OPTIMISATION OF POSTHARVEST DRENCH APPLICATION OF FUNGICIDES ON CITRUS FRUIT

by

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Charmaine Christie

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SUMMARY

South Africa is the 2nd largest exporter of fresh citrus, after Spain, worldwide. Delays to the packline, i.e. degreening, can result in substantial postharvest decay such as green mould caused by *Penicillium digitatum* (PD). Pre-packline aqueous fungicide drench application is an important tool to minimize postharvest losses before degreening, which provides a favourable environment for infection. Sour rot, caused by *Geotrichum citri-aurantii* (GC), becomes an infection risk after rainfall and the availability of effective fungicides against this pathogen is limited. Thiabendazole (TBZ), pyrimethanil (PYR), guazatine (GZT) and 2,4-dichlorophenoxyacetic acid (2,4-D) are applied during drenching in South Africa for the control of postharvest diseases on citrus, although this application has not yet been standardized and guazatine use is restricted to certain export markets; GZT is the only fungicide in the drench mixture that is effective against sour rot. Therefore the aim of this study was to improve our understanding of drench application in terms of the influence of infection age, fruit orientation (pole), treatment exposure time and the addition of adjuvants and sanitisers on disease control.

Lemon, Satsuma mandarin and navel orange fruit were drenched with TBZ and PYR (1000 µg.mL^{-1} each) at different exposure times (14 s, 28 s and 56 s) and inoculated with PD 0, 6, 12, 18, 24, 30, 42, 48 and 54 h before (curatively) and 24 h after (protectively) treatment. Sporulation inhibition and residue loading were evaluated. Lemon and Satsuma mandarin fruit were exposed to a lower drench volume compared to navel orange fruit (26.5 and 64.3 L.min^{-1}, respectively). Batch differences played a significant role in green mould control with lemon and Satsuma mandarin fruit requiring treatment by 33.1 to 44.5 h and 23.8 to 32.1 h infection age, respectively, to gain 90% control. Exposure time only became significant with ≥ 30 h old infections on navel orange fruit at the higher drench volume used, with control declining more rapidly for fruit drenched at shorter exposure times. Control on navel orange fruit differed as much as 30.2% between exposure times with 54 h old infections and > 90% control was achieved by drenching fruit before 27 h. Protective control was generally effective (> 90%). These results support the proposition to drench all citrus types ≤ 24 h in order to reduce the risk for green mould decay development as sporulation inhibition was poor (< 50%) and fruit batches differed as much as 8 to 12 h in infection age for similar control levels. Valencia orange fruit were drenched with TBZ, PYR and 2,4-D (1000, 1000 and 250 µg.mL^{-1}, respectively; calyx-end facing upward, sideways and downwards) at 41.0 L.min^{-1} for 18 s with different adjuvant concentrations (0.0, 0.025, 0.05, 0.1 and 0.2 µl.mL^{-1}). Almost no differences were evident between concentrations, other than a negative effect on residue loading, deposition quantity and green mould control at the

highest adjuvant concentration tested. Fruit orientation was however significant, with fruit facing calyx-end upward resulting in higher residue levels, curative green mould control, deposition quantity and quality compared to the stylar-end.

Since sour rot inoculum levels can accumulate in the drench solution with dirt from fruit during drenching, Chlorine (Cl; 80 µg.mL^{-1}) and hydrogen peroxide/peracetic acid (HPPA; 0.6%) efficacy was compared for the control of GC spores (CFU.mL^{-1}) in solution without reducing fungicide persistence and efficacy. Wounded navel orange fruit were drenched with TBZ, PYR, GZT and 2,4-D (1000, 1000, 500 and 250 µg.mL^{-1}, respectively) during commercial packhouse trials with Cl or HPPA (80 µg.mL^{-1} and 0.6%, respectively) used as shock treatments at each bin stack (two bins) containing bin no. 1, 50, 100 and 150. Fungicide persistence and green mould infection (environmental inoculum) was similar regardless of whether sanitisers were present or not. Green mould infection increased by bin 150 (4.6 – 5.4% difference). Different sanitiser concentrations (0, 20, 40, 60 and 80 µg.mL^{-1} Cl or 0.00, 0.01, 0.10, 0.30 and 0.60% HPPA) were combined with a mixture of TBZ, PYR and 2,4-D (1000, 1000 and 250 µg.mL^{-1}, respectively) and GC spores (≈ 3.175 × 10^{4} spores.mL^{-1}) for 1, 3 and 60 min exposure during *in vitro* trials. Fungicide concentration was generally not influenced by sanitisers although sanitisers, however, did not persist after 60 min in solution exposed to fungicides. Only HPPA could completely reduce sour rot inoculum (0.0 CFU.mL^{-1}) after 1 – 3 min as Cl was not as effective at the high pH levels (> 10) of the solution. During *in vivo* trials, green mould inoculated (24 h before treatment) and wounded fruit were drenched with TBZ, PYR and 2,4-D (1000, 1000 and 250 µg.mL^{-1}, respectively) and GC spores (similar to *in vitro* trials) containing either 80 µg.mL^{-1} Cl or 0.3% HPPA with the addition of 0, 500 or 1000 µg.mL^{-1} kaolin, used to simulate dust accumulation during drenching. Sanitiser addition mostly did not affect solution concentration and green mould control, although HPPA treatments improved sour rot control on Valencia and Nadorcott mandarin fruit and resulted in improved green mould control on Nadorcott mandarin fruit; the lower level of kaolin (500 µg.mL^{-1}) tested in this study improved green mould and sour rot control in some cases.

Timeous drench application (≤ 24 h) provides effective green mould control whereas exposure time and adjuvant concentration requires further investigation in order to improve fungicide retention and distribution throughout highly congested fruit bins. Since drench pH is not regulated, HPPA was superior to Cl at high pH levels (> 10) for reducing sour rot infection and inoculum levels in solution, although further research is required to determine shock treatment intervals (within 60 min) required and potential side effects.

OPSOMMING

Suid-Afrika is wêreldwyd die tweede grootste uitvoerder van vars sitrus vrugte. Vertragings vanaf oes na die paklyn, vir onder andere ontgroening, kan lei tot aansienlike na-oes verliese, veral weens groenskimmel wat deur *Penicillium digitatum* (PD) veroorsaak word. ‘n Voor-paklyn stortstelsel is 'n belangrike instrument om na-oes verliese te beperk voor ontgroening, wat 'n gunstige omgewing vir infeksie ontwikkeling bied. Suurvrot, wat veroorsaak word deur *Geotrichum citri-aurantii* (GC), raak ‘n probleem in tye van hoë reënval en die beskikbaarheid van doeltreffende swamdoders teen hierdie patogeen is beperk. Thiabendazole (TBZ), pyrimethanil (PYR), guazatine (GZT) en 2,4-dichlorofenoksie-asynsuur (2,4-D) word aangewend in die voor-paklyn stortstelsels in Suid-Afrika vir die beheer van na-oes siektes op sitrus. Hierdie proses is nog nie gestandariseer nie en GZT gebruik is tot sekere uitvoer markte beperk; GZT is die enigste swamdoder wat effektief in die stortstelselmengsel teen suurvrot is. Die doel van hierdie studie was om die begrip van stortaanwending te verbeter in terme van die invloed van infeksie ouderdom, vrugoriëntasie, blootstellingstyd aan behandeling en die toevoeging van benatters en ontsmettingsmiddels om siektebeheer te verbeter.

Suurlemoen, Satsuma manderyn en navel lemoen vrugte is gestort met TBZ en PYR (1000 µg.mL^{-1} elk) met verskillende blootstellingstye (14 s, 28 s en 56 s) en geïnokuleer met groenskimmel 0, 6, 12, 30, 42, 48 en 54 h voor (kuratief) en 24 uur na (beskermend) behandeling. Spoorvormingsinhibisie en residu-lading is ook geëvalueer. Suurlemoen en Satsuma manderyn vrugte is aan 'n laer stortingsvolume in vergelyking met navel lemoen vrugte blootgestel (26.5 en 64.3 L.min^{-1}, onderskeidelik). Vruglotverskille het ‘n beduidende rol in groenskimmel beheer met suurlemoen en Satsuma mandaryn vrugte gespeel, en behandelings van onderskeidelik 33.1 – 44.5 en 23.8 – 32.1 h oue infeksies was nodig om 90% beheer te kry, afhangende van die vruglot. Blootstellingstyd het eers beduidend geraak met ≥ 30 h ou infeksies in navel lemoen vrugte, met die hoër stortvolumes, met beheer wat vinniger afneem vir vrugte gestort met korter blootstellingstyd. Beheervlakke het verskil van 30,2% op 54 h oue infeksies en > 90% beheer is behaal op vrugte wat binne 27 h na infeksie behandel is. Beskermende beheer was oor die algemeen effektief (> 90%). Hierdie resultate ondersteun die aanbeveling om alle sitrus tipes ≤ 24 h na-oes te stort om so die risiko vir groenskimmel ontwikkeling te beperk. Spoorvorminginhibisie was in die algemeen swak (<50%). Om die effek van ‘n benatter te bepaal, is Valencia lemoen vrugte met TBZ, PYR en 2,4-D (1000, 1000 en 250 µg.mL^{-1}, onderskeidelik) teen 41.0 L.min^{-1} vir 18 s met verskillende benatter konsentrasies (0.0, 0.025, 0.05, 0.1 en 0.2 µl.mL^{-1}) gestort. Geen verskille is ondervind behalwe 'n negatiewe uitwerking op residu-lading, neerslag

hoeveelheid en groenskimmel beheer teen die hoogste getoetste benatter konsentrasie. Vrug oriëntasie het egter ‘n beduidende rol gespeel, met hoër residu-vlakke, kuratiewe groenskimmel beheer, neerslag hoeveelheid en kwaliteit op vrugte wat kelk-end opwaarts gewys het, in vergelyking met die teenoorgestelde end van dieselfde vrug.

Siende dat suurvrot inokulumvlakke in die stortstelsel oplossing saam met stof van vrugte tydens stortaanwending kan opbou, is chloor (Cl; 80 µg.mL^{-1}) en waterstofperoksied / asynsuur (HPPA; 0,6%) se doeltreffendheid vir beheer van GC spore (CFU.mL^{-1}) in oplossing vergelyk, sowel as om te toets dat die swamdoderkonsentrasie en doeltreffendheid daarvan nie verminder word nie. Gewonde navel lemoen vrugte is met TBZ, PYR, GZT en 2,4-D (1000, 1000, 500 en 250 µg.mL^{-1}, onderskeidelik) gedurende kommersiële pakhuisproewe gestort, met Cl of HPPA (80 µg.mL^{-1} en 0.6%, onderskeidelik) wat toegedien is as skokbehandelings in die oplossing by elke vrugkratstapel (twee kratte) wat kratnommers 1, 50, 100 en 150 ingesluit het. Swamdoderbehoud en groenskimmel infeksie (vanweë omgewingsinokulum) was soortgelyk ongeag die eenwoordigheid van ontsmettingsmiddel. Groenskimmel infeksie het verhoog by krat 150 (4.6 – 5.4% verskil). Tydens *in vitro* proewe is verskillende ontsmettingsmiddel konsentrasies (0, 20, 40, 60 en 80 µg.mL^{-1} Cl of 0.00, 0.01, 0.10, 0.30 en 0.60% HPPA) met 'n mengsel van TBZ, PYR en 2,4-D (1000, 1000 en 250 µg.mL^{-1}, onderskeidelik) en GC spore (≈ 3,175 × 104 spore.mL^{-1}) gekombineer vir 1, 3 en 60 min blootstellingtyd. Swamdoderkonsentrasies is oor die algemeen nie beïnvloed deur ontsmettingsmiddels nie, maar ontsmettingsmiddels het egter nie in oplossing bly voortbestaan na 60 min blootstelling nie. HPPA kon suurvrot inokulum heeltemal uitwis (0,0 CFU.mL^{-1}) na 1 – 3 min en Cl was nie so effektief in die hoë pH vlak (> 10) van die oplossing nie. Tydens *in vivo* proewe is groenskimmel geïnokuleerde (24 h voor behandeling) en gewonde vrugte gestort met ‘n mengsel van TBZ, PYR en 2,4-D (1000, 1000 en 250 µg.mL^{-1}, onderskeidelik) en GC spore (soortgelyk aan *in vitro* proewe) wat 80 µg.mL^{-1} Cl of 0,3% HPPA bevat het, asook 0, 500 of 1000 µg.mL^{-1} kaolin. Die ontsmettingsmiddel het meestal geen negatiewe invloed op swamdoder konsentrasie en groenskimmel beheer gehad nie, alhoewel HPPA behandelings suurvrotbeheer op Valencia en Nadorcott manderyn vrugte verbeter het, asook verbeterde groenskimmelbeheer op Nadorcott manderyn vrugte. In sommige gevalle het die laer vlak van kaolin (500 µg.mL^{-1}) gelei tot verbeterde groenskimmel en suurvrot beheer.

Tydige stortbehandeling (≤ 24 h) lewer doeltreffende groenskimmel beheer, terwyl blootstellingstyd en benatter konsentrasie verder ondersoek moet word om swamdoderwerking en verspreiding deur dig-verpakte vrugkratte te verbeter. Met die wete dat die pH vlakke van stortstelseloplossings nie gereguleer word nie, is HPPA ‘n beter ontsmettingsopsie teen die hoë pH-vlakke (> 10) in stortoplossings. Verdere navorsing is nodig om skokbehandelingsintervalle en moontlike newe-effekte van ontsmettingsmiddels te

bepaal. Behoorlike vermenging van oplossings is ook noodsaaklik vir verbeterde swamdodereenvormigheid in oplossing en die daaropvolgende residu-lading.

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CONTENTS

CHAPTER 1

An introduction to postharvest pathogens on citrus and chemical methods of control in South Africa, with emphasis on green mould and sour rot

THE SOUTH AFRICAN CITRUS INDUSTRY

South Africa was rated 10th in terms of citrus production and 2nd in export next to Spain during the 2013/14 citrus season, having exported approximately 1 750 000 tons (Edmonds, 2015). The citrus industry in South Africa contributed R8.3 billion to the gross value of agriculture during the 2012/13 production season (Directorate Marketing, 2014) and provides employment for over 100 000 people making up 15% of the agricultural labour force (Potelwa, 2015). Major citrus export destinations include the Middle and Far East (21 and 10%, respectively), Northern Europe (20%), Russia (12%) and Asia (10%) during the 2014 citrus season (Edmonds, 2015).

Citrus is planted over more than 60 000 ha, with major production areas located in Limpopo, Mpumalanga, and the Western and Eastern Cape provinces (Edmonds, 2015). Due to its ecologically distinct citrus growing regions and diverse climatic conditions, which include tropical, sub-tropical and Mediterranean, a range of citrus cultivars are grown in South Africa (Pelser and la Grange, 1981; Mather, 1999; Ndou and Obi, 2013). Grapefruit and Valencia oranges are cultivated in the warmer climates of Limpopo, Mpumalanga and Kwazulu-Natal, whereas navel oranges, lemons and soft citrus are mainly grown in the cooler climates of the Eastern and Western Cape (Directorate Marketing, 2014), with ± 63% grown for export (Edmonds, 2015).

POSTHARVEST DISEASES OF CITRUS

Quality control is challenging as citrus can be stored for extended periods between harvest and consumption, this being mostly dictated by market demand (Bancroft *et al*., 1984; Eckert and Eaks, 1989). Postharvest losses occur primarily due to green and blue mould [*Penicillium digitatum* (Pers.: Fr.) Sacc. and *P. italicum* Wehmer, respectively], and sour rot [*Geotrichum citri-aurantii* E.E. Butler (*G. candidum* Link)] (Eckert and Eaks, 1989). The above-mentioned wound pathogens typically require nutrients from a fresh fruit injury site for infection and disease initiation (Brown, 1979; Barkai-Golan, 2001), although *Geotrichum* often requires more extensive damage to the fruit rind for infection to take place (Pers. comm. A. Erasmus). Other postharvest pathogens affecting citrus include: Diplodia (*Diplodia natalensis*) and Phomopsis stem-end rot (*Phomopsis citri*), anthracnose (*Colletotrichum gloeosporioides*), brown rot (various *Phytophthora* spp.), Alternaria stem-end

rot (black rot; *Alternaria citri*) (Zhang and Timmer, 2007; Montesinos-Herrero *et al*., 2009; Van Zyl *et al*., 2013; Kellerman *et al*., 2014) and Rhizopus spp. (Lesar, 2013).

Diplodia natalensis and *P. citri* can survive saprophytically on dead plant material in the orchard and are able to infect surrounding fruit through rain splash of pycnidia (water-borne spores) (Eckert and Eaks, 1989), infecting all citrus varieties (Ritenour *et al*., 2003). Stem-end rot pathogens establish in the button of the fruit (calyx and disk), remaining inactive (quiescent stage) until senescence of the button occurs, providing an entry point for infection (Eckert and Eaks, 1989; Barkai-Golan, 2001). After anthracnose spores contaminate developing fruit by rain or wind, spores germinate and give rise to appressoria within 12 hours of infection and remain quiescent until the fruit peel over-matures or is injured (Eckert and Eaks, 1989). Green fruit become susceptible to infection after prolonged exposure to ethylene during degreening (Barkai-Golan, 2001).

Phytophthora brown rot zoospores are water-borne, germinating immediately upon fruit contact and spreading rapidly from infected to adjacent uninjured fruit (Pelser, 1977; Smith, 1979). Extensive decay occurs mainly in humid coastal areas where this soil-borne fungus is more prevalent (McCornack, 1970). *Alternaria citri* mostly occurs in pome fruits, (Barkai-Golan, 2001) with some cases of disease on oranges and grapefruit (Ritenour *et al*., 2003), through the release of airborne spores with subsequent colonization occurring at the stem end or underneath the button (Barkai-Golan, 2001). *Rhizopus* spp. were isolated more often on berries and only on 5% of contaminated tangerine fruit out of a number of citrus varieties tested (Tournas and Katsoudas, 2005). *Rhizopus* spp. also only made up < 9.3% of fungal genera isolated from a packhouse in 2004/2005 (Fischer *et al*., 2009). This pathogen is able to survive as a saprophyte on debris, with spores carried by wind or water onto fruit surfaces (Lesar, 2013).

GREEN MOULD

Disease incidence and development

Green mould is the most common type of decay (McCornack, 1970) with blue mould only becoming a concern under conditions where green mould is suppressed (Smith, 1988). Montesinos-Herrero *et al*. (2009) noticed that 41% of untreated, naturally infected ‘Marisol’ mandarins decayed after 30 days at 20°C, with 62% of decay attributed to green mould, approximately eight times higher than blue mould occurrence. Lesar (2013) also found that green mould was involved in 80 – 90% of citrus losses during export. Green mould control proves to be challenging due to the millions of dust-like spores produced on infected fruit (Pelser, 1977). Decay occurring during the postharvest period results in not only direct monetary losses, but also loss of investment in production, packing, harvesting, transportation and handling, and negatively impacts consumer confidence (Brown and Miller,

1999). Secondary losses occur due to 'soilage', which results from decaying fruit depositing spores on the surface of adjacent healthy fruit, necessitating the removal and repacking of healthy fruit (Pelser, 1977; Eckert and Eaks, 1989; Smilanick *et al*., 1999).

Initial symptoms of disease involve the formation of a water-soaked lesion, visible within 24 - 36 h of infection (Barmore and Brown, 1982). The water-soaked lesion expands rapidly followed by growth of white mycelium over the lesion (Benhamou, 2004). Olive green conidiophores later form in the centre of the green mould lesion with the entire fruit invaded after 5 days (Benhamou, 2004).

Disease epidemiology

The peel of citrus fruit consists of two layers, namely the compact cells of the flavedo/exocarp (outer coloured rind), and the spongy parenchymatous albedo/mesocarp consisting of the inner white tissue (Hyodo and Nishino, 1981; Benhamou, 2004). The flavedo contains a uniform distribution of oil glands, which extend into the albedo (Kavanagh and Wood, 1967).

Penicillium digitatum requires damage to the fruit rind before infection can take place, with wounds being inflicted during harvesting, improper handling or insect activities in the orchard (Kavanagh and Wood, 1967; Shellie and Skaria, 1988; Brown, 2003). Deep injuries extending into the susceptible mesocarp, and minor injuries involving individual oil glands of the flavedo, both resulted in green mould infection (Kavanagh and Wood, 1967; Brown and Ismail, 1978; Brown *et al*., 2000). Shallow injuries between oil glands remained resistant to infection since the release of essential oils are required to destroy resistant flavedo cells in order for infection to take place (Kavanagh and Wood, 1967). Brown *et al*. (2000) states that minor punctures are frequent sites for infection as they are often overlooked during grading, and that these can result from rough handling, twigs within the tree canopy, or from sand grains accumulating in picking bags or on conveyor belts in the packline. Moisture and epicarp extracts from wounds increase infection and facilitate green mould development (Arimoto *et al*., 1995).

Penicillium spp. can rapidly produce billions of spores after 7 days at 25°C, which are highly dispersible via air currents (Gardner *et al*., 1986; Holmes and Eckert, 1995). Airborne spores can contaminate packhouses and orchards and survive between seasons as conidia (Gardner *et al*., 1986; Smilanick and Mansour, 2007). Temperature, moisture and humidity influence spore longevity, with conidia declining quicker in groves than in the protected packhouse environment (Smilanick and Mansour, 2007). Inoculum levels therefore build up over time with increasing decay and emergence of resistant isolates in packhouses where sanitation measures are not carried out properly (Gardner *et al*., 1986; Smilanick and Mansour, 2007). Fruit are more likely to become contaminated in high risk areas that

contain elevated spore concentrations, such as those within or adjacent to the fruit packing and dumping site (Gardner *et al*., 1986). Soiled healthy fruit can also develop green mould under conditions where fruit are tightly packed and wet, and the rind of the fruit is slightly damaged; accumulation of galacturonic acid in infected wounds induce rind pitting in the healthy fruit, providing entry for the pathogen (Kavanagh and Wood, 1967; Barmore and Brown, 1982).

Penicillium digitatum has been shown to acidify host tissue namely through the production of organic acids (citric and gluconic) and NH_4^+ utilization associated with H^+ influx. The pH level in healthy tissue was reduced from ± 4.7 to ± 3.1 in decayed tissue, which suggests that acidification compromises plant defences (Prusky *et al*., 2004; Smilanick *et al*., 2005). Green mould develops optimally at 25°C, with growth increasing from 10 to 25°C. Growth is retarded at temperatures from 25 to 30°C, and is inhibited above 35°C (Zhang and Swingle, 2005). Smoot *et al*. (1983) revealed that green mould growth slows between 4.5° - 10°C and is arrested below 1°C (Barkai-Golan, 2001).

Pathogenicity of *Penicillium digitatum* relies upon several mechanisms, which lead to infection and subsequent disease formation, namely: 1) pectin transeliminase (PTE), which is present in *P. digitatum,* but not *P. notatum*, a related mould lacking macerating activity (Bush and Codner, 1968); 2) accumulation of citric and gluconic acid involved in host tissue acidification (Prusky *et al*., 2004); 3) production of catalase, an enzyme that increases pathogenicity through the removal of hydrogen peroxide involved in host defence (Macarisin *et al*., 2007); and 4) exopolygalacturonase, which leads to the accumulation of galacturonic acid, both of which are responsible for peel maceration (Eckert and Eaks, 1989).

SOUR ROT

Disease incidence and development

Sour rot on citrus, tomatoes, carrots and other fruits and vegetables are caused by *Geotrichum* (Agrios, 2005). Fruit and vegetables kept under high humidity conditions are increasingly susceptible to sour rot (Agrios, 2005) and disease becomes more prevalent with increasing maturity (Brown, 1979). Sour rot on postharvest citrus fruit is caused by *G. citri-aurantii* and, although it is less common than green mould, significant losses can occur in areas or years of high rainfall (Eckert and Eaks, 1989; Mercier and Smilanick, 2005).

This disease can be controlled on the domestic (South African) market with guazatine, but not in several export markets where this fungicide is prohibited (Cunningham and Taverner, 2006). Sour rot can therefore be considered a serious postharvest disease of citrus after rainfall since it cannot be controlled by any other currently registered fungicides, such as imazalil (IMZ) and thiabendazole (TBZ) (Cunningham and Taverner, 2006; Horuz and Kmay, 2010) which effectively controls green mould. It is also difficult to detect incipient

infections during grading, with sour rot developing rapidly after shipment once fruit are transferred to ambient temperatures during marketing (Eckert and Eaks, 1989).

Following infection, a small soft water-soaked lesion develops within two days and is almost indiscernible from other decays (Eckert and Eaks, 1989). As the lesion rapidly spreads, a compact, cream-coloured fungal growth develops over the fruit surface while the inside of the fruit turns into a sour-smelling watery mass (Agrios, 2005) with a yeasty, fruit odour (Eckert and Eaks, 1989). Secondary infections can result without a wound, allowing this pathogen to spread from infected fruit to neighbouring healthy fruit (Pelser, 1977; Brown, 1979; Eckert and Eaks, 1989).

Disease epidemiology

Arthroconidia of *G. citri-aurantii* (Smilanick and Mansour, 2007) survive in soil and debris and accumulate on fruit surfaces through wind action, splash or direct contact with the soil; fruit nearest to the ground become easily contaminated (Brown, 1979). *Geotrichum citri-aurantii* inoculum can build up in dip tanks or drenchers with dirt and debris, infecting injured fruit (Brown, 1979). Sour rot can also spread from infected to adjacent healthy fruit, resulting in large nests of decay during storage and transport (Mercier and Smilanick, 2005), with severely diseased fruit disintegrating in the packline and further spreading inoculum (Pelser, 1977; Brown, 1979).

Initially, this pathogen requires a substantial injury into the albedo for penetration and subsequent infection, which often occurs due to fruit piercing insects in South Africa (Pelser, 1977; Brown, 1979) or due to damage during harvesting or handling of fruit (Brown, 2003). Damage to the oil glands in the fruit peel increases the chance of decay by 25 – 50% (Baudoin and Eckert, 1982).

Sour rot can grow and develop at temperatures between 4 – 30°C with optimal growth between 25 and 30°C; growth slows down considerably from 10 to 4°C (Plaza *et al*., 2003). Sour rot rapidly develops within a 5 day incubation period at 25°C in the case of an active rot, otherwise a dry lesion (2 – 3 mm in diameter) results if infection is arrested (Baudoin and Eckert, 1982). The chances of an active rot developing from an arrested infection into an active rot is slim after a 5 day incubation period (Baudoin and Eckert, 1982).

From previous work (Barash, 1968), Barash (1969) explains how polygalacturonase synthesis increased during germination and growth of *G. citri-aurantii*, which is responsible for galacturonic acid accumulation and subsequent peel maceration (Eckert and Eaks, 1989). The extracellular endopolyglacturonase produced by *G. citri-aurantii* reduced with decreasing osmotic potential of the growth medium, which could explain why turgid lemons are more susceptible to sour rot due to the high water potential of the fruit (Davis and Baudoin, 1986).

HOST SUSCEPTIBILITY

Despite the presence of wounds or sufficient quantities of inoculum, citrus fruit can still resist disease development, depending on susceptibility of the individual fruit (Eckert and Eaks, 1989; Prusky, 1996). Cultivar type plays a role in the susceptibility of fruit to green mould, with Erasmus *et al*. (2013) observing that 'Eureka' lemon and Valencia orange fruit were more resistant to disease than navel orange and Clementine mandarin fruit. Smilanick *et al*. (2008) inoculated mandarin fruit with lower concentrations of *P. digitatum* than lemon and navel orange fruit due to its greater susceptibility to infection, and D'Aquino *et al*. (2006) found that all the wounded, non-inoculated, untreated Satsuma fruit were infected after 5 days of storage at 20°C, mainly by *P. digitatum*. Differences in disease susceptibility is also found between fruit of the same cultivar, with a lower decay incidence found in wounded, noninoculated Valencia late orange fruit (60.1%) compared to Tarocco (89.2%) and Sanguinello oranges (76.6%), when dipping fruit at 20°C as a control treatment and stored at 20°C for 12 days (D'Aquino *et al*., 2006).

Referring to work of others, Montesinos-Herrero *et al*. (2009) explains that the physical and physiological condition of fruit also influences susceptibility to decay, with mature fruit being more prone to decay due to lower levels of antifungal compounds being produced in response to fungal attack. Fruit susceptibility increases with increasing maturity and fruit stored for long periods of time, such as lemons, become increasingly more prone to sour rot development (Brown, 1979; Baudoin and Eckert, 1982). Also, fruit exposed to storage treatments, simulating export conditions, resulted in significantly higher decay levels on untreated early season lemon fruit (65%) compared to late season harvested fruit (42.5%) (Venditti *et al*., 2010).

Rootstock, harvest season, fruit condition in the orchard and postharvest environment also influence host susceptibility (Eckert and Eaks, 1989). Fruit resistance to sour rot decay increases when harvested during dry, sunny periods and decreases when harvested after periods of rainfall (Baudoin and Eckert, 1982).

Plants can resist pathogen infection through induced defences, such as: 1) the hypersensitive response (HR). Macarisin *et al*. (2007) observed that plants initially react to pathogens through a hydrogen peroxide (H_2O_2)-oxidative burst followed by HR and tissue lignification, which was suppressed by *P. digitatum* through the production of catalase; 2) cell wall modifications; 3) formation of phenolic compounds, such as lignin, which accumulate in the exocarp (Benhamou, 2004); (4) production of phytoalexins/anti-microbial compounds such as scoparone, found to reduce pathogen germination and germ-tube elongation - synthesis of this compound increases in response to UV light and heat treatment (Kim *et al*., 1991; Venditti *et al*., 2010); 5) accumulation of pathogenesis-related (PR) proteins (e.g. chitinase and β-1,3-glucanase), stimulated by UV treatment (Porat *et al*.,

1999); and elicitors of plant defences such as chitosan, found to reduce green mould infection (Benhamou, 2004) by inducing the accumulation of chitinases and other defence related compounds (El Ghaouth *et al*., 1992).

Synthesis of lignin or lignin-like polymers, which act as a physical barrier to germ-tube penetration, can be induced in wounds exposed to high temperatures (30°C) and relative humidity (RH: 95 – 100%); under these conditions, shallow injuries (flavedo) can lignify and develop resistance to *P. digitatum* infection (Brown and Ismail, 1978). Deeper injuries to the albedo remain susceptible to *P. digitatum* as a result of no lignin being produced in the presence of peel oil (Kavanagh and Wood, 1967; Brown and Ismail, 1978). Although, Baudoin and Eckert (1985) found that resistance development in the fruit coincided with the formation of a barrier-like zone in both the flavedo and albedo, with this lignin-like substance stimulated more significantly in wounds inoculated with *G. citri-aurantii*. Phenylalanine ammonia lyase (PAL) is also induced in response to pathogen infection, with activity found to be higher in the flavedo than the albedo (Ballester *et al*., 2006). PAL activity can also be inhibited in the presence of *P. digitatum* infection, confirming the pathogens ability to suppress various defence responses (Ballester *et al*., 2006).

CONTROL

In reference to work done by other researchers', Brown and Chambers (1996) remarks on the extensive research that has gone into the development of biological agents for the replacement of fungicides due to the cost of registering and re-registering fungicides. Alternatives to fungicides are also developed due to the rate that pathogens develop resistance to chemicals (Lesar, 2006) and consumer safety concerns (Tournas and Katsoudas, 2005) necessitating the need for integrated postharvest management (IPHM); this term was introduced by Taverner (2014).

According to Eckert and Eaks (1989) and Eckert (1995), postharvest decay control involves several integrated strategies such as: orchard and packhouse sanitation, appropriate handling of fruit, washing fruit with broad–spectrum chemicals, treatment of fruit with selective fungicides and growth regulators and cold storage.

Non-chemical control methods

Sanitation

Penicillium spp. can rapidly produce large numbers of highly dispersible conidia (Holmes and Eckert, 1995), which can survive in the orchard and packhouse between seasons (Gardner *et al*., 1986; Smilanick and Mansour, 2007). Cleaner packhouses resulted in fewer decayed fruit (Bancroft *et al*., 1984) and fungicide resistant spores only emerged in packhouses during periods where sanitation programs were not carried out diligently

(Gardner *et al*., 1986). Sanitary practices should therefore be stringently enforced to limit the spread of airborne spore populations and the emergence of resistant strains (Gardner *et al*., 1986; Brown, 2003).

Since *G. citri-aurantii* can survive in soil and debris on the orchard floor, dirt accompanying harvested fruit in the orchard bin needs to be removed as soon as possible (Brown, 1979). Partial control of sour rot can be obtained through sanitation and cold storage practices after harvest, although temperature variations during transport and marketing and potential chilling injury to the fruit limit the success of this strategy (Mercier and Smilanick, 2005).

Daily removal of all possible inoculum sources, such as fruit, leaves and other debris, from the packhouse is essential. Orchard bins require thorough cleaning before each trip to the field and equipment need to be washed regularly with hot water or approved sanitising agents (Ritenour *et al*., 2003). Fruit dumping and re-packing should be performed in remote areas (isolated from the packinghouse and storage areas) and exhaust fans installed to remove spores from the environment (Brown, 2003). After sorting, decayed fruit must be removed as far away as possible from the packhouse to prevent further contamination of the line as spores can be carried into the packhouse by insects or the wind (Bancroft *et al*., 1984; Ritenour *et al*., 2003). Orchard sanitation involves removing fallen fruit to prevent contamination of the tree canopy, and careful fruit handling practiced during harvesting to avoid fruit wounding (Eckert and Eaks, 1989; Carstens *et al*., 2012).

Cultural control practices

Physical treatments show direct or indirect activity against pathogens, and include ultraviolet light (UV-C, 254 nm) and heat treatment, cold storage, and controlled atmosphere, as discussed below. Ultraviolet treatment was shown to reduce green mould decay from 80% to 30% 7 days after inoculation (Porat *et al*., 1999). Venditti *et al*. (2010) provided a more comprehensive treatment by combining UV-C with sodium bicarbonate (SBC), resulting in phytoalexin production in the flavedo and scoparone in the albedo. Treatments performed with different concentrations of potassium sorbate (KS) and sodium bicarbonate (SBC) gave variable results in terms of sour rot control, depending on the temperature of the treatment solution. At 25°C the control exhibited ± 90% sour rot incidence, which was reduced to between 40 and 60% when treating fruit with KS and SBC; further significant reductions in disease incidence was obtained at each treatment when heating the solution to 50°C (Smilanick *et al*., 2008). Fruit should be stored under conditions where disease development and fruit senescence is retarded (Barkai-Golan, 2001), making cold storage vital as *Penicillium digitatum* and *G. citri-aurantii* germination is delayed and growth slowed down below 10°C (Kassim and Khan, 1996; Plaza *et al*., 2003). Erasmus *et al*. (2011) found that

P. digitatum infection was reduced by 20% on control treatments exposed to cold-stored incubation periods (21 days at 7°C followed by 7 days at 23°C) in comparison to fruit placed under ambient storage conditions (14 days at 23°C). Although, the effect of cold storage on fruit should also be kept in mind as lemons and grapefruit cannot be stored below 10°C for long periods of time due to the risk of chilling injury (Barkai-Golan, 2001).

Heat treatment involves exposing fruit to either wet (hot water treatments) or dry treatments at high temperatures (curing). Curing comprises placing fruit at 35°C for 48 h with ± 95% RH for complete reduction in green mould incidence, although stem-end rot incidence increased (Zhang and Swingle, 2005). Lemon fruit exposed for 3 days at 36°C had no signs of decay for 2 months during storage at 17°C (Kim *et al*., 1991). Fruit subjected to lower temperatures (< 35°C) during curing resulted in increased green mould decay (Zhang and Swingle, 2005). Curing at 35°C should also control sour rot, which grows optimally between 25 – 30°C at 0.99 a_w (Plaza *et al*., 2003). Reduction in postharvest decay during curing is a result of thermal inhibition (Kinay *et al*., 2005), wound healing (induced lignin formation) and stimulation of antifungal compounds such as scoparone (Brown and Ismail, 1978; Kim *et al*., 1991). Stange *et al*. (1994) found that curing was effective at reducing green mould incidence, but was not always reliable and provided no antisporulant action.

Biological control

Biological agents involve the use of microbial antagonists, which control pathogens through competition, antibiosis, direct parasitism and induced host resistance (Janisiewicz *et al*., 2000; Barkai-Golan, 2001; Benhamou, 2004). The biological control products Aspire (*Candida oleophila)* and BioSave™ 1000 (*Pseudomonas syringae*) have been registered by Ecogen and EcoScience Corporation, respectively, to control postharvest pathogens on citrus fruit by competing for nutrients released by wounded fruit, although neither product will prevent green mould from sporulating if infection is successful (Brown and Chambers, 1996). Aspire and BioSave™ 1000 significantly reduced green mould decay in most treatments applied by Brown and Chambers (1996), although it was still not comparable to IMZ and TBZ efficacy, which was significantly better. Droby *et al*. (2002) found the ability of *C. oleophila*to to induce host resistance to green mould was dependant on distance from wounds, time of wound-inoculation and yeast concentration (10^8 and 10^9 cells/ml).

Verticillium lecantii (Zimm.) is another promising mycoparasite shown to antagonize *P. digitatum* and induce host resistance, resulting in reduced disease incidence under experimental conditions (Benhamou, 2004). Although *V. lecantii* has good prospects for disease control, Benhamou (2004) states that it cannot be registered until questions of human safety, and its ability to be used on a commercial scale and within an integrated

disease management system, has been answered. A significant drop in sour rot disease incidence (%) from the control was seen when treating *G. citri-aurantii* inoculated and naturally infested fruit with the biological control agent *Bacillus amyloliquefaciens* or with tea saponin (TS), a natural surfactant, and storing fruit at 25°C for 5 days and 4 weeks, respectively; disease incidence (%) was further significantly reduced when combining both treatments, again presenting effective protective capabilities, although curative action was not assessed (Hao *et al*., 2011).

Biological agents can be an important part of a resistance management program, controlling resistant pathogen strains unaffected by prevailing synthetic fungicides (Wisniewski *et al*., 2001) and protecting fruit against potential infections (Benhamou, 2004; Taverner, 2014), which is especially important during in-line packhouse treatments in preparation for fruit export. Chemical control is still vital in industry for consistent disease management, with no viable biological agents practically used in industry.

Chemical control methods

Fungicides can be applied to fruit using several methods, namely: dip, in-line aqueous spray, wax and bin drench treatment (Kaplan and Dave, 1979; Förster *et al*., 2007). Dip treatment involves soaking fruit in a fungicide solution bath, where fungicide concentration, exposure time and pH should be carefully monitored and adjusted for optimal fungicide residue loading (Brown and Miller, 1999; Erasmus *et al*., 2013). An in-line aqueous spray system or in-line drencher (flooder) is used in California, and is a viable alternative to dip application in South Africa (Erasmus, unpublished data). Fruit move through the flooder over rotating brushes, passing through a recirculating low-pressure high- or low-volume aqueous fungicide solution; solution temperature is adjustable (Smilanick *et al*., 2003; Förster *et al*., 2007; Kanetis *et al*., 2008b) and are overall more manageable compared to dip application (Erasmus, unpublished data). Following dip or in-line aqueous spray treatment, fruit should be dried using hot air and/or brushes before subsequent wax treatment (Brown and Miller, 1999). Commercial fungicide-containing waxes are added to fruit to improve appearance and water retention, as well as providing additional protection against infection through the incorporation of fungicides into the waxes (Eckert and Eaks, 1989; Brown and Miller, 1999).

Bin drench application will be discussed in further detail in the following sections and represents pre-packline fungicide application, which supplements the aforementioned in-line fungicide application methods already covered.

DRENCH APPLICATION

Description and use

Lemons and oranges harvested early in the season usually require degreening to improve fruit peel colour, as do late harvested Valencia oranges due to regreening on the tree (Eckert and Eaks, 1989). Optimal carotenoid accumulation, necessary for desired fruit colour, occurs in the flavedo between 15 and 25°C (Nigg *et al*., 1956; Wheaton and Stewart, 1973). Since green mould grows optimally at 25°C (Plaza *et al*., 2003; Zhang and Swingle, 2005) and sour rot grows optimally at 25 and 30°C (Plaza *et al*., 2003), this temperature range is not ideal during degreening as it increases the risk of postharvest decay. Degreening, however, involves exposing fruit to temperatures ranging from 18° to 25°C at 94 - 96% RH, with the addition of 1 – 5 ppm ethylene gas, for several days in South Africa in order to achieve acceptable external rind colouring at these higher temperatures (Krajewski and Pittaway, 2010). Other countries may degreen at temperatures ranging from 27° to 33°C at 90 - 96% RH (Eckert and Eaks, 1989; Zhang and Swingle, 2005; Sdiri *et al*., 2012). Fruit cannot be subjected to ethylene for too long as it become increasingly susceptible to calyx senescence with prolonged ethylene exposure (Sdiri *et al*., 2012). Also, storage of fruit at 25°C increased fruit susceptibility over time to sour rot infection, which was further hastened with the addition of ethylene (20 – 50 µl/L) (Baudoin and Eckert, 1982). Fruit therefore need to be stored in cooler environments as soon as possible following harvest, which is delayed during degreening.

An impossible balance therefore needs to be achieved between degreening and disease control, so it is not surprising that Smilanick *et al*. (2006b) observed losses during degreening ranging between 2 – 30%, depending on environmental conditions in the orchard prior to harvest. Fungicide treatment is therefore necessary due to the potentially high losses that can occur during degreening, with green mould and stem-end rot incidence reduced when drenching fruit with TBZ (500 ppm) prior to curing (Zhang and Swingle, 2005).

Soil pathogens, such as *GC*, survive in soil and debris and contaminate fruit hanging near the ground through wind action, splash or direct contact (Brown, 1979). Since harvested fruit are drenched directly in field bins with a re-circulating fungicide solution (Brown and Miller, 1999), sour rot inoculum and dirt can accumulate in dip tanks or drenchers, potentially infecting vulnerable wounded fruit (Brown, 1979; Barkai-Golan, 2001). Since no information has been found in literature regarding adequate dirt removal with current methods involving dumping tanks regularly and replenishing it with clean water and a new fungicide solution (Cunningham and Taverner, 2006), disinfectants could be used to reduce the microbial load (Brown, 1979) and sour rot inoculum due to the lack of registered fungicides available to control sour rot.

Fruit should be drenched if packline fungicide treatment is delayed for more than 24 hours after harvest, which is the case when degreening fruit, a process that can take up to 5 days (Brown and Miller, 1999; Smilanick *et al*., 2006b). It is important to drench fruit destined for degreening immediately after harvest (Pers. comm. A. Erasmus) while the fruit are still in the field bins (Dodd *et al*., 2010), ready for the degreening chamber.

Brown *et al*. (1988) describes one drench system, constructed by Waverly Growers Cooperative, where ± 3500 litres of solution was pumped, using two pumps at about 1300 L/min, through spray nozzles over an entire trailer loaded with fruit orchard bins for 3 min. Solution run-off was then recirculated through PVC piping, 15 cm in diameter, back into the spray nozzles (Brown *et al*., 1988). An orchard bin containing 380 kg of fruit removed approximately 4 to 5 litres of drench solution, necessitating the monitoring and maintenance of fungicide concentrations in the drench tank (Brown *et al*., 1988). Coverage also needs to be monitored when stacking fruit bins (Brown and Miller, 1999). Fungicide mixes should also be constantly agitated in the treating tank of drenching systems in order to keep wettable powders from settling out of solution and to provide uniform application (Brown and Miller, 1999).

Zhang and Swingle (2005), Erasmus *et al*. (2011) and Kellerman *et al*. (2014) used experimental drench systems during trials designed to simulate industry best practice, with these *in vivo* drench systems containing a solution reservoir tank, showerhead or spray manifold and pumps for re-circulating the solution through the system at a specified flow rate over crates containing treatment fruit. Zhang and Swingle (2005) and Erasmus *et al*. (2011) drenched 3-crate stacks during treatments in order to simulate a commercial bin stack, whereas Kellerman *et al*. (2014) drenched bins individually.

Fungicides used for the control of green mould and sour rot

Fungicides registered for the control of postharvest diseases on citrus in South Africa (Pers. comm. K. Lesar; Taverner, 2001; Erasmus *et al*., 2011; Kellerman *et al*., 2014) include; thiabendazole (TBZ), imazalil (IMZ), guazatine (GZT; certain markets) and the new 'reduced risk fungicide' pyrimethanil (PYR). The plant growth regulator 2,4-D is also used to enhance fruit resistance when applied during degreening or prior to storage by delaying button senscence (Pers. comm. K. Lesar; Pelser, 1977; Barkai-Golan, 2001).

Imazalil and TBZ are fungicides most widely used for the control of postharvest decay in citrus, providing effective curative control and sporulation inhibition of green mould, but provides practically no control against sour rot (Kaplan and Dave, 1979; Schirra *et al*., 2000; Smilanick *et al*., 2006a; Liu *et al*., 2009). It is not recommended to include IMZ during drenching, especially if it is going to be applied more than 24 h later in the packhouse, as part of a resistant management protocol, which is why TBZ and PYR is the main component

of drench application (Pers. comm. A. Erasmus). Guazatine has been included in drench application for the control of sour rot, blue mould and benzimidazole sensitive and resistant strains of green mould (Eckert and Eaks, 1989; Wild, 1994). The plant growth regulator 2,4-D reduces stem-end rot caused by Diplodia, Phomopsis (Pelser, 1977; Barkai-Golan, 2001) and Alternaria (Brown and Miller, 1999) indirectly by delaying stem-end button senescence of citrus fruit (Brown and Miller, 1999; Barkai-Golan, 2001). Pyrimethanil is also added to a drench solution as it is able to effectively control TBZ and IMZ resistant strains of *P. digitatum* due to its different mode of action (Smilanick *et al*., 2006a). Other fungicides that fall under the 'reduced risk' category include Propiconazole (PPZ) (European Food Safety Authority, 2012) and Fludioxonil (FLU) (Zhang and Timmer, 2007; D'Aquino *et al*., 2010).

Penicillium digitatum resistant-biotypes rapidly emerge following each successive fungicide introduction, leaving the industry with a limited selection of fungicides effective against green mould and other postharvest diseases of citrus. IPHM techniques therefore need to be implemented in order to prevent losing efficacy of major postharvest fungicides such as TBZ and IMZ (Taverner, 2014) by combining available fungicides with different modes of action for optimal control of postharvest citrus diseases. The simultaneous registering of three 'reduced risk' fungicides from different chemical classes provides the opportunity to better manage the risk of resistance development and to more effectively control IMZ and TBZ resistant *P. digitatum* isolates (Kanetis *et al*., 2008a). Propiconazole may also play an important role in sour rot management (McKay *et al*., 2012a) whether or not GZT is removed from the market.

Thiabendazole

Thiabendazole was developed in 1971 as a systemic (2(4-thiazol-4-yl) benzimidazole fungicide (Dodd *et al*., 2010) that inhibits microtubule assembly during mitosis and adversely affects respiration of the pathogen (Barkai-Golan, 2001). Allen and Gottlieb (1970) found that TBZ targeted the terminal electron transport system of the pathogen, with secondary effects resulting in decreased lipid, nucleic acid and protein synthesis. Standard rates of TBZ control Diplodia and Phomopsis stem-end rots (Brown and Chambers, 1996). Thiabendazole was able to effectively reduce stem-end rots on fruit stored at 21°C for up to two weeks (Brown and Chambers, 1996), although it is not active against *Rhizopus, Phytophthora, Alternaria* and *Geotrichum* (Barkai-Golan, 2001).

This fungicide is able to control sporulation and protect fruit from subsequent infection (Schirra *et al*., 2008) due to persistent residues (Smilanick *et al*., 2006b). Thiabendazole has a MRL tolerance of 10 ppm in the USA, Canada and Japan, and this is reduced to 5 ppm in Europe (Ritenour *et al*., 2003). A TBZ residue of ≥ 0.2 $\mu g.g^{-1}$ is required on fruit before degreening in order to effectively control green mould and Diplodia stem-end rot

(Smilanick *et al*., 2006b). Thiabendazole is not very soluble in water, requiring constant agitation to ensure uniform application and to prevent the chemical from settling out of solution (Ritenour *et al*., 2003), with improved solubility in dilute acids and alkalis (Barkai-Golan, 2001).

McCornack (1970) referring to previous studies states that TBZ is more effective than other postharvest fungicides in that it requires no pH control and is effective at ambient temperatures. A lower dose of TBZ (200 $\mu g.ml^{-1}$) is needed when applied at 50°C as opposed to TBZ (1200 $\mu g.ml^{-1}$) at ambient temperature in order to load the same residues (Schirra *et al*., 2000). When drenching fruit with TBZ (500 ppm) before curing at 35°C (95% RH for 48 h), green mould and stem end rot incidence were reduced from 29.6 and 10.9% to 0.3 and 0.7%, respectively (Zhang and Swingle, 2005), demonstrating the advantage of drenching fruit before degreening. In naturally infected orange fruit drenched with a mixture of TBZ, sodium bicarbonate and chlorine, green mould incidence was reduced from 11% among untreated fruit, to 2% (Smilanick *et al*., 2006b).

Benomyl is a benzimidazole fungicide that was developed shortly after TBZ, in 1973, and was used in South Africa as a pre-harvest spray for the control of *Guignardia citricarpa* (Kiely) (Pelser, 1977; Dodd *et al*., 2010). Due to the same mode of action of these benzimadazole fungicides (Lyr, 1995), *P. digitatum* resistant biotypes have occurred in South Africa due to the routine pre- and postharvest application of these fungicides for controlling black spot and green mould on citrus, respectively (Pelser, 1977; Dodd *et al*., 2010). Thiabendazole should therefore not be used alone during drench application in order to lower selective pressure for the development of *P. digitatum* resistant biotypes, combining TBZ with other fungicides with different modes of action.

Imazalil

IMZ was developed and introduced in the 1970s (Pelser, 1977; Kaplan and Dave, 1979; Barkai-Golan, 2001) and shown to effectively control green mould decay and sporulation of both TBZ sensitive and resistant isolates of *P.digitatum* (Kaplan and Dave, 1979), with only partial control of Diplodia and Phomopsis stem-end rots (Brown and Chambers, 1996) and no activity against sour rot (Schirra *et al*., 2000) and *Alternaria* (Brown and Miller, 1999). Shortly after the introduction of IMZ in South Africa in 1980, *P. digitatum* strains resistant to IMZ was reported by Keith Lesar in 1999 (Pers. comm. K. Lesar; Dodd *et al*., 2010), which further necessitates the development of new chemicals (Barkai-Golan, 2001). The primary mode of action of IMZ is inhibiting C-4-desmethyl sterol synthesis, which inhibits ergosterol synthesis, and was speculated to precede impaired membrane synthesis and function (Siegel and Ragsdale, 1978).

Dip treatment with IMZ at 500 ppm reduced decay caused by green and blue mould by 80 – 95% as well as reducing sporulation by 85 – 100%, with drench treatment giving slightly reduced control (Kaplan and Dave, 1979). Spraying provided good results when using 1000 ppm IMZ and only after the brushes became saturated with the spray-solution (Kaplan and Dave, 1979). Imazalil (1000 $\mu g.mL^{-1}$) reduced *P. digitatum* decay from 50% on untreated fruit to 0% for up to 30 days after treatment (Dore *et al*., 2010). Imazalil effectiveness is related to solution pH (Smilanick *et al*., 2005) with Erasmus *et al*. (2013) showing that fruit dipped with IMZ sulphate at pH 6 and 3 for 45 and 90 s, respectively, resulted in optimal residue loading without the risk of exceeding the maximum residue limit (MRL) of 5 $\mu g.g^{-1}$.

Pyrimethanil

Following the development of Imazalil resistant strains (Pers. comm. K. Lesar; Dodd *et al*., 2010), PYR was used as part of a more efficient resistance management strategy (Kanetis *et al*., 2008a). Pyrimethanil is able to effectively control sodium o-phenylphenate, IMZ, and TBZ resistant strains of *P. digitatum* (Smilanick *et al*., 2006a) as well as Diplodia stem-end rot (Pelser, 1977) due to its different mode of action (Smilanick *et al*., 2006a). PYR was originally developed to control *Botrytis cinerea* (Rosslenbroich and Stuebler, 2000). Heye *et al*. (1994) described the mode of action of pyrimidines using other researcher's results, indicating that these chemicals interfere with methionine biosynthesis and are involved in the inhibition of various fungal hydrolytic enzymes.

The MRL for PYR on citrus fruit is 10 and 8 $mg.kg^{-1}$ in the USA and as general export tolerance, respectively (The European Commision, 2014; Hattingh and Hardman, 2015). Increased PYR residue levels were loaded with increasing temperature from 20 to 50°C (D'Aquino *et al*., 2006).

Dip treating fruit, inoculated the day before, with 200, 400 and 600 $\mu g.ml^{-1}$ PYR at 20°C followed by storage at 20°C for 12 days was found to control 92, 97 and 100% of green mould infections , respectively (D'Aquino *et al*., 2006). Green mould on oranges inoculated 36 h before treatment was effectively controlled when combining heat (50°C) and potassium sorbate (KS) with very low rates of PYR (50 $\mu g.ml^{-1}$) (Smilanick *et al*., 2008). Pyrimethanil has good curative action with effective control attained up to 24 h after inoculation, however poor protective activity with 24 and 48 h old infections (Smilanick *et al*., 2006a) and poor antisporulant action (Kanetis *et al*., 2007).

Pyrimethanil is able to effectively control IMZ and TBZ resistant strains of *P. digitatum* due to its different mode of action (Smilanick *et al*., 2006a). It is therefore reasonable to combine both PYR and TBZ during drenching, as this combination will effectively control TBZ- and IMZ resistant *Pencillium* isolates and inhibit sporulation, IMZ can therefore be applied later during packhouse treatments with a reduced risk of resistance build up. Also,

PYR efficacy is not affected by pH and therefore does not require pH adjustment during treatment (Smilanick *et al*., 2006a), making it desirable for drench usage.

Kanetis *et al*. (2008b) recommends using new compounds, such as PYR, judiciously in order to ensure lasting efficacy. PYR-resistant isolates of *P. digitatum* were found in citrus groves situated in California (Kinay *et al*., 2007), although there has been no reports in South Africa.

Guazatine

Guazatine (1,17-diguanidino-9-aza-hepta-decane acetate; GZT) (Rippon and Morris, 1981) is a broad-spectrum, water-soluble fungicide that has been found to be effective against sour rot, blue and green mould, with no activity against *Alternaria* or *Phomopsis* stem-end rots (Eckert and Eaks, 1989; Wild, 1994; Barkai-Golan, 2001). At 24°C, sour rot decay development in Satsuma mandarin fruit, inoculated 24 h before treatment, decreased from 84.07% (control) to 46.1 and 22.2% when treating fruit with GZT (900 ppm) and a combination of GZT and sodium bicarbonate (SBC), respectively (Horuz and Kmay, 2010). Further trials performed by Horuz and Kmay (2010) on different GZT, IMZ, FLU, TBZ and azoxystrobin concentrations established that only GZT, which is highly effective against both GZT sensitive and resistant sour rot isolates, and FLU can control sour rot, although results for FLU were inconsistent. Eckert and Eaks (1989) uses other researchers work to discuss GTZs ability in controlling sensitive and resistant isolates of green and blue mould between 250 and 1000 $\mu g.ml^{-1}$, although GZT shows no protective or anti-sporulation activity and therefore cannot be used on its own. A minimum concentration of GZT (50 ppm) was found to completely control benzimidazole-resistant strains of green mould after 7 days of storage in another article by Hartill *et al*. (1977).

Within the fungicide mixture adopted in South Africa, only GZT is most effective against sour rot while other treatments merely reduce sour rot incidence, although use is becoming increasingly restricted in several export markets (Lesar, 2006; Cunningham and Taverner, 2006; Smilanick *et al*., 2008). Guazatine is no longer permitted in the USA, Japan and Korea (Hattingh and Hardman, 2015) and its use is partly restricted due to difficulty in choosing a method for residue analysis as it consists of several derivatives with quantification techniques focused on one or two components (Dreassi *et al*., 2007).

The removal of GZT is a concern as sour rot infections are difficult to detect during grading, resulting in rapid sour rot development once fruit are transferred to ambient temperatures during marketing if not controlled (Eckert and Eaks, 1989). Propiconazole has recently been registered for postharvest application on citrus and is shown to be highly effective against sour rot (McKay *et al*., 2012a), but imazalil (IMZ) and PPZ can lead to resistance build-up against this group of fungicides and cross resistance due to the same

mode of action (demethylation inhibitors) (Lyr, 1995). It is therefore not advisable to apply PPZ during drench application as a pre-cursor to IMZ packline treatment.

Thiabendazole has very limited activity against sour rot (Barkai-Golan, 2001) and is therefore used in conjunction with GZT during drench application (Eckert and Eaks, 1989; Wild, 1994; Barkai-Golan, 2001). Guazatine cannot be used on its own for the control of green mould as it lacks protective action and the ability to inhibit sporulation (Eckert and Eaks, 1989), hence the incorporation of thiabendazole in the drench mix.

Propiconazole

Propiconazole (PPZ) is a triazole fungicide that acts as a ergosterol biosynthesis inhibitor (European Food Safety Authority, 2012). Propiconazole has been registered previously for preharvest application on several crops over several years, and has recently been registered for postharvest application of stone fruit and citrus, among other fruit crops, as it is highly effective against sour rot (McKay *et al*., 2012a). The MRL for PPZ on citrus fruit is 6 mg/kg (The European Commision, 2013).

Treating lemon fruit curatively (14h) with PPZ as an aqueous in-line drench application, followed by an incubation period of 6 days at 20°C, resulted in sour rot decay incidence reduction from 83.8 (control) to 0% (McKay *et al*., 2012a). Propiconazole also protected lemon fruit by providing 95% decay control when treating fruit 8h after inoculation, and control was reduced to 44% if treated 24h after inoculation (Adaskaveg, 2008).

Due to concerns surrounding the possible withdrawal of GZT, PPZ could replace GZT in fungicide mixtures for the control of sour rot. McKay *et al*. (2012a) found that decay incidence (%) of sour rot reduced from 52.5 (control) to 0 and 2.8% when treating lemon fruit with PPZ (256 µl/L) or a combination of FLU (300 µl/L), azoxystrobin (300 µl/L) and PPZ (256 µl/L), respectively; green mould (sensitive and mildly resistant) was also effectively controlled with the combination. Green mould incidence was also reduced, when treated with PPZ in an in-line drench, system from 77.5 to less than 6.3% and from 80 to 32% when inoculated with *P. digitatum* sensitive- and resistant-isolates, respectively (Adaskaveg, 2008).

Both imazalil and propiconazole should not be used together as they are demethylation inhibitors (DMIs) and therefore have the same mode of action, which can lead to resistance build-up against this group of fungicides and cross resistance (Lyr, 1995). McKay *et al*. (2012b) found IMZ-resistant *P. digitatum* isolates cross resistant to PPZ during laboratory selection studies, although resistance has not been evaluated before.

Fludioxonil

Fludioxonil belongs to the phenylpyrroles chemical group with a mode of action different from that of IMZ and TBZ, which involves inhibiting mycelium growth, spore germination and germ tube elongation in *B. cinerea* with non-systemic properties and protective action (Rosslenbroich and Stuebler, 2000; Zhang and Timmer, 2007; D'Aquino *et al*., 2010). Fludioxonil was registered for federal use on postharvest fruit in the United Stated in 2003 (Förster *et al*., 2007).

Fludioxonil (600 $\mu g.mL^{-1}$) shows good green mould curative control, similar to IMZ (600 $\mu g.mL^{-1}$), when treating Satsumas and lemons picked under low disease pressure conditions 24h after inoculation or wounding. However, significantly more decay occurred when treating Satsumas picked during conditions favourable to disease development with FLU than IMZ (D'Aquino *et al*., 2010). Similar green mould decay control was seen when simultaneously wounding and treating fruit with FLU and IMZ 24h before inoculation, although FLU provided little control when inflicting wound-infections 24h following treatment (D'Aquino *et al*., 2010). Fludioxonil effectively protects fruit when inoculation occurs shortly after treatment, although efficacy diminished from 9 to 21 h, unlike pyrimethanil which provided effective control over the entire time frame tested (Kanetis *et al*., 2007). By combining half rates of TBZ and FLU, decay incidence only increased slightly over time, demonstrating potential additive effects (Kanetis *et al*., 2007). Low sour rot decay incidence (%) similar to GZT was seen when treating fruit with FLU, showing some potential of this fungicide for the control of sour rot. However results were inconsistent across the different *G. citri-aurantii* isolates and FLU concentrations used (Horuz and Kmay, 2010).

2,4-D

The plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) enhances fruit resistance to stem-end rot when applied prior to degreening or storage, as it inhibits ethylene action and delays aging and deterioration of the stem-end button (Barkai-Golan, 2001). The delayed dropping of the button also hinders the transition of saprophytic fungi (Diplodia and Phomopsis stem-end rot pathogens) present in the button from a quiescent to an active state, retarding decay development (Barkai-Golan, 2001). This growth regulator can also be incorporated into a wax applied before storing fruit in order to retard senescence of the calyx, thereby controlling Alternaria stem-end rot (Brown and Miller, 1999). The rate of disease spread is ultimately reduced by application of 2,4-D as this growth regulator sustains the natural resistance of the host tissue to invasion by pathogens (Eckert, 1990).

Enhancing drench application

Adjuvants

Improved performance chemicals are added to formulation ingredients in order to enhance efficacy through improved chemical distribution and uptake (Gisi, 1996). Surfactants that cause a physical change at the surface of liquids include wetting agents, emulsifiers, dispersants, spreaders, penetrants, stickers and detergents (Karnok *et al*., 2004).

Ryckaert *et al*. (2007) concluded from work done using several adjuvants on lettuce and *Triticale* that adjuvants improve residue loading and can be exploited to reduce doses of the active ingredient used. Bower *et al*. (2003) sprayed trees using a formulated phosphorous acid product (Phytex®) containing a wetter to aid application. Eradicant and antisporulant activity of Benlate against green mould and stem-end rot was, in some cases, improved slightly through the addition of the spray adjuvant Pinolene (3%) (Brown and Albrigo, 1970). The adjuvant, Nu-film-17®, was used as a preharvest spray by Rheinländer and Fullerton (2007) to assist coverage and retention of the chemical on fruit surfaces. Van Zyl *et al*. (2014) found that the addition of adjuvants during the spraying of citrus canopies improved deposition quantity, efficiency and uniformity in pruned and less-dense canopies, particularly at reduced volume applications, although it provided little improvement to deposition quality. Some adjuvants improved spray deposition on grapevine foliage at higher concentrations, whereas other adjuvants reduced deposition at higher concentrations due to run-off (van Zyl *et al*., 2010).

The use of adjuvants that increase solution run-off during drenching may have a beneficial effect on overall fruit coverage throughout the harvest bin, although volume and adjuvant combination will be important as enough of the fungicide active still needs to be retained on the fruit surface.

Antifoamers

Foaming of drenching systems must be properly managed to prevent encapsulation of the fungicide by foam, which is then excessively removed by the fruit resulting in a more dilute concentration of the fungicide (Brown and Miller, 1999), and reduced efficacy. Antifoams are therefore used to prevent foaming of the solution (Darby *et al*., 1962) and must have limited solubility, low surface tension and low interfacial tension with the foaming liquid (Schwarz and Reid, 1964). Drench application can result in excessive foaming depending on the specific systems specifications (Pers. comm. C. Muller). Kellerman *et al*. (2014) used 0.04 $\mu g.ml^{-1}$ antifoaming adjuvant (Antifoam, Chempac, Paarl, South Africa) in a fruit wax coating.

Excessive foaming of a fungicide solution remains an operational issue. Therefore the consequence is yet to be determined on residue loading and disease control in terms of availability of fungicides within the drench solution.

Sanitizers

Standard practice for commercial packhouses involves the use of broad-spectrum chemicals applied during the fruit cleaning process, such as chlorine (Eckert, 1995) and hydrogen peroxide/peroxyacetic acid (HPPA) (Kanetis *et al*., 2008a).

Chlorine is used during sanitation of fruit and packhouse equipment (Kanetis *et al*., 2008a), with sodium hypochlorite and calcium hypochlorite used as the main source of chlorine (Hewett, 2014). Chlorine must be maintained in solution at a pH of between 6.8 – 7.2 in order to reduce microbial populations and inoculum build-up of resistant isolates (Hewett, 2014) and the lethal action of chlorine is time dependant with maximum efficacy against propagules occurring in a 2 min exposure (Brown and Miller, 1999). Calcium hypochlorite is the most popular form of chlorine used in South Africa, formulated as chips or tablets (Pers. comm. K. Lesar). Dirt and debris reduce the activity of chlorine (Hewett, 2014).

Chlorine effectively kills spores in solution, preventing spore build-up, and removes surface populations of *P. digitatum* and *G. citri-aurantii* (Smilanick *et al*., 2002; Cunningham and Taverner, 2006). Sanitizing agents need to be used in combination with fungicides due to a lack of residual effect although incompatibility issues exist and need to be kept in mind (Cunningham and Taverner, 2006). Chlorine is not compatible with most fungicides, such as PYR and IMZ (Smilanick *et al*., 2006a; Kanetis *et al*., 2008b), and is therefore not combined with the drench mixture due to the inclusion of PYR (Pers. comm. A. Erasmus). However, chlorine is compatible with TBZ, FLU and PPZ (Adaskaveg, 2008; Kanetis *et al*., 2008a). To overcome incompatibility issues, Brown *et al*. (1988) discussed re-charging a drench solution with benomyl during chlorine application. Incompatibility with chlorine can therefore be addressed by adjusting the fungicide concentration with each consecutive chlorine application. A fungicide top-up protocol could be established when incorporating chlorine during drench application in order to negate adverse effects on drench applied fungicides, although this protocol will need to be established.

The sanitizer paracetic acid or peroxyacetic acid (PAA) is commercially available as a mixture of acetic acid (CH_3CO_2H), hydrogen peroxide (H_2O_2), PAA (CH_3CO_3H) and water (H_2O) in equilibrium, as shown by the following equation: $CH_3CO_2H + H_2O_2 \rightarrow CH_3CO_3H + H_2O$ (Taverner, 2004). Hydrogen peroxide/peroxyacetic acid is compatible with IMZ, PYR, TBZ and FLU, whereas IMZ and PYR are not compatible with chlorine (Kanetis *et al*., 2008a). Another benefit of HPPA is that it has a larger effective pH range (pH 5 – 8) than chlorine (6.8 – 7.2) and isn't as sensitive to the presence of organic matter, although it can be corrosive to certain metals or surfaces (Taverner, 2004; Hewett, 2014). Examples of HPPA available are Citrocide PC and Citrocide PLUS from Citrosol S.A. (Valencia, Spain), and Tsunami® 100 (Ecolab). Hydrogen peroxide/peroxyacetic acid could be an alternative

to chlorine as a sanitizing agent, although very little research is currently available on HPPA products, necessitating further research on its potential use and corrosive nature within a drench application system on citrus and effectiveness at these shorter exposure times.

Filtering

Drenching involves spraying a recirculating fungicide solution over fruit in field bins, directly from the orchard, which can result in soil accumulating in the tank during treatment (Brown and Miller, 1999). Fungicides can also be bound by dirt, silt and organic matter settling on the bottom of the treatment tank if not properly mixed. Litres of the solution is lost over time during drenching, allowing the microbial load to build up while the desired fungicide concentration becomes more dilute (Brown and Miller, 1999). A truck bin drenching system, used by Brown *et al*. (1988), required cleaning of the tank after treating 17 500 pallets of fruit. Organic material was manually removed daily and a commercial septic tank company hired to pump out sludge and water (Brown *et al*., 1988). Very little information was found in literature concerning methods for removing dirt and debris from drench tanks other than completely dumping tanks regularly and replenishing it with clean water and a new fungicide solution (Cunningham and Taverner, 2006). Filtering is therefore an option that should be investigated to improve drench application, although no literature could be found to support this statement.

RESISTANCE MANAGEMENT

The high rate of emerging fungicide resistant (R)-biotypes is as a result of: poor sanitation practices and subsequent build-up of resistant populations, poor fruit handling practices, which result in damaged fruit and an entry point for pathogen infection (Gardner *et al*., 1986), continuous selection pressure exerted due to the year round processing of fruit in packinghouses in combination with fungicide residue persistence, intensive or inappropriate use of a limited number of registered postharvest chemicals (Brown and Miller, 1999; Holmes and Eckert, 1999), the removal of diseased fruit and subsequent repacking leading to aerial dispersal of fungicide-resistant conidia to recently harvested fruit (Kinay *et al*., 2007) and a short life cycle that results in a large number of dispersible *P. digitatum* conidia (Gardner *et al*., 1986; Holmes and Eckert, 1995) increasing the risk of fungicide-resistant isolates emerging due to spontaneous mutation in fungal populations (Brown and Miller, 1999). Strategies to limit the number of R-biotypes detected include practices such as: implementing sanitation measures, careful handling of fruit, routine monitoring of resistant populations, spatial separation of decay-elimination areas and combining or rotating fungicides with different modes of action (Bancroft *et al*., 1984; Gardner *et al*., 1986; Kanetis *et al*., 2007), which necessitates the development of new generation pesticides. Registering

postharvest chemicals is limited due to the cost involved (Brown and Chambers, 1996) and due to the small volumes needed to produce high residue levels during postharvest application (Eckert, 1995), which may easily exceed the MRL for that fungicide.

Kaplan and Dave (1979) mentioned that Decco routinely surveyed citrus packhouses for resistance, with several TBZ and Benomyl resistant green mould isolates found. IMZ was developed and shown to effectively control green mould decay and sporulation for both TBZ sensitive and resistant isolates (Kaplan and Dave, 1979). *Penicillium digitatum* strains resistant to IMZ was first reported by Keith Lesar (Pers. comm. K. Lesar; Dodd *et al*., 2010). Pyrimethanil, FLU and azoxystrobin are newer fungicides belonging to different chemical classes from the current chemicals used for green mould control. This allows for a more efficient resistance management strategy involving the combination or rotation of all registered fungicides, reducing the risk of resistance development to the newer fungicides (Kanetis *et al*., 2008a). Despite its recent introduction, Kinay *et al*. (2007) collected PYR resistant *P. digitatum* isolates from Californian citrus groves and Kanetis *et al*. (2010) found natural populations resistant to FLU at Californian packhouses. Therefore this signifies a high risk of resistance development to these fungicides.

The rate at which *P. digitatum* develops resistance to fungicides (Lesar, 2006) is challenging and necessitates the implementation of a resistance management program. It is not advisable to apply IMZ in both drench and dip/wax treatments as loss of control and sporulation inhibition can increase the risk of resistance development against IMZ (Erasmus *et al*., 2013). Loss of control can result from inadequate residue loading during drenching (Pers. comm. A. Erasmus) before fruit reach the degreening chamber with favourable conditions conducive to green mould development (Erasmus *et al*., 2011). Fungicide treatments with different modes of action to IMZ (Bancroft *et al*., 1984; Erasmus *et al*., 2015) are therefore necessary before degreening in order to (Zhang and Swingle, 2005) reduce green mould inoculum levels, sporulation (Eckert and Eaks, 1989; Eckert, 1995) and, ultimately, resistance development and spread.

CONCLUSION

Postharvest losses occur primarily due to green mould decay, which can be problematic due to its rapid growth and spread. Various integrated approaches are used to control postharvest diseases, with fungicides still the most widely used and trusted method for managing green mould and sour rot on citrus. Fungicide treatments are applied in the packline as a dip, aqueous spray and/or wax treatment, although this may not be sufficient if treatment is delayed for more than 24 hours after harvest, such as when fruit are degreened. Degreening is necessary to improve fruit peel colour although green mould incidence increases as conditions are also optimal for disease development. Degreening can take up

to 5 days which allows infections to take place and inoculum levels to build up, reducing the efficacy of subsequent packline fungicide applications.

Drench application provides a pre-packline fungicide treatment for curing orchard borne infections and reducing the inoculum load before and after degreening. It is imperative to treat fruit soon after harvest for more effective green mould control, which is possible when drenching fruit directly after harvest in orchard bins. Several chemicals are used together in the drench mixture to broaden the spectrum of action and to control isolates resistant to various prevailing fungicides. Despite the efficacy of IMZ, it is not advisable to include IMZ during drenching in order to limit the risk of resistance development due to its intensive application in the packline.

Regardless of the benefit of drenching fruit, this application method has not yet been standardized and very little information has been published concerning ideal drench specifications, with even experimental drench designs varying considerably. Drench application essentially resembles in-line aqueous sprays, although it differs in the fact that fruit are drenched in field bins meaning that the fungicide solution has to move through layers of tightly stacked fruit, which severely limits spread and coverage. Another challenge surrounding drench application concerns the quantity of soil and debris that accumulates in the drench tank, binding to fungicides and sanitizers and ultimately reducing their efficacy. Currently the entire solution needs to be discarded with no techniques for improvement mentioned. This necessitates the standardisation of the drench with new strategies for optimising drench fungicide application in order to overcome various shortcomings.

The aim of this study therefore was to investigate methods that could improve drench application on citrus and subsequent disease control by analysing various factors that impact upon fungicide efficacy and residue loading, such as flow rate, exposure time and timing of application after harvest (infection age). The addition of an adjuvant to the drench mixture was investigated in order to evaluate its effect on the performance of chemicals in terms of chemical distribution and uptake. Residue loading, green mould infection (curative and protective treatments) and sporulation incidence were some of the main factors measured during these laboratory drench trials.

Sanitisers (Cl and HPPA) were also compared for the control of sour rot inoculum in drench solutions. Their effect on fungicide persistence and subsequent green mould control in the presence of kaolin (to simulate ‘dirt’) was also studied. Fungicide concentration, residue loading, green mould and sour rot control and colony forming units were some of the main factors measured, as applicable to each specific trial.

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CHAPTER 2

Postharvest fungicide drench application for the control of citrus green mould: effects of exposure time, infection age and adjuvant

ABSTRACT

Drench fungicide application can be an important tool for curing orchard-borne infections and protecting early season fruit requiring degreening, although this application has not been standardized and information concerning conditions for optimal coverage and disease control is limited. This study evaluated the influence of infection age, treatment exposure time and adjuvant concentration during drench application for the control of green mould (*Penicillium digitatum*) on postharvest citrus fruit. Assessment included infection ratings, fungicide residue loading and deposition quality and quantity of a yellow fluorescent pigment in the adjuvant treatments. Lemon, Satsuma mandarin and navel orange fruit were drenched with thiabendazole (TBZ), pyrimethanil (PYR) and 2,4-dichlorophenoxyacetic acid (2,4-D). Fruit batch differences and not exposure time were significant for both lemon and Satsuma mandarin fruit, with > 90% control achieved by drenching fruit before 33.1 – 44.5 and 23.8 – 32.1 h, respectively, considering batch differences. Exposure time and batch differences only became significant with ≥ 30 h old infections on navel orange fruit, which were subjected to a higher volume compared to lemon and Satsuma mandarin fruit, with control declining more rapidly for fruit drenched at the shorter exposure time. More than 90% control was achieved by drenching fruit before 27 h. Although protective control was effective (> 90%), overall sporulation inhibition was poor (< 50%) and fruit batches differed as much as 8 to 12 h in infection age for similar control levels, therefore supporting previous recommendations made to treat all citrus types within 24 h to reduce risk for decay development. Fungicide residue loading appeared to be dependent on specific citrus type × fungicide combination, with superior TBZ residue levels linked to improved sporulation inhibition. This study also showed that the calyx-end of fruit loaded higher or equal TBZ, PYR and 2,4-D residue levels compared to the stylar-end resulting in equal or improved curative control, deposition quantity and quality. Valencia orange fruit were drenched with the addition of an adjuvant (0.0, 0.025, 0.05, 0.1 and 0.2 µl.mL^{-1}), with the highest concentration negatively impacting on residue loading, deposition quantity and disease control.

INTRODUCTION

South Africa was rated 10th in the world in terms of citrus production and 2nd in export next to Spain during the 2013/14 citrus season, making the citrus industry a major contributor to the gross value of agriculture in this country (Directorate Marketing, 2014; Edmonds, 2015). Due to the length of shipping storage required to export fruit to faraway countries, quality control becomes challenging with 80 – 90% of postharvest losses to citrus predominantly occurring as a result of green mould decay (*Penicillium digitatum*) (McCornack, 1970; Montesinos-Herrero *et al*., 2009; Lesar, 2013). *Penicillium digitatum* requires damage to the fruit rind for infection, with many orchard-borne infections occurring during the harvesting process (Kavanagh and Wood, 1967; Brown, 2003). Disease symptoms occur rapidly (24 – 36 h) after infection with optimal growth at 25°C (Barmore and Brown, 1982; Plaza *et al*., 2003), followed by the formation of highly dispersible conidiospores (Gardner *et al*., 1986; Benhamou, 2004) that can cause secondary losses as a result of 'soilage', i.e. decaying fruit deposit spores on the surface of adjacent healthy fruit, necessitating the repacking of healthy fruit (Pelser, 1977; Eckert and Eaks, 1989).

Many early season citrus varieties require degreening for desired fruit colour (Wheaton and Stewart, 1973; Sdiri *et al*., 2012), which in South Africa mainly involves exposing fruit placed in a degreening chamber to temperatures ranging from 18 to 25°C (depending on citrus type) at 94 – 96% relative humidity, with the addition of 1 – 5 µg.mL^{-1} ethylene gas (Krajewski and Pittaway, 2010), for 2 – 3 days (Pers. comm. P. Cronje; Dodd *et al*., 2010). Other countries may degreen at temperatures ranging from 27° to 33°C at 90 – 96% RH (Eckert and Eaks, 1989; Zhang and Swingle, 2005; Sdiri *et al*., 2012). Conditions in a degreening chamber correspond with optimal green mould development (25°C) (Plaza *et al*., 2003; Zhang and Swingle, 2005) leading to losses that range from 2 – 30% depending on pre-harvest environmental conditions (Smilanick *et al*., 2006b). This highlights the necessity for a fungicide application before degreening. Dodd *et al*. (2010) mentioned that drenching fruit before degreening resulted in a significant reduction in decay incidence in the degreening room. Drench application is applied as a pre-packline fungicide treatment directly over the fruit in a field bin before degreening, and is widely used in South Africa (Lesar, 2006).

Both imazalil (IMZ) and thiabendazole (TBZ) are widely used due to effective curative action against green mould (Kaplan and Dave, 1979; Schirra *et al*., 2000; Smilanick *et al*., 2006a; Erasmus *et al*., 2013; Kellerman *et al*., 2014), although use of each fungicide should be confined to either packline or drench application as part of a resistant management protocol (Erasmus, *et al*., 2012). Thiabendazole (2(4-thiazol-4-yl) is a systemic benzimidazole fungicide developed in the early 1970s (Barkai-Golan, 2001; Dodd *et al*., 2010) and found to control *Diplodia* and *Phomopsis* stem-end rots on fruit stored at 21°C for

up to two weeks (Brown and Chambers, 1996), while action was inadequate against *Rhizopus, Phytophthora, Alternaria* and *Geotrichum* (Barkai-Golan, 2001). Thiabendazole is ideal for drench application as it is not affected by solution pH and is effective against green mould at ambient temperature (McCornack, 1970) and relatively low concentrations. Smilanick *et al.* (2006b) determined that ≥ 0.2 $\mu g.g^{-1}$ TBZ is necessary for effective green mould control and Kellerman *et al.* (2014) stated that 75% curative control could be achieved with a residue of between 0.06 – 0.22 $\mu g.g^{-1}$, depending on citrus type. Zhang and Swingle (2005) found that drenching fruit with 500 $\mu g.mL^{-1}$ TBZ reduced green mould and stem-end rot disease incidence, although a concentration of 1000 $\mu g.mL^{-1}$ TBZ is recommended for bin drench application (Anonymous, 2003).

Due to extensive pre- and postharvest use of benzimidazole fungicides for the control of black spot and green mould on citrus, resistance to TBZ has emerged (Pelser, 1977; Dodd *et al*., 2010). The development of resistant biotypes can be reduced by combining fungicides with different modes of action (Kanetis *et al.*, 2007). Thiabendazole is therefore mixed with pyrimethanil (PYR) and/or guazatine (GZT) during drenching, which provides a broader spectrum of control against several postharvest pathogens (Erasmus *et al*., 2012). The plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) is often included for its ability to delay stem-end button senescence of citrus fruit (Brown and Miller, 1999; Barkai-Golan, 2001), and thus indirectly reducing stem-end rots caused by *Diplodia*, *Phomopsis* (Pelser, 1977; Barkai-Golan, 2001) and *Alternaria* (Brown and Miller, 1999). Pyrimethanil provides good curative action against green mould up to 24 h after inoculation (E. Liebenberg, unpublished data) as well as controlling TBZ resistant strains of *P. digitatum* due to a different mode of action, although protective activity (Smilanick *et al*., 2006a) and anti-sporulant action is poor (Kanetis *et al*., 2007). Pyrimethanil also doesn't require pH adjustment (Smilanick *et al*., 2006a) and is therefore favourable for drench application. The MRL tolerance for TBZ is 10 ppm in the USA, Canada and Japan (Ritenour *et al*., 2003), 10 and 8 $mg.kg^{-1}$ for PYR in the USA and as general export tolerance, respectively, and the general export tolerance for 2,4-D is 1.0 $mg.kg^{-1}$ (The European Commision, 2014; Hattingh and Hardman, 2015).

Various formulation ingredients, such as adjuvants, can also be added with active ingredients in order to improve chemical distribution and uptake (Gisi, 1996) by causing a change to the surface tension of liquids (Karnok *et al*., 2004). Adjuvants have the potential to improve deposition quantity and quality, although excessive run-off caused by some adjuvant and spray volume combinations reduced spray deposition on grapevine (Van Zyl *et al*., 2010) and citrus tree foliage (Van Zyl *et al.,* 2014). Fruit need to dry promptly within the fruit bin after drench treatment in order to avoid possible phytotoxic damage during degreening (Erasmus, *et al*., 2012). Adjuvants could optimise fruit drying and fungicide

distribution throughout a tightly packed fruit bin without hindering sufficient fungicide uptake. No references or published work has been found to support this statement.

Very little information exists concerning drench application, with several researchers only mentioning similar in-line drench systems, modifications to the bin-drenching system, or experimental drench applicators that all involve re-circulating a fungicide solution from a reservoir tank through nozzles or weirs placed directly over the fruit (Brown *et al*., 1988; Brown and Miller, 1999; Zhang and Swingle, 2005; Erasmus *et al*., 2011; Kellerman *et al*., 2014). Drench treatments consisted of exposure times of 30, 60 and 90 s with 1000 or 2000 $\mu g.mL^{-1}$ TBZ. Average TBZ residues were 2.14 $\mu g.g^{-1}$ for Clementine mandarin fruit and 3.50 $\mu g.g^{-1}$ for navel orange fruit. Green mould control on navel orange fruit resulted in 66–92%, 34–90% and 9–38% control for curative treatments after 6 and 24 h and protective treatments, respectively, depending on fruit batch (Kellerman *et al*., 2014).

Challenges to drench application mainly involve poor and inconsistent fungicide deposition as a result of poor agitation (Brown and Miller, 1999), stacking of fruit bins during drenching, tightly packed fruit within the fruit bin and/or inadequate flow rate or distribution.

Shortcomings of drench application can be rectified through improved design of drenching systems. Previous research (Kellerman *et al*., 2014) and practical experience have indicated minimum specifications for drenching systems, which includes minimum flow rate of pump (250 $L.min^{-1}$), minimum exposure time (60 s leading to dose of 250 $L.bin^{-1}$), and limitation to the stacking of bins (better green mould control following single bin drenching).

The objectives of this study were to determine the effect of infection age, exposure time and the addition of an adjuvant on curative control after drench treatments, as well as the proficiency of this application method to inhibit sporulation when using an optimal drenching system as described above.

MATERIALS AND METHODS

Fungal isolates and culture preparation

A *P. digitatum* isolate (STE-U 6560) from the culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa, was used throughout the trials. This isolate is known to be sensitive to IMZ, GZT, TBZ and PYR (Erasmus *et al.,* 2015). Inoculum for biological tests was obtained by plating the isolate onto chloramphenicol amended potato dextrose agar medium (PDA^{+}) (PDA, Difco™, Becton, Dickinson and Company, Sparks, MD 21152, USA; chloramphenicol, Chlorcol, 250 mg CAP 500, Adcock Ingram, Midrand, Gauteng, South Africa), and incubated at 25°C.

Spore suspensions were prepared shortly (2 – 4 h) before the commencement of fruit inoculation by harvesting conidia from approximately 2-week-old cultures. The surface of a culture was washed with sterile deionised water amended with ≈ 0.01 $\mu l.mL^{-1}$ Tween 20

(Merck, Wadeville, Gauteng, South Africa) in order to dislodge the conidia into solution (Erasmus *et al*., 2011), followed by filtration through two layers of autoclaved cheesecloth and further dilution until a concentration of 1 x 10^6 spores.mL^{-1} was reached using a spectrophotometer (0.1 absorbance at 420 nm; Cecil CE 1011 1000 series, Cecil Instruments Limited, Cambridge, England) (Morris and Nicholls, 1978; Eckert and Brown, 1986; Kellerman *et al*., 2014). A uniform distribution of spores was maintained in suspension using magnetic stirrers.

Fruit

Untreated export quality citrus fruit were obtained during the 2014 season from packhouses in the Limpopo and Mpumalanga provinces of South Africa, according to seasonal availability. Fruit were collected shortly after harvest, washed over rotating brushes using chlorine (75 µg.mL^{-1} HTH; Arch Chemicals (Pty) Ltd., Bergvlei, Gauteng, South Africa) and stored at 4°C until use (± 3 days). Fruit were transferred to ambient temperature (≈ 22°C) a day before commencing trial preparation in order to allow the evaporation of any condensation formed.

Inoculation and incubation

Depending on the specific trial, fruit were inoculated curatively (prior to treatment) and protectively (following treatment) for evaluating green mould control. A cylindrical rod, with a 2 mm x 1 mm protruding tip, was dipped into a spore suspension of *P. digitatum* (1 x 10^6 spores.mL^{-1}) and used to pierce the rind of each fruit four separate times equidistantly around the calyx for curative and protective inoculations (Kellerman *et al*., 2014). For the assessment of sporulation control, additional fruit were injected (Brown *et* al., 1983; Brown and Dezman, 1990) with 0.2 ml of spore suspension, 1 cm deep, shortly (≈ 30 min) before treatment. Control fruit were inoculated and left untreated.

Following treatment, fruit were left in the treatment units (crates) overnight to dry and were subsequently packed into table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd., Atlantis, South Africa) before being covered with transparent polyethylene bags punctured twice on each end for gaseous exchange. Fruit were left for several days (≈ 4) to incubate, and were rated once controls displayed high levels of infection (Erasmus *et al*., 2011).

Disease evaluation

A UV light (UV-A at 365 nm, Labino Mid-light; www.labino.com) was used to evaluate the number of infected wounds per fruit as a rating out of four, visible as yellow fluorescent lesions. Sporulation was rated after an incubation period of ± 14 days using a sporulation

index of 1 – 6, where 0 = no disease; 1 = infection but no sporulation; 2 = sporulation area covers less than a quarter of the fruit surface; 3 = sporulation area covers between a quarter and half of the fruit surface; 4 = sporulation area covers between a half and three quarters of the whole fruit; 5 = sporulation area covers over three quarters of the fruit surface; 6 = 100% sporulation coverage (Erasmus *et al*., 2011; Erasmus *et al*., 2015).

Residue analysis

Six residue fruit per treatment replicate were combined during maceration with small fruit chopped in its entirety, while larger fruit were cut into four or eight equal pieces, i.e. from the stylar- to the calyx-end, with a single piece selected to be macerated from each fruit. Fruit were chopped and diluted with ± 0.19, 0.40 and 0.50 $mL.g^{-1}$ distilled water (in accordance to the weight of the fruit) on Satsuma mandarin, Eureka lemon and Palmer navel orange fruit, respectively, before being macerated to a fine pulp in a blender for 2 min and stored at -20°C (Erasmus *et al*., 2011; Kellerman *et al*., 2014).

Samples were sent for residue analysis by an accredited analytical laboratory (Hearshaw and Kinnes Analytical Laboratory, Westlake, Cape Town, South Africa). Acetonitrile, followed by a matrix solid phase dispersion extraction, was used to obtain sample extracts. These extracts were further analyzed using tandem liquid chromatography mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA). Residue results were adjusted based on the individual dilution factor of each batch of macerated pulp samples.

Experimental drench applicator

An experimental stainless steel drench applicator was custom-built (Citrus Research International, Nelspruit, South Africa) to simulate industry best-practice. The drench reservoir was filled with 125 L of municipal water and amended with TBZ (Thiazole® 500SC; Villa Crop Protection (Pty) Ltd., Aston Manor, Gauteng, South Africa) and PYR (Protector® 400SC; ICA International Chemicals (Pty) Ltd., Stellenbosch, Western Cape, South Africa). As industry standard in drench mixtures, 2,4-dichlorophenoxyacetic acid (2,4-D Amine 480; Plaaskem (Pty) Ltd., Witfield, Gauteng, South Africa or Deccomone®, Citrashine (Pty) Ltd., Booysens, Gauteng, South Africa) was also added in all treatments at specified concentrations.

The drench applicator pump was left to run for 5 min allowing the amendment of fungicides to properly mix into solution. Fruit were packed into plastic fruit crates (325 x 505 x 245 mm) used to simulate the standard 800 L commercial orchard bin. A weir (Figure 1) moved back and forth over the fruit for a specified amount of time (exposure time) at a speed

of 0.056 – 0.072 $m.s^{-1}$, drenching fruit with a re-circulating solution flowing through the crate into the reservoir and then back through the weir.

The effects of infection age and treatment exposure time

Infection age is defined as hours-post-inoculation throughout this study. Curative control (fruit infected 0, 6, 12, 18, 24, 30, 42, 48 and 54 hours prior to treatment), protective control (fruit infected 24 hours after treatment) and sporulation control were investigated in this trial. All crates contained 12 fruit from each curative incubation period, the protective treatment and the sporulation control treatment. Six fruit for residue analysis were added to the first and last replicate of each treatment combination. Three replications per treatment combination were carried out. The drench solution flow rate was ± 26.5 and 64.3 $L.min^{-1}$ over fruit crates, which at 28 and 14 s, respectively, exposure time related to an industry dosage of 250 and 305 $L.bin^{-1}.min^{-1}$, respectively.

Satsuma mandarin and Eureka lemon fruit

Fruit were drenched for 14 s, 28 s and 56 s at 26.5 $L.min^{-1}$ with 1000 $\mu g.mL^{-1}$ TBZ and PYR, respectively and 4800 $\mu g.mL^{-1}$ 2,4-D (2,4-D Amine 480; this was an erroneous concentration as the registered concentration is 250 $\mu g.mL^{-1}$). Trials were conducted twice on each citrus type.

Palmer navel orange fruit

Fruit were drenched for 14 s, 28 s and 56 s at 64.3 $L.min^{-1}$ with 1000 $\mu g.mL^{-1}$ TBZ and PYR, respectively and 250 $\mu g.mL^{-1}$ 2,4-D (Deccomone). Batches were harvested 3 days apart and drenched together in the same drench solution. Protective control was not evaluated.

The effects of an adjuvant and fruit orientation on fungicide drench treatment

Each crate contained 12 fruit from each curative incubation period, the protective treatment, and 10 additional residue fruit. Treatment fruit were arranged randomly in the crate with a third placed with the calyx facing upward, a third downward and the remaining third on its side relative to the flow of the fungicide solution from the weirs (fruit pole). Half of the residue fruit were placed with the calyx facing upward and the other half downward, with calyx-end and stylar-end halves of fruit macerated separately for individual residue analysis. A layer of non-inoculated buffer fruit was placed at the top and bottom of the crate, with treatment fruit in the middle. Fruit were drenched at a flow rate of 41.0 $L.min^{-1}$ and exposure time of 18 s, which relates to an industry dosage of 250 $L.bin^{-1}.min^{-1}$. The drench mixture included 1000 $\mu g.mL^{-1}$ TBZ, 1000 $\mu g.mL^{-1}$ PYR, 250 $\mu g.mL^{-1}$ 2,4-D (Deccomone) and

several concentrations of an experimental adjuvant (0.00, 0.025, 0.050, 0.100 and 0.200 µl.mL^{-1}) (ICA, batch number: W004; isotridecanol, ethoxylated; ICA International Chemicals (Pty) Ltd., Stellenbosch, Western Cape, South Africa). Fluorescent pigment (1 µl.mL^{-1}; Yellow Fluorescent Pigment SC, SARDI, Loxton Research Centre, Loxton SA 5333, Australia) was also added to the drench solution in order to visualize deposition on the fruit surface following treatment. Three replications per treatment combination were carried out with two different harvest batches of Valencia orange fruit (sourced from different farms) treated simultaneously. Adjuvant concentration and fruit orientation was assessed concurrently in these trials.

Biological treatments

Fruit were inoculated with *P. digitatum* 24 and 48 h before treatment (curatively) and 24 h following treatment (protectively).

Deposition

Fluorescent pigment deposition on fruit was evaluated by using the methods of van Zyl *et al*. (2013, 2014). Treated fruit were individually placed in the centre of a back-illuminated red Perspex box (300 x 210 x 110 mm). Evaluation was done inside a dark room in order to reduce potential shadowing and to enhance fruit edging during image capturing. A ultra-violet light source (UV-A; ≈ 365 nm; Labino Mid-Light; www.labino.com) was used to illuminate the fluorescing pigment coating fruit surfaces. A Canon EOS 40D camera (60 mm macro lens), fixed in position directly above the fruit using a tripod, was used to capture digital images of the calyx- and stylar-end of the fruit surface in Canon RAW file format (.CR2 ≈ 10 MB). Digital Photo Professional version 3.1.0.0 (Canon INC.; www.canon.com) was used to convert RAW image files to 8-bit Exif-TIFF (.TIF ≈ 30 MB). Deposition quality and quantity per fruit were determined using Image PRO PLUS software version 7.0 (Media Cybernetics, www.mediacy.com).

Percent fruit area covered by pigment particles (percentage fluorescent particle coverage; FPC%) was determined and used to quantify deposition. A higher FPC% indicates improved pigment retention. Deposition quality, i.e. uniformity of deposition on the fruit surface, was assessed by dividing the fruit area image into equally-sized squares [100 × 100 pixels (10000 pixels)] with percentage area covered by fluorescent pigment particles determined per square. Deposition quality per fruit was measured using the Interquartile Coefficient of Dispersion (ICD%) [((3rd quartile – 1st quartile)/(3rd quartile + 1st quartile))*100]). Low interquartile coefficient of dispersion values were indicative of better deposition quality as lower variation in pigment deposition was measured between blocks over the fruit surface.

Statistical analysis

For all of the trials, wound infection and sporulation inhibition data were normalized by calculating percentage control relative to the untreated controls. Analysis of variance (ANOVA) was done using XLSTAT version 2014.4.03 (www.xlstat.com). Fisher's least significant difference test (95% confidence interval) was used to identify significant differences between treatments. For the infection age trials percentage control data of each fruit batch was regressed against infection age using non-linear regression with the function, Y = pr3/(1+Exp(-pr1-pr2×X1)). The coefficient of determination (R^2) was used to demonstrate goodness of fit. The effective infection age for both 50 and 90% curative control were calculated from the model for each fruit batch. In terms of deposition quantity (FPC%) and quality (ICD%), data obtained were subjected to analysis of variance (ANOVA) using XLSTAT and SAS version 8.2 statistical software (www.SAS.com), respectively.

RESULTS

The effects of infection age and treatment exposure time

Since no adverse effects were noticed on the Satsuma mandarin and Eureka lemon trials, due to the erroneous 2,4-D concentration, results were reported on all evaluated variables except 2,4-D residue levels.

Satsuma mandarin fruit

Residue loading

Analysis of variance for TBZ and PYR residue data presented no significant interaction, with batch significant as main effect (*P* = 0.011 and 0.023, respectively; ANOVA tables not shown). Exposure time did not affect TBZ or PYR residue loading significantly (*P* = 0.879 and 0.719, respectively). Batch 2 loaded significantly higher residues than Batch 1 (2.24 and 1.70 $\mu g.g^{-1}$ PYR and 0.70 and 0.38 $\mu g.g^{-1}$ TBZ, respectively).

Green mould control

Analysis of variance for percentage curative control data indicated a significant batch × infection age interaction (*P* = 0.006) with exposure time significant as main effect (*P* = 0.043).

Mean curative green mould control values of the 3 replications per batch were subjected to non-linear regression statistics. The model predicted that 90% control can be obtained by treating fruit 32.1 and 23.8 h (Figure 2; Table 1) after inoculation for Batch 1 and 2, respectively, and 50% control at 54.3 and 53.2 h incubation, respectively. In terms of exposure time, drenching for 56 s significantly improved control compared to 28 and 14 s (85.8, 83.0 and 82.8%, respectively).

Analysis of variance for percentage protective control data revealed no significant effects for exposure time or batch (P = 0.820 and 0.712, respectively). High levels of control (average of 97.8%; results not shown) were obtained by all treatments.

Sporulation inhibition

Analysis of variance for sporulation inhibition data presented no significant interaction, with batch significant as main effect (P < 0.0001). Exposure time was not significant as main effect (P = 0.863). Significantly higher sporulation inhibition levels were measured on Batch 2 than on Batch 1 (54.2 and 40.4%, respectively).

Eureka lemon fruit

Residue loading

Analysis of variance for the TBZ and PYR residue data revealed no significant effects for exposure time or batch (P = 0.658 and 0.341 for TBZ, respectively, and P = 0.358 and 0.743 for PYR, respectively). Residue levels of 0.26 – 0.737 µg.g^{-1} for TBZ and 1.99 – 4.5 µg.g^{-1} for PYR were loaded (results not shown).

Green mould control

Analysis of variance for percentage curative control data indicated a significant batch × infection age interaction (P < 0.0001) with exposure time significant as main effect (P = 0.005).

Mean curative green mould control values of the 3 replications per batch were subjected to non-linear regression statistics. The model predicted that 90% control can be obtained by treating fruit 44.5 or 33.1 h (Figure 3; Table 1) after inoculation for Batch 1 and 2, respectively, and 50% control at 53.2 and 54.5 h, respectively. An exposure time of 14 s showed significantly lower levels of control compared to 28 and 56 s (84.3, 88.0 and 88.0%, respectively).

Analysis of variance for percentage protective control data presented no significant effect for exposure time or batch (P = 0.375 and 0.651, respectively). High levels of control (average of 95.1%; results not shown) were obtained by all treatments.

Sporulation inhibition

Analysis of variance for sporulation inhibition data showed exposure time to be significant as main effect (P = 0.019), while batch was not significant (P = 0.140). Fruit treated at 28 s showed significantly higher inhibition levels compared to 56 s (36.1 and 29.9%, respectively), whereas the 14 s treatment resulted in intermediate levels (34.3%).

Navel orange fruit

Residue loading

Analysis of variance for TBZ, PYR and 2,4-D residue data indicated a significant batch effect for PYR only (P = 0.002). Batch was not significant for TBZ and 2,4-D (P = 0.753 and 0.111, respectively) and exposure time not significant for TBZ, PYR and 2,4-D (P = 0.161, 0.374 and 0.776, respectively). Batch 1 loaded significantly higher PYR (3.97 $\mu g.g^{-1}$) compared to Batch 2 (2.73 $\mu g.g^{-1}$). Mean residue levels of 0.48 – 1.31 $\mu g.g^{-1}$ for TBZ, 2.05 – 4.45 $\mu g.g^{-1}$ for PYR and 0.26 – 0.51 $\mu g.g^{-1}$ for 2,4-D were loaded on the fruit (results not shown).

Green mould control

Analysis of variance for percentage curative control data indicated a significant batch × infection age × exposure time interaction (P < 0.0001).

Mean curative green mould control values of the 3 replications per batch were subjected to non-linear regression statistics. Similar control levels (≥ 88.9%; Figure 4) were seen with the majority of exposure time treatments and batches up to ± 30 h incubation, after which control declined more rapidly with decreasing exposure time and batch differences became more prominent. The model predicted that 90% control levels at 27.4, 29.9 and 33.0 h (Table 1) incubation for 14, 28 and 56 s exposure times for Batch 1, respectively, and at 29.9, 28.5 and 29.1 h incubation for Batch 2, respectively. A 50% control level can be achieved by treating fruit at 48.5, 56.8 and 66.1 h incubation for Batch 1 and at 45.1, 47.8 and 53.3 h incubation for Batch 2 for the respective exposure times.

Sporulation inhibition

Analysis of variance for sporulation inhibition data presented no significant interaction, with exposure time and batch significant as main effects (P = 0.002 and 0.001, respectively). The 14 s exposure time treatment showed significantly higher levels of sporulation inhibition (54.6%) compared to 28 and 56 s (48.6 and 45.9%, respectively). Batch 1 had significantly higher levels of sporulation inhibition than Batch 2 (53.2 and 46.2%, respectively).

The effects of an adjuvant and fruit orientation on fungicide drench treatment

Fruit batch was ignored during statistical analysis and interpretation of the adjuvant trials to reduce the complexity of the dataset and to improve robustness of the outcomes.

Residue loading

Analysis of variance for TBZ, PYR and 2,4-D residue data indicated a significant fruit pole × fruit orientation interaction for TBZ and PYR (P < 0.0001 and P = 0.019, respectively) with adjuvant concentration significant as main effect for TBZ, PYR and 2,4-D residue data (P =

0.003, = 0.0002 and < 0.0001, respectively). Fruit pole was significant as main effect for 2,4-D residue data (P = 0.0003). Fruit orientation × adjuvant concentration was not significant for TBZ, PYR and 2,4-D (P = 0.162, 0.331 and 0.193, respectively). Fruit pole × adjuvant concentration was not significant for TBZ, PYR and 2,4-D (P = 0.532, 0.628 and 0.357, respectively).

Upward facing calyx-end halves loaded significantly higher TBZ and PYR residues (1.04 and 2.19 $\mu g.g^{-1}$, respectively; Table 2) compared to stylar-end halves of the same fruit (0.76 and 1.74 $\mu g.g^{-1}$, respectively) as well as calyx- (0.60 and 1.83 $\mu g.g^{-1}$, respectively) and stylar- (0.56 and 1.75 $\mu g.g^{-1}$, respectively) end halves of calyx-end downward facing fruit; the latter halves loaded similar residue levels. For 2,4-D, however, downward facing stylar-end halves loaded significantly lower 2,4-D residues (0.21 $\mu g.g^{-1}$) compared to upward or downward facing calyx-end halves (0.27 $\mu g.g^{-1}$). Upward facing stylar-end halves loaded intermediate levels (0.24 $\mu g.g^{-1}$).

Fruit treated with solution containing 0.2 $\mu l.mL^{-1}$ adjuvant loaded significantly lower TBZ and PYR residues (0.54 and 1.52 $\mu g.g^{-1}$, respectively; Table 3) than when the adjuvant concentrations was between 0 and 0.1 $\mu l.mL^{-1}$ (0.78 – 0.80 and 1.87 – 2.01 $\mu g.g^{-1}$, respectively). A significant reduction in 2,4-D residues was seen from 0 (0.29 $\mu g.g^{-1}$) to 0.05 and 0.1 $\mu l.mL^{-1}$ adjuvant (0.25 $\mu g.g^{-1}$), while 0.2 $\mu l.mL^{-1}$ resulted in significantly lower residues (0.17 $\mu g.g^{-1}$).

Curative green mould control

Analysis of variance for percentage control data indicated a significant infection age × fruit orientation (P = 0.008) interaction, while adjuvant concentration appeared not to have any significant effect (P > 0.345). Adjuvant concentration × fruit orientation was not significant (P = 0.883)

Percentage control was significantly higher on 24 h (78.4 – 87.8%; Table 4) than 48 h (32.2 – 53.2%) old infections. For the majority of cases, fruit orientated with the inoculated calyx-end upward resulted in similar or higher levels of control compared to calyx-end sideways or downward fruit (87.8, 79.8 and 78.4%, respectively, for 24 h and 53.2, 48.1 and 32.2%, respectively, for 48 h).

Protective green mould control

Analysis of variance for percentage protective control data demonstrated significant effects for fruit orientation and adjuvant concentration (P = 0.0004 and 0.002, respectively). Adjuvant concentration × fruit orientation was not significant (P = 0.956). Fruit oriented calyx-end upward showed significantly lower levels of control compared to calyx-end sideways and downward facing fruit (66.1, 75.9 and 80.3%, respectively). Similar control

levels were measured on fruit treated with 0 to 0.1 µl.mL^{-1} adjuvant concentrations (72.7 to 82.3%; Figure 5), but the 0.2 µl.mL^{-1} treatment displayed significantly lower levels of control (63.5%).

Pigment deposition

Analysis of variance of deposition quantity (percentage fluorescent particle coverage; FPC%) data showed significant effects for adjuvant concentration and fruit orientation (P < 0.0001 and 0.0001). Adjuvant concentration × fruit orientation was not significant (P = 0.367). Deposition quantity was significantly higher on fruit orientated calyx-end upward (3.44 FPC%) than downward (0.13 FPC%). The 0.200 µl.mL^{-1} adjuvant concentration resulted in significantly lower pigment coverage (1.85 FPC%; Figure 6) compared to fruit drenched at 0 – 0.1 µl.mL^{-1} adjuvant concentration (2.66 – 3.20 FPC%).

Analysis of variance of deposition quality (Interquartile Coefficient of Dispersion; ICD%) data demonstrated a meaningful adjuvant concentration × fruit orientation interaction (P = 0.0694). Upward facing calyx-end halves of fruit drenched with no adjuvant included had the lowest ICD values (81.10 ICD%; i.e. best deposition quality) compared to treatments with adjuvant (86.92 – 89.23 ICD%). Significantly poorer deposition quality values were observed on downward facing calyx-end halves of fruit with no difference observed for adjuvant concentration (96.97 – 99.62 ICD%).

DISCUSSION

This study evaluated the influence of infection age, treatment exposure time and the addition of an adjuvant during fungicide drench application for the postharvest control of citrus green mould. The importance of timeous drench application for effective curative control was clearly demonstrated, especially in light of the poor sporulation inhibition provided by this application. The general recommendation to drench within 24 h after harvest is supported and practical guidelines for packhouse use are provided. For the first time the effect of an adjuvant on residue loading and subsequent disease control during drench application was investigated.

Due to delays between harvest and subsequent in-line packhouse treatments (Erasmus *et al*., 2013) and the favourability of degreening conditions for green mould development (Plaza *et al*., 2003; Krajewski and Pittaway, 2010), an application such as drenching is required to cure orchard-borne infections and inhibit sporulation, which ultimately reduces the inoculum load and the potential development of fungicide resistant *Penicillium* populations (Brown and Miller, 1999). Sporulation inhibition following drench treatment was poor in our trials (average < 50%), as well as < 75 and 60% on navel orange and Satsuma mandarin fruit during TBZ (1000 µg.mL^{-1}) drench trials performed by Kellerman *et al*. (2014).

Curative green mould control therefore becomes more important during drench application. Despite TBZ residue loading always being > 0.20 µg.g^{-1} (the threshold value determined by Smilanick *et al*. (2006b) to effectively control green mould during dip application) throughout these trials, control on both Satsuma mandarin and Eureka lemon fruit declined with increasing infection age. The efficacy of drench application was significantly affected by infection age, and curative control rapidly declined to unacceptable levels (< 90%) when infections were older than 23.8 to 44.5 h, depending on citrus type and batch. Fruit are often only drenched after 24 h, or as long as 2 – 5 days (Erasmus *et al*., 2013) after harvest, which can seriously compromise or negate the effectiveness of drench treatment. Previous work done by Erasmus *et al*. (2015) using an extensive exposure time (15, 45, 90, 180 and 540 s) and infection age (0, 6, 12, 18, 24, 36 and 48 h) range on IMZ dip application led to the South African recommendation to treat fruit within 24 h of harvest.

Previous drench studies investigated single product drenches, while the current study investigated the combination of TBZ and PYR. Satsuma mandarin fruit loaded higher TBZ residue levels compared to Eureka lemon fruit (≈ 0.54 and ≈ 0.39 µg.g^{-1}, respectively) whereas the opposite was true with PYR residue levels loaded (≈ 1.97 and ≈ 3.08 µg.g^{-1}, respectively). This shows that not only can residue loading be dependent on citrus type, but also on a specific citrus type × fungicide combination. Curative control was superior on Eureka lemon fruit compared to Satsuma mandarin fruit, as > 90% control was achieved on infections as old as 33.1 to 44.5 h, compared to 23.8 to 32.1 h for Satsuma, depending on fruit batch. This confirms observations by Smilanick *et al*. (2008) and Erasmus *et al*. (2013) that Eureka lemon fruit were more resistant to green mould than soft citrus mandarin fruit. Although Eureka lemon may be hardier than Satsuma mandarin fruit, requiring a longer time period for initial infection development, similar levels of control were observed when fruit were treated 54 h after inoculation (44.3 – 51.4 and 48.7 – 50.9%, respectively).

Similar protective control was achieved on both citrus types (≈ 97.8 and ≈ 95.1%, respectively) although sporulation inhibition was superior on Satsuma mandarin fruit compared to Eureka lemons (≈ 47.3 and ≈ 33.4%, respectively), which could be ascribed to the higher TBZ residue levels loaded on Satsuma mandarin fruit as this fungicide has been shown to control sporulation (Smilanick *et al*., 2006a). Both TBZ and PYR provide effective curative control (Smilanick *et al*., 2006a; Schirra *et al*., 2008), but PYR is inferior to TBZ in terms of protective control and sporulation inhibition (Smilanick *et al*., 2006a; Kanetis *et al*., 2007). Effective sporulation inhibition requires 4.10 µg.g^{-1} PYR (Smilanick *et al*., 2006a). Whilst residue loading was unaffected, exposure time (14, 28 and 56 s) had a significant effect on curative control (82.8, 83.0 and 85.8%, respectively) on Satsuma mandarin fruit and on curative control (84.3, 88.0 and 88.0%, respectively) as well as sporulation inhibition (34.3, 36.1 and 29.9%, respectively) on Eureka lemon fruit.

The navel oranges were treated at a higher drench flow rate (64.3 L.min^{-1}) than the lemons and Satsumas (26.5 L.min^{-1}). Curative control was predicted to decline < 90% after 27.4 to 33.0 h. Whilst exposure time (and dosage per crate) did not affect curative control at that stage, it had a significant effect on subsequent treatment stages, with longer exposure times leading to improved curative control. The higher flow rate used in the navel trials resulted in higher TBZ and PYR residue levels (≈ 0.94 µg.g^{-1} and ≈ 3.35 µg.g^{-1}, respectively) compared to Eureka lemon and Satsuma mandarins. However, curative control levels were similar to those on Satsuma mandarin fruit and sporulation inhibition levels similar to those on Eureka lemon fruit.

Longer exposure time on navel orange fruit improved curative control on > 24 h old infections and concomittantly resulted in a reduction in sporulation inhibition. Curative green mould control increased with longer exposure times in a range between 28.0 – 58.2% at 54 h old infections and sporulation decreased from 54.6 to 45.9%. Similar observations were made by Kellerman *et al*. (2014) following drench treatment with 1000 µg.mL^{-1} TBZ on navel orange fruit, with an improvement in curative and protective disease control at 60 and 90 s exposure (67.2 – 69.0%) compared to 30 s (62%), but in contrast, reduced sporulation inhibition at 90 s (53.5% incidence) compared to 30 and 60 s (37.2 – 38.9% incidence); residue loading was unaffected. These results suggest that longer exposure times could be used to improve curative control, and if done on younger infections it would result in very high levels of control and could potentially remove the need for sporulation control, which was unreliable in this study.

Often different fruit batches are combined for a more robust interpretation of the data (Kellerman *et al*., 2014; Erasmus *et al*., 2015), but batch differences also need to be considered for practical application in a packhouse setting since results can vary considerably due to innate differences. Batch differences for TBZ and PYR residue levels on Satsuma mandarin fruit were 0.32 and 0.54 µg.g^{-1}, respectively, while sporulation inhibition differed as much as 13.8%. An infection age difference of 8.3 and 11.36 h was seen between batches for 90% control on Satsuma mandarin and Eureka lemon fruit, respectively. Control on young infections (< 24 h) were comparable between navel orange batches (90% control; differences of 2.5, 1.4 and 3.9 h at 14, 28 and 56 s, respectively), whilst older infections clearly highlighted differences in green mould susceptibility with increasing exposure time (50% control; differences of 3.5, 9.0 and 12.8 h at 14, 28 and 56 s, respectively), accentuating inherent variability in fruit batches and not just between fruit cultivars and kinds (D'Aquino *et al*., 2006; Smilanick *et al*., 2008; Erasmus *et al*., 2013). The ability of fruit to resist disease development, despite the presence of wounds or sufficient quantities of inoculum, is dependent on the susceptibility of the individual fruit (Eckert and Eaks, 1989; Prusky, 1996) as a result of fruit maturity or physiological age (Brown, 1979;

Baudoin and Eckert, 1982), rootstock, harvest season, fruit condition in the orchard and postharvest environment (Eckert and Eaks, 1989).

Adjuvant trials focused on residue loading, green mould control and pigment distribution over the fruit rind as affected by orientation relative to the flow of the drench solution and adjuvant concentration. Fourie *et al*. (2009) and Van Zyl *et al*. (2010) found that the appropriate adjuvant concentration or spray volume is needed to ensure optimal fungicide spreading and retention without excessive run-off. From this study it was found that an upward orientation of the calyx during drenching resulted in equal or higher TBZ and PYR residue levels loaded at each fruit pole compared to its counterparts on fruit with the opposite orientation, which was also seen on work done by Kellerman *et al*. (2014) using TBZ only; an upward orientation of the calyx also resulted in superior curative green mould control and deposition quantity and quality. Although adjuvant concentration did not seem to affect residue loading, disease control or deposition in these trials, it was obvious that too high a concentration (0.2 $\mu l.mL^{-1}$) was detrimental, likely due to excessive run-off (Fourie *et al.*, 2009). Fruit stacking in the crates used in this study was also not as congested as in a commercial fruit bin, requiring further trials using commercial drench application for more conclusive results.

These findings have practical implications for packhouses, providing tools to determine expected control according to citrus type and infection period, assuming that wounding and infection that occur during harvest are similar to those wounds made in our trials. In fact, the wound inducer used in our trials simulates pressure wounds from long stems on harvested fruit. It was determined from our trials that Satsuma mandarin, Eureka lemon and navel orange fruit should be drenched before 27, 39 and 29 h, respectively, after harvest to obtain > 90% green mould control when drenching with the fungicide mixture of TBZ, PYR and 2,4-D. This information is invaluable to packhouses and highlights the need to ideally drench-treat within a day after harvest, especially on fruit destined for degreening. Another reason for the recommendation to drench within a day after harvest is the poor sporulation inhibition results in this study, making effective curative control imperative before reaching the disease favourable environment of the degreening chamber.

Whilst drench application was shown not to be as effective as dip application (Erasmus *et al.*, 2011), it is clearly an effective fungicide application method to control green mould when delays occur on the way to the packline or on fruit destined for degreening. Further work needs to study improved fungicide retention and distribution throughout highly congested fruit bins. Adjuvants might be beneficial in optimising drench application and fungicide retention, but should be researched carefully as its inclusion in drench mixes might have detrimental effects, such as excessive run-off and phytotoxicity.

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Table 1. Infection age for predicted 50 and 90% curative green mould control on two batches of Satsuma mandarin, Eureka lemon and navel orange fruit drenched at different exposure times (14, 28 and 56 s) at various infection ages (0 to 54 h) of *P. digitatum*.

		Model parameter values and goodness of fit[b]						Infection age[c]	
Citrus type	**Exposure time[a]**	**Batch**	**Pr1**	**Pr2**	**Pr3**	**SSE**	**R^2**	**90% control**	**50% control**
Satsuma	n/a	1	6.018	-0.110	97.489	4170.637	0.828	32.1	54.3
	n/a	2	3.500	-0.067	103.476	4277.575	0.857	23.8	53.2
Lemon	n/a	1	16.294	-0.304	95.705	5475.901	0.796	44.5	53.2
	n/a	2	6.601	-0.120	96.455	5154.726	0.785	33.1	54.5
Navel	14	1	4.542	-0.095	102.854	2115.317	0.863	27.4	48.5
		2	8.293	-0.182	95.094	1899.060	0.918	29.9	45.1
	28	1	4.077	-0.073	103.528	1977.851	0.772	29.9	56.8
		2	5.098	-0.107	101.763	919.777	0.942	28.5	47.8
	56	1	3.729	-0.058	104.525	829.518	0.813	33.0	66.1
		2	4.158	-0.080	104.292	3818.612	0.705	29.1	53.3

[a]A significant exposure time x batch interaction occurred for navel orange fruit, but not for Satsuma mandarin nor Eureka lemon fruit

[b]Data were subjected to non-linear regression statistics using the function Y = pr3/(1+Exp(-pr1-pr2*X1))

[c]Infection age for a specified level of green mould control following drench treatment

Table 2. Mean thiabendazole (TBZ), pyrimethanil (PYR) and 2,4-D residue levels determined on calyx- or stylar-end halves (fruit pole) of Valencia orange fruit drenched with a combination of TBZ, PYR (at 1000 µg.mL^{-1} each) and 2,4-D (250 µg.mL^{-1}) at 41.0 L.min^{-1} for 18 s at ambient, with fruit placed at different orientations, i.e. calyx- or stylar-end upward or downward.

	Residue loaded (µg.g^{-1})		
Fruit orientation and pole	**TBZ[a]**	**PYR[b]**	**2,4-D[c]**
Calyx-end upward	1.04a	2.19a	0.27a
Stylar-end downward	0.76b	1.74b	0.21b
Calyx-end downward	0.60c	1.83b	0.27a
Stylar-end upward	0.56c	1.75b	0.24ab

[a]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.139)

[b]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.211)

[c]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.03)

Table 3. Mean thiabendazole (TBZ), pyrimethanil (PYR) and 2,4-D residue levels measured on Valencia orange fruit drenched with a combination of TBZ, PYR (each at 1000 µg.mL^{-1} each) and 2,4-D (250 µg.mL^{-1}) and the addition of an adjuvant (0, 0.025, 0.05, 0.1 and 0.2 µl.mL^{-1}) at 41.0 L.min^{-1} for 18 s at ambient.

Adjuvant concentration	Residue loaded (µg g^{-1})		
(ml/L^{-1})	**TBZ[a]**	**PYR[b]**	**2,4-D[c]**
0	0.78a	1.98a	0.29a
0.025	0.78a	1.87a	0.28ab
0.05	0.79a	2.01a	0.25b
0.1	0.80a	2.00a	0.25b
0.2	0.54b	1.52b	0.17c

[a]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.156)

[b]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.236)

[c]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.034)

Table 4. Mean percentage green mould control on Valencia orange fruit inoculated with *Penicillium digitatum* 24 and 48 h before drenching with a combination of thiabendazole, pyrimethanil (each at 1000 µg.mL^{-1}) and 2,4-D (250 µg.mL^{-1}) at 41.0 L.min^{-1} for 18 s at ambient, with one third of the fruit placed calyx-end upward, downward and sideways, and incubated at ambient temperature for ± 4 days.

	Green mould control (%)[a]	
Fruit orientation	**24h**	**48h**
calyx-end up	87.8a	53.2c
calyx-end side	79.8b	48.1c
calyx-end down	78.4b	32.2d

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 6.851)

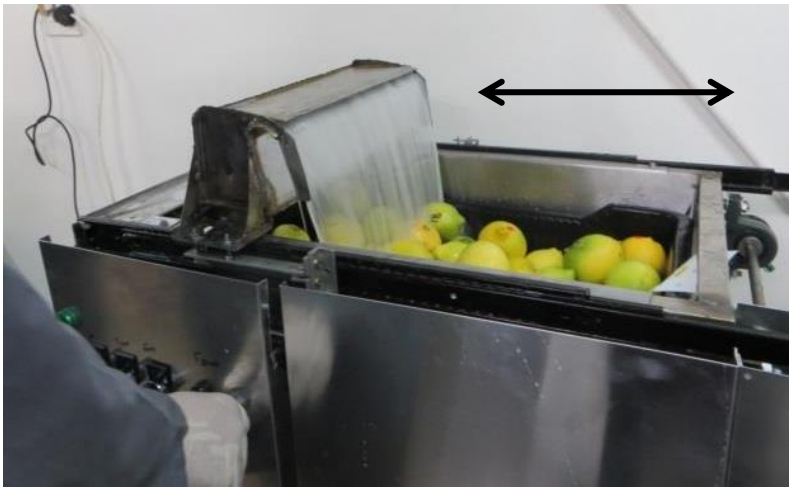

Figure 1. Experimental drench system, pumping the re-circulating fungicide solution through a weir as a laminar flow, moving back and forth over stationary fruit at a speed of 0.056 - 0.07 $m.s^{-1}$ before flowing back into the solution reservoir; the drench solution flow rate was between ± 26.5 – 64.3 $L.min^{-1}$ over fruit crates.

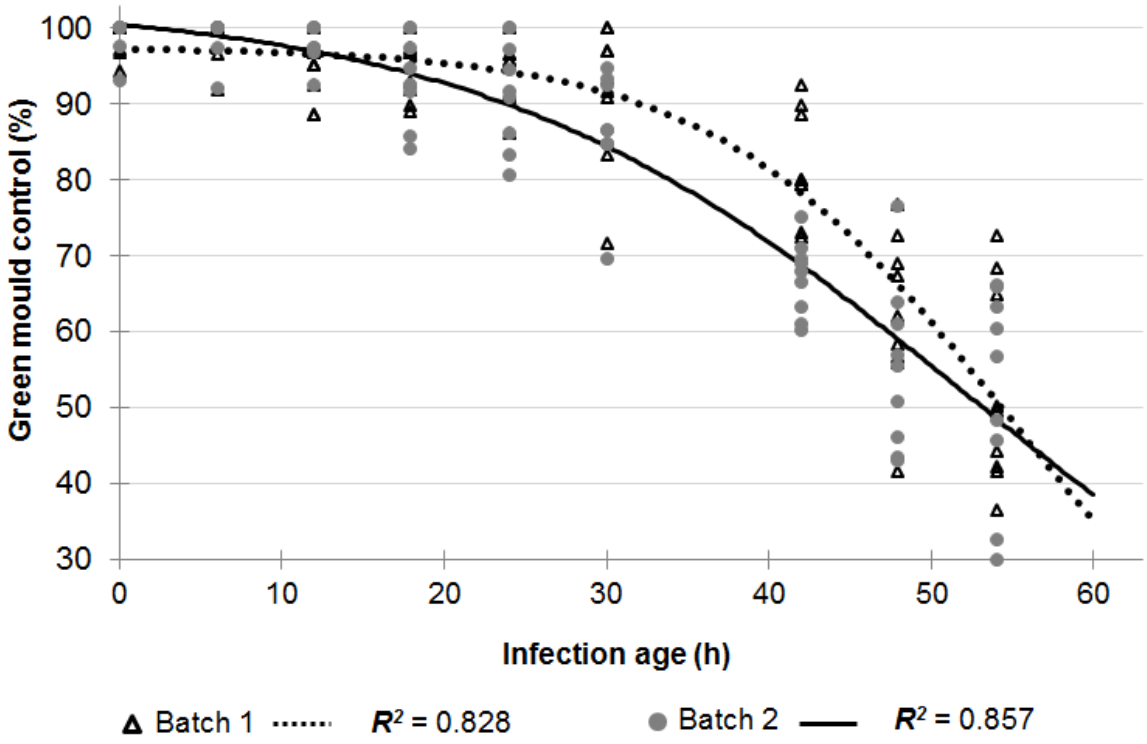

Figure 2. Predicted and measured percentage green mould control on two batches of Satsuma mandarin fruit drenched with a combination of pyrimethanil and thiabendazole (1000 µg.mL^{-1} each) at 26.5 L.min^{-1} for 14 - 56 s at ambient after inoculation with *P. digitatum* at various infection ages (0 to 54 h). Data were fitted on the model Y = pr3/(1+Exp(-pr1-pr2*X1)) using mean values of three replicates per batch.

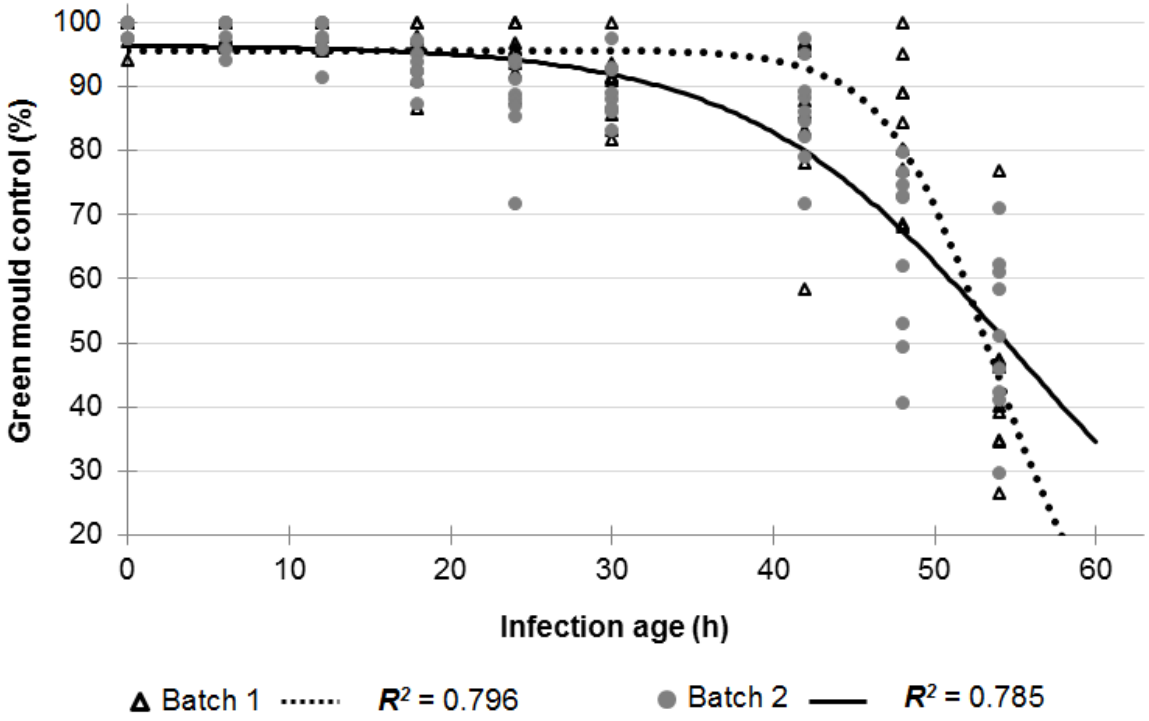

Figure 3. Predicted and measured percentage green mould control on two batches of Eureka lemon fruit drenched with a combination of pyrimethanil and thiabendazole (1000 µg.mL^{-1} each) at 26.5 L.min^{-1} for 14 - 56 s at ambient after inoculation with *P. digitatum* at various infection ages (0 to 54 h). Data were fitted on the model Y = pr3/(1+Exp(-pr1-pr2*X1)) using mean values of three replicates per batch.

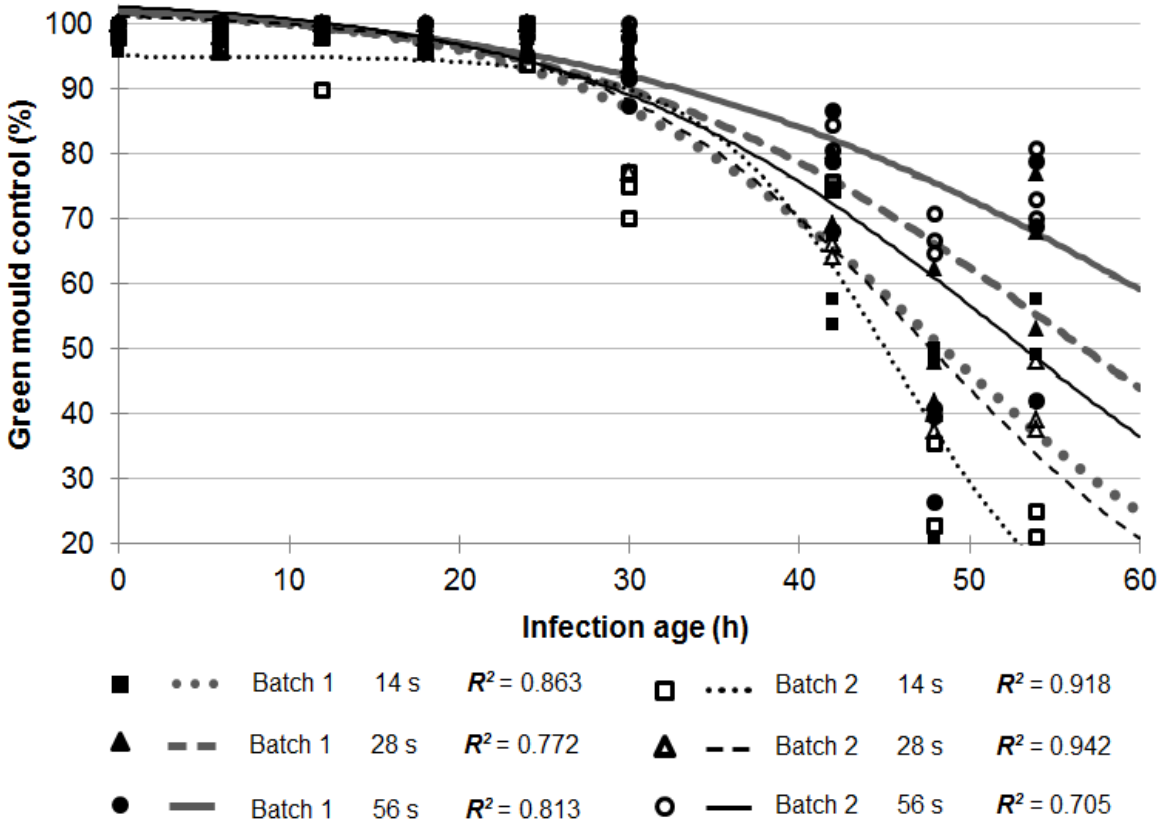

Figure 4. Predicted and measured percentage green mould control on two batches of Palmer navel orange fruit drenched at different exposure times (14, 28 and 56 s) with a combination of pyrimethanil, thiabendazole (1000 µg.mL^{-1} each) and 2,4-D (250 µg.mL^{-1}) at 64.3 L.min^{-1} at ambient after inoculation with *P. digitatum* at various infection ages (0 to 54 h). Data were fitted on the model Y = pr3/(1+Exp(-pr1-pr2*X1)) using mean values of three replicates per batch.

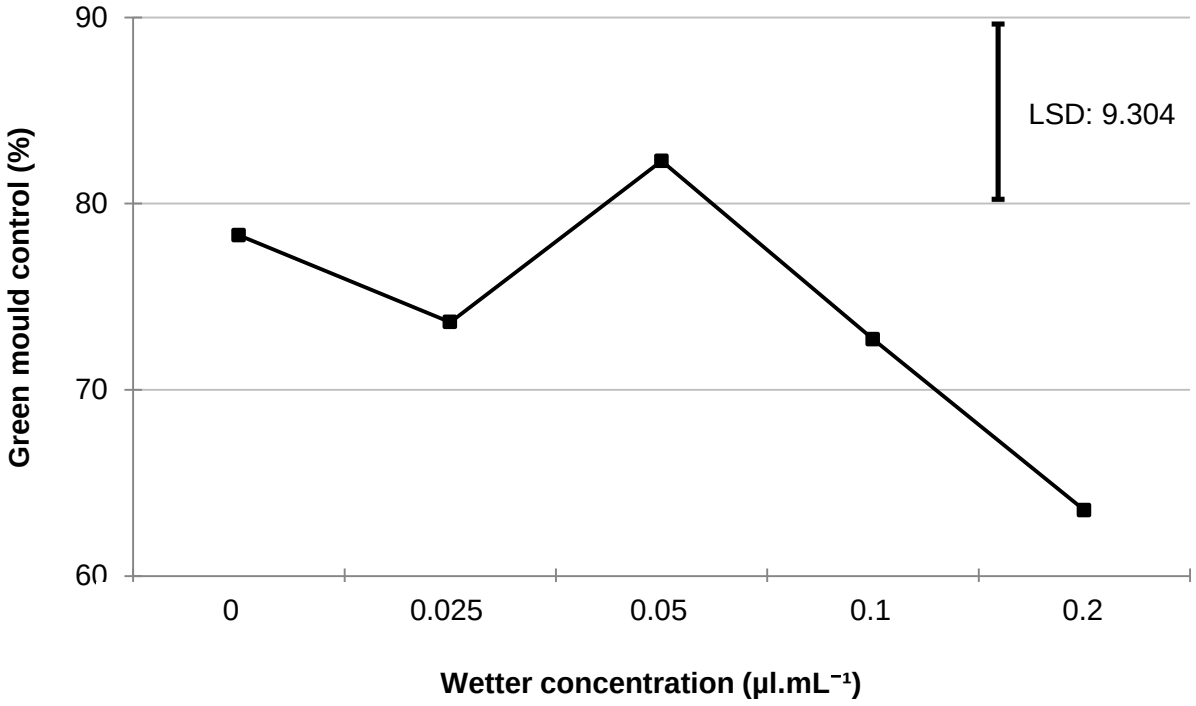

Figure 5. Mean percentage green mould control on Valencia orange fruit inoculated with *Penicillium digitatum* 24 h after drenching with thiabendazole, pyrimethanil (each at 1000 µg.mL^{-1}) and 2,4-D (250 µg.mL^{-1}) and the addition of an adjuvant (0, 0.025, 0.05, 0.1 and 0.2 µl.mL^{-1}) at 41.0 L.min^{-1} for 18 s at ambient and incubated at ambient temperature for ± 4 days.

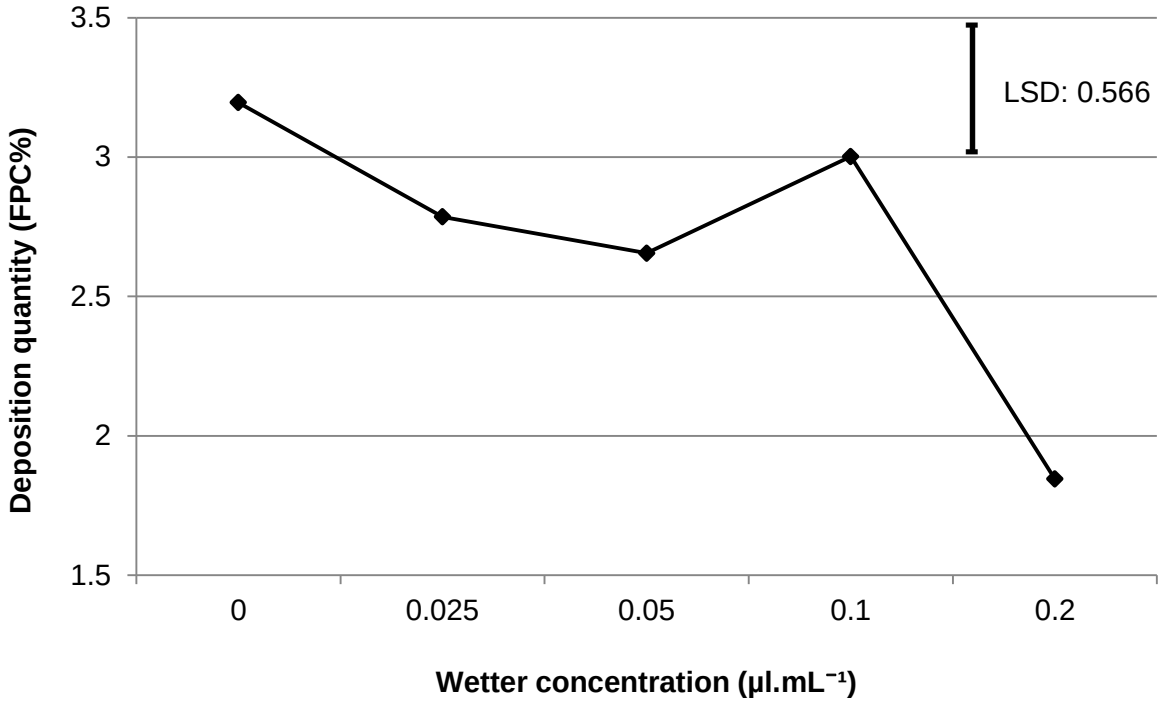

Figure 6. Mean deposition quantity data (FPC%) on Valencia orange fruit drenched with thiabendazole, pyrimethanil (each at 1000 µg.mL^{-1}) and 2,4-D (250 µg.mL^{-1}) and the addition of an adjuvant (0, 0.025, 0.05, 0.1 and 0.2 µl.mL^{-1}) at 41.0 L.min^{-1} for 18 s at ambient.

CHAPTER 3

Sanitisation of fungicide drench solution and effects on green mould and sour rot control

ABSTRACT

Green mould (PD; caused by *Penicillium digitatum*) is the most important postharvest disease of citrus, while sour rot (GC; caused by *Geotrichum citri-aurantii*) becomes more of a decay concern after rainfall, especially since guazatine use is restricted to certain export markets. Sanitisers can be added to drench solutions to reduce sour rot inoculum levels that accumulate with dirt from fruit. The effect of two sanitisers was compared during *in vitro*, *in vivo* and commercial packhouse trials. Variables investigated included green mould and sour rot control and ability of the sanitisers to reduce microbial load ($CFU.mL^{-1}$) in the drench solution while maintaining fungicide persistence for effective green mould control. In commercial packhouse trials, wounded navel orange fruit were drenched with thiabendazole (TBZ), pyrimethanil (PYR), guazatine (GZT) and 2,4-dichlorophenoxyacetic acid (2,4-D) drench-mix and either chlorine (Cl) or hydrogen peroxide/peracetic acid (HPPA) were added every 50 bins during a drenching run of 150 fruit bins. Green mould infection was reduced from ≥ 78.3% to ≥ 67.7% following fungicide drench application. Infection and fungicide persistence were similar regardless of sanitiser treatment, although green mould infection levels increased significantly by bin 150 (10.6 vs. 5.2 – 6.0%). Sanitiser concentrations (0, 20, 40, 60 and 80 $\mu g.mL^{-1}$ Cl or 0.00, 0.01, 0.10, 0.30 and 0.60% HPPA) were combined with TBZ, PYR and 2,4-D and GC spores (≈ 3.175×10^4 $spores.mL^{-1}$) mixture for 1, 3 and 60 min exposure and plated out. The sanitisers did not affect fungicide concentration levels. HPPA completely reduced sour rot inoculum (0.0 $CFU.mL^{-1}$) after 1 – 3 min at the high pH levels (> 10) of the mixture. *In vivo* trials involved exposing 24 h *P. digitatum* inoculated and uninoculated wounded fruit to TBZ, PYR and 2,4-D and GC spores (similar to *in vitro* trials) containing either 80 $\mu g.mL^{-1}$ Cl or 0.3% HPPA with the addition of 0, 500 or 1000 $\mu g.mL^{-1}$ kaolin, used to simulate dust accumulation during drenching. Residue levels, solution concentration and green mould control were similar between sanitiser and kaolin treatments. HPPA treatments improved sour rot control on Valencia and Nadorcott mandarin fruit and improved green mould control on Nadorcott mandarin fruit. Exposure to 0.3% HPPA for 3 min was superior to Cl treatment at high pH levels.

INTRODUCTION

Postharvest losses on citrus fruit occur primarily due to green mould (*Penicillium digitatum* [Pers.: Fr.] Sacc.) and sour rot (*Geotrichum citri-aurantii* E.E. Butler [*G. candidum* Link]) (Eckert and Eaks, 1989). Green mould is responsible for 80 – 90% of citrus losses occurring during export (Lesar, 2013), although sour rot becomes more of a decay concern after high rainfall, especially since so few fungicides are registered for the control of this disease (Cunningham and Taverner, 2006; Horuz and Kmay, 2010). In South Africa, guazatine (GZT) and propiconazole (PPZ) are the only fungicides registered for sour rot control, while several actives registered for green mould control include imazalil (IMZ), thiabendazole (TBZ), pyrimethanil (PYR) and GZT (Pers. comm. K. Lesar; Taverner, 2001; Erasmus *et al*., 2011; Kellerman *et al*., 2014), with IMZ being the most effective and commonly included in inline dip and wax applications (Erasmus *et al*., 2011; Njombolwana *et al*., 2013). The use of GZT is becoming increasingly restricted, necessitating greater (Pers. comm. K. Lesar; Cunningham and Taverner, 2006) focus on alternative methods of controlling sour rot.

Early season citrus fruit requiring degreening for desired fruit colour (Sdiri *et al*., 2012) are exposed to 1 – 5 $\mu g.mL^{-1}$ ethylene gas at 18 to 25°C (depending on fruit type) and 94 - 96% relative humidity (Krajewski and Pittaway, 2010) for 2 – 3 days in South Africa (Pers. comm. P. Cronje). These conditions correspond with optimum conditions for growth and development of green mould (25°C) (Zhang and Swingle, 2005) and sour rot (25 to 30°C) (Plaza *et al*., 2003). Since green mould has been shown to be effectively controlled with timely drench application (< 24 h after harvest) (Chapter 2), this study focused on further optimising drench applications and to improve sour rot control to markets where GZT use is restricted.

Geotrichum citri-aurantii is able to survive in soil and debris, easily contaminating fruit near the ground through wind action, splash or direct contact. Consequently sour rot inoculum can build up with dirt and debris in dip tanks or drenchers, infecting injured fruit (Brown, 1979). Mature to over-mature fruit with high peel moisture are more susceptible to sour rot development (Ismail and Zhang, 2004). Substantial albedo injuries, caused by fruit piercing insects or during harvest, are initially required for infection (Pelser, 1977; Brown, 1979; Brown, 2003). Damage to oil glands in the fruit peel can increase the chance of decay by 25 – 50% (Baudoin and Eckert, 1982). Following initial infection and decay, sour rot can spread from diseased to adjacent healthy fruit resulting in large nests of decay during storage and transport (Mercier and Smilanick, 2005). Optimal sour rot growth occurs between 25 and 30°C while growth slows down considerably from 10 to 4°C (Plaza *et al*., 2003).

Thiabendazole is ideal for drench application as both pH and temperature adjustment is unnecessary (McCornack, 1970) and relatively low residue levels are required to control

green mould. Smilanick *et al.* (2006b) determined that a residue level of ≥ 0.2 µg.g^{-1} TBZ is sufficient for effective control, and Kellerman *et al.* (2014) found that > 75% control can be achieved with 0.06 – 0.22 µg.g^{-1} TBZ, depending on fruit type. Pyrimethanil is able to effectively control TBZ resistant strains of *P. digitatum* due to a different mode of action (Smilanick *et al*., 2006a) with PYR residue values of 0.905 µg.g^{-1} required for 75% curative green mould control (E. Liebenberg, unpublished data). Thiabendazole and PYR provide effective curative control of green mould (Smilanick *et al*., 2006a; Schirra *et al*., 2008), although TBZ remains superior to PYR in terms of protective control and sporulation inhibition (Smilanick *et al*., 2006a; Kanetis *et al*., 2007), with neither fungicide providing effective sour rot control (Ismail and Zhang, 2004; Liu *et al*., 2009). The plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) is often added to drench mixtures and does not have direct fungicidal action, but reduces decay indirectly by delaying stem-end button senescence and subsequently enhancing fruit resistance (Eckert and Eaks, 1989). The MRL tolerance for TBZ is 10 ppm in the USA, Canada and Japan (Ritenour *et al*., 2003) and 10 and 8 mg.kg^{-1} for PYR in the USA and as general export tolerance, respectively, and the general export tolerance for 2,4-D is 1.0 mg.kg^{-1} (The European Commision, 2014; Hattingh and Hardman, 2015).

Within the fungicide drench mixture commonly adopted in South Africa (TBZ, PYR, GZT and 2,4-D), only GZT is highly effective against sour rot while other treatments merely reduce sour rot incidence. If GZT is not an option for a specific market, sour rot becomes a concern as incipient infections are difficult to detect during grading, resulting in rapid sour rot development once fruit are transferred to ambient temperatures during marketing (Eckert and Eaks, 1989). Propiconazole was shown to be effective against sour rot (McKay *et al*., 2012) but is not yet available for postharvest use on citrus in many countries. Imazalil (IMZ) and PPZ also have the same mode of action (demethylation inhibitors), which can lead to resistance build-up against this group of fungicides (Lyr, 1995). To limit DMI resistance development, it is therefore not advisable to apply PPZ during drench application as a pre-cursor to IMZ dip and/or wax application in the packline. Alternative methods such as sanitising drench solutions should be investigated to lessen the reliance on fungicides for the control of sour rot.

Drenching involves application of fungicide solution over fruit in field bins by means of a waterfall in a recirculating system. As the bins and fruit come directly from the orchard, soil (Brown and Miller, 1999) and soil-borne pathogens, such as *Geotrichum*, can accumulate in the tank during treatment necessitating disinfectants to reduce the microbial load (Brown, 1979). Very little information is available in literature concerning methods for removing dirt, debris and contamination from drench tanks other than regularly replenishing it with clean water and a new fungicide solution (Cunningham and Taverner, 2006). Due to the lack of

registered or available fungicides for the control of sour rot and accumulation of dirt during drenching, other methods need to be evaluated to extend the effective life of a drench solution, specifically because a high microbial load in the solution increases the risk of inducing infection to vulnerable wounded fruit (Barkai-Golan, 2001).

Standard sanitation practice for commercial packhouses involves the use of broad-spectrum sanitisers applied during the fruit cleaning process, such as chlorine (Cl) (Taverner, 2004; Fischer, 2009) and hydrogen peroxide/peracetic acid (HPPA) (Kanetis *et al*., 2008a). Several packhouses in Spain and South Africa already apply HPPA as part of a fungicide dosage system (Pers. Comm. J.C. Martin-Loeches) where it acts as a solution sanitiser. The sanitiser peracetic acid or peroxyacetic acid (PAA) is commercially available as a mixture of acetic acid (CH_3CO_2H), hydrogen peroxide (H_2O_2), PAA (CH_3CO_3H) and water (H_2O) in equilibrium, as shown by the following equation: $CH_3CO_2H + H_2O_2 \rightarrow CH_3CO_3H + H_2O$ (HPPA; Hydrogen peroxide/peroxyacetic acid) (Taverner, 2004). Calcium hypochlorite is the main form of Cl used in South Africa (Pers. comm. K. Lesar; Hewett, 2014) in fruit washing systems, and is applied to kill spores in bulk dip and re-circulating washes, preventing inoculum build-up and removing surface populations of *P. digitatum* and *G. citri-aurantii* (Smilanick *et al*., 2002; Cunningham and Taverner, 2006). Ismail and Zhang (2004) also mentions that Cl can be added to TBZ during drenching to control *G. citri-aurantii* and TBZ resistant *Penicillium* strains. For optimal Cl use, this sanitiser must be maintained in solution at a pH of between 6.8 – 7.2 (Hewett, 2014) for a time interval of at least 2 min for maximum efficacy against propagules (Brown and Miller, 1999). Dirt and debris also reduce Cl activity (Hewett, 2014). HPPA has a larger effective pH range (pH 5 – 8) than Cl and is not as sensitive to the presence of organic matter, but can be corrosive to certain metals or surfaces (Taverner, 2004; Hewett, 2014).

Sanitising agents need to be used in combination with fungicides due to a lack of residual effect, although incompatibility issues exist and need to be considered (Cunningham and Taverner, 2006). Chlorine incompatibility has been linked to fungicides such as PYR and imazalil (IMZ) (Kanetis *et al*., 2008b; Smilanick *et al*., 2006a), which were unaffected by HPPA (Kanetis *et al*., 2008a). To overcome incompatibility issues, Brown *et al*. (1988) advised re-charging a drench solution with benomyl during Cl application. Incompatibility issues can therefore be addressed by adjusting the fungicide concentration following sanitiser application, although the effect of shock treatments on fungicide concentration and residue loading needs to be evaluated.

Due to the accumulation of dirt and contaminants in drench mixtures during drench application and the restricted use of GZT, sanitisers could be used through shock treatments to not only control sour rot, but to extend the effective use of a drench solution in spite of increasing dirt levels. Therefore, the objectives of this study were to compare the effect of

two sanitisers (Cl and HPPA) during *in vitro*, *in vivo* and commercial packhouse trials on green mould and sour rot control, the ability of the sanitisers to reduce microbial load in the drench solution and the effect on residue loading and breakdown of fungicide actives in the solution.

MATERIALS AND METHODS

General protocols and information

Fungal isolates and culture preparation

A *P. digitatum* (PD) isolate (STE-U 6560) known to be sensitive to IMZ, GZT, TBZ and PYR (Erasmus *et al.,* 2015) was obtained from a Satsuma orchard on the Stellenbosch University experimental farm, Welgevallen, Stellenbosch, Western Cape, South Africa. A GC isolate (CRI360) from an orchard at Joubert and Sons farm, Schoemanskloof, Mpumalanga, South Africa, was also used during these trials. Inoculum cultures of PD and GC were incubated on amended potato dextrose agar medium (PDA^{+}; Difco™, Becton, Dickinson and Company, Sparks, MD, USA amended with chloramphenicol; Chlorcol; 250 mg CAP 500, Adcock Ingram, Midrand, Gauteng, South Africa) at 25 and 28°C, respectively. Other medium used in this study was PDA amended with 1 µg.mL^{-1} IMZ (PDA^{IMZ}) (IMZ; Imazacure® 750SG, ICA International Chemicals (Pty) Ltd., Stellenbosch, Western Cape, South Africa).

Conidia were harvested from ± 2-week-old cultures and prepared as spore suspensions by washing the surface of a culture with sterile deionised water amended with ≈ 0.01 µl.mL^{-1} Tween 20 (Merck, Wadeville, Gauteng, South Africa) followed by filtration through two layers of autoclaved cheesecloth (Erasmus *et al*., 2011; Kellerman *et al*., 2014) and appropriate adjustment with a spectrophotometer. A reading of 0.1 at 420 nm absorbance (Cecil CE 1011 1000 series, Cecil Instruments Limited, Cambridge, England) equivalent to a concentration of 1 × 10^6 spores.mL^{-1} of PD (Morris and Nicholls, 1978; Eckert and Brown, 1986) whereas 0.14 absorbance at 420 nm was ≈ 3.175 × 10^6 spores.mL^{-1} of GC (confirmed by means of haemocytometer for this study).

Inoculation, incubation and evaluation

In order to evaluate green mould control during the *in vivo* trials, PD spore suspensions were prepared shortly before inoculating fruit 24 h prior to treatment (curative control). Green mould inoculations involved dipping a cylindrical stainless steel rod with a 2 mm protruding tip, 1 mm wide, into a spore suspension of PD (1 × 10^6 spores.mL^{-1}) and used to pierce the rind of each fruit four separate times equidistantly around the calyx. Control fruit were similarly inoculated and left untreated. Several 1.2 L units containing GC spore suspension (≈ 3.175 × 10^6 spores.mL^{-1}) were prepared a day before use and stored at ± 4°C; 1.2 L spore

suspension was later diluted into the 120 L drench reservoir to make up ≈ 3.175 × 10^4 spores.mL^{-1}. Sour rot infection and control was assessed by wounding fruit four times equidistantly around the calyx using the screw end (25 mm long and 3 mm diameter) of a round cup hook (Product code 2E45; Eureka Park, Lea Glen Ext. 2, Roodepoort, South Africa) 3 mm deep through the albedo 30 min before treating with drench solution containing GC spores (≈ 3.175 × 10^4 spores.mL^{-1}) and other specific treatment combination products. Control fruit were similarly wounded and drenched with water containing GC spores only.

Following treatment, PD inoculated fruit were packed into table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine pulp trays (Huhtamaki South Africa (Pty) Ltd., Atlantis, South Africa) and covered with transparent polyethylene bags (perforated four times using the screw end of a round cup hook and incubated at ambient temperature (≈ 22°C) for 4 – 6 days. The GC inoculated fruit were incubated similarly to PD with two exceptions: the pulp trays were moistened with ± 100 mL muncipal water before placed in the cover bag and the incubation regime was ± 28°C for 5 - 7 days. All treatments were rated when control fruit were sufficiently infected. The number of infected wounds per fruit was determined by rating water soaked lesions that were soft to the touch.

Chemicals

The fungicide mixture used throughout these trials, unless stated otherwise, was 1000 µg.mL^{-1} TBZ (ICA - Thiabendazole® 500SC, ICA International Chemicals (Pty) Ltd., Stellenbosch, Western Cape, South Africa), 1000 µg.mL^{-1} PYR (Protector® 400SC, ICA International Chemicals (Pty) Ltd.) and 250 µg.mL^{-1} 2,4-D (Deccomone®, Citrashine (Pty) Ltd., Booysens, Gauteng, South Africa). Antifoam (50 mL Biologix AF 720: Foamfix®; Moreleta Park, Gauteng, South Africa) was added at the beginning of each treatment during the *in vivo* trials. Sanitisers used separately throughout this study were calcium hypochlorite (Cl; HTH, Arch Chemicals (Pty) Ltd., Bergvlei, Gauteng, South Africa) and a combination of hydrogen peroxide and peracetic acid (HPPA; Citrocide® PC, Citrosol S.A., Portries, Valencia, Spain). Concentrations varied according to trial. Citrocide® is available as 5% peracetic acid, 8% acetic acid and 23% hydrogen peroxide (Citrocide® Technical data sheet). Sodium thiosulfate pentahydrate (STP; $Na_2S_2O_3 \cdot 5H_2O$ Emparta® ACS, Merck Specialities Private Limited, Worli, Mumbai, India) and Sodium metabisulfite (SMB; Pyrosulfurous acid, disodium salt [$Na_2S_2O_5$], Houston, Texas, USA) were used to de-activate each sanitiser, respectively, in selected trials (Pers. comm. J. Breto; Pers. comm. S. Serfontein).

A pH meter (Waterproof Tester pH·EC·TDS·ORP·°C/°F; Hanna Instruments® Inc., Woonsocket, USA) and test paper strips for Cl (Cl strip; 10 – 200 µg.mL^{-1}, LaMotte, Chestertown, Maryland, USA) and peracetic acid (HPPA strip; 5 – 50 µg.mL^{-1} Peracetic Acid

Test [MQuant^{TM}]; Merck (Pty) Ltd., Gauteng, South Africa) were used to measure solution pH and sanitiser concentration, respectively. The pH level was not adjusted. According to the Citrosol procedure for testing the concentration of HPPA (Pers. comm. J.C. Martin-Loeches), 1 mL of solution is added to 20 mL de-ionized water (1/20 dilution) before using the 5 – 50 µg.mL^{-1} HPPA strips with ≤ 5, 5 – 10, 10 – 20, ≈ 20, > 20 µg.mL^{-1} approximately relating to ≤ 0.2%, 0.2 – 0.4%, 0.4 – 0.8% (low for drencher), ≤ 0.8 (optimal for drencher), > 0.8% (overdose) peracetic acid, respectively.

Residue analysis

The preparation process involved macerating the fruit sampled for residue analysis from each treatment combination, using either wholly chopped small fruit or a section from larger fruit, i.e. fruit were cut into four or eight equal pieces from the stylar- to the calyx-end. Fruit were chopped and diluted with measured amounts of distilled water (in accordance to the weight of the fruit) before being macerated to a fine pulp in a blender for 2 min and stored at -20°C; ± 0.58 mL.g^{-1} water was used to dilute Navel oranges in the commercial packhouse trials and ± 0.56 and ± 0.40 mL.g^{-1} for the Valencia orange and Nadorcott mandarin fruit, respectively, in the *in vivo* trials (Erasmus *et al*., 2011; Kellerman *et al*., 2014).

Preparation of solution samples for fungicide concentration analyses involved preparing a 1 (solution sample) in 10 mL dilution with methanol (99.5% CH_3OH: 32.04; Merck (Pty) Ltd., Gauteng, South Africa), followed by another 50 µL (from previously diluted solution) in 10 mL dilution with methanol. A final 1 mL is then removed from this dilution for concentration analysis.

Samples were analyzed by an accredited analytical laboratory (Hearshaw and Kinnes Analytical Laboratory, Westlake, Cape Town, South Africa) using acetonitrile, matrix solid phase dispersion extraction and tandem liquid chromatography mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA). All results received from the analytical laboratory were converted according to each individual dilution factor in order to obtain the actual residue value.

Commercial packhouse trials

This trial was conducted during a commercial packing program for export at a packhouse near Nelspruit (Mpumalanga province, South Africa). Untreated control data were analyzed first in order to provide an indication of disease pressure followed by bin 1, which demonstrates starting solution inoculum levels. Bins 50, 100 and 150 were then evaluated to study the effect of an aging solution.

Drench applicator

The reservoir of the drench applicator was filled with 1000 L water and amended with 1000 µg.mL^{-1} TBZ (Tecto 500 SC; Syngenta Crop Protection AG, Postfach, Basel, Switzerland), 1000 µg.mL^{-1} PYR (Protector® 400SC; both TBA and PYR were pre-mixed in cold water), 500 µg.mL^{-1} guazatine (GZT; Kenopel® 200 SL; Adama SA (Pty) Ltd., Brackenfell, Cape Town, South Africa) and 250 µg.mL^{-1} 2,4-D (Deccomone®). For each drench run (one treatment combination), 150 double-stacked commercial fruit bins were drenched at 1066 L.min^{-1} for ± 30 s exposure time per double-stack, after which the drench mixture was discarded and replaced with a fresh mixture. Fruit were treated several hours after harvest when enough bins were accumulated for a drench run (150 bins). Each drench run commenced after a fresh fungicide mixture was prepared and circulated for several minutes in the drench reservoir by pumping the solution through the weirs and back into the tank.

Protocol

Navel orange fruit were collected in field bins directly from the orchard. All fruit came from the same farm and were harvested on the same day of treatment. Trial commencement was dependent on the daily operation of the packhouse. The capacity of the packhouse allowed for 300 bins to be drenched per day therefore, trials had to be run over two consecutive days (within 48 h) for Cl and HPPA treatments, respectively. Each trial day consisted of one control (drench mixture with no sanitizer; 150 bins) and one sanitiser treatment (150 bins). Each control and sanitiser treatment was conducted twice. All solution samples and treatment fruit were taken from stacked bins at number 1, 50, 100 and 150. The sanitiser treatments involved adding 80 µg.mL^{-1} Cl or 0.6% HPPA to bin 50 and 100, as well as bin 150 for Cl only.

Thirty-six fruit per treatment bin were wounded four times equidistantly around the calyx using the round cup hook (as explained above) within 30 min before drenching each treatment combination and were placed randomly on both the top and bottom bins at numbers 1, 50, 100 and 150. The same number of fruit were wounded and left untreated, in the vicinity of the drench applicator exposed to the environment, for each treatment combination that served as untreated controls. After each drench run the thirty-six fruit per treatment bin were randomly divided into three replicates of twelve fruit each.

Sampling and evaluation

A sample from each solution was collected at bins 1, 50, 100 and 150 in a 500 mL polyethylene container directly from the weir at each treatment for HPPA concentration measurements (where required), plating out and solution fungicide concentration analysis. The sanitisers were not deactivated in this trial. Approximately 24 hours after sampling, 100

µL were pipetted from each of the samples onto each of three PDA^{+} and three PDA^{IMZ} plates and were spread using a glass hockey-shaped rod and stored at 25°C for ± 2 days before determining total $CFU.mL^{-1}$ (colony forming units).

Following drench application, treated fruit were left for approximately 15 min to dry in harvest bins before packing both untreated control and treated fruit in cartons and covering. Six additional fruit were removed for residue analysis from the top bin and bottom bin of every stacked containing fruit bin 1, 50, 100 and 150. The wounded fruit were prepared and stored according to the incubation regime for green mould as described above.

In vitro **sanitiser trials**

Spore suspension, fungicide solution and chlorine stock solution

A 1 L fungicide solution was prepared with municipal water and agitated for 1 min on a magnetic stirrer followed by the addition of 10 mL GC spore suspension and another 1 min of agitation. The fungicide solution contained 1000 $\mu g.mL^{-1}$ TBZ, 1000 $\mu g.mL^{-1}$ PYR and 250 $\mu g.mL^{-1}$ 2,4-D. The final GC spore concentration was ≈ 3.175×10^{4} $spores.mL^{-1}$.

Two separate 1 L stock solutions were prepared for Cl and STP, at a concentration of 10 000 $\mu g.mL^{-1}$ each.

Trial protocol

In the first trial the fungicide and spore combination was exposed to 0, 20, 40, 60 and 80 $\mu g.mL^{-1}$ Cl or 0.00, 0.01, 0.10, 0.30 and 0.60% HPPA for 1 and 60 min. In the second trial the fungicide and spore combination was exposed to 0, 40 and 80 ppm Cl or 0, 0.1 and 0.3% HPPA for 1 and 3 min. Each trial was conducted three times. The active Cl was deactivated by adding 0, 1, 2, 3 and 4 mL from the STP stock solution to the respective Cl treatments. Similarly HPPA was deactivated by adding 0.000, 0.014, 0.140, 0.420 and 0.840 g SMB to respective HPPA treatments. Three replications per treatment combination were carried out.

Evaluation

Following each exposure time period, two samples were removed: one for measuring pH and sanitiser concentration and one 100-mL sample for deactivation and subsequent plating out and concentration analysis. Plating out of samples involved pipetting 1 mL of the deactivated sample solution into 9 mL sterile de-ionised water (1/10 dilution) with 50 µL removed from the diluted sample solution and pipetted onto PDA^{+} plates and spread using a glass hockey-shaped rod and stored at 28°C for ± 2 days before determining GC $CFU.mL^{-1}$. $CFU.mL^{-1}$ was determined by the following formula: (dilution factor × number of colonies counted)/amount plated out. Three and two PDA^{+} plates were used for Cl and HPPA,

respectively, during the 1 and 60 min exposure trials, whilst six plates were used for Cl and HPPA treatments during the 1 and 3 min exposure trials.

In vivo sanitiser trials

Fruit

Untreated export quality Late Valencia orange and Nadorcott mandarin fruit were obtained for *in vivo* trials from packhouses in the Limpopo and Mpumalanga provinces of South Africa shortly after harvest. Fruit were washed over rotating brushes and sprayed with ozone treated tap water (ArcAqua patented Ozone applicator; 24 $L.min^{-1}$ of Ozone at 2 $g.h^{-1}$ using 8 $L.min^{-1}$ tap water at 3 bar through four nozzles; ArcAqua (Pty) Ltd., Westlake Business Park 7945, Cape Town, South Africa) before being stored at 4°C for 5 and 7 days (Batch 1 and 2, respectively). Fruit were transferred to ambient temperature (≈ 22°C) 1 day before commencing trial preparation in order to allow evaporation of any condensation.

Experimental drench applicator

The reservoir of a custom-built stainless steel drench applicator (Citrus Research International, Nelspruit, South Africa) was filled with 120 L of municipal water and amended according to treatment combination (Chapter 2). Fruit were packed randomly into plastic fruit perforated packing crates (Kaap-Agri, 65 Voortrekker road, Malmesbury; 325 × 505 × 245 mm), used to simulate the standard 800 L commercial orchard bin, containing a wire mesh 75 mm from the bottom to prevent fruit from being immersed in the fungicide solution that might accumulate in the crate. A weir moved back and forth over the fruit crate at a speed of 0.06 $m.s^{-1}$, drenching fruit with a re-circulating fungicide solution pumped (Salflo pumps V230 H250; Stewarts & Lloyds pumps, Longmeadow, Edenvale, Johannesburg, South Africa) from the reservoir to the weirs at ± 31.04 $L.min^{-1}$, which at ≈ 24 s exposure time applies 12.5 $L.crate^{-1}$, which relates to the industry-recommended dosage of 250 $L.bin^{-1}$.

Protocol

For each treatment the drench solution contained TBZ, PYR, 2,4-D (at 1000, 1000 and 250 $\mu g.mL^{-1}$, respectively) and GC spores (≈ 3.175×10^4 $spores.mL^{-1}$). Kaolin (mineral dust formed by weathering of aluminum silicates; Protea Chemicals, Milnerton, Cape Town, South Africa) was added to each treatment combination at 0, 500 and 1000 $\mu g.mL^{-1}$ concentrations and mixed for 1 min before drenching fruit crates in order to simulate dirt accumulation in a commercial drench applicator. Each crate acted as one treatment replicate unit and contained 12 PD inoculated and 12 wounded fruit from each batch of Nadorcott mandarin and Valencia orange fruit per treatment combination. Ssix fruit per

citrus type was added to the first and last replicate of each treatment combination for residue analysis. Three replications per treatment combination were carried out, and the trial was conducted twice on each fruit type.

Following drenching the afore mentioned treatments, 80 µg.mL^{-1} Cl or 0.3% HPPA were added to the drench solution and circulated for 3 min. The sanitiser solution was then de-activated using STP (400 µg.mL^{-1}) or SMB (4200 µg.mL^{-1}), respectively, and circulated for 1 min before drenching fruit.

Evaluation

A solution sample of 100 mL was taken from each specific drench treatment for pH and sanitiser concentration assessment and for sour rot CFU.mL^{-1} and concentration analysis; each evaluation set was conducted before and after sanitiser de-activation. Evaluation protocols were similar to those described in the *in vitro* sanitiser trials.

Following drench treatment of crates, green mould inoculated fruit and wounded fruit were incubated and evaluated as described in the inoculation, incubation and evaluation section. Six additional fruit were removed for residue analysis from the first and third replicate of each treatment combination.

Statistical analysis

Infection ratings were converted to percentage infection in the commercial packhouse trials by combining data in this study even though sanitisers were tested on different days. Green mould and sour rot infection data from the *in vivo* trial were normalised by calculating percentage control relative to the untreated controls. XLSTAT version 2014.4.03 (www.xlstat.com) was used for analyses of variance (ANOVA) and Fisher's least significant difference test was used to identify significant differences between treatments. A 90% confidence interval was used to assess residue and concentration level data in the commercial packhouse trials while a 95% confidence interval in the other trials. Experiments involving different citrus types were analyzed separately.

RESULTS

Commercial packhouse trials

HPPA solution concentration

Following the first 0.6% HPPA dosage at fruit bin no. 50, the solution concentration was 0.8% (results not shown), which increased to > 0.8% following the second dose at bin 100; this is considered an overdose and HPPA was therefore not applied again at bin 150 and the concentration remained > 0.8%. The chlorine concentration was not measured.

Residue loading and fungicide solution concentration

Analysis of variance for TBZ, PYR and 2,4-D concentration levels measured at bins 1, 50, 100 and 150 presented no significant interaction but the number of bins was meaningful as main effect for TBZ (P = 0.0762; ANOVA tables not shown) and treatment was significant as main effect for PYR (P = 0.0396). Treatment was not significant as main effect for TBZ (P = 0.470), bin was not significant for PYR (P = 0.238), while bin and treatment did not significantly affect 2,4-D concentrations (P = 0.788 and 0.483, respectively). A lower TBZ concentration (730.0 µg.mL^{-1}) was measured in bin 1 compared to bin 50, 100 and 150 (1350.0, 1215.0 and 1512.5 µg.mL^{-1}, respectively). Solutions containing Cl resulted in significantly higher PYR concentration levels compared to the HPPA treatment (2195.0 and 1385.0 µg.mL^{-1}, respectively) and each corresponding control (1477.5 and 1370.0 µg.mL^{-1}, respectively). Mean concentration levels of 1191.0 µg.mL^{-1} for TBZ, 1618.7 µg.mL^{-1} for PYR and 820.7 µg.mL^{-1} for 2,4-D were obtained.

Analysis of variance for TBZ, PYR and 2,4-D fruit residue levels measured at bins 1 – 150 indicated that number of fruit bins was significant as main effect for TBZ (P = 0.0004) and that bin-stack and treatment were meaningful as main effects for PYR (P = 0.0930 and 0.0976, respectively). Bin stack and treatment was not significant as main effects for TBZ (P = 0.494 and 0.722, respectively), number of bins for PYR (P = 0.260) and bin stack, number of bins and treatment for 2,4-D (P = 0.481, 0.871 and 0.830, respectively). Bin 150 resulted in significantly higher TBZ residue levels (1.30 µg.g^{-1}) compared to bin 1, 50 and 100 (0.40, 0.69 and 0.72 µg.g^{-1}, respectively). When stacking fruit bins, the top bin loaded higher PYR residue levels compared to the bottom of the two-bin stack (1.90 and 1.61 µg.g^{-1}, respectively). Solution amended with Cl resulted in significantly higher PYR residue levels compared to the HPPA treatment (2.05 and 1.44 µg.g^{-1}, respectively) while controls resulted in intermediate levels (1.75 and 1.76 µg.g^{-1}, respectively). Mean residue levels of 0.77 µg.g^{-1} for TBZ, 1.74 µg.g^{-1} for PYR and 0.38 µg.g^{-1} for 2,4-D were obtained on treated fruit.

Total colony forming units

Analysis of variance for total CFU.mL^{-1} data determined at bins 1, 50, 100 and 150 indicated a significant sanitiser × bin interaction for both PDA^{+} and PDA^{IMZ} media (P < 0.0001 and < 0.0001, respectively). The addition of Cl (bin 150) resulted in significantly lower total CFU.mL^{-1} levels (0.0) on both PDA^{+} and PDA^{IMZ} compared to corresponding control treatments (1703.3 and 5431.7, respectively). Only bin 1 during the HPPA treatment also resulted in significantly higher total CFU.mL^{-1} levels on PDA^{IMZ} media (3645.0 CFU.mL^{-1}) compared to other treatments. The majority of CFU.mL^{-1} was as a result of a combination of fungal and bacterial growth.

Green mould infection

High infection levels in the untreated dry controls (≥ 78.3%; results not shown) indicated a high inoculum load for the majority of treatments.

Analysis of variance for percentage infection data at bins 1 – 150 presented no significant interactions, with the number of bins significant as main effect ($P < 0.0001$). Bin 150 resulted in significantly higher infection levels (10.6%) compared to bin 1 (6.0%), 50 (5.7%) and 100 (5.2%). No sanitiser treatment effect was observed ($P = 0.310$).

In vitro sanitiser trials

One and 60 min exposure time trial

Fungicide solution pH remained similar over the addition of different Cl concentrations (pH 10.29 – 10.45; results not shown), although a reduction was seen with increasing HPPA concentrations from 0, 0.01, 0.1, 0.3 and 0.6% (pH > 10, > 10, ± 7.21, 5.15 and 4.6, respectively; results not shown). The sanitiser concentrations measured the same after 1 min, but did not persist in solution after 60 min (results not shown).

Analysis of variance for the TBZ, PYR and 2,4-D solution concentration levels measured indicated a significant treatment × sanitiser concentration interaction for TBZ, PYR and 2,4-D ($P = 0.0012$, $P < 0.0001$ and $P = 0.0165$, respectively) and a significant concentration × time interaction for PYR ($P = 0.0003$). Since these significant interactions are largely due to anomalously low concentration at 0.0% HPPA and at 60 µg.mL^{-1} Cl, main effects were discussed further. Analysis of variance for the TBZ, PYR and 2,4-D concentration levels measured showed sanitiser concentration and treatment significant as main effects for TBZ ($P < 0.0001$ and $P < 0.0001$, respectively), PYR ($P < 0.0001$ and $P < 0.0001$, respectively) and 2,4-D ($P = 0.0005$ and $P < 0.0001$, respectively). Exposure time was not significant for TBZ, PYR and 2,4-D ($P = 0.593$, 0.944 and 0.357, respectively). A concentration of 80 µg.mL^{-1} Cl and 0.3% for HPPA resulted in significantly higher TBZ, PYR and 2,4-D concentration (792.5, 667.5 and 270.0 µg.mL^{-1}, respectively) compared to the other concentrations (661.7 – 641.7, 523.3 – 566.7 and 205.0 – 225.0 µg.mL^{-1}, respectively), whereas 0 µg.mL^{-1} sanitiser resulted in the lowest TBZ and PYR concentration levels (485.0 and 460.8 µg.mL^{-1}, respectively). Chlorine resulted in significantly higher TBZ, PYR and 2,4-D concentration levels (811.0, 648.7 and 266.0 µg.mL^{-1}, respectively) compared to HPPA treatments (486.0, 464.7 and 190.0 µg.mL^{-1}, respectively).

Analysis of variance for GC CFU.mL^{-1} data on PDA^{+} indicated a significant treatment × sanitiser concentration × exposure time interaction ($P = 0.0035$). After 1 min exposure time, the CFU.mL^{-1} count of 11222.2 – 12433.3 decreased over the concentration range to a count of 0.0 at 0.1% for HPPA, while the lowest count (1288.9 CFU.mL^{-1}) for Cl was at 80 µg.mL^{-1} (Figure 1). After 60 min exposure time, GC CFU.mL^{-1} decreased more rapidly over

the concentration range and a count of 0.0 was reached at 0.1% for HPPA and at 20 µg.mL^{-1} for Cl.

One and 3 min exposure time trial

Fungicide solution pH remained similar over the addition of different Cl concentrations (pH 10.45 – 10.89), and a reduction was seen with increasing HPPA concentrations from 0, 0.1 and 0.6% (pH 10.56, 7.39 and 5.18, respectively; results not shown). Concentrations of the sanitisers persisted after 1 and 3 min in solution.

Analysis of variance for TBZ, PYR and 2,4-D concentration levels measured presented no significant effects with exposure time significant as main effect for 2,4-D (P = 0.0568). Exposure time, sanitiser concentration and treatment was not significant for TBZ (P = 0.169, 0.433 and 0.402, respectively) and PYR (P = 0.115, 0.372 and 0.279, respectively), with sanitiser concentration and treatment not significant for 2,4-D (P = 0.236 and 0.137, respectively). 2,4-D concentrations levels were higher after 3 min solution agitation compared to 1 min (249.5 and 225.6 µg.mL^{-1}, respectively). Average concentration levels were 1046.8, 1025.0 and 237.5 µg.mL^{-1}, respectively.

Analysis of variance for GC CFU.mL^{-1} data indicated a significant treatment × sanitiser concentration × exposure time interaction (P = 0.0002). After 1 min exposure time, the GC CFU.mL^{-1} count of 9133.3 – 10033.3 decreased over the concentration range to a count of 0.0 at 0.1% for HPPA, while the lowest count (4844.4 CFU.mL^{-1}) for Cl was at 80 µg.mL^{-1} (Figure 2). After 3 min exposure time, GC CFU.mL^{-1} decreased more rapidly over the concentration range and a count of 0.0 and 22.2 was reached at 0.1% HPPA and 80 µg.mL^{-1} Cl, respectively.

In vivo sanitiser trials

Concentration levels

Municipal water pH ranged from 7.3 – 7.96 (results not shown) and which increased to 9.94 – 10.33 with the addition of the fungicide mixture. The solution pH remained similar with the addition of Cl (pH 10.1 – 10.28), but was reduced with the addition of HPPA (pH 4.89 – 5.05).

Analysis of variance for the TBZ, PYR and 2,4-D concentration levels measured showed that treatment was significant for TBZ (P = 0.0001). Kaolin concentration was not significant for TBZ (P = 0.227). Kaolin and treatment was not significant for PYR (P = 0.571 and 0.288, respectively) and 2,4-D (P = 0.848 and 0.461, respectively), with concentration levels not declining significantly in the presence of sanitiser or kaolin treatments relative to the control treatment and average concentration levels of 870.2 and 214.3 µg.mL^{-1}, respectively, were measured. Thiabendazole concentration levels were significantly higher during HPPA

treatment (806.9 µg.mL^{-1}) compared to the control (585.0 µg.mL^{-1}) and the Cl treatment (461.2 µg.mL^{-1}), which was significantly lower than the control.

Residue levels

Analysis of variance for TBZ, PYR and 2,4-D residue levels measured presented no significant effects, with sanitiser and kaolin concentration not influencing fungicide residue loading. Average TBZ, PYR and 2,4-D residue levels of 1.94, 1.88 and 0.40 µg.mL^{-1}, respectively, were loaded on Nadorcott mandarin fruit and 0.98, 1.35 and 0.29 µg.mL^{-1}, respectively, on Valencia orange fruit (results not shown).

Sour rot colony forming units

Analysis of variance for GC CFU.mL^{-1} data indicated that sanitiser treatment was significant as main effect ($P < 0.0001$). HPPA and Cl reduced sour rot inoculum in solution from 5327.8 CFU.mL^{-1} counted in the control treatment to 0.0 and 155.6 CFU.mL^{-1}, respectively.

Curative green mould control

Late Valencia orange fruit

Very high infection levels (± 96.9%) were observed on untreated control fruit (results not shown) and curative control levels were generally very high (mean of 91.3% control). Analysis of variance for percentage curative control data showed a meaningful effect for sanitiser treatment ($P = 0.1005$), and no effect for kaolin treatment ($P = 0.364$). The addition of a sanitiser (HPPA or Cl) resulted in improved green mould control compared to the control treatment (92.7, 92.2 and 90.2%, respectively).

Nadorcott mandarin fruit

Very high infection levels (± 92.5%) were observed on untreated control fruit (results not shown) and curative control levels were generally high (mean of 70.5% control). Analysis of variance for percentage curative control data indicated a significant sanitiser × kaolin concentration interaction ($P = 0.0224$). HPPA treatments improved the fungicides' ability to cure 24 h old infections (> 83.5%; Table 2), differing significantly from most of the Cl treatments (73.7 – 81.5%) and the control treatments (70.6 – 79.3%). Kaolin (500 µg.mL^{-1}) appeared to improve green mould control in the non-sanitiser control treatments. This beneficial effect was not obvious for the sanitiser treatments, nor was any detrimental effect observed.

Sour rot control

Late Valencia orange fruit

Analysis of variance for percentage curative control data indicated a significant sanitiser treatment × kaolin concentration interaction ($P < 0.0001$). The fungicides alone resulted in 10.9 – 59.0% (Table 3) sour rot control (69.0% infection levels on untreated control fruit), which was improved significantly with the addition of sanitisers (80.1 – 100.0%). HPPA treatments (98.6 – 100.0%) provided significantly better control compared to Cl treatments (80.1 – 85.8%) except for at 500 µg.mL^{-1} koalin (94.3%, respectively). A significant improvement in disease control was seen between 0 and 500 µg.mL^{-1} koalin for the control and Cl sanitiser treatments (48.1 and 14.2% improvement, respectively).

Nadorcott mandarin fruit

Analysis of variance for percentage curative control data indicated a significant treatment × kaolin concentration interaction ($P < 0.0001$). The fungicides alone resulted in 15.7 – 55.6% (Table 4) sour rot control (83.5% infection levels on untreated control fruit), which was improved significantly with the addition of sanitisers (82.7 – 99.0%). HPPA treatments (95.2 – 99.0%) provided significantly improved control compared to Cl treatments (82.7 – 89.4%). A significant improvement in disease control was seen between 0 and 500 µg.mL^{-1} koalin for the control treatment (39.9% improvement).

DISCUSSION

This study aimed to compare the ability of two different sanitisers (Cl and HPPA) to reduce sour rot inoculum in solution while maintaining fungicide concentration and residue levels for effective green mould control. Incompatibility between sanitisers and fungicides was not observed in this study, with both sanitisers providing effective sour rot control in the presence of different concentrations of koalin clay. Although both Cl and HPPA reduced sour rot inoculum and infection, HPPA is effective at a short exposure time (1 – 3 min) at the high pH used in this study (> 10), which indicates it can be incorporated with commercial drenching when pH is not regulated.

Commercial drench treatments are focussed on preventing fungal pathogens from reaching a point of infection where they can no longer be controlled (Brown and Miller, 1999) before fruit reach the favourable environment of degreening chambers (Plaza *et al*., 2003; Krajewski and Pittaway, 2010). In the commercial drench trials in this study, a re-circulating fungicide solution was applied over 150 fruit bins directly from the orchard, and sanitiser shock treatments were applied to reduce inoculum that may accumulate with soil (Brown and Miller, 1999). Solution concentration and/or residue levels were measured over the various trials to assess persistence in light of incompatibility concerns between sanitisers and

fungicides (Taverner, 2014). The addition of sanitisers throughout these trials did not appear to reduce fungicide persistence in the drench solution, even at relatively high Cl and HPPA concentrations (80 µg.mL^{-1} and 0.3%, respectively) used in the Commercial packhouse trials. Kanetis *et al*. (2008b) found that 100 µg.mL^{-1} PYR was reduced to 60 and 45% after 30 min and 8 h exposure to 100 µg.mL^{-1} Cl, respectively. Initial pH of the aqueous fungicide solution was 6.5 - 7. The pH of the solution during the *in vitro* and *in vivo* trials was similar (pH 9.94 – 11.08) regardless of whether Cl was added or not. Our results most probably differ from Kanetis *et al*. (2008b) as Cl is less effective at higher pH levels (Hewett, 2014).

A difference in PYR residue loading was also seen between the top and bottom bin levels during commercial packhouse trials with the upper level loading higher residue levels compared to the bottom level. This could be due to poor solution coverage associated with drenching (Brown and Miller, 1999), especially when stacking fruit bins, and due to the bin perforation not being optimally designed for drench application (Pers. comm. A. Erasmus). Thiabendazole residue loading and solution concentration increased with drench age during the commercial packhouse trials, which was likely a result of insufficient solution agitation in the initial phase of the drenching system. It is known (Ritenour *et al*., 2003) that TBZ precipitates from solution when not effectively agitated.

Although fungicide concentration and residue levels were mostly unaffected in this study over the various trials, Cl and HPPA did not persist in solution after 60 min exposure during *in vitro* exposure time trials, although both were still present after 3 min. Sanitisers also persisted in solution after 3 min exposure during the *in vivo* trials in the presence of various kaolin concentrations (results not shown). Smilanick *et al*. (2006a) found that 200 µg.mL^{-1} Cl reduced to 10 µg.mL^{-1} after 3 hours exposure to 500 µg.mL^{-1} PYR. This information supports the use of regular sanitiser shock treatments of drench mixtures. Sanitisers did not persist after 60 min and should therefore be added at least every hour although these trials have not conclusively demonstrated at what intervals these shock treatments should be administrated in terms of efficacy, therefore more work is required.

In the commercial drench trial, total CFU.mL^{-1} levels were mostly between 0.0 to 8.3 CFU.mL^{-1}in freshly prepared mixtures (sampled at fruit bin 1 in this study). In one trial, the high initial total CFU.mL^{-1} levels (3645.0 CFU.mL^{-1}) could be a result of high inoculum pressure in a certain orchard or remnants in the drench reservoir that was not cleaned properly. The total CFU.mL^{-1} level range was generally the highest at bin 150, which was reduced to 0.0 total CFU.mL^{-1} with Cl application

Geotrichum citri-aurantii is able to survive in soil and debris, so it stands to reason that inoculum can build up in dip tanks or drenchers with the accumulation of dirt (Brown, 1979), especially in the absence of GZT given its restrictions in various export markets (Lesar, 2006; Cunningham and Taverner, 2006). This study proved that sanitisers were able to

reduce sour rot inoculum in solution before infection can occur. *In vitro* trials showed that a low HPPA concentration of 0.1% was sufficient to completely eliminate sour rot spores at all exposure times, whereas 3 min exposure using the highest Cl concentration (80 µg.mL^{-1}) still could not eradicate sour rot spores completely at these high pH levels (> 10). Brown and Miller (1999) reported that a time interval of at least 2 min is required for maximum efficacy of Cl against fungal propagules. In our study, relatively poor Cl efficacy could be ascribed to the solution pH of ± 10.4, which is markedly higher than the optimal pH of 6.8 – 7.2 for Cl (Hewett, 2014). In packhouses, pH would be difficult to manage during drench application due to the volumes of fruit drenched and accumulation of dirt. Pyrimethanil and TBZ are regarded as good drenching fungicides as pH adjustment is not required (McCornack, 1970; Smilanick *et al*., 2006a). The addition of these fungicides to a sour rot containing solution during *in vivo* trials did not reduce sour rot inoculum levels compared to the unamended control solution (5327.8 CFU.mL^{-1}), whereas spores were reduced in the presence of Cl (155.6 CFU.mL^{-1}) and eradicated with HPPA (0.0 CFU.mL^{-1}), which supports results seen in the *in vitro* trials. It is expected that Cl would have provided improved results at optimally adjusted pH levels, whilst the pH-insensitive HPPA provided excellent results.

As on untreated control in the commercial drench trials, fruit were only wounded and left exposed in the drench area. High green mould infection levels on these fruit were indicative of high inoculum load surrounding the drench area and emphasize the importance of timely fungicide application (Chapter 2) as risk of infection will increase if treatment of wounded fruit is delayed. Sanitation of packhouse environments is a crucial control strategy, as *Penicillium* spp. can rapidly produce billions of spores after 7 days at 25°C, which are highly dispersible via air currents, contaminating packhouses and orchards (Gardner *et al*., 1986; Holmes and Eckert, 1995; Smilanick and Mansour 2007). It may also not be ideal to have drench application in the vicinity of the degreening rooms where higher levels of decay is often observed, which explains the high levels of green mould inoculum in this study.

Fungicide application in the commercial drench trials reduced decay by > 67.7% regardless of whether sanitisers were present or not. In the *in vivo* trials, green mould control on Valencia orange and Nadorcott mandarin fruit was also unaffected by the addition of sanitising agents, although HPPA improved the ability of fungicides to cure 24-h-old green mould infections.

The addition of Cl in the commercial drench trials did not improve green mould control levels, which might be due to high solution pH or, alternatively, to the presence of organic matter in the drench mixture. Barkai-Golan (2001) reported that Cl is too unstable in the presence of organic matter and therefore is not effective in killing microorganisms embedded within injured tissue, and merely reduces inoculum present in solution that may infect vulnerable wounded fruit. Kanetis *et al*. (2008b) found that exposing 100 µg.mL^{-1} Cl to 250

µg.mL^{-1} PYR for 0 and 8 h reduced the efficacy of this fungicide during a 30 s dip treatment, resulting in increased green mould decay incidence on lemons inoculated 14 – 16 h before treatment from 5.5 – 10% to 49.5 and 72.4%, respectively. In contrast, this study found that green mould control was unaffected by the presence of Cl, which may be due to the higher PYR concentrations (1000 µg.mL^{-1}) used, combining PYR with TBZ or the high pH levels in the drench mixtures. In the *in vivo* trials, fungicides provided effective green mould control (± 91.3%) on 24-h-old infections on Valencia orange fruit, which was comparable to similar trials by Smilanick *et al*. (2006a). On Nadorcott mandarin fruit, however, control levels following the fungicides-only treatment and fungicides with Cl treatment were lower with 70.6 – 81.5% green mould control on 24 h old infections, which was improved to > 83.5% with the addition of HPPA. Effective green mould control is associated with effective residue loading (Smilanick *et al.,* 2006b; Erasmus *et al*., 2011; Njombolwana *et al*., 2013; Kellerman *et al.,* 2014), although application method (Erasmus *et al*., 2011) and infection age also plays an important role in fruit susceptibility to disease (Chapter 2). In this study TBZ and PYR residue levels and PYR concentration levels averaged well above the recommended levels for effective green mould control.

In the absence of sour rot specific fungicides in the drench mixture control ranged from 10.9 – 59.0%. Shock treatments with sanitiser improved sour rot control on Valencia orange and Nadorcott mandarin fruit through a reduction of sour rot inoculum levels in drench mixtures (80.1 – 100.0%) depending on fruit type. HPPA was superior to Cl at shorter exposure times in *in vitro* trials, but a more optimal pH might have resulted in improved Cl efficacy.

The addition of kaolin during the *in vivo* trials was aimed at simulating dirt accumulation during drenching (Brown and Miller, 1999). Unexpectedly, green mould and sour rot control was mostly improved during control treatments in the presence of 500 and 1000 µg.mL^{-1} kaolin concentrations (up to 48.1 and 15.5%, respectively, depending on fruit type), with the 500 µg.mL^{-1} kaolin treatment leading to significantly better control than the 1000 µg.mL^{-1} kaolin treatment. Surround® WP is derived from kaolin clay and creates a physical barrier on fruit (Engelhard Surround WP Crop Protectant Product Label, Engelhard Corporation, 101 Wood Avenue, P.O. Box 770, Iselin, NJ 08830-0770 USA). These results show that Cl still effectively controlled sour rot, despite the presence of high clay content in the mixture. Dirt and debris reduce Cl activity (Hewett, 2014). From our study it appears that the organic matter content might be more detrimental to Cl activity than the clay (dirt) matter. HPPA was not affected by clay, and was also reported to be insensitive to the presence of organic matter (Taverner, 2004; Hewett, 2014).

Kanetis *et al.* (2008b) found that green mould germination was completely inhibited after exposure to 50 µg.mL^{-1} Cl and 2700 µg.mL^{-1} HPPA at pH 7, but that inhibition levels reduced

at pH 8. Brown *et al*. (1988) also highlighted the recommendation of Cl treatment at a pH level of 6.5 – 7.5 to prevent the accumulation of green mould and sour rot propagules. In our trials, pH levels of the unamended solution during these trials was 7.3 – 7.39, which increased to above 10 with the addition of fungicides and Cl. Solution pH was likely increased from 7 – 8 to > 10 due to the addition of the specific 2,4-D formulation used in this study, although this is not the case with other formulations (unpublished data). Exposure at these high pH levels is most probably why longer Cl exposures times were required to reduce sour rot inoculum and why this sanitiser was not as effective as HPPA in our study. Smilanick *et al.* (2002) demonstrated the significant effect of pH on Cl efficacy and sour rot spores had to be exposed to 200 $\mu g.mL^{-1}$ Cl for 114 s at pH 10 to obtain a similar level of control as a 3 s exposure at pH 7. HPPA should therefore be preferred to Cl in cases where the pH of the mixture is not adjusted to 7.

HPPA treatment at 0.3% for 3 min eliminated sour rot inoculum in solution and prohibited infection of wounded citrus fruit, which was superior to 80 $\mu g.mL^{-1}$ Cl at the high pH levels. Fungicide residue and concentration levels were comparable between sanitiser and non-sanitiser treatments as well as subsequent green mould control, although HPPA treatments in the *in vivo* trials resulted in superior green mould control. HPPA can cause some discomfort due to its strong astringent odour and can result in fruit burning if concentrations are not properly regulated (Taverner, 2004). Several HPPA formulations exist and should be tested before a specific recommendation can be made concerning application. Regular spiking of the drench solution with sanitisers is necessary since both HPPA and Cl did not persist in solution, and continuous sanitation is essential in a commercial packhouse due to regular contamination of the mixture through drenching of dirty field-bins and fruit. Proper solution agitation is also essential for improved fungicide uniformity in solution and subsequent residue loading.

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Table 1. Total Colony forming units (CFU.mL^{-1}) determined on PDA^{+} and PDA^{IMZ} at fruit bin no. 50, 100 and 150 determined during commercial packhouse trials combining a solution containing thiabendazole (1000 µg.mL^{-1}), pyrimethanil (1000 µg.mL^{-1}), 2,4-dichlorophenoxyacetic acid (250 µg.mL^{-1}) and guazatine (500 µg.mL^{-1}) to the drench reservoir and drenching on a variety of navel orange fruit cultivars at 1066 L.min^{-1} for ± 30 s with the addition of either chlorine (Cl; 80 µg.mL^{-1}) or hydrogen peroxide/peracetic acid (HPPA; 0.6%) every 50 bins.

		[a]PDA^{+}		[b]PDA^{IMZ}	
Bin	**Treatment**	**Cl**	**HPPA**	**Cl**	**HPPA**
1	**Control**	0.0 b	0.0 b	5.0 b	8.3 b
	Treatment	8.3 b	1.7 b	5.0 b	3645.0 a
50	**Control**	23.3 b	1.7 b	55.0 b	75.0 b
	Treatment	0.0 b	0.0 b	0.0 b	145.0 b
100	**Control**	130.0 b	0.0 b	103.3 b	98.3 b
	Treatment	0.0 b	0.0 b	0.0 b	68.3 b
150	**Control**	1703.3 a	28.3 b	5431.7 a	973.3 b
	Treatment	0.0 b	1.7 b	0.0 b	328.3 b

[a]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 353.082)

[b]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 2056.0)

Table 2. Percentage green mould control on Nadorcott mandarin fruit inoculated 24 h before drenching in a solution amended with thiabendazole (1000 µg.mL^{-1}), pyrimethanil (1000 µg.mL^{-1}), 2,4-dichlorophenoxyacetic acid (250 µg.mL^{-1}) and *Geotrichum citri-aurantii* conidia (3.175 × 10^4 spores.mL^{-1}) and treated with either with hydrogen peroxide/peracetic acid (HPPA; 0 or 0.30%) or Chlorine (Cl; 0 or 80 µg.mL^{-1}) with accumulating levels of kaolin clay (0, 500 and 1000 g.mL^{-1}).

	Green mould control (%)[a]		
Kaolin (g.mL^{-1})	**Fungicide solution plus no sanitiser**	**Fungicide solution plus HPPA**	**Fungicide solution plus Cl**
0	70.6 d	86.2 ab	74.7 cd
500	73.6 d	89.9 a	81.5 bc
1000	79.3 bc	83.5 ab	73.7 cd

[a]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 6.945)

Table 3. Percentage sour rot control on Valencia orange fruit wounded within 30 min of drenching in a solution amended with thiabendazole (1000 µg.mL^{-1}), pyrimethanil (1000 µg.mL^{-1}), 2,4-dichlorophenoxyacetic acid (250 µg.mL^{-1}) and *Geotrichum citri-aurantii* conidia (3.175 × 10^4 spores.mL^{-1}) treated with either hydrogen peroxide/peracetic acid (HPPA; 0.30%) or Chlorine (Cl; 80 µg.mL^{-1}) with accumulating levels of kaolin clay (0, 500 and 1000 g.mL^{-1}).

	Sour rot control (%)[a]		
Kaolin (g.mL^{-1})	**Fungicide solution plus no sanitiser**	**Fungicide solution plus HPPA**	**Fungicide solution plus Cl**
0	10.9 f	100.0 a	80.1 c
500	59.0 d	98.6 a	94.3 ab
1000	26.4 e	99.6 a	85.8 bc

[a]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 7.44)

Table 4. Percentage sour rot control on Nadorcott mandarin fruit wounded within 30 min of drenching in a solution amended with thiabendazole (TBZ; 1000 µg.mL^{-1}), pyrimethanil (PYR; 1000 µg.mL^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D; 250 µg.mL^{-1}) and *Geotrichum citri-aurantii* spore conidia (3.175 × 10^4 spores.mL^{-1}) with treated with either hydrogen peroxide/peracetic acid (HPPA; 0 or 0.30%) or Chlorine (0 or 80 µg.mL^{-1}) with accumulating levels of kaolin clay (0, 500 and 1000 g.mL^{-1}).

	Sour rot control (%)[a]		
Kaolin (g.mL^{-1})	**Fungicide solution plus no sanitiser**	**Fungicide solution plus HPPA**	**Fungicide solution plus Cl**
0	15.7 e	95.2 ab	82.7 c
500	55.6 d	99.0 a	89.4 bc
1000	19.0 e	96.3 ab	86.4 c

[a]Means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 6.731)

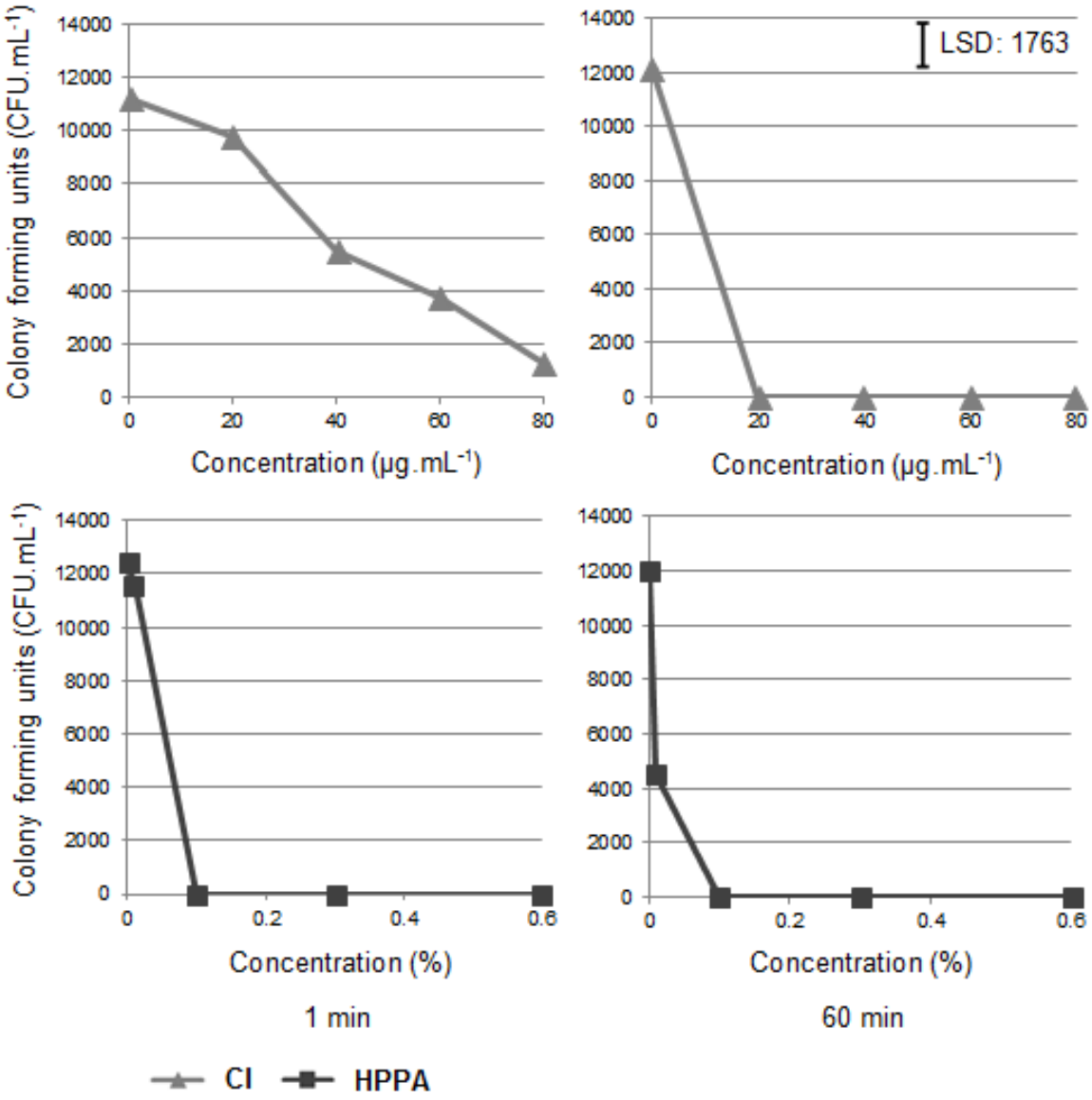

Figure 1. Colony forming units (CFU.mL^{-1}) determined on PDA^{+} during *in vitro* trials following treatment of *Geotrichum citri-aurantii* spore suspension (3.175 × 10^{4} spores.mL^{-1}) with a mixture of thiabendazole (1000 µg.mL^{-1}), pyrimethanil (1000 µg.mL^{-1}), 2,4-dichlorophenoxyacetic acid (250 µg.mL^{-1}) containing either 0.0, 0.01, 0.10, 0.30 and 0.60% hydrogen peroxide/peracetic acid (HPPA) (bottom graphs) or 0, 20, 40, 60 or 80 µg.mL^{-1} chlorine (Cl) (top graphs) for 1 and 60 min.

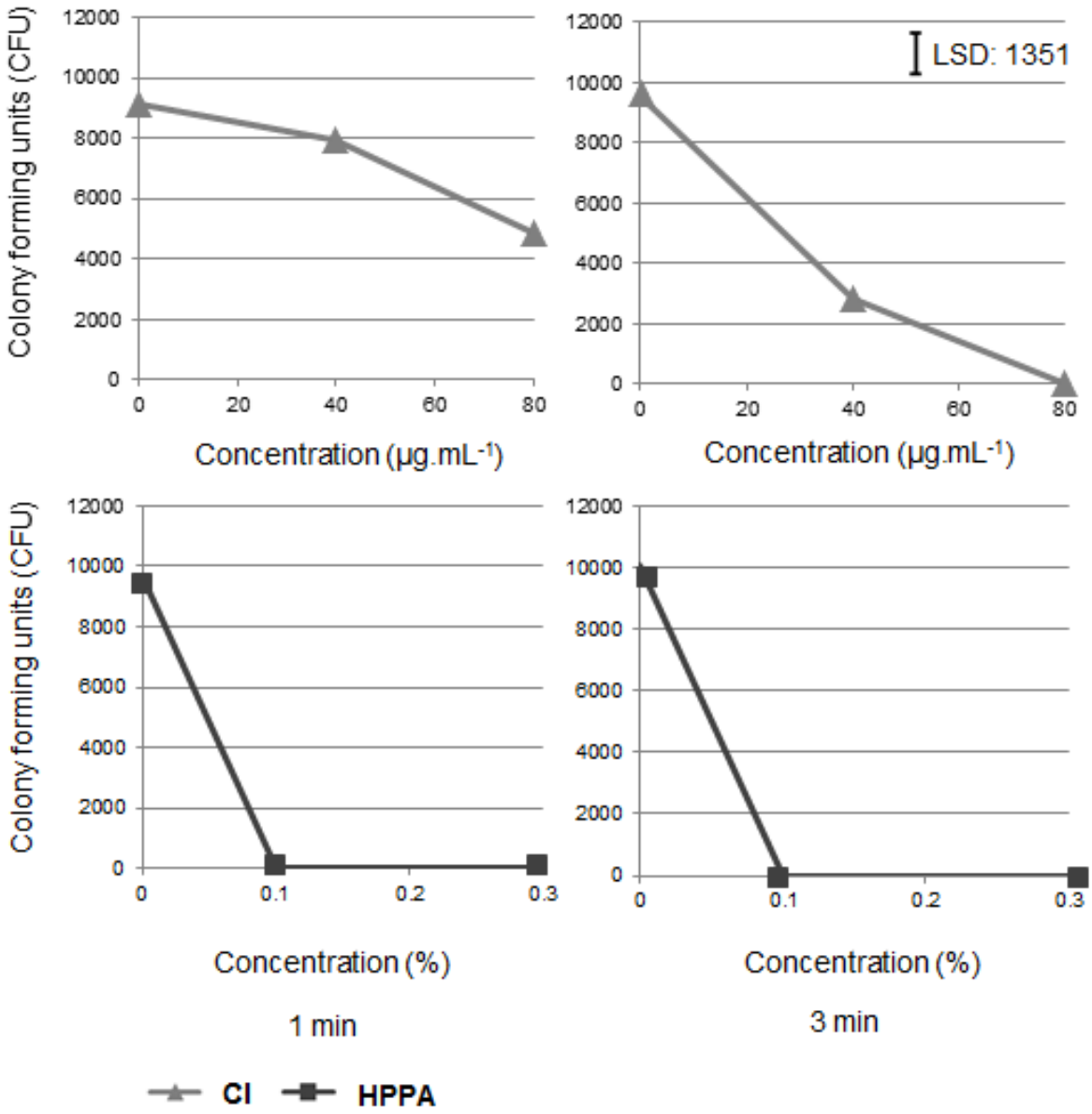

Figure 2. Colony forming units (CFU.mL^{-1}) determined on PDA^{+} during *in vitro* trials following treatment of *Geotrichum citri-aurantii* spore suspension (3.175 × 10^{4} spores.mL^{-1}) with a mixture of thiabendazole (1000 µg.mL^{-1}), pyrimethanil (1000 µg.mL^{-1}), 2,4-dichlorophenoxyacetic acid (250 µg.mL^{-1}) containing either 0.0, 0.01, 0.10, 0.30 and 0.60% hydrogen peroxide/peracetic acid (HPPA) (bottom graphs) or 0, 20, 40, 60 or 80 µg.mL^{-1} chlorine (Cl) (top graphs) for 1 and 3 min.