experiments, at 97% and 74 equivalent lesions per fruit, respectively (Table 1.3.6). Freckle spot was the most common symptom type, followed by hard spot, then virulent spot. However, there was no significant difference between treatments for the assessments of hard spot or virulent spot. For this reason the best measure for comparison between treatments is the incidence and severity of freckle spot. In this case both fungicides significantly reduced CBS, with mancozeb reducing the incidence of freckle spot significantly more than Compound 4. Unlike experiment 1.3.2, no signs of phytotoxicity were observed in association with Compound 4, or the in the experiment altogether.

Discussion

In this study we set out to continue work started under project CT07012 (Miles and Drenth, 2013) to identify alternative fungicides for the control of CBS in Queensland. Based on our experiments the most promising fungicide able to match the efficacy of the standard fungicide, mancozeb, was the multisite fungicide Compound 4. However, in one trial Compound 4 treatments were associated with significant phytotoxicity, the possible cause of which is discussed below. The next best performing fungicide treatment was the SDHI fungicide Compound 6 at the 2× rate. The fungicides

iprodione and Compound 7 failed to significantly reduce CBS compared to the control. The sanitiser Comp 10 did not show useful CBS control, but did show significant disease reductions compared to the control by some measures. Furthermore, there was no evidence for a synergistic effect between Comp 10 and reduced rates of mancozeb. Finally, no evidence was found to show any efficacy of the biological product Compound 3 against CBS, nor was there evidence for enhanced efficacy of reduced rates of mancozeb when tank mixed with Compound 3. In general, it was more commonly observed for the various treatments to show significant differences in disease severity, rather than disease incidence.

To our knowledge, Compound 4 is not widely used for CBS control, but it is reported to be used to manage CBS in Taiwan (Tsai, 1981). It is also reported to be effective against other diseases of citrus including scab (Whiteside, 1990) and melanose (Jwu-guh and Tsai-young, 1989; Whiteside, 1977). A wide range of efficacy against citrus pathogens, along with a reduced likelihood of resistance from the multisite mode of action of Compound 4 is a good fit with citrus production. However, the occurrence of phytotoxicity in experiment 1.3.2 is a concern. While it is not possible to be certain of the cause of the phytotoxicity, phytotoxicity of Compound 4 (and captan) has been reported in applications with minreral oils (Koller, 1999). Review of the orchard spray records shows two pest oil sprays that occurred during the experiment which may be responsible for inducing the Compound 4 phytotoxicity. These oil applications occurred on the 19th Dec 2014 and 20th Feb 2016, each within a few days of application numbers 3 and 5 of the experiment. Considering that no phytotoxicity was observed in any of the other experiments, including those detailed in ***chapter 2*** of this report, it is likely that the issue was related to the oil applications. Experiment 1.3.3 was undertaken largely to confirm any phytotoxicity in a soft rind variety (Imperial) during the hottest time of the year, and no phytotoxicity was observed; noting that no oil applications where made during this experiment. While in general the efficacy of Compound 4 looked promising, an atypical rate response was observed in experiment 1.3.2 that cannot be readily explained. While the Comp 4 2× (Compound 4 1.4 g/L) treatment was consistently equivalent to the mancozeb standard, experiment 1.3.2 suggests lower rates may be sufficient. Additional experiments are need to optimise the rate of Compound 4 for CBS control and confirm the role of oil in phytotoxicity to citrus.

The SDHI fungicide included in our experiment generally reduced CBS significantly compared to the control, but was not consistently equivalent to the mancozeb standard. In previous trials evaluating SDHI fungicides against CBS, similar results were observed, with high rates being required for equivalent efficacy to mancozeb (Miles and Drenth, 2013). However, the high rate of Compound 6 required for acceptable control would potentially become cost prohibitive, have a high residue potential, and be likely to exceed good agricultural practice limits. It may therefore be preferable to investigate the use of the SDHI fungicides in tank mixes with other fungicides. The use of Compound 6 at the end of the fruit susceptibility period (the Comp 6 late treatments in experiment 1.3.1) only modestly reduced CBS, and is therefore unlikely to be useful as a late eradicant treatment as was once the case with benomyl for CBS control (Kiely, 1976).

Iprodione is an important fungicide used in citrus for the control of ‘Emperor’ brown spot caused by *Alternaria alternata* (Hutton, 1989; Miles *et al.*, 2005; Pegg, 1966). Cultivars susceptible to the disease are at risk whenever environmental conditions are suitable (Canihos *et al.*, 1999). As these environmental conditions may overlap with

the period of time that fruit are susceptible to CBS, iprodione may be applied during the period of CBS susceptibility. However, as we have found the efficacy of iprodione against CBS to be very poor for CBS, it will be important for additional fungicides such as mancozeb to also be applied to maintain protection against both diseases during this time. As resistance of *A. alternata* to iprodione has already been observed (Hutton, 1989; Erklc *et al.*, 1999), combining iprodione with an additional fungicide of an alternative resistance activity group will also assist in reducing further resistance risks.

The evaluation in experiment 1.3.1 of tank mixes of reduced rates of mancozeb with the sanitiser Comp 10 or biological product Compound 3 did not show any synergistic benefits for CBS efficacy. While Comp 10 alone significantly reduced CBS relative to the control for some specific measures, the effect was only modest. Compound 3 was never significantly different from the control. Varied CBS efficacy has been reported with similar biological products (Roberts *et al.*, 2012; Kupper *et al.*, 2006). The most interesting result from the assessment of any potential synergism was that mancozeb at half the standard rate gave equivalent efficacy to mancozeb at the standard rate. Reduced rates of copper fungicides for Alternaria brown spot control have been shown to be effective (Vicent *et al.*, 2009), and similar investigations for mancozeb are warranted.

While Compound 4 was found to be the most promising of the fungicides tested in our experiments in this project and previous projects (Miles and Drenth, 2013), the experiments have also demonstrated that the range of fungicides offering equivalent efficacy to mancozeb is very limited. It is particularly concerning as it places a heavy reliance on fungicides such as mancozeb. In the absence of alternate fungicides it will be important to increase research efforts to more efficiently use the fungicides available, as well as improve the efficacy of biological and cultural control options. Long term CBS management is likely to rely on a combination of judicious fungicide applications, inoculum management, and potentially host resistance.

Table 1.3.4. Effects of various fungicides in experiment 1.3.1 on the incidence and severity of citrus black spot (caused by *Phyllosticta citricarpa*) and its various symptom types, in 'Imperial' mandarin fruit harvested during the 2013-14 season[a].

	Total CBS[c]		Freckle spot		Hard spot		Virulent	
Treatment[b]	**Incidence (%)[d]**	**Severity[e]**	**Incidence (%)**	**Severity**	**Incidence (%)**	**Severity**	**Incidence (%)**	**Severity**
Control	58 a	0.58 (4.78) abc	37 a	1.25 (1.55) ab	0.7 (40.8) a	1.23 (1.87) a	0.26 (6.83) bcd	0.16 (0.40) ab
Mz standard	6 d	0.27 (2.85) def	4 de	0.38 (0.14) defg	0.1 (0.7) d	0.17 (0.00) g	0.09 (0.88) efgh	0.07 (0.04) cde
Full program	12 cd	0.27 (2.87) def	9 de	0.51 (0.26) def	0.2 (4.8) cd	0.56 (0.18) def	0.08 (0.56) fgh	0.05 (0.01) cde
Comp 6 2×	6 d	0.22 (2.66) def	4 de	0.22 (0.05) efg	0.2 (2.5) d	0.29 (0.03) fg	0.07 (0.54) fgh	0.05 (0.01) cde
Comp 6	24 c	0.35 (3.22) de	11 de	0.47 (0.22) defg	0.4 (15.1) b	0.68 (0.31) cde	0.14 (2.04) defg	0.09 (0.08) bcd
Comp 6 0.5×	24 c	0.39 (3.46) cde	13 cd	0.57 (0.32) de	0.4 (13.6) bc	0.70 (0.34) cde	0.21 (4.18) bcde	0.11 (0.13) bc
Comp 6 late	52 ab	0.56 (4.63) abc	31 ab	0.99 (0.97) bc	0.6 (32.1) a	1.01 (1.02) abc	0.30 (8.45) ab	0.17 (0.48) ab
Mz 0.5×	7 d	0.15 (2.41) f	4 de	0.20 (0.04) fg	0.1 (2.1) d	0.25 (0.02) fg	0.05 (0.25) fgh	0.04 (0.00) cde
Mz 0.5× + Comp 10	10 cd	0.14 (2.37) f	6 de	0.29 (0.08) efg	0.2 (3.7) d	0.37 (0.05) efg	0.02 (0.06) gh	0.01 (0.00) e
Comp 10	41 b	0.40 (3.53) bcd	23 bc	0.73 (0.53) cd	0.5 (26.2) ab	0.86 (0.63) bcd	0.17 (2.78) cdef	0.10 (0.11) bcd
Comp 4 2×	5 d	0.09 (2.22) f	2 e	0.12 (0.01) g	0.1 (2.0) d	0.29 (0.02) fg	0.00 (0.00) h	0.00 (0.00) e
Mz late	56 ab	0.62 (5.20) a	32 ab	1.14 (1.30) ab	0.6 (36.3) a	1.08 (1.28) ab	0.40 (15.47) a	0.21 (0.89) a
Mz 0.5× (14 day)	5 d	0.12 (2.32) f	1 e	0.14 (0.02) fg	0.1 (2.1) d	0.31 (0.03) fg	0.05 (0.24) fgh	0.03 (0.00) de
Mz 0.5× Comp 3	3 d	0.21 (2.61) ef	2 e	0.15 (0.02) fg	0.1 (1.6) d	0.35 (0.04) fg	0.09 (0.73) efgh	0.06 (0.02) cde
Comp 3	54 ab	0.59 (4.89) ab	37 a	1.41 (1.98) a	0.6 (36.2) a	1.27 (2.03) a	0.29 (8.07) abc	0.16 (0.40) ab
Transformation	-	Log_{10}	-	Square root	Arcsine angular	3rd root	Arcsine angular	3rd root
LSD	16	0.19 (2.55)	11	0.37 (0.14)	0.2 (3.1)	0.33 (0.04)	0.13 (1.58)	0.08 (0.05)
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Covariate	0.019	n.s.	n.s.	n.s.	<0.001	<0.001	n.s.	n.s.

[a]Mean values within columns followed by the same letter are not significantly different at $p \leq 0.05$. Numbers in parentheses are back transformed means.

[b]Mz = mancozeb, Full program = monthly program of cuprous oxide/Mz/azoxystrobin/Mz/Compound 6, Comp 10 = Compound 10, Comp 4 = Compound 4, Late = treatments commenced 17th December 2016. Values followed by "×" refer to a multiple of the standard rate according to table 1.3.1.

[c]Total CBS is the total of all lesion types; freckle spot, hard spot, virulent spot and speckled blotch (data not shown).

[d]Incidence refers to the proportion of fruit with symptoms of CBS.

[e]Severity refers to the number of spots per fruit. In the case of Total CBS, the measures of fruit surface area were converted to a number of spots based on the assumption that 1% of fruit surface area was equivalent to 10 spots of 3mm diameter.

Table 1.3.5. Effects of various fungicides in experiment 1.3.2 on the incidence and severity of citrus black spot (caused by *Phyllosticta citricarpa*) and its various symptom types, as well as phytotoxicity, in 'Arnold' blood orange fruit harvested during the 2014-15 season[a].

	Total CBS[c]		Freckle spot		Virulent	Phytotoxicity
Treatment[b]	**Incidence (%)[d]**	**Severity[e]**	**Incidence (%)**	**Severity**	**Incidence (%)**	**Incidence (%)**
Control	54 a	2.4 (14.2) a	0.54 (53.60) a	1.7 (2.8) ab	0.20 (3.85) ab	1 c
Mz standard	6 b	0.7 (0.3) b	0.06 (6.25) b	0.3 (0.1) c	0.03 (0.07) c	1 c
Comp 4 2×	6 b	0.8 (0.4) b	0.05 (5.34) b	0.4 (0.1) c	0.04 (0.13) c	61 a
Comp 4	20 b	1.5 (3.2) ab	0.18 (18.38) b	0.8 (0.7) bc	0.14 (1.92) abc	45 b
Comp 4 0.5×	9 b	0.6 (0.2) b	0.08 (8.47) b	0.4 (0.1) c	0.07 (0.53) bc	40 b
Comp 4 use pattern	7 b	0.7 (0.3) b	0.07 (7.23) b	0.5 (0.2) c	0.03 (0.07) c	39 b
Comp 7 2×	49 a	1.3 (2.4) ab	0.48 (48.27) a	1.1 (1.2) abc	0.12 (1.45) abc	0 c
Comp 7	42 a	1.5 (3.7) ab	0.41 (41.20) a	1.5 (2.2) ab	0.15 (2.17) abc	2 c
Comp 7 0.5×	47 a	2.1 (8.6) a	0.43 (43.44) a	1.6 (2.4) ab	0.26 (6.51) a	1 c
Ipr	43 a	2.5 (15.7) a	0.42 (41.94) a	1.9 (3.5) a	0.26 (6.80) a	0 c
Transformation	-	3rd root	-	Square root	Asin	-
LSD	18	1.2 (1.6)	0.17 (17.36)	0.9 (0.7)	0.15 (2.20)	14
P	<0.001	0.008	<0.001	<0.001	0.01	<0.001
Covariate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a]Mean values within columns followed by the same letter are not significantly different at $p \leq 0.05$. Numbers in parentheses are back transformed means.

[b]Mz = mancozeb, Comp 4 = Compound 4, Comp 7 = Compound 7, Ipr = iprodione. Values followed by "×" refer to a multiple of the standard rate according to table 1.3.1.

[c]Total CBS is the total of all lesion types; freckle spot, hard spot, virulent spot and speckled blotch (data not shown).

[d]Incidence refers to the proportion of fruit with symptoms of CBS.

[e]Severity refers to the number of spots per fruit. In the case of Total CBS, the measures of fruit surface area were converted to a number of spots based on the assumption that 1% of fruit surface area was equivalent to 10 spots of 3mm diameter.

Table 1.3.6. Effects of mancozeb and Compound 4 in experiment 1.3.3 on the incidence and severity of citrus black spot (caused by *Phyllosticta citricarpa*) and its various symptom types, in 'Imperial' mandarin fruit harvested during the 2015-16 season[a].

	Total CBS[c]		Freckle spot		Hard spot		Virulent	
Treatment[b]	**Incidence (%)[d]**	**Severity[ef]**	**Incidence (%)[f]**	**Severity[f]**	**Incidence (%)**	**Severity[g]**	**Incidence (%)**	**Severity**
Control	97 a	74 a	95 a	21 a	54	2	45	5
Mz standard	83 b	17 b	62 c	2 b	65	2	30	1
Comp 4 0.5×	92 ab	39 ab	80 b	7 b	62	3	34	3
LSD	9	39	9	5	16	1	20	4
P	0.023	0.028	<0.001	<0.001	n.s.	n.s.	n.s.	n.s.
Covariate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a]Mean values within columns followed by the same letter are not significantly different at $p \leq 0.05$.

[b]Mz = mancozeb, Comp 4= Compound 4. Values followed by "×" refer to a multiple of the standard rate according to table 1.3.1.

[c]Total CBS is the total of all lesion types; freckle spot, hard spot, virulent spot and speckled blotch (data not shown).

[d]Incidence refers to the proportion of fruit with symptoms of CBS.

[e]Severity refers to the number of spots per fruit. In the case of Total CBS, the measures of fruit surface area were converted to a number of spots based on the assumption that 1% of fruit surface area was equivalent to 10 spots of 3mm diameter.

1.4 Summary

The aims of this chapter were to: 1) improve our understanding of the response of *P. citricarpa* to existing postharvest fungicides; and 2) evaluate alternative options for field control of the fungus. Addressing these aims has shown that:

1) *P. citricarpa* is sensitive to existing postharvest fungicides, supporting the hypothesis that variable efficacy may be due to poor contact between the fungicide and the pathogen. It may therefore be necessary to focus future efforts for developing a postharvest solution to CBS on ways of overcoming this issue, such as penetrants/surfactants.
2) The only fungicide identified from the study, and previous related studies (Miles and Drenth, 2013), that can offer similar efficacy to mancozeb against CBS was Compound 4. Even then, potential phytotoxicity issues may exist in some cases for this fungicide, possibly when used with, or close to, oil applications. However, given that few other options exist, and that Compound 4 is also effective against EBS (see ***chapter 2***), it may well be worth undertaking studies to better understand the phytotoxicity risk with Compound 4 and oil, or other possible causes.

As an additional summary point, the reducing number of fungicide options for CBS management, and reliance on mancozeb, is particularly concerning considering that mancozeb is a priority group 1 fungicide for review by the APVMA. Without adequate alternatives to this fungicide, the loss of mancozeb would be highly problematic for growers affected by the disease. It is highly recommended that CBS management research continues in order to mitigate the potential risk of losing mancozeb.

Chapter 2
'Emperor' brown spot

2.1 Introduction

'Emperor' brown spot (EBS), caused by the fungus *Alternaria alternata* (Pegg, 1966), is the most directly damaging fungal disease of citrus in Queensland orchards. EBS is estimated to cost the Australian citrus industry approximately $5M annually (Miles *et al.*, 2011). The disease was first reported from coastal Queensland in the 1960's, and has since become widespread where susceptible varieties are grown in subtropical regions (Pegg, 1966; Miles *et al.*, 2015). These varieties include mandarins (e.g. 'Emperor'), tangors and tangor hybrids (e.g. 'Murcott'). Symptoms on leaves and shoots are typically large necrotic areas, surrounded by a chlorotic halo and often associated with vein darkening, premature senescence and entire shoot death (Fig. 2.1.1) (Pegg, 1966; Swart *et al.*, 1998; Timmer *et al.*, 2000). Symptoms on fruit are expressed as sunken, brown lesions, observed reaching up to 5 mm in diameter. A chlorotic halo often surrounds lesions on green fruit, with the halo becoming indistinguishable as fruit colour. If conditions become unfavourable for the pathogen, lesions can be dry, corky scars which may or may not spread when conditions are again favourable.

EBS has a relatively simply disease cycle whereby the fungus sporulates on dead tissues in the tree canopy and on abscised leaves and twigs on the orchard floor (Timmer *et al.*, 1998a). The conidia are then dispersed by wind, then adhere to susceptible tissue and germinate, before the toxin produced by the fungus initiates cell necrosis within as little as 30 hours. The nature of this disease cycle means that control has tended to rely on the protective application of fungicides such as copper, mancozeb, azoxystrobin and iprodione (Hutton, 1989; Vicent *et al.*, 2007; Miles *et al.*, 2005). While these fungicides represent a relatively high number of options, the theoretical number of weeks of protection achievable with the four existing fungicides and their use patterns is approximately 23 weeks (Miles 2011; Vicent et al. 2007), out of an approximately 40-week-long season. Furthermore, the real-world number of weeks of protection is likely to be far less than 23 weeks due to declining fungicide coverage due to rainfall and fruit expansion (Timmer *et al.*, 1998b). Copper fungicides can also cause rind stippling (Schutte *et al.*, 1997) and mancozeb can be disruptive to the IPM predator *Amblyseius victoriensis* (Smith and Papacek, 1991). Resistance development in *Alternaria* sp. has also been shown to be a risk for iprodione in citrus (Hutton, 1989) and azoxystrobin in other crops (Luo *et al.*, 2012; Rosenzweig *et al.*, 2008). For these reasons additional control options are still required.

Further to simply evaluating fungicides for efficacy against EBS in the field, the duration of efficacy after an application of a fungicide is rarely investigated. The result of this are typically arbitrary re-application intervals that do not consider issues of rain fastness or fruit expansion (Timmer *et al.*, 1998b; Vicent *et al.*, 2007). It is therefore important to gain an understanding of how long different fungicides provide high levels of fruit protection.

While fungicides present a short term approach to EBS management, breeding for resistance to the disease presents a robust, long term solution to the disease. Resistance screening has become routine for the breeding program based at the

Bundaberg Research Facility (Miles *et al.*, 2015). This hybridisation breeding program is ideally suited to breeding for resistance to EBS, as the genetics of resistance to EBS is based on a single, dominant gene causing sensitivity to the toxin produced by the fungus (Akimitsu, 2009; Pegg, 1966; Dalkilic *et al.*, 2005). Hybrids lacking this gene should carry a long and stable resistance to EBS, as for varieties such as 'Imperial'.

This chapter aims to address the need for improved management strategies for EBS that are cost effective, and comply with food safety requirements by: 1) providing technical support for screening for EBS resistance; 2) investigating the potential to improve resistance screening methods; 3) evaluating fungicides for the control of EBS; and 4) determining the duration of efficacy of fungicices. Addressing these aims will provide short term management options (fungicides) and long term control measures (resistance) for the Qld citrus industry.

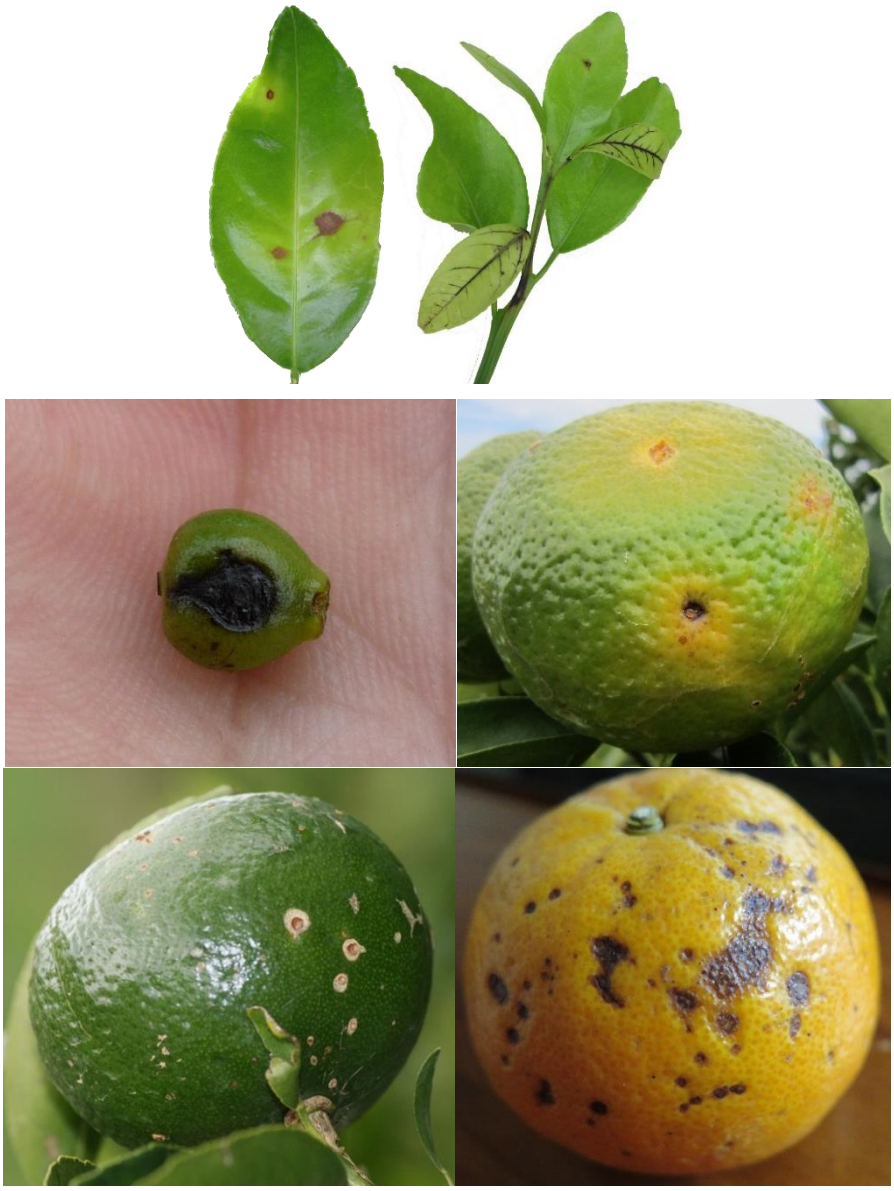

Figure 2.1.1. Symptoms of 'Emperor' brown spot on leaves and fruit.

2.2 Screening for resistance to 'Emperor' brown spot

Introduction

Many current commercial varieties are susceptible to EBS, necessitating costly fungicide inputs in order to ensure satisfactory pack out. New varieties that are resistant to the disease would be highly beneficial to the industry. The reduced fungicide use from resistant varieties would significantly improve pack out and reduce fungicide residues, in line with the industry's commitment to food safety. Reduced fungicide input would also improve efficiency through reduced chemical and labour costs.

EBS presents as lesions on fruit and shoots of susceptible mandarins (e.g. Emperor, Taylor Lee), tangelos (e.g. Minneola) and tangors (e.g. Murcott) (Pegg 1966). The disease is highly destructive, causing symptoms on fruit and shoots in as little as 30 hours, then spreading and initiating new infections. As damage occurs so quickly, disease control relies heavily on repeated fungicide sprays to protect fruit and shoots from damage. This fungicide reliance is expensive and risky, as a single poorly timed fungicide can result in very high losses of fruit. One orchard has reported losses of $35/tree, even though fungicides were applied. Reliance on fungicides also makes the industry vulnerable when chemical registrations change, chemicals are withdrawn from market, and presents difficulty in complying with export market residue requirements.

In order to develop a robust, long term solution to EBS, a resistance screening step in project CT09014 *Early-season replacement for Imperial mandarin* based in Bundaberg was developed and implemented in collaboration with citrus breeder Malcolm Smith and plant pathologist Andrew Miles under project CT07012 (Miles and Drenth, 2013). The screening program has proven effective, having produced ~20,000 resistant hybrids from the 2011, 2010, and 2009 hybrid pollinations (Miles *et al.*, 2015).

The hybridisation breeding program is ideally suited to breeding for resistance to EBS. The genetics of resistance to EBS is based on a gene causing sensitivity to the toxin produced by the fungus (Akimitsu 2009; Pegg 1966). The inheritance of toxin sensitivity is related to a single dominant gene (Dalkilic *et al.* 2005). Therefore hybridisation results in progeny that is either resistant or susceptible, depending on the parents used. This form of resistance is likely to be very robust, as it is most probably the same resistance mechanism as in varieties such as Imperial and Ellendale, which have remained completely resistant to EBS for many decades.

To have the best chance of commercialising EBS resistant mandarin varieties, the resistance screening process needs to be applied to every generation of hybridising. Throughout this project the resistance screening has continued in the mandarin breeding program at Bundaberg with material and technical support from project CT13020 as required.

Methods

The EBS resistance screening process has been described thoroughly (Miles *et al.*, 2015; Miles and Drenth, 2013), and remains a routine practice in the mandarin breeding program.

Results and discussion

At the conclusion of project CT07012 (Miles and Drenth, 2013) in August 2013, the breeding program had field planted just over 20,000 resistant hybrids. From August 2013 until the conclusion of project CT13020 in December 2016, a further 12,750 resistant hybrids have been field planted for horticultural evaluation. More specifically, 2,560, 6,730, and 3,460 resistant hybrids from pollinations made in 2012, 2013, and 2014, respectively. Since the commencement of the resistance screening program in 2011 the total number of resistant hybrids is ~32,000. It should be noted that the resistance screening has become routine for the breeding program, requiring minimal outside expertise. Furthermore, the breeding program team has implemented an additional screen for resistance to scab, caused by *Elsinoë fawcettii* (Smith *et al.*, 2016).

2.3 Alternative approaches to screening for resistance

Introduction

While the existing screening method is yielding excellent results for the breeding program, there is still potential to improve the efficiency of the method. Two novel approaches to achieving this are: 1) direct seed inoculation; and 2) toxin sensitivity testing of germinating seed. The major advantage of both approaches is screening for resistance at the earliest possible stage in the breeding program. Raising only resistant hybrids means time and space is not dedicated to susceptible progeny that will ultimately be culled. This also creates the opportunity to screen larger numbers of hybrids, knowing that resources currently used for raising susceptible progeny, could be used for resistant progeny instead. Pilot experiments conducted by Andrew Miles and Malcolm Smith have shown that peeled citrus seed can be inoculated with spores of *Alternaria alternata*, and segregate as resistant and susceptible. However, further work is needed to confirm that the seed reaction is consistent with the field reaction. Similarly, it is expected that sensitivity to the 'ACT' toxin produced by *A. alternata* is a trait expressed in seed (Kohmoto et al 1993; Pegg 1966; Dalkilic 2005). Therefore testing the sensitivity of seed to purified toxin may provide an effective method in the absence of pathogen itself. Therefore the aim of these experiments was to confirm if seed testing by either method is an accurate and viable option for resistance screening.

Methods

To further investigate direct seed inoculation, seed of sweet orange (resistant), Weikiwa (susceptible) and Murcott (susceptible) was harvested, dried, vacuumed packed and stored at 4˚C. When needed, the seed were peeled and stored in petri dishes sealed inside a bag containing wet paper towel at 4˚C for no longer than 48 hours. The seed was weighed into three replicate batches of 2.5 g each. Three replicates of seed of each cultivar were dipped for 30 seconds in either distilled water, or conidia suspensions of AKM452 (mandarin strain) or AKM470 (core rot strain) prepared to 2,500 conidia/mL. The seeds were tipped through a tea strainer, then spread evenly over the base of a petri dish. The presence of conidia on the seed surface was confirmed using a dissecting microscope. The petri dishes were then incubated at room temperature in a sealed plastic container lined with blotting paper

saturated in tap water. The seed were observed every 24 hours for disease development.

In order to produce host specific toxin from isolates of *Alternaria alternata* from citrus, isolates of the fungus from mandarin 'Emperor' brown spot lesions, rough lemon brown spot lesions, and navel orange core rot were grown in Czapek's Dox solution. Liquid cultures of isolate AKM 452, 459 (mandarin), 461 (rough lemon) and 470 (core rot) were grown for 14 days, then filtered progressively from Whatman No. 1 filters, to 0.2 µm Millipore filters to produce sterile culture filtrates. Filtrate from each isolate was stored in ~5mL aliquots and stored at both -20˚C and 4˚C. The subsequent production of toxin was tested by immersing the cut end of a young, susceptible terminal shoot of both rough lemon and Murcott into the culture filtrate. The shoots were then observed for symptoms.

In order to determine the uptake of the rough lemon toxin through the tap roots of seedlings of rough lemon and susceptible mandarin, seed were first germinated in rockwool for 3 weeks. The seed were transferred to 5 replicate vials each, containing ~1.5 mL of either rough lemon filtrate or core rot filtrate prepared as 1:0, 1:1 or 1:10 vol:vol filtrate and water. Water only was used as an additional control. The tap root only was immersed in the liquid. Shoots from the same batch of germinated seed were cut above the seed, and the cut end immediately immersed in filtrate or water. All the vials were transferred to a humid chamber for incubation and observation of symptoms over 2 weeks.

Results and discussion

After inoculation of seed, conidia were visible on the surface of the seed. During incubation, no necrosis of the seed was observed. After 6 days, all of the inoculated seed were covered in a mat of mycelium, indicating that incubation conditions were favourable for fungal growth. However, previous pilot studies have shown necrosis to develop in compatible inoculations. It is therefore suspected that the isolates used which should have produced necrosis are possibly no longer pathogenic.

The isolate used was AKM 452, which has been shown previously to be highly pathogenic (Miles *et al.*, 2015). In subsequent studies the BRS team reported good symptom development from inoculations with AKM 452. However, storage conditions of this isolate at BRS and in Brisbane are significantly different. At BRS the isolate is stored as mycelium on agar at 4˚C, while in Brisbane it has been stored as a conidia suspension in glycerol at -20˚C and -80˚C (standard herbarium process). The loss of pathogenicity may be due to sufficient genetic variation occurring within conidia. Cultures of AKM 452 from BRS were obtained for subsequent work, and were routinely stored using the BRS method without further issues regarding pathogenicity.

The bioassay to determine if the culture filtrates contained host specific toxin indicated that toxin symptoms were only produced in the shoots of rough lemon immersed in the filtrate from the rough lemon isolate (AKM 461). All other shoots remained healthy. This showed that only the rough lemon toxin was produced, and the rough lemon toxin was specific to rough lemon shoots.

Using the lemon toxin and rough lemon seed, uptake of the toxin via roots was tested. In the root uptake assay after 7 days, only the cut shoots immersed in the rough lemon filtrate showed symptoms of wilting and necrosis, while all other shoots (cut or with tap root intact) remained healthy. At the end of the incubation period, some signs of wilting

were appearing in the rough lemon filtrate, but all other seedlings remained healthy in appearance (except for the cut shoots previously mentioned). This suggests that movement of the toxin might occur very slowly through the tap root at high concentrations. Using the rough lemon toxin as a model, it appears that using a root-upwards approach with culture filtrate as a screening method would not be highly useful to the breeding program. In addition to the studies outlined in this project an Honours student project proposal was developed and submitted to collaborators at the University of Queensland. However, a suitable candidate student could not be found.

A highly efficient disease screening system has been developed for EBS. It is now standard procedure in the Australian breeding program and substantial field plantings of genetically resistant new hybrids have been produced using this screening system. Efforts to further improve the system through the use of seed inoculation and direct toxin application have been unsuccessful and require more research before they can be applied in the breeding program. However the incorporation of the additional pathogen causing Scab disease (*Elsinoë fawcettii)* has enable the co-inoculation of seedlings and culling of hybrids for two important diseases prior to field planting, thus increasing the efficiency of the existing system.

2.3 Field evaluation of fungicides

Introduction

As discussed in ***chapter 1*** of this report, overcoming the limitations of the fungicides currently used for controlling citrus diseases in Qld requires the expansion of the range of active ingredients. As previously discussed for CBS, a short list of potential options was developed around three main criteria: 1) high efficacy potential based on existing studies; 2) active ingredients outside of the resistance activity groups already used in citrus; and 3) have favourable residue profiles for domestic and export markets. This short listing process is thoroughly explained in, and commenced under, the previous project CT07012 (Miles and Drenth, 2013) The succinate dehydrogenase inhibitor (SDHI) fungicides have been idenfitied as a good fit with these criteria for EBS control. The SDHI group of fungicides target fungal respiration through inhibition of the ubiquinone-binding sites in the mitochondrial complex II (Avenot and Michailides, 2010). The SDHI fungicide, Compound 6, is used to manage EBS in Florida (Vega and Dewdney, 2014), suggesting efficacy under Queensland conditions is likely. However, like the strobilurin fungicides, the specific mode of action of the SDHI group increases the risk of resistance development. The best approach to managing this risk would be to also pursue a multisite activity fungicide for alternating with an SDHI fungicide in the field. Multisite activity fungicides disrupt cell function across a range of processes, therefore resistance is unlikely to develop from any single point mutation such as for strobilurins or SDHI fungicides. For example, the pthalimide fungicide, captan, and quinone fungicide, Compound 4, disrupt enzyme function (Hewitt, 1998). Previous studies have suggested efficacy of captan against EBS (Miles *et al.*, 2005; Timmer and Zitko, 1997), making captan a promising candidate for use in Qld orchards. Similarly, Compound 4 is reported to be efficacious against several citrus diseases including CBS (Tsai, 1981), scab (Whiteside, 1990) and melanose (Jwu-guh and Tsai-young, 1989; Whiteside, 1977).

Also critical to successful commercial use is ensuring that any new fungicide options do not negatively impact on the internal fruit quality. The Australian citrus industry has recently introduced the Australian Citrus Quality Standards in order to ensure acceptable fruit eating quality for consumers. It would be counterproductive if any new control measure for EBS resulted in reduced internal characters such as juice content, Brix and % acid.

Independent field evaluations of alternative fungicides for the control of EBS in Australia have not been undertaken since 2003 (Miles *et al.*, 2005). While this previous work resulted in the registration of azoxystrobin for EBS control in Australia, management of this disease has remained problematic. The aim of this study is to directly continue efforts started under project CT07012 (Miles and Drenth, 2013) to evaluate the efficacy of alternative fungicides for the control of EBS in Australia.

Methods

Treatment application and disease assessment

In order to determine the efficacy of various fungicides against EBS (Table 2.3.1), field experiments were conducted in high disease pressure commercial orchards in the Central Burnett and Wide Bay regions of Queensland, Australia. Various treatment applications were made initially throughout the entire season, but later focused on the autumn/winter period when EBS has been observed to be most prevalent in our studies. The treatments were applied to four individual replicate trees in commercial orchards using a custom built hand lance sprayer with dual D4 hollow cone nozzles, operating at 50 psi delivered by a 6.0 horsepower Subaru Robin EX17 gas engine-driven pressure pump (Subaru, Japan). All experiments included an untreated control, and mancozeb at the standard rate as a positive control.

Disease was assessed when fruit reached commercial maturity. One hundred fruit were arbitrarily selected from each data tree, comprising of approximately 50 fruit from each row-side of the canopy. To determine the disease severity, the numbers of EBS lesions were counted on each fruit. The incidence of EBS in each data tree was then calculated as the proportion of fruit with one or more lesions. To determine the mean fruit weight, the total weight of the sampled fruit from each data tree was measured and divided by the total number of sampled fruit.

In order to assess any impact of the fungicide treatments on external and internal fruit quality, fruit samples from each replicate tree were taken for assessment in experiments conducted in 2013-14 (experiment 2.3.2) and 2014-15 (experiment 2.3.3). For external quality any notable fruit defects were recorded during disease assessment. For internal quality juice content, Brix, % acid, the Brix acid ratio, and final ACQ standard were determined according to the Australian Citrus Quality Standards Manual.

Table 2.3.1. Product names, active ingredients, fungicide group, and standard rates used in EBS control experiments carried out in Queensland.

Product name	Active ingredient	Group (FRAC code)	Standard rate of product	Standard rate of active ingredient
Amistar 250 SC	25% azoxystrobin	Quinone outside inhibitors (C3)	0.40 mL/L	0.100 g/L
Antracol	70% propineb	Dithiocarbamate (multi-site)	2.00 g/L	1.400 g/L
Captan 800WG	80% captan	Phthalimides (multi-site)	1.25 g/L	1.000 g/L
Chief Aquaflo	50% iprodione	Dicarboximide (E3)	1.00 mL/L	0.500 g/L
Compound 1	Undisclosed	Succinate dehydrogenase inhibitors (C2)	0.20 mL/L	0.100 g/L
Compound 2	Undisclosed	Undisclosed (multi-site)	0.50 g/L	0.350 g/L
Compound 4	70% a.i.	Quinone (multi-site)	0.70 g/L	0.500 g/L
Compound 5	20% a.i.	Succinate dehydrogenase inhibitors (C2)	0.75 mL/L	0.150 g/L
Compound 6	50% a.i.	Succinate dehydrogenase inhibitors (C2)	0.30 g/L	0.150 g/L
Lorsban 500 EC	50% chlorpyrifos	N/A – insecticide	1.00 mL/L	0.500 g/L
Penncozeb 750DF	75% mancozeb	Dithiocarbamate (multi-site)	2.00 g/L	1.500 g/L
Red copper WG	50% cuprous oxide	Inorganic (multi-site)	1.35 g/L	0.675 g/L
Compound 7	40% a.i.	Anilino-pyrimidines (D1)	1.00 g/L	0.400 g/L
Compound 8	12.5% a.i.	Succinate dehydrogenase inhibitors (C2)	0.80 g/L	0.100 g/L
Compound 9	30% a.i.	Succinate dehydrogenase inhibitors (C2)	0.25 mL/L	0.075 g/L
SprayPhos 620	62% phosphorus acid	Unknown (Unknown MOA)	2.25 mL/L	1.395 g/L

Table 2.3.2. Treatment schedule applied to trees in experiment 2.3.2.

	Application date												
Treatment[a]	**14/10/13**	**6/11/13**	**19/11/13**	**4/12/13**	**16/12/13**	**2/1/14**	**13/1/14**	**29/1/14**	**11/2/14**	**25/2/14**	**18/3/14**	**8/4/14**	**29/4/14**
Control	-	-	-	-	-	-	-	-	-	-	-	--	-
Mz standard	Mz	-	Mz	-	Mz	-	Mz	-	Mz	-	Mz	Mz	Mz
All options	Cu	Mz + Ipr	Azo	Mz	Comp 6 2×	Azo	Ipr	Cap 2×	Comp 6 2×	Cap 2×	Ipr	Comp 6 2×	Cap 2×
Comp 6 2×	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	-	Comp 6 2×	Comp 6 2×	Comp 6 2×
Comp 6	Comp 6	-	Comp 6	-	Comp 6	-	Comp 6	-	Comp 6	-	Comp 6	Comp 6	Comp 6
Comp 6 0.6×	Comp 6 0.6×	-	Comp 6 0.6×	-	Comp 6 0.6×	-	Comp 6 0.6×	-	Comp 6 0.6×	-	Comp 6 0.6×	Comp 6 0.6×	Comp 6 0.6×
Comp 81.5×	Comp 8 1.5×	-	Comp 8 1.5×	-	Comp 8 1.5×	-	Comp 8 1.5×	-	Comp 8 1.5×	-	Comp 8 1.5×	Comp 8 1.5×	Comp 8 1.5×
Comp 8	Comp 8	-	Comp 8	-	Comp 8	-	Comp 8	-	Comp 8	-	Comp 8	Comp 8	Comp 8
Cap 2×	Cap 2×	-	Cap 2×	-	Cap 2×	-	Cap 2×	-	Cap 2×	-	Cap 2×	Cap 2×	Cap 2×
Cap 1.5×	Cap 1.5×	-	Cap 1.5×	-	Cap 1.5×	-	Cap 1.5×	-	Cap 1.5×	-	Cap 1.5×	Cap 1.5×	Cap 1.5×
Cap	Cap	-	Cap	-	Cap	-	Cap	-	Cap	-	Cap	Cap	Cap
Ipr 2×	Ipr 2×	-	Ipr 2×	-	Ipr 2×	-	Ipr 2×	-	Ipr 2×	-	Ipr 2×	Ipr 2×	Ipr 2×
Ipr	Ipr	-	Ipr	-	Ipr	-	Ipr	-	Ipr	-	Ipr	Ipr	Ipr
Ipr 0.5×	Ipr 0.5×	-	Ipr 0.5×	-	Ipr 0.5×	-	Ipr 0.5×	-	Ipr 0.5×	-	Ipr 0.5×	Ipr 0.5×	Ipr 0.5×
Comp 4 2×	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	-	Comp 4 2×	Comp 4 2×	Comp 4 2×
Comp 4	Comp 4	-	Comp 4	-	Comp 4	-	Comp 4	-	Comp 4	-	Comp 4	Comp 4	Comp 4

[a]Mz = mancozeb, Cu = cuprous oxide, Ipr = iprodione, Azo = azoxystrobin, Comp 6 = Compound 6, Cap = captan, Comp 8 = Compound 8, Comp 4 = Compound 4. Values followed by "×" refer to a multiple of the standard rate according to table 2.3.1.

Experiment 2.3.1

In 2012-13 fungicide efficacy was evaluated in a field trial located near Wallaville, Qld (-25.113479, 151.992485). The trial comprised of 'Murcott' tangor (*Citrus* × *aurantium*) trees on 'Benton' rootstock (*C.* × *aurantium* × *C. trifoliata*), planted in 2009 at a 7 m × 4 m spacing. Treatments were applied eight times at approximately monthly intervals (28/9/12, 2/11/12, 30/11/12, 3/1/13, 6/2/13, 7/3/13, 10/4/13, and 8/5/13). Mancozeb and phosphorus acid were applied at the standard rate. Captan, Compound 1, Compound 2, Compound 5, Compound 6, Compound 9 and iprodione were all applied at the standard and double the standard rate. Treatment applications were made at 10 L per tree. Fruit were harvested on the 19/7/13. Following disease assessment the overall visual appearance of the fruit collectively from each data tree was ranked on a 1-10 scale, whereby 1 = the poorest presentation and 10 = the best presentation.

Experiment 2.3.2

In 2013-14 fungicide efficacy was evaluated in a field trial located near Mundubbera, Qld (-25.628433, 151.219270). The trial comprised of 'Daisy' mandarin (*Citrus reticulata*) trees on alternating 'Troyer' (*C. sinensis* × *Poncirus trifoliata*) and 'sweet orange' (*C. sinensis*) rootstocks, planted in 2005 at a 7.3 m × 5.5 m spacing. Treatments were applied according the schedules in table 2.3.2. Treatment applications were made at 10 L per tree. Fruit were harvested on the 15/5/14. At harvest samples for internal fruit quality assessment were taken from the control, mancozeb standard, 'All options', Compound 6, Compound 8 1.5×, captan 2×, iprodione 2×, and Compound 4 2× treatments.

Experiment 2.3.3

In 2014-15 fungicide efficacy was evaluated in a field trial near Mundubbera, Qld (-25.654136, 151.185448). The trial comprised of 'IrM2' Murcott tangor (*Citrus* × *aurantium*) trees on 'Troyer' rootstock (*C. sinensis* × *Poncirus trifoliata*), planted in 2006 at a 7.3 m × 4 m spacing. Treatments were applied according the schedules in table 2.3.3. Treatment applications were made at 10 L per tree. Fruit were harvested on the 1/7/15. At harvest samples for internal fruit quality assessment were taken from the control, mancozeb standard, Compound 6 2×, Captan 2×, Compound 4 2×, Compound 9, and the Compound 6/captan use pattern.

Experiment 2.3.4

In 2015-16 fungicide efficacy was evaluated near Mundubbera, Qld (-25.600309, 151.298271). The trial comprised of 'IrM2' Murcott tangor (*Citrus* × *aurantium*) trees on 'Volkameriana' rootstock (*C. volkameriana*), planted in 2000 at a 6 m × 3 m spacing. Treatments were applied according the schedules in table 2.3.4. Treatment applications were made at 10 L per tree. Fruit were initially harvested on the 19/7/16, but due to a lack of disease a 5th treatment application was made and the trial harvested again on the 16/8/16.

Statistical analysis

The mean disease incidence and severity was determined for each data tree. The mean values was then subjected to analysis of variance (ANOVA) in GenStat 16th Edition (VSN International, UK). Arcsine angular transformation was applied to incidence data, and square root or fourth root transformations applied to severity data, where required to normalise the data. Fruit presentation data were also analysed by ANOVA. In order to account for any effects of fruit size, mean fruit weight was included as a covariate. The % juice, Brix and Brix acid ratio data were were also analysed by ANOVA.

Table 2.3.3. Treatment schedule applied to trees in experiment 2.3.3.

	Date of application							
Treatments[a]	**2/10/14**	Grower applied 4/11/14	Grower applied 9/12/14	**13/1/15**	**4/3/15**	**31/3/15**	**28/4/15**	**4/6/15**
Control	-	Cu	Mz	-	-	-	-	-
Mz standard	-	Cu	Mz	-	Mz	Mz	Mz	Mz
Comp 6 2×	-	Cu	Mz	-	Comp 6 2×	Comp 6 2×	Comp 6 2×	Comp 6 2×
Comp 6	-	Cu	Mz	-	Comp 6	Comp 6	Comp 6	Comp 6
Comp 6 0.5×	-	Cu	Mz	-	Comp 6 0.5×	Comp 6 0.5×	Comp 6 0.5×	Comp 6 0.5×
Cap 2×	-	Cu	Mz	-	Cap 2×	Cap 2×	Cap 2×	Cap 2×
Cap 1.5×	-	Cu	Mz	-	Cap 1.5×	Cap 1.5×	Cap 1.5×	Cap 1.5×
Cap	-	Cu	Mz	-	Cap	Cap	Cap	Cap
Comp 4 2×	-	Cu	Mz	-	Comp 4 2×	Comp 4 2×	Comp 4 2×	Comp 4 2×
Comp 4	-	Cu	Mz	-	Comp 4	Comp 4	Comp 4	Comp 4
Comp 4 0.5×	-	Cu	Mz	-	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×
Comp 7	-	Cu	Mz	-	Comp 7	Comp 7	Comp 7	Comp 7
Comp 7 0.1×	-	Cu	Mz	-	Comp 7 0.1×	Comp 7 0.1×	Comp 7 0.1×	Comp 7 0.1×
Comp 9	-	Cu	Mz	-	Comp 9	Comp 9	Comp 9	Comp 9
Comp 9 0.6×	-	Cu	Mz	-	Comp 9 0.6×	Comp 9 0.6×	Comp 9 0.6×	Comp 9 0.6×
Comp 9 0.4×	-	Cu	Mz	-	Comp 9 0.4×	Comp 9 0.4×	Comp 9 0.4×	Comp 9 0.4×
Comp 6 2× / cap 1.5×	-	Cu	Mz	-	Comp 6 2×	Cap 1.5×	Comp 6 2×	Cap 1.5×
Ipr permit[b]	Ipr	Cu	Mz	Ipr	Mz	Ipr	Mz	Mz

[a]Cu = cuprous oxide, Mz = mancozeb, Comp 6 = Compound 6, Cap = captan, Comp 4 = Compound 4, Comp 7 = Compound 7, Comp 9 = Compound 9, Ipr = iprodione. Values followed by "×" refer to a multiple of the standard rate according to table 2.3.1.

[b]Application timing according APVMA permit number 14772.

Table 2.3.4. Treatment schedule applied to trees in experiment 2.3.4.

	Application date				
Treatments[a]	**8/3/16**	**6/4/16**	**2/5/16**	**3/6/16**	**20/7/16**
Control	-	-	-	-	-
Mz standard	Mz	Mz	Mz	Mz	Mz
Comp 9	Comp 9	Comp 9	Comp 9	Comp 9	Comp 9
Comp 9 0.6×	Comp 9 0.6×	Comp 9 0.6×	Comp 9 0.6×	Comp 9 0.6×	Comp 9 0.6×
Comp 9 0.4×	Comp 9 0.4×	Comp 9 0.4×	Comp 9 0.4×	Comp 9 0.4×	Comp 9 0.4×
Cap/Comp 9x Use pattern	Comp 9 0.6×	Cap 1.5×	Comp 9 0.6×	Cap 1.5×	Cap 1.5×
Cap 1.5×	Cap 1.5×	Cap 1.5×	Cap 1.5×	Cap 1.5×	Cap 1.5×
Cap+Comp 9 Tank Mix	Cap + Comp 9 0.4×	Cap + Comp 9 0.4×	Cap + Comp 9 0.4×	Cap + Comp 9 0.4×	Cap + Comp 9 0.4×
Comp 1 2×	Comp 1 2×	Comp 1 2×	Comp 1 2×	Comp 1 2×	Comp 1 2×
Comp 1 1×	Comp 1 1×	Comp 1 1×	Comp 1 1×	Comp 1 1×	Comp 1 1×
Comp 4	Comp 4	Comp 4	Comp 4	Comp 4	Comp 4
Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×	Comp 4 0.5×
Comp 4 0.3×	Comp 4 0.3×	Comp 4 0.3×	Comp 4 0.3×	Comp 4 0.3×	Comp 4 0.3×
Ipr/Mz/Chlor	Mz + Ipr	Mz + Ipr	Mz + Ipr	Mz + Ipr + Chlor	-
Pro	Pro	Pro	Pro	Pro	Pro

[a]Mz = mancozeb, Comp 9 = Compound 9, Cap = captan, Comp 4 = Compound 4, Ipr = iprodione, Chlor = chlorpyrifos, Pro = propineb. Values followed by "×" refer to a multiple of the standard rate according to table 2.3.1.

Results

The incidence and severity of EBS was >90% and >8 lesions per fruit, respectively, in experiments 2.3.1, 2.3.2, and 2.3.3 (Table 2.3.5). Experiment 2.3.3 saw particularly high severity of EBS at nearly an average of 30 lesions per fruit. Experiment 2.3.4 was exceptional in contrast, with the EBS levels being too low to be able to make any meaningful assessment of the treatments, hence no data are shown.

In most cases the mancozeb standard significantly reduced EBS relative to the control. However, most of the SDHI and multisite fungicide treatments showed further significant reductions in EBS compared to mancozeb. Outside of the SDHI and multiste treatments phosphorous acid and Compound 7 showed very poor control of EBS, with the exception of iprodione which was very effective in most cases.

Excellent control of EBS was generally observered across all the SDHI fungicides, with Compound 8 being marginally less promsing than the others based on comparisons of efficacy relative to Compound 6 across the three experiments. Compound 6 has shown consistently high efficacy across all three experiments, as has Compound 9 in experiments 2.3.1 and 2.3.3. The consistency of performance of Compound 1 cannot be ascertained due to the low disease pressure in experiment 2.3.4. In direct comparison of Compound 6 and Compound 9, Compound 9 showed equivalent efficacy to Compound 6 at generally half the rate of active ingredient.

Similar to the SDHI fungicides, all the multisite activity fungicides showed very promsing efficacy against EBS. Captan in particular showed consistent efficacy across all three experiments, and typically not significantly different from the SDHI fungicides. Compound 4 was typically not signficiantly different from captan, except for some of the lower rates. While Compound 2 looked promising, this fungicide was withdrawn by the manufacturer.

When considering some of the use patterns included in the experiments, the efficacy of the SDHI fungicides and captan is particularly good. In experiment 2.3.2 most of the captan treatments were equivalent to the “All options” treatment, which included a higher frequency and overall number of fungicide applications. Similarly, most of the SDHI and captan treatments in experiment 2.3.3 were equivalent to the “grower” program which was based on a much higher frequency of fungicide applications; 15 fungicides at appromxately 3 week intervals throughout the season. More specifically, the Compound 6/captan use pattern achieved equivalent control to this “grower” treatment with a total of six fungicide applications (copper, mancozeb, Compound 6, captan, Compound 6, captan). The alternation of Compound 6 and captan (Comp 6/cap treatment in experiment 2.3.3) should also offer good resistance management. Alternating Compound 9 with captan would also most likely offer excellent EBS control. Interestingly, the efficacy of the iprodione permit use pattern was very poor.

The external fruit quality assessments noted no negative effects of the fungicide treatments on external fruit appearance. The internal fruit quality assessments from experiments 2.3.2 and 2.3.3 generally found no significant differences among the treatments for % juice, Brix, Brix acid ratio, and ACQ standard. The only exception was for Brix in experiment 2.3.2, whereby the captan 2×, Compound 4 2×, ‘All options’ and bocalid treatments had significantly lower Brix than the control. However, this did not result in significant differences in the Brix acid ratio or final ACQ result between

the different treatments. In both experiments the average ACQ values were above the established mandarin ACQ of 110, at 125 and 120, respectively.

Table 2.3.5. Results of fungicide efficacy experiments 2.3.1, 2.3.2 and 2.3.3[a].

		Experiment 2.3.1		Experiment 2.3.2		Experiment 2.3.3	
Treatment	**g (a.i.)/L**	**Incidence**	**Severity**	**Incidence**	**Severity**	**Incidence**	**Severity**
Control	-	96 a	2.9 (8.2) a	1.29 (92.33) a	1.7 (8.9) a	100 a	5.4 (29.1) a
Mz standard	1.500	71 bc	1.6 (2.6) c	0.59 (30.53) c	0.9 (0.6) cd	90 ab	2.7 (7.4) c
SDHI							
Comp 6 2×	0.300	44 ef	0.9 (0.8) efgh	0.32 (9.65) ef	0.6 (0.1) f	68 cde	1.5 (2.3) g
Comp 6	0.150	53 de	1.1 (1.2) def	0.52 (24.70) cd	0.8 (0.4) de	66 de	1.6 (2.5) fg
Comp 6 0.6×	0.100			0.54 (26.54) cd	0.8 (0.4) de		
Box 0.5×	0.075					78 bcd	1.8 (3.2) efg
Comp 1	0.200	35 fgh	0.8 (0.6) fgh				
Comp 1 0.5×	0.100	66 cd	1.3 (1.7) cd				
Comp 9 2×	0.150	28 gh	0.7 (0.4) gh				
Comp 9	0.075	27 h	0.6 (0.4) h			70 cde	1.7 (2.9) efg
Comp 9 0.6×	0.050					67 de	1.6 (2.6) efg
Comp 9 0.4×	0.033					75 bcde	1.8 (3.4) defg
Comp 8 1.5×	0.150			0.78 (49.84) b	1.0 (1.1) bc		
Comp 8	0.100			0.81 (52.52) b	1.2 (2.0) b		
Comp 5 2×	0.300	40 efgh	0.8 (0.7) efgh				
Comp 5	0.150	63 cd	1.2 (1.4) de				
Multisite							
Cap 2×	2.000	42 efg	1.0 (1.1) defg	0.37 (12.77) ef	0.7 (0.2) ef	62 e	1.6 (2.5) efg
Cap 1.5×	1.500			0.34 (11.22) ef	0.6 (0.1) f	61 e	1.4 (2.1) g
Cap	1.000	61 cd	1.3 (1.8) cd	0.53 (25.22) cd	0.9 (0.7) cd	76 bcde	1.8 (3.2) efg
Comp 2	0.070	36 fgh	0.8 (0.7) efgh				
Comp 2 0.5×	0.035	42 efg	1.0 (1.0) defgh				
Comp 4 2×	1.000			0.33 (10.43) ef	0.6 (0.1) f	75 bcde	1.8 (3.2) efg
Comp 4	0.500			0.41 (15.98) de	0.7 (0.2) ef	83 bc	2.3 (5.3) cde
Comp 4 0.5×	0.250					88 ab	2.3 (5.2) cdef
Other							
Ipr 2×	1.000	31 fgh	0.7 (0.5) gh	0.41 (16.09) de	0.7 (0.2) ef		
Ipr	0.500	34 fgh	0.8 (0.6) fgh	0.53 (25.68) cd	0.8 (0.5) de		
Ip2 0.5×	0.250			0.50 (23.19) cd	0.9 (0.6) cd		
Comp 7	0.400					100 a	4.5 (20.5) b
Pry 0.1×	0.040					99 a	4.7 (22.4) ab
Phos	1.395	84 ab	2.0 (4.0) b				
Use							
Comp 6/cap[b]	0.300/1.500					67 de	1.6 (2.5) fg
Ipr permit[c]	0.500					87 ab	2.5 (6.5) cd
All options[d]	-			0.25 (6.16) f	0.5 (0.1) f		
Grower[e]	-					67 de	1.7 (3.0) efg
Trans		Nil	Sqrt	ASIN Angular	4th root	Nil	sqrt
p-value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
95% LSD		15	0.4 (0.1)	0.13 (1.73)	0.2 (0.0)	15	
Covariate[f]		0.001	<0.001	n.s.	n.s.	n.s.	n.s.

[a]Means are followed in parentheses by back transformed means where appropriate, and means followed by the same letter are not significantly different (P = 0.05). Values followed by "×" refer to a multiple of the standard rate according to table 2.3.1.

[b]Two applications of Compound 6 2× alternated with two applications of captan 1.5× at monthly intervals.

[c]Iprodione applications according APVMA permit PER14444 and alternated with Mz.

[d]All existing (Cu, Mz, Az, Ip) and potential new fungicide options (Bo, Ca) applied throughout the season.

[e]Fruit from adjacent to the trial site and subjected to the grower's standard treatment schedule: 24/9/14 Cu, 8/10/14 Cu, 29/10/14 mancozeb, 18/11/14 mancozeb, 9/12/14 mancozeb, 22/12/14 mancozeb + iprodione, 12/1/15 mancozeb, 6/2/15 mz, 26/2/15 mz, 25/3/15 mz, 15/4/15 mz + ip, 5/5/15 cu + mz, 19/5/15 mz, 9/6/15 mz, 26/6/15 mz.

Discussion

In this study we aimed to identify promising fungicides for the control of EBS in Queensland orchards. The SDHI fungicides and two multisite fungicides gave very promising results for the control of EBS, typically at levels far superior to the over-relied on mancozeb. Commercial access to a respresentative of each of these fungicide groups would provide an excellent resistance management strategy, while also meeting disease management requirements.

Of the SDHI fungicides, Compound 6 and Compound 9 are the two options best placed for commercial use based on the data generated herein. Both options have their pros and cons. Compound 6 is an older generation product already in the market place, therefore giving the option of an industry-driven registration, or lower prices if the product becomes available from a generic supplier. There are also maximum residue limits set in some export markets. However, Compound 6 residues can be persistent in soils (Environmental Protection Agency, 2003), and manufacturer support may be reduced as the product becomes generic. Compound 6 is also used in Florida for the control of EBS (Vega and Dewdney, 2014), likely increasing the amount of pre-existing efficacy and residue data available for assisting registration in Australia. On the other hand, Compound 9 is a relatively new fungicide and will require high levels of manufacturer support to achieve registration. The efficacy at lower rates of active ingredient is also highly benficial in terms of environmental and fruit residues. However, being a new product there are fewer established MRLs in export markets, which may be an impediment to use on export fruit.

Among the multsite activity fungicides captan and Compound 4, captan would be best placed for commercial use based on the data we have generated. This fungicide has performed consistently throughout all the experiments and offers a significant improvement in EBS control over mancozeb. This fungicide has residue limits in some export markets and has shown no evidence of phytotoxicity in our trials. However, it should be noted that phytotoxicity of captan and Compound 4 has been reported in the presence of mineral oil sprays (Koller, 1999). This is probably the case where phytotoxicity was associated with Compound 4 in experiment 1.3.2 (Table 1.3.5). However, an important advantage of Compound 4 is that it has been the only commercially available fungicide included in this project that is sufficiently efficacious against both EBS and CBS. This makes Compound 4 the best candidate as a replacement for mancozeb, should mancozeb ever be withdrawn from use. For this reason it may be justified to further investigate the factors associated with the oil-related phytotoxicity risk of Compound 4, or other possible causes.

Apart from the phytotoxicity associated with Compound 4 on Blood oranges in experiment 1.3.2 for CBS, the external and internal quality assessments have not found any issues with any of the evaluated treatments. This is also keeping in mind that in most cases the highest rates of the various fungicides, and higher frequency of application, were assessed for quality issues. Therefore registration at lower rates and frequency of application should provide assurance of crop safety compliance.

Based on the results of these experiments gaining commercial use of an SDHI (preferably Compound 9) and a multisite (preferably captan) for EBS control would yield an impressive cost benefit to growers of varieties susceptible to EBS in subtropical areas. An ideal use pattern would be alternated applications of Compound 9 and captan during autumn/winter in susceptible varieties, when EBS pressure is typically highest.

2.4 Duration of fungicide efficacy

Introduction

Protective fungicide applications are crucial for the management of citrus diseases such as EBS and CBS. As such, significant effort goes into evaluating fungicides for efficacy against these diseases, including ***chapters 1*** and ***2*** of the report, as well as many other studies (Willingham *et al.*, 2003; Miles and Drenth, 2013; Miles *et al.*, 2005; Miles *et al.*, 2004; Silva Junior *et al.*, 2016; Roberts *et al.*, 2012; Colturato *et al.*, 2009; Schutte, 2008; Rosenzweig *et al.*, 2008; Swart *et al.*, 1998). While these studies focus on determining which fungicides are efficacious or otherwise, it is rare that studies investigate the duration of time after application for which the fungicides remain effective in the field. This is surprising, as the re-application interval is a very important piece of information for growers to know to ensure effective and efficient disease control. One example of a study investigating this issue used the fungus causing EBS, *Alternaria alternata*, as an effective bioassay for determining the duration of efficacy of various fungicides after application (Vicent *et al.*, 2007).

A. alternata is an ideal candidate for use in a bioassay to determine the duration of efficacy of fungicides for several reasons. Firstly, it is responsible for causing one of the most damaging diseases in Qld orchards (EBS), costing an estimated $5M in losses annually (Pegg, 1966; Miles *et al.*, 2011). Secondly, the fungus sporulates readily in culture and is relatively easy to use reliably in the laboratory. Thirdly, and perhaps most importantly, the fungus can induce symptoms within as little as 30 hours on detached fruit (Timmer *et al.*, 1998a; Miles and Drenth, 2013), facilitating rapid results for a bioassay. A contrast to this final point would be attempting to use *P. citricarpa*, the cause of CBS, for a bioassay due to the pathogen's very long latent phase between infection and disease development.

Previous work by Vicent *et al.* (2007) utilised these favourable traits of *A. alternata* to investigate the rainfastness of several fungicides used to manage EBS in the field. The fungicides included in this study were a number of copper formulations, mancozeb, difenoconazole, iprodione, famoxodone, and pyraclostrobin. Of these, mancozeb and iprodione are most frequently used in Qld, but there remains interest in the fungicides azoxystrobin, captan, Compound 4, Compound 6, Compound 9; all of which have been found to be effective in reducing EBS (see ***chapter 2***). The main findings of Vicent *et al.* (2007) were that copper fungicides tended to offer the longest duration of efficacy under field conditions, with the other fungicides having shorter times. However, copper fungicides are less often used in Qld for EBS control due to concerns over phytoxicity. It was also reported that the duration of efficacy was most significantly reduced by the 71mm of rainfall that occurred during one season of the experiment, with fruit expansion considered to have had little impact on efficacy during the study. As such, Vicent *et al.* (2007) recommended a 4-weekly strategy consisting of copper fungicides during weather conditions favourable to EBS, with more frequent re-application required only after heavy, wind-driven, rain events. However, considering that copper fungicides are not readily used in Qld for EBS control, and that a number of different fungicides are being used, or are of future interest, it is necessary to investigate the duration of efficacy of these fungicides under Qld conditions.

Understanding the duration of efficacy of fungicides under field conditions is crucial to efficiently and effectively managing diseases such as EBS in Qld. Without a better understanding of re-application intervals, a grower is at risk of applying fungicides too frequently and wasting fungicide and labour costs, or too infrequently and risking disease losses. Therefore, the aim of these experiments was to determine the duration of efficacy of existing, and promising future, fungicides for the management of EBS in Qld. The results of these experiments will greatly assist in making recommendations regarding the re-application intervals of these fungicides.

Methods

In order to determine the duration of efficacy of various fungicides, the unsprayed trees adjacent to those in experiment 2.3.3 were treated with a single application of the following fungicides: mancozeb, Compound 6, Compound 9 (1.5×), captan, Compound 4, iprodione and amistar. No fungicides were applied to the untreated control trees. The fungicides were applied on the 5/2/15 when fruit were approximately 45mm diameter, using the same application methods as per experiment 2.3.3. Fruit were sampled for bioassay at 1, 11, 21, 33 and 64 days after treatment. At each sampling time 10 fruit were arbitrarily sampled from around the canopy of each of the four replicate data trees, taking care to avoid handling the fruit surface that will be inoculated in the bioassay. The ten fruit were affixed to a HDPE board to minimise movement of fruit and physical disturbance of any fungicide residues. The boards where then placed inside a plastic container filled to a depth of approximately 10mm of distilled water, and the container sides lined with saturated blotting paper. The fruit were lightly misted with distilled water, then each fruit inoculated with four 5 mm diameter blotting paper discs soaked in spore suspension of 1×10^5 conidia/mL of an *A. alternata* isolate previously determined to be the 'Tangerine' pathotype (Miles *et al.*, 2015). The blotting paper discs were equidistantly spaced around the stem end of the fruit at a distance of approximately 15 mm. Following inoculation the containers were thoroughly sealed to maintain high humidity, then incubated at approximately 23 ˚C for 72 h. After incubation the paper discs were removed and the fruit inspected under a dissecting microscope for the development of lesions under the discs. Disease severity was rated as: 0 = no lesions; 1 = 1 to 5 lesions; 2 = >5 lesions for each disc. At the time of fruit rating, the diameter of each fruit was measured, than converted to fruit surface area assuming fruit were spherical.

The experiment was repeated the following season on trees located near Mundubbera, Qld (-25.603754, 151.301719). The trial comprised of 'Royal Honey' Murcott tangor (*Citrus* × *aurantium*) trees on 'Troyer' rootstock (*C. sinensis* × *Poncirus trifoliata*), planted in 2013 at a 6 m × 3 m spacing. The methods were as above, but fruit were sampled 1, 12, 21, 29 and 45 days after treatment.

Statistical analysis

Plot averages (i.e. for the batches of 10 fruit) for disease severity and fruit surface area data were calculated for each experiment, then the plot-average data was analysed with a repeated-measures restricted maximum likelihood (REML) analysis in GenStat Version 18.2. The mean data from the REML analysis was then used to generate plots over time of % disease control relative to the untreated control. Plots of fruit surface over time were also produced.

Results

In experiment 1, mean disease severity ratings were only significantly lower than the untreated control at 1 and 11 days after treatment (Table 2.4.1). More specifically, at day 1 only captan, Compound 4, Compound 9, iprodione and mancozeb significantly reduced disease ratings compared with the untreated control, while at 11 days only iprodione and captan were significantly lower than the untreated control. From 21 days onwards there were no significant differences amongst treatments. In experiment 2, all the fungicides were found to have significantly lower disease severity scores than the untreated control from days 1 to 29 (Table 2.4.1). However, at day 45 there were no significant differences among the treatments. Average fruit surface area increased significantly with time in both experiments (Table 2.4.1), showing very similar rates of increase in both experiments (Fig. 2.4.1).

Figure 2.4.2. shows that the overall disease control was much lower in experiment 1 than experiment 2. In general, the fungicides were more efficacious, for a longer period of time, in experiment 2. Figure 2.4.2 also shows that on day 16 of experiment 1 a significant rainfall event of 104 mm occurred, but other than this event rainfall was generally minimal for the majority of both experiments. Maximum temperatures were also not consistently different between experiments (Fig. 2.4.2).

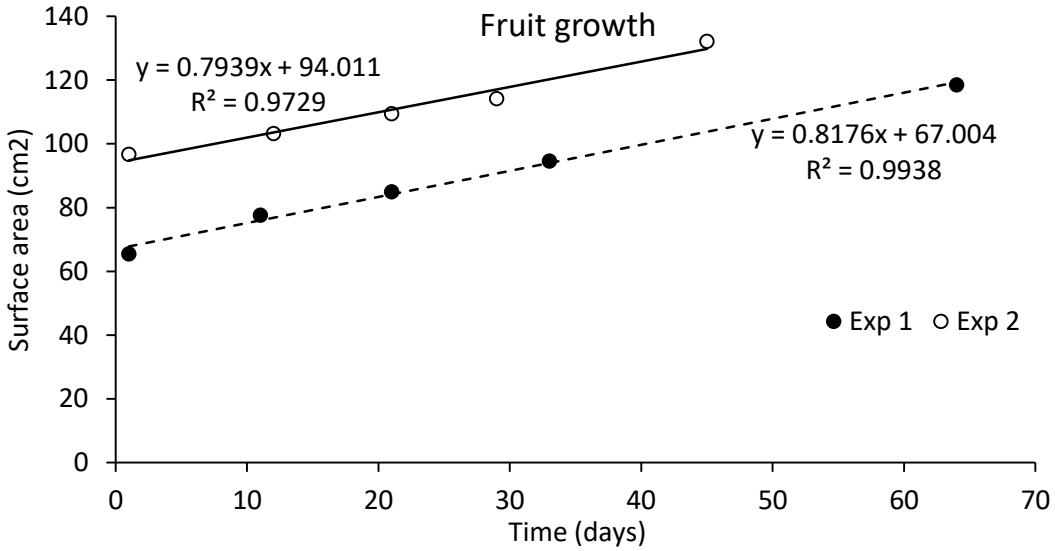

Figure 2.4.1. Increase in fruit surface over time during fungicide efficacy decline experiments.

Table 2.4.1. Average disease-severity ratings for seven fungicides and the untreated control, and average fruit surface-area averaged across treatments, Experiment 1, 2015.

Days after application	Average disease-severity rating for fungicide treatments								Average fruit surface area[2] ± SE (mm^2)[b]
	Azoxystobin	Compound 6	Captan	Compound 4	Compound 9	Iprodione	Mancozeb	Untreated	
	Multiple comparisons between fungicides within dates (across rows)[a]								
Experiment 1									
1	1.12 ab	0.98 abc	0.23 de	0.78 bcd	0.48 cde	0.18 e	0.67 bcde	1.52 a	6553±93 a
11	1.68 a	1.48 a	0.76 c	1.44 ab	1.48 a	0.86 bc	1.43 ab	1.82 a	7768±104 b
21	1.54 a	1.22 a	1.32 a	1.24 ab	1.63 a	1.11 a	1.08 ab	1.50 a	8500±132 c
33	1.35 a	1.38 a	1.66 a	1.61 ab	1.55 a	1.41 a	1.29 ab	1.84 a	9462±129 d
64	0.69 a	0.57 a	0.50 a	0.63 ab	0.57 a	0.71 a	0.73 ab	0.79 a	11848±192 e
Experiment 2									
1	0.34 b	0.20 b	0.07 b	0.14 b	0.08 b	0.05 b	0.45 b	1.24 a	9674±151 a
12	0.46 bc	0.07 c	0.08 c	0.12 c	0.10 c	0.05 c	0.64 b	1.38 a	10325±147 b
21	0.34 bc	0.18 bc	0.21 bc	0.12 c	0.12 c	0.11 c	0.56 b	1.26 a	10945±209 c
29	0.34 b	0.21 b	0.15 b	0.07 b	0.32 b	0.19 b	0.22 b	1.02 a	11516±163 d
45	0.29 a	0.18 a	0.24 a	0.18 a	0.20 a	0.12 a	0.34 a	0.25 a	13220±198 e

[a]Within sampling days after application (across rows), average disease-severity rating means followed by the same letters are not significantly different (Protected LSD Test with sequential Bonferroni correction (Family-wise probability = 0.05)). Because the uniform model was fitted, all average disease-severity means in experiment 1 have the same SE (0.1378) and every comparison has the same SED (0.1934) and LSD value (0.3839) prior to the application of the Bonferroni probability correction. Similarly, for experiment 2 all average disease-severity means the same SE (0.1001) and every comparison has the same SED (0.1357) and LSD value (0.2694) prior to the application of the Bonferroni probability correction.

[b]Within the average fruit surface area column, means followed by the same letters are not significantly different (Protected LSD Test with sequential Bonferroni correction (Family-wise probability = 0.05)). Because a second order antedependence model was used, SEs are different for each fruit surface area mean and SEDs and LSDs are different for every pair of means. The table gives SE for each mean and the average LSD and the range of values of the LSD.

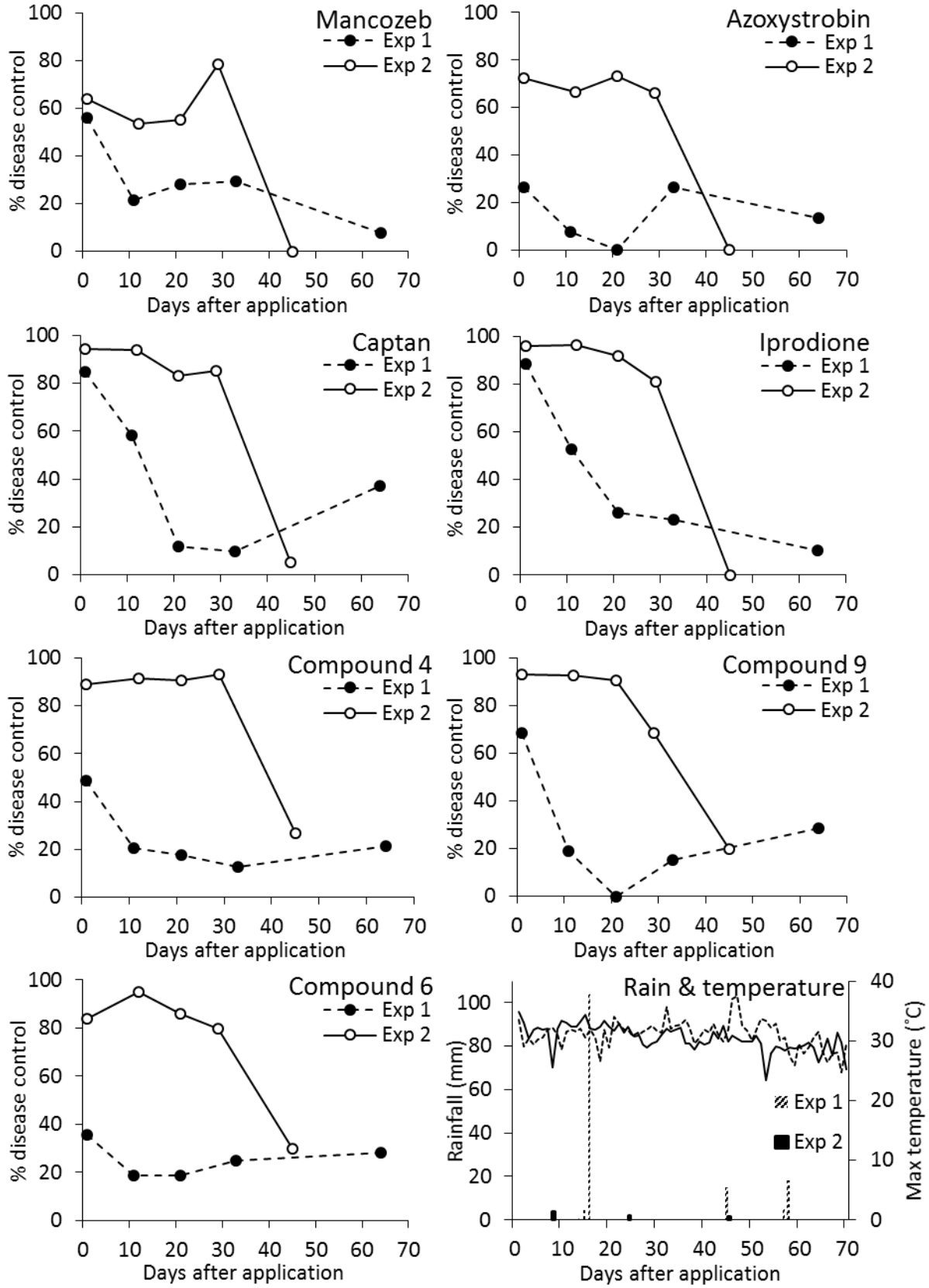

Figure 2.4.2. Summary plots of % disease over time after single applications of various fungicides, and rainfall and temperature over time during experiments 1 (2014-15) and 2 (2015-16).

Discussion

In this experiment we aimed to determine the duration of efficacy of existing, and promising future fungicides for the management of EBS in Qld. Overall, the results of the *A. alternata* bioassay have shown the potential for large variation between years/experimental sites, suggesting a useful efficacy lifespan within only 11 days, but possibly up to 29 days, depending on the particular fungicide and circumstances. These results suggest that the typically arbitray re-application interval of 4 weeks is most likely a best case scenario for the duration of efficacy of the various fungicides. This is especially the case when considering fruit expansion was relatively low during the the experiments, and rainfall was not a factor in the first 15 days of both experiments. However, in spite of this there were large differences between the experiments that are likely to be due to factors other than fruit growth and rainfall. Among the different fungicides, captan and iprodione appeared to be the most durable fungicides, while according to the bioassay, the fungicides axozystrobin and Compound 6 appear quite poor. However, as all the fungicides included in this experiment are known to be relatively effective in the field (e.g. ***chapter 2***), consideration may need to be given to how fungicide mode of action may influence disease control during this particular bioassay.

The potential for much shorter durations of efficacy than generally thought is of significant importance to growers, as it suggests that a monthly fungicide program will often be insufficient to protect fruit. While this may be taken as a need to simply apply more fungicides at closer intervals throughout the season, this would not be a practice supported by existing product use patterns (Miles, 2011). Moreso, these results suggest a need for better timing of the available fungicide label use patterns to target forecast conditions likely to be favourable for disease development. Doing this will require improvements in two main areas: 1) the speed and efficiency of existing spray equipment; and 2) a better understanding of disease forecasting and extension of forecast warnings. The main impediment to a prompt reaction to a disease forecast would be the tendency for growers to adopt high volume spray applicators that may results in a single fungicide application to the whole orchard taking upwards of a week to complete. It would be anticipated that an accurate weather forecast-based disease warning system may require the same spray application to be completed in 48 hours. The second impediment to a disease forecasting system is a good understanding of the critical weather events likely to drive key diseases such as EBS and CBS, as well as a way to readily extend that information to growers and consultants. Infection timing studies have been commenced for CBS under collaborating project CT13021 *Joint Florida and Australia Citrus Black Spot Research Initiative* to better understand the weather factors driving infection. Similar studies may be needed for EBS. Once the various weather factors are understood, a smart phone/web-based platform could be utilised to provide the necessary information to growers.

The results from experiment 1 and 2 differ considerably. There are several possible explanations, or combinations of explanations, for this difference. Two different scion varieties, ‘IrM2’ Murcott and ‘Royal Honey’ Murcott were used in the two experiments, with a difference in susceptibility possibly explaining the differences in disease severity. This appears to be the case when comparing the untreated control results for the two experiments in Table 2.4.1., whereby the IrM2 in experiment 1 had higher severity ratings than the Royal Honey in experiment 2. However, the difference in % disease control (Fig. 2.4.2) would have to be the result of a fungicide rate × cultivar interaction; i.e. different cultivars may require different fungicide rates for the same

level of efficacy. However, no comparative studies of EBS susceptiblility for these varieties are known of. Another possibility is inconsistent virulence of the *A. alternata* isolate used in the study. While all efforts were made to maintain the culture in a way to minimise mutation or loss of virulence, this possibility cannot be completely removed. Finally, a large range of variables between years cannot be completely controlled or measured within the scope of this study. These may include impacts from dew (hydrolysis), ultra violet light intensity (photolysis), and temperature (primarily volatilisation), all of which could impact on the persistence of the fungicides in the field (Wightwick *et al.*, 2010).

The fungicides captan and iprodione resulted in consistently lower disease severity ratings / higher % disease control in both experiments, relative to the other fungicides. This is in keeping with field efficacy evaluations in ***chapter 2***, as well as previous efficacy studies (Miles and Drenth, 2013; Miles *et al.*, 2005), which have shown iprodione and captan to provide excellent field control of EBS. When considering only experiment 2, all the fungicides gave results consistent with the results of field efficacy experiments that show all these fungicides to be very useful for EBS. More specifically, all the fungicides have been shown to be very effective, with mancozeb slightly less so (***chapter 2***), and axozystrobin generally equivalent to mancozeb (Miles *et al.*, 2005). However, the results from experiment 1 suggest nil efficacy against EBS for azoxystrobin and Compound 6 (Table 2.4.1) which is in contrast to the field evaluations of efficacy. One possibility is that the *A. alternata* bioassay used in this study may favour different fungicides on the basis of their mode of action. As the bioassay studies only the first 72 hours of the infection process, it may then favour fungicides that inhibit spore germination and germ tube growth, over mycelial growth. While generally azoxystrobin is reported to be effective against spore germination (Bertelsen *et al.*, 2001; Bartlett *et al.*, 2002) (Sauter *et al.*, 1995), one study showed lower sensitivity to azoxystrobin for spore germination of *A. alternata* (Reuveni and Sheglov, 2002). Compound 6 also is reported to inhibit germination, and other early infection processes such as germ tube growth and mycelial growth (Avenot and Michailides, 2007), so would have been expected to perform well in the bioassay. In contrast, iprodione has been reported to be relatively more effective against mycelial growth than spore germination (Pappas and Fisher, 1979), but was one of the better performing fungicides in the bioassay. More consistent with other studies is the good performance of captan in the bioassay, considering reports of higher sensitivity to captan for spore germination than mycelial growth for various fungi (Everett *et al.*, 2005). Another possibility for inconsistency between the bioassay and field evaluation trials are differences in the ability of fungicides to inhibit sporulation, which would be more benficial in the field than the bioassay. For example, strobilurin fungicides such as azoxystrobin have been shown to suppress sporulation from EBS lesions on leaves (Reis *et al.*, 2006).

This study has shown that the duration of fungicide efficacy in the field can be highly variable, ranging from within only 7 days of application or potentially up to 29 days from application. This result provides some explanation of why disease control can often fail in the orchard when relying on arbitrary monthy application intervals. The success of a routine application will depend on the proximity of that application to a subsequent infection event. Instead, it is proposed that a more targeted approach to fungicide application is needed, whereby spray applications are in response to forecast infection events. However, successful implementation of such a system will

need faster, more efficient spray application technology, and verified forecast systems readily available to growers and consultants.

2.5 Summary

This chapter aims to address the need for improved management strategies for EBS that are cost effective, and comply with food safety requirements by: 1) providing technical support for screening for EBS resistance; 2) investigating the potential to improve resistance screening methods; 3) evaluating fungicides for the control of EBS; and 4) determining the duration of efficacy of fungicices. Addressing these aims has shown:

1) The ongoing screening for EBS resistance has produced another 12,750 resistant hybrids during the life of this project, consistently improving the likelihood of developing a resistant variety with desirable horticultural traits.
2) The EBS resistance screening methods could be improved with seed-based inoculation systems, but developing these methods at this stage may require more resources than they are likely to save in the short term.
3) The SDHI fungicides Compound 9 and Compound 6, and multsite activity fungicides, captan and Compound 4, have shown excellent efficacy against EBS. All these fungicides typically offered superior EBS control to mancozeb The ideal use pattern would be applications of Compound 9 alternated with captan to provide excellent disease control and resistance management.
4) The duration of efficacy of fungicides can be highly variable, with 29 days after application being a best case scenario. Rather on relying on arbitrary assumptions of reapplication interval, a disease forecasting system may be more beneficial. Nevertheless, the fungicides iprodione and captan were found to be the most robust based on the bioassay used in the study.

Chapter 3
Residues and APVMA engagement

3.1 Introduction

Agrichemical residues are an emerging technical barrier to trade. As Australia's citrus exports become increasingly important to the value of the industry, any impediments to export market access become increasingly costly. Agrichemical residues pose a significant risk, as the MRLs for citrus agrichemicals vary massively between export markets. The fungicide iprodione is an excellent example of this problem, whereby its use domestically is associated with an MRL of 5 mg/kg, but the extremely low MRL of 0.05 mg/kg in Taiwan effectively disqualifies fruit treated with iprodione from this important export market. In this example a likely reason for this problem arising was insufficient consideration of export MRLs during the development of iprodione, bearing in mind the lower export focus of the citrus industry during the developmental phase. However, in this present project it is important that export MRLs have been considered during the evaluation phase, alongside the efficacy evaluation such as detailed in ***chapters 1*** and ***2***.

In addition to the giving consideration to the residue profiles of the alternative fungicides/use patterns from ***chapters 1*** and ***2*** when considering registration potential. Other impediments to registration may exist in terms of environmental impact, health and safety, export MRL compliance, and other possible data requirements. It is therefore important to engage with the Australian Pesticides and Veterinary Medicines Authority early in the process to identify any unforeseen issues. This can be done through the Pre-Application Assistance process.

As well as seeking APVMA advice on issues such as export MRL compliance, additional approaches to ensuring export MRL compliance may be needed. One approach to export MRL compliance is the "zero residue concept", which is an approach to citrus production aiming to meet the need for agrichemical use, while ultimately resulting in a final product with nil or very low residues. HIA project CT14001 Zero residue concept – scoping study for citrus (Cunningham *et al.*, 2015) has been investigating the feasibility of producing citrus fruit in Australia which at the time of sale has negligible agrichemical residues. Two key findings of CT14001 were i) the lack of residue survey data for fruit from Qld, and ii) the need to determine the potential for postharvest practices such as high pressure washing to significantly reduce residues of preharvest chemical applications; in particular iprodione and dithiocarbamate (mancozeb) which have caused disruptions to trade in the past. The first of the CT14001 findings hinders the accurate assessment of the potential for ultra-low residue production in Qld, as the residue priorities are not well known. The second of the scoping study's findings can be well addressed through integration into other activities detailed above; namely the fungicide evaluation trials in ***chapter 2***. Through the fungicide evaluation trials, fruit can be treated with agrichemicals of interest to the ultra-low residue concept, then used for postharvest residue removal experiments.

The aims of this chapter are to: 1) evaluate the residue profile likely to result from alternative fungicides and use patterns; 2) engage with the APVMA for pre-application assistance; 3) expand participation form Qld in the NRS and evaluate the existing residue situation; and 4) evalute the efficacy of postharvest processes for reducing

residues in citrus. Addressing these aims will greatly assist the Australian citrus industry in dealing with the emerging technical barrier to trade that is export MRLs.

3.2 Preliminary residue studies for alternative fungicides

Introduction

In order to determine the likely residues associated with the fungicides and use patterns identified from ***chapters 1*** and ***2*** of this report, fruit samples were strategically taken from the various fungicide efficacy trials and the residues determined. The residues in fruit were determined by Symbio Alliance using the National Residue Survey screen, with or without dithiocarbamates where required. This approach offered a cost effective means for determining whether any of the identified fungicides and/or use patterns would result in impractically high residues for commercial use. Using the NRS screen also meant additional residue information was provided for some existing fungicides at no extra cost, which will assist in determining the feasibility of producing low residue citrus fruit in the future.

The aim of collecting preliminary residue data was to avoid undertaking further research on fungicides or use patterns that would result in residues unsuitable for key export markets.

Methods

Fruit for residue samples were harvested from the data trees from the fungicide efficacy trials described in ***chapters 1*** and ***2***. Each residue sample comprised of 4 fruit collected from each replicate tree of each specific treatment, giving a total of 12 fruit per residue sample. Where residues were determined for different numbers of days after application, the samples were collected at different times after application; as opposed to making the applications at different times and then taking all the samples on the same day. Upon sampling the fruit were double bagged and either immediately frozen and stored before delivery, or cooled and delivered to Symbio Alliance with 24 hours of sampling.

Results and discussion

Residue results are provided in table 3.2.1. The four most promising fungicides from ***chapters 1*** and ***2*** of this report were captan, Compound 4, Compound 6 and Compound 9. Residues for these fungicides were generally less than 1 mg/kg, with a few exceptions. Interestingly, in no cases were residues of Compound 4 reported. However, the APVMA advised that Compound 4 residues can decline rapidly in storage. Even so, several of the Compound 4 samples were provided to Symbio Alliance within 24 hours, but once submitted to the laboratory the storage conditions are out of the project team's control. Also of note are the consistently low Compound 9 residues (<0.30 mg/kg), and the Compound 9 residue levels relative to Compound 6, which was over 1 mg/kg in some cases. This suggest Compound 9 would be preferable over Compound 6 for commercial use. Compound 6 appears to give less predicatable residues.

In terms of export market residues, these results show compliance with several export markets. For Compound 4, Taiwan and Japan have suitable MRLs, while for captan,

China, Japan and Singapore would be compliant. Compound 6 residues would be compliant with Thailand, Hong Kong, Japan, Singapore, Netherlands and Malaysia. Due to Compound 9 being relatively new to market, no MRLs are currently know of in export destinations.

Overall, these preliminary residue results suggest that all of the fungicides and use patterns identified from ***chapters 1*** and ***2*** result in manageable residues that are unlikely to disqualify these options for future commercial use.

Table 3.2.1. Preliminary fungicide residues in various citrus fruits treated in fungicide efficacy trials[a].

Residue	Form	Trial details	kg a.i./ha	kg a.i./hl	No. of sprays	Days	Residues (mg/kg)
Dithiocarbamate	Penncozeb 750 DF	2.3.4 Mundubbera, IrM2 Murcott 2015/16	42	0.150	5	15	8.60
						22	4.20
		1.3.2 Gayndah, Imperial, 2013/14	15	0.150	2	73	<0.10
						89	<0.10
						110	<0.10
		2.3.2 Mundubbera, Daisy, 2013/14	7	0.150	2	163	<0.10
						169	<0.10
Dithiocarbamate (propineb)	Antracol 700 WG	2.3.4 Mundubbera, IrM2 Murcott 2015/16	39	0.140	5	15	4.10
						22	3.00
Captan	**Captan 800 WG**		**42**	**0.150**	**5**	**22**	**3.60**
			25	**0.150**	**3**	**22**	**4.60**
			28	**0.100**	**5**	**22**	**3.90**
		2.3.2 Mundubbera, Daisy, 2013/14	**15**	**0.200**	**3**	**17**	**0.22**
						23	**0.45**
						42	**0.22**
						65	**0.27**
		2.3.3 Mundubbera, IrM2 Murcott, 2014/15	**10**	**0.150**	**2**	**27**	**0.49**
Compound 4	**Compound 4**	**2.3.4 Mundubbera, IrM2 Murcott 2015/16**	**7**	**0.025**	**5**	**15**	**<0.01**
						22	**<0.01**
		1.3.2 Mundubbera, Arnold Blood, 2014/15	**6**	**0.025**	**5**	**121**	**<0.01**
		1.3.3 Mundubbera, Imperial 2015/16	**5**	**0.025**	**4**	**50**	**<0.01**
		2.3.3 Mundubbera, IrM2 Murcott, 2014/15	**3**	**0.025**	**4**	**27**	**<0.01**
Compound 6	**Compound 6**	**2.3.3 Mundubbera, IrM2 Murcott, 2014/15**	**2**	**0.030**	**2**	**64**	**0.38**
		2.3.2 Mundubbera, Daisy, 2013/14	**2**	**0.030**	**3**	**38**	**<0.01**
						44	**<0.01**
						63	**1.00**
						86	**1.40**
		1.3.2 Gayndah, Imperial, 2013/14	**1**	**0.015**	**1**	**45**	**<0.01**
						61	**<0.01**
						82	**<0.01**
Compound 9	**Compound 9**	**2.3.4 Mundubbera, IrM2 Murcott 2015/16**	**1**	**0.005**	**5**	**22**	**0.27**
			1	**0.003**	**5**	**22**	**0.12**
		2.3.3 Mundubbera, IrM2 Murcott, 2014/15	**1**	**0.005**	**4**	**27**	**0.22**
		2.3.4 Mundubbera, IrM2 Murcott 2015/16	**1**	**0.005**	**2**	**101**	**0.04**
Iprodione	Chief Aquaflo SC	2.3.3 Mundubbera, IrM2 Murcott, 2014/15	5	0.050	3	92	0.79
		2.3.2 Mundubbera, Daisy, 2013/14	4	0.050	3	59	0.41
						65	0.24
						84	0.09
						107	0.10
Azoxystrobin	Amistar 250 SC	2.3.2 Mundubbera, Daisy, 2013/14	0.5	0.010	2	134	0.03
						140	0.01

Residue	Form	Trial details	kg a.i./ha	kg a.i./hl	No. of sprays	Days	Residues (mg/kg)
						159	0.03
						182	0.01
		1.3.2 Gayndah, Imperial, 2013/14	1	0.010	1	104	<0.01
						120	<0.01
						141	<0.01
Compound 7	Compound 7	1.3.2 Mundubbera, Arnold Blood, 2014/15	10	0.040	5	121	0.39

[a]Table formatting according to The Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Bold face type identifies the promising alternative fungicides identified for use in Queensland orchards.

3.3 National residue survey – expanding Qld data

Introduction

Past project CT14001 (Cunningham *et al.*, 2015) has identified the low participation of Qld citrus in the National Residue Survey (NRS) as an impediment for evaluating the feasibility of a low residue program for citrus produced in this region. In order to meet this data gap, this project worked directly with industry to boost participation in the NRS program. To draw further value from the program, this project also saught to obtain spray application records corresponding to the submitted samples, where possible. Combining the spray records and residue data significantly increases the knowledge that can be gained from the NRS program, as it shows what residues are being detected, but importantly also shows which agrichemicals and use patterns are resulting in low to nil residues in fruit at harvest. This kind of information is crucial for identifying which agrichemicals should potentially be avoided, or which alternatives may be preferable, in orchards wanting to move towards a low residue future.

Methods

During the 2015-16 production season the project team directly collected fruit samples from growers and retailers. Approximately 12 fruit were collected for each sample, the fruit double bagged and either immediately frozen and stored before delivery, or cooled and delivered to Symbio Alliance within 48 hours of sampling. Where possible, the spray application records were also obtained for each sample. All the received spray application and residue information were compiled in the table 3.3.1. with all grower identifiers removed for confidentiality.

Results and discussion

Table 3.3.1 shows results of the residue testing, and the corresponding spray application schedule. In terms of field applied fungicides, the three applied fungicides were azoxystrobin, iprodione and mancozeb. As anticipated, iprodione and dithiocarbamate (mancozeb) were readily detected. Azoxstrobin residues were below the LOR. Iprodione was well within the domestic MRL of 5 mg/kg, while dithiocarbamates where typically well within the temporary MRL of 7 mg/kg. However, in all cases where dithiocarbamates were above the limit of reporting (LOR), the level was greater than the previous MRL of 0.2 mg/kg. This was the case even when mancozeb was applied at ½ the label rate and 206 days after the final application. This suggests that compliance with the previous MRL of 0.2 mg/kg would be challenging regardless of the use pattern. Regarding the postharvest fungicides, imazalil, Compound 7 and thiabendazole were detected, but at levels well below the MRLs.

The main insecticides applied to the sampled fruit were abamectin, chlorpyrifos, spirotetramat, methomyl, and dimethoate. However, residues were only detected for chlorpyrifos and dimethoate. This is generally in keeping with the citrus NRS results more broadly, where chlorpyrifos residues are detected with high frequency in citrus, and dimethoate with moderate frequency (Cunningham *et al.*, 2015).

When comparing the NRS data obtained for Qld to the wider NRS data for the rest of Australia, the residue profile is generally very similar. The exceptions are most notably the dithiocarbamate and iprodione residues resulting from the application of these

fungicides for the control of CBS and EBS. At present this does not represent a domestic MRL issue, but it is important for several export markets where MRLs are low or not established for either fungicide. The fungicides identified in ***chapters 1*** and ***2*** of this report will go some way to improving the ease of export market compliance by providing a broader range of options to growers, but not nearly as far as a low residue program would potentially go. However, for the Queensland situation the low residue concept will remain hindered primarily by the need to control EBS in susceptible varieties (such as Murcott) in the second half of the season. Until varieties resistant to EBS become available, the low residue concept will remain challenging for Qld's main export variety, Murcott.

Table 3.3.1. Details of agrichemicals, citrus varieties, applications and residue results from Queensland.

Agrichemical	Variety	Application time/s (days before sampling)	Residue (mg/kg)	MRL (mg/kg)
Field fungicides				
Azoxystrobin	Imperial	84	<LOR	3
	Imperial	84	<LOR	
	Murcott	108 + 70	<LOR	
Iprodione	Murcott	127 + 66	0.330	5
Mancozeb	Daisy	164 + 150 + 122 + 87 + 24	0.420	7
	Imperial	248 + 223 + 190 + 162 + 140 + 119 + 84	0.450	
	Imperial	218 + 188 + 166 + 139 + 118 + 84	0.640	
	Murcott	236 + 189 + 165 + 127 + 66 (field sample)	1.400	
	Murcott	273 + 158 + 135	<LOR	
	IrM2 Murcott	153 + 121	0.420	
	IrM2 Murcott	153 + 121	0.280	
	IrM2 Murcott	153 + 121	<LOR	
	IrM2 Murcott	153 + 121	0.380	
Mancozeb ½ rate	Valencia	248 + 227 + 206	0.410	
Postharvest fungicides				
Imazalil	Daisy	Postharvest treatment	1.100	10
	Valencia	Postharvest treatment	0.620	
	Ellendale (organic)	Postharvest treatment (market purchase)	0.012	
	Murcott	Posthavrest treatment	1.100	
Compound 7	Valencia	Posthavrest treatment	0.016	7
Thiabendazole	Daisy	Postharvest treatment	0.140	10
Insecticides				
Abamectin	Daisy	164	<LOR	0.01
	Imperial	162	<LOR	
	Imperial	166	<LOR	
	Murcott	236	<LOR	
	IrM2 Murcott	250 + 190	<LOR	
	IrM2 Murcott	250 + 190	<LOR	
	IrM2 Murcott	250 + 190	<LOR	
	IrM2 Murcott	250 + 190	<LOR	
Chlorpyrifos	Daisy	115	0.069	0.5
	Valencia	248 + 115	0.140	
	Imperial	223 + 140	0.041	
	Imperial	218 + 139	0.029	
	Murcott	189 (field sample)	0.019	
	IrM2 Murcott	190	0.030	
	IrM2 Murcott	190	0.053	
	IrM2 Murcott	190	0.029	
	IrM2 Murcott	190	0.031	
Dimethoate	Imperial	Unknown (market purchase)	0.340	5
Methomyl	Imperial	61	<LOR	1
	Imperial	60	<LOR	
Omethoate	Imperial	Postharvest treatment	0.012	5
Spirotetramat	Valencia	94	<LOR	1
	Imperial	197	<LOR	
	Imperial	195	<LOR	
	IrM2 Murcott	177	<LOR	
	IrM2 Murcott	177	<LOR	
	IrM2 Murcott	177	<LOR	
	IrM2 Murcott	177	<LOR	
	IrM2 Murcott	140	<LOR	
Other				
Dichlorprop-p	Daisy	199	<LOR	0.2

3.4 Postharvest residue removal

Introduction

Maximum residue limits for agrichemicals vary widely between countries. For example, the MRLs for dithiocarbamate in Australia, Thailand, and Canada are 7, 2, and 0.1 mg/kg, respectively. This means that an export citrus orchard needs to manage agrichemical residues according to the export destination, rather the domestic MRL and associated label use pattern. However, as export destinations are often not known well in advance, managing export MRL compliance during the season becomes even more challenging. One possible approach to improving the ease of export MRL compliance is the postharvest treatment of fruit to remove as much of the field applied residues as possible. The most likely component of postharvest processing to reduce residues is high pressure washing of fruit. It may also be possible that treatment of fruit with specific residue removal steps might also result in measurable residue reductions.

The three main residues of interest are chlorpyrifos, dithiocarbamate, and iprodione based on ***section 3.3*** of this report, and project CT14001 (Cunningham *et al.*, 2015). Of these agrichemicals, several reports have shown reductions in residues of dithiocarbamate and iprodione in various fruits and vegetables after postharvest washing (Sharma *et al.*, 1994; Patsakos *et al.*, 1992; Hwang *et al.*, 2001; Lozowicka *et al.*, 2016; Cabras *et al.*, 1998). It is therefore likely that optimising the high pressure washing step, in particular the dwell time, might be of benefit. In contrast, chlorpyrifos residues may not be as likely to be removed by washing (Krol *et al.*, 2000). Characteristics likely to influence the efficacy of washing include solubility in water, which is relatively poor for the three agrichemicals (Extension Toxicology Network, 1993a; Extension Toxicology Network, 1983; Extension Toxicology Network, 1993b). It may be of value to consider a fruit treatment in something other than water that will provide better solubility. Preliminary evidence from a grower project collaborator indicated that the sanitiser Hygiene Plus may assist in removing residues. One active ingredient in Hygiene Plus is the inorganic acid, phosphoric acid, which may improve solubility.

The aim of this experiment was to determine the efficacy of postharvest fruit processing, particularly high pressure washing, for reducing residues of chlorpyrifos, dithiocarbamate, and iprodione in fruit. Methods that consistently reduce residues in fruit would be of benefit to achieving compliance with export MRLs that are typically more limiting than domestic equivalents.

Methods

In order to determine the efficacy of postharvest fruit processing for removing residues from fruit, fruit from experiment 2.3.4 were treated with various postharvest treatments and then sent for residue analysis. At commercial maturity “untreated” fruit were sampled from the untreated control treatment of experiment 2.3.4, while “spiked” fruit were sampled from the “Ipr/Mz/Chlor” treatment of experiment 2.3.4. The latter provided fruit of high residues of iprodione, mancozeb and chlorpyriphos for experimental purposes. Fruit were sampled in batches of 15 fruit each, in four replicates corresponding with the four replicate trees in experiment 2.3.4. The fruit were assigned to the following treatments:

T1) spiked fruit + no postharvest treatment

T2) spiked fruit + standard postharvest treatment (SPT)

T3) spiked fruit + SPT + high pressure wash at double the standard dwell time*

T4) spiked fruit + pre-dip for 1 min in Hygiene Plus (100 mL/100 L) + SPT

T5) untreated fruit + no postharvest treatment

T6) untreated fruit + SPT

*high pressure wash consisted of 10 brushes and 8 rows of 12 nozzles operating at 625 psi and a standard dwell time of 13 seconds.

The postharvest experiment was undertaken at the Abbotsleigh Citrus fruit packing shed. The treatments were applied to the replicate batches of fruit in a completely randomised order. Following treatment the fruit were dried, sealed in sample bags, and then frozen in a -20 ˚C freezer. Samples were delivered in separate replicate batches to Symbio Alliance for residue analysis using the National Residue Survey Screen + dithiocarbamates.

Statisical analysis

Residue analysis data provided by Symbio Alliance were compared using analysis of variance (ANOVA) in GenStat 16th Edition (VSN International, UK). Square root transformation was applied to the dithiocarbamate and iprodione data to normalise the data. The raw data were also summarised using box and whisker plots to show the spread of the data.

Results and discussion

Results from the experiment showed readily detectable residues of dithiocarbamate, irpodione and chlorpyrifos in the “spiked” fruit, and close to nil residues in the “untreated” fruit, with a few exceptions. In some replicate samples from the untreated control treatment trees from experiment 2.3.4, residues of dithiocarbamate and chlorpyrifos were detected (Fig. 3.4.1). Spray records from the trial block showed that mancozeb and chlorpyrifos applications had been made four months prior to commencing experiment 2.3.4. However, in both cases the spiked - PHT fruit showed significantly higher residues than the untreated - PHT fruit. Also impotant to note is that no residues of dithiocarbamate, iprodione or chlorpyrifos were detected in fruit of the “untreated + PHT” treatment. This indicates that detectable residues of these fungicides were not acquired from the packing line itself. As expected, residues of 2-phenylphenol and imazalil were significantly higher following postharvest treatment.

All the treatments incporating some form of postharvest treatment were found to significantly reduce residues of dithiocarbamate and iprodione, but there were not significant differences among these postharvest treatments for these two residues (Table 3.4.1). Dithiocarbamate residues were reduced by a factor of approximately five, while iprodione residues were reduced by a factor of about two. None of the postharvest treatments significantly reduced residues of chlorpyrifos, but trendwise

the hygiene plus pre-wash resulted in the lowest residues of the various postharvest treatments. These findings suggest that the standard packline procedures have a large part to play in reducing residues of dithiocarbamate, and to a slightly lesser extent, iprodione. A reduction in these residues as a result of the packingline was anticipated, as significant reductions of dithiocarbamate have been reported for various vegetables washed in tap water (Sharma *et al.*, 1994), apricots agitated in water (Patsakos *et al.*, 1992), as well as apples dipped in various compounds (Hwang *et al.*, 2001). Similarly, iprodione residues have been reduced in broccoli after washing in chlorinated or ozonated water (Lozowicka *et al.*, 2016) and prunes after washing in tap water (Cabras *et al.*, 1998). In contrast, washing did not appear to significantly reduce iprodione residues in apples (Rasmusssen *et al.*, 2003). While more broadly rinsing has been shown to reduce iprodione residues in a range of produce, it is of interest that chlorpyrifos residues were not significantly reduced in the same study (Krol *et al.*, 2000), as was the case in our experiment.

No significant differences in residues between fruit receiving the standard pressure wash and the double pressure wash dwell time might be due to an effect of diminishing returns with increased dwell (Table 3.4.1). This has been shown in other studies, whereby washing prunes for 25 mins did not significantly decrease the residues over washing for 5 mins (Cabras *et al.*, 1998). Failures to reduce residues have then been explained by possible barrier effects of the fruit cuticle, acting to prevent the washing substrate to make contact with the residue (Cabras *et al.*, 1998; Riederer and Schreiber, 1995). In the case of chlorpyrifos, it may be possible that the pre-dip in Hygiene Plus was overcoming this effect to some degree, resulting in the trendwise reduction in chlorpyrifos. While statistically there was no significant reduction in residues from increasing the pressure wash dwell, it is worth noting that Fig. 3.4.1 shows that the dithiocarbamate residue results had a much tighter spread of results at twice the standard dwell time, which might suggest there is a benefit of a thorough high pressure washing step in the packingline. Particularly if the longer dwell time is reducing the occurrence of outlier fruit with higher than average residues, which could be fruit that lead to a breach of MRL.

It can also be seen from Fig. 3.4.1 that the spread of residue data across the replicate samples is generally quite large, and demonstrates a high level of variability between samples. This could arise from many different variables, ranging from fungicide application in the field, through to the laboratory analysis. The critical point is that any future experiments further exploring the issue of residue removal will require several replicate samples to ensure meaningful results are obtained.

The results of this experiment show that the packingline has a significant role to play in reducing residues on fruit. It may be possible to significantly improve postharvest residue removal, but further studies would be needed, and would possibly demonstrate diminishing returns as previously discussed. Instead, the greatest reductions in fruit residues will most likely come from a systems approach combining pre- and postharvest steps. Preharvest components would mostly like be judicious use of pesticides through thorough monitoring and pest forecasting, alternative pest control tools such as biological control and Generally Regarded As Safe (“GRAS”) compounds, and eventually varieties resistant to diseases such as EBS and CBS. Reducing agrichemical residues in citrus has few downsides, and is a goal worth pursuing.

Table 3.4.1. Residues of pesticides in “IrM2” Murcott fruit following various postharvest treatments (PHT)[a].

	Residue (mg/kg)				
Treatment	Dithiocarbamate	Iprodione	Chlorpyrifos	2-phenylphenol	Imazalil
Spiked - PHT	1.8 (3.3) a	1.3 (1.8) a	0.44 a	0.00 c	0.0 c
Spiked + PHT	0.4 (0.2) b	0.7 (0.5) b	0.33 a	0.14 ab	3.3 ab
Spiked + 2× wash	0.5 (0.2) b	0.7 (0.4) b	0.32 a	0.16 ab	3.8 a
Spiked + hygiene	0.6 (0.4) b	0.6 (0.3) b	0.24 ab	0.19 a	2.7 ab
Untreated – PHT	0.4 (0.2) b	0.0 (0.0) c	0.04 bc	0.00 c	0.0 c
Untreated + PHT	0.0 (0.0) c	0.0 (0.0) c	0.02 c	0.11 b	2.5 b
P-value	<0.001	<0.001	0.003	<0.001	<0.001
LSD	0.3 (0.1)	0.5 (0.2)	0.21	0.05	1.2

[a]Means are followed in parentheses by back transformed means where appropriate, and means followed by the same letter are not significantly different (*P* = 0.05).

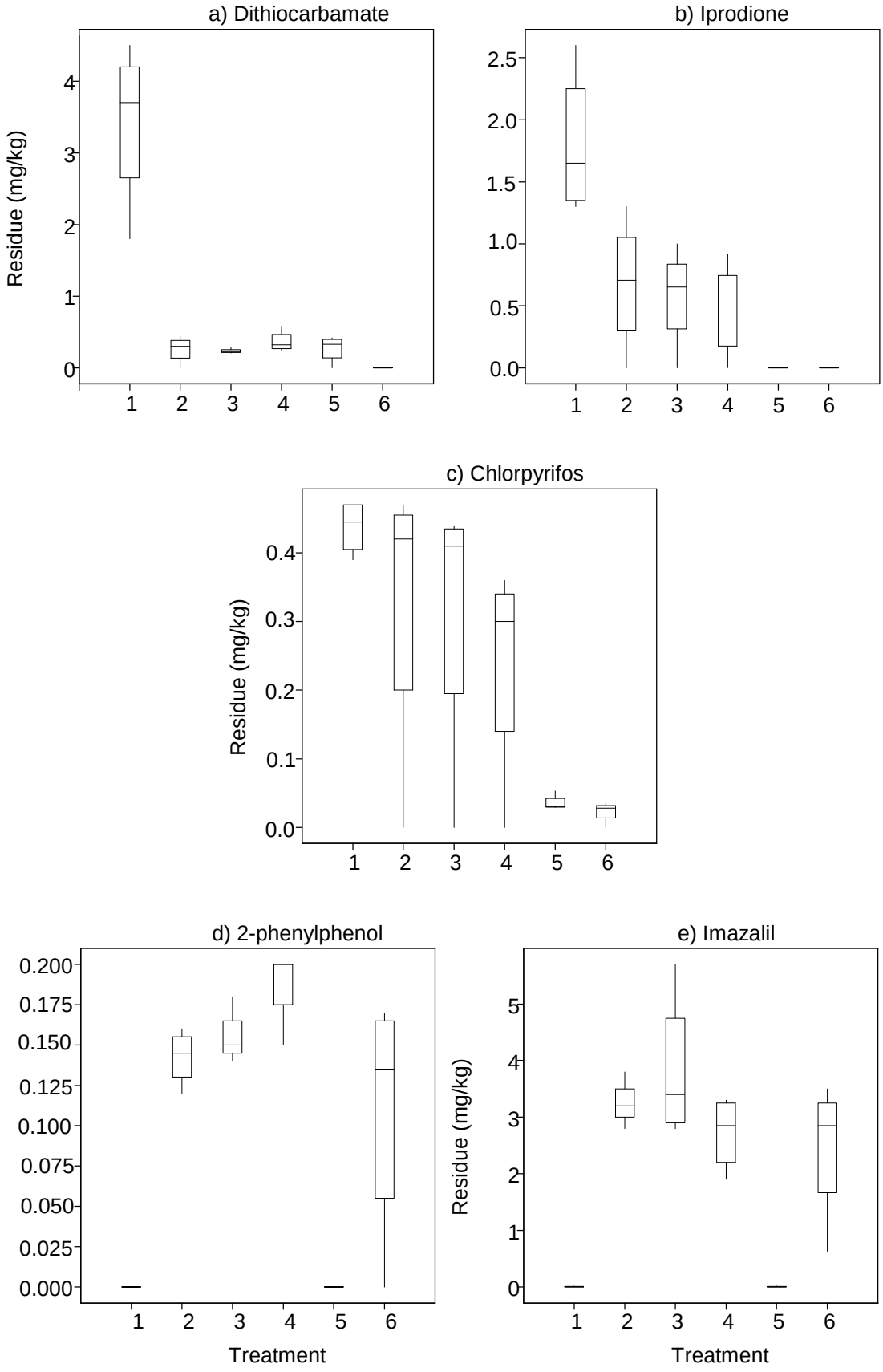

Figure 3.4.1. Box and whisker plots of residue analysis results. Treatment 1) spiked + no postharvest treatment, 2) spiked + standard postharvest treatment (SPT), 3) spiked + SPT + high pressure 2× dwell time, 4) spiked + pre-dip for 1 min in Hygiene Plus + SPT, 5) untreated fruit + no postharvest treatment, 6) untreated fruit + SPT

3.5 Summary

The aims of this chapter were to: 1) evaluate the residue profile likely to result from alternative fungicides and use patterns; 2) engage with the APVMA for pre-application assistance; 3) expand participation form Qld in the NRS and evaluate the existing residue situation; and 4) evalute the efficacy of postharvest processes for reducing residues in citrus. Addressing these aims has shown:

1) The new fungicides and use patterns evaluated in ***chapters 1*** and ***2*** of this project have resulted in manageable residues, compliant with several, but not all, citrus export markets.
2) Engagement with the APMVA has provided an outline of the further work needed in order to achieve registration for the various fungicides identified in ***chapters 1*** and ***2***. The only noteable issue being the need for Compound 4 residues to remain below 0.3 mg/kg in dried citrus pulp, otherwise costly animal feeding studies would be required. However, preliminary residue studies in ***section 3.2*** have so far failed to exceed the LOR for Compound 4, which is promising.
3) Increasing the participation of Qld in the NRS has shown the residue profile of Qld citrus to be very similar to the wider industry, with the exception of dithiocarbamate and iprodione residues. It is hoped that the new fungicides and use patterns evaluated in this project, as well as the long term aim of developing disease resistant varieties will greatly reduce dithiocarbamate and iprodione residues in Qld citrus.
4) Postharvest treatment of fruit makes a large contribution to reducing residue in citrus, but making further improvements would require more studies, and would possibly demonstrate diminishing returns i.e. standard procedures including high pressure washing already remove most of what can be removed. Instead, the greatest reductions in fruit residues will most likely come from a systems approach combining pre- and postharvest steps.

Chapter 4
Extension and communication

4.1 Industry presentations

Miles AK, Papacek D, 2016. Citrus pathology update. In. *Citrus Australia Regional Forum.* Gayndah, 1st March.

Miles AK, Papacek D, 2015. New fungicides for new challenges. In. *Citrus Australia Technical Forum.* Mildura, Australia, 16-17 March: Citrus Australia Limited.

Miles AK, 2015. Update on EBS and CBS management research. In. *Citrus Australia Regional Forum, 24th March.* Gayndah, Queensland.

Bodnaruk K, Griffin D, Harty A, Miles AK, Papacek D, 2014. Plan for registration of iprodione and abamectin. In. *Citrus Australia Regional Forum.* Gayndah, Queensland, 12th February.

Miles AK, Papacek D, 2013. Fungicide evaluation trials. In. *Strategic Agrichemical Review Process, 11th September.* Ibis Hotel, Melbourne.

4.2 Industry publications

Miles AK, 2016. Promising 'Emperor' brown spot fungicide getting closer. *Australian Citrus News* **Autumn**, 26.

Miles AK, 2014. MRL minefield? Plan ahead and tread carefully. *Australian Citrus News* **89**, 14-5.

4.3 Conferences

Smith MW, Gultzow DL, Newman TK, Parfitt SC, Miles AK. A co-inoculation technique to rapidly screen citrus hybrids for resistance to both scab and alternaria diseases. *Proceedings of the 13th International Citrus Congress*, *2016*. Foz do Iguacu, Brazil: International Society of Citriculture, 104.

Miles AK, Papacek D. Persistence of fungicide efficacy on mandarin fruit. *Proceedings of the Australasian Plant Pathology Society Conference, 14-16 September*, *2015*. Fremantly, Western Australia: Australasian Plant Pathology Society, 24.

Miles AK, 2014. Trip Report: Citrus Research International, 8th Citrus Research Symposium, 17th-20th August 2014, Central Drakensberg, Republic of South Africa. In., 29.

4.4 Project steering committee

Project steering committee meetings were undertake via phone on the following dates:

- **24th March 2014:** Ben Callaghan (HAL), Andrew Harty (CAL), Michael McMahon (Abbostleigh Citrus), Andrew Miles (R&DPI), Dan Papacek (Bugs for Bugs), Malcolm Smith (DAFF).

- **24th June 2014:** Kevin Bodnaruk (AKC Consulting), Ben Callaghan (HAL), Michael McMahon (Abbostleigh Citrus), Andrew Miles (R&DPI), Dan Papacek (Bugs for Bugs), Malcolm Smith (DAFF).
- **9th December 2014:** Kevin Bodnaruk (AKC Consulting), Ben Callaghan (HIA), Dale Griffin (Crop Protection Research), Andrew Harty (CAL), Michael McMahon (Abbostleigh Citrus), Andrew Miles (R&DPI), Dan Papacek (Bugs for Bugs).
- **22nd June 2015:** Kevin Bodnaruk (AKC Consulting), Ben Callaghan (HIA), Andrew Harty (CAL), Andrew Miles (R&DPI), Malcolm Smith (DAF), Peter Taverner (SARDI).
- **15th December 2015:** Kevin Bodnaruk (AKC Consulting), Michael McMahon (Abbostleigh Citrus), Ben Callaghan (HIA), Andrew Harty (CAL), Andrew Miles (R&DPI).

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