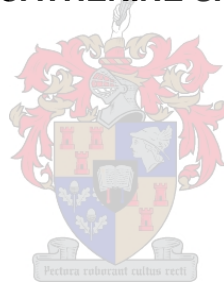


**FURTHER OPTIMISATION OF IN-LINE AQUEOUS APPLICATION OF
IMAZALIL TO CONTROL CITRUS GREEN MOULD CAUSED BY *PENICILLIUM
DIGITATUM***

by

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Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science in the Faculty of AgriSciences at the University of Stellenbosch

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March 2017

DECLARATION

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SUMMARY

South Africa has a successful citrus export industry. A threat to fresh citrus fruit is the fungal pathogen *Penicillium digitatum* causing green mould. Imazalil (IMZ) is the most important fungicide to combat green mould. Solution pH and temperature, and exposure time of the fruit to the solution, are important when using the sulphate form of IMZ. Research has increased our understanding of IMZ use, but further variables need to be investigated, along with an alternative application method.

The control of green mould infection and sporulation by IMZ were tested using a heated floodler. Solution variables included the effects of pH (3; 4; 5; 6), temperature (45; 55; 65°C), and concentration (250 or 500 µg.mL⁻¹) in a time of 8 s. Residues increased with increasing pH, temperature and concentration. The majority average residues loaded were between 0.4 and 3.0 µg.g⁻¹. Treatments at pH 6 loaded higher residues at 55 and 65°C, where the maximum residue limit (MRL) of 5 µg.g⁻¹ was almost always exceeded. The floodler loaded adequate residues, offering good curative and protective control. Sporulation inhibition of green mould was also linked to residues, and complete inhibition was achieved at the higher residue levels. The floodler was an effective applicator of IMZ.

The fungicide bath is the most common IMZ application method in South Africa. The ability of IMZ to control green mould was investigated in a cold bath of 10°C and compared to ambient temperature and 35°C baths. Solution temperature had no significant effect on IMZ's ability to cure 24 hr old green mould infections with all temperatures providing control above 80%. Sporulation inhibition and residue loading increased as solution pH, temperature, and exposure time increased. Sporulation inhibition was < 50% in pH 3 baths, irrespective of temperature, complete inhibition was obtained at 35°C and pH 6, but the IMZ MRL was exceeded at longer exposure times (> 45 s).

The survival of *Rhizopus stolonifer* was studied *in vitro* at various water temperatures (10°C to 65°C) for exposure times of 1 or 60 min, and after a pasteurisation step. Sub-treatments included the addition of IMZ fungicide or green mould spores, with IMZ seemingly having a significant effect on *Rhizopus* spore survival. The same was not true for solutions at temperatures below 35°C, however, temperatures of 45, 55 and 65°C, particularly after a 60 min exposure, caused a significant reduction in *Rhizopus* spore viability. Complete *Rhizopus* eradication was achieved with 65°C and the pasteurisation step. In order to control fungal contaminants in the fungicide bath, packhouses need to apply IMZ in heated solutions (*circa* 45°C) and/or pasteurize fungicide baths overnight.

Imazalil residue levels on citrus can be increased by increasing solution pH, temperature, concentration or exposure time. Most treatments gave excellent infection

control and only a low residue is necessary to cure or prevent a green mould infection. Residue levels were closely linked to the level of sporulation inhibition achieved. Both the flooder and dip tank offered good green mould control. Contaminants that build up in solution can be eradicated at high temperatures.

OPSOMMING

Suid Afrika het 'n suksesvolle sitrusbedryf. *Penicillium digitatum*, die swampatogeen wat groenskimmel veroorsaak, is 'n bedreiging vir vars sitrusvrugte. Imazalil (IMZ) is die belangrikste swamdoder in die bekamping van hierdie patogeen. Die pH en temperatuur van die oplossing, asook blootstellingstyd van die vrugte aan die oplossing is belangrik wanneer die sulfaat vorm van IMZ gebruik word. Navorsing het ons kennis van IMZ verbreed, maar verdere ondersoek van toepaslike veranderlikes is nodig, asook 'n alternatiewe aanwendingsmetode.

Die beheer van groenskimmelinfeksies en sporulasie deur IMZ na aanwending met 'n verhitte vloedtoediener is ondersoek. Verskillende oplossingsveranderlikes het ingesluit pH (3; 4; 5; 6), temperatuur (45; 55; 65°C) en konsentrasie (250 of 500 $\mu\text{g}\cdot\text{mL}^{-1}$), na 'n blootstellingstyd van 8 s. Residue het toegeneem met toenemende pH, temperatuur en konsentrasie. Die meeste residuwaardes was tussen 0.4 en 3.0 $\mu\text{g}\cdot\text{g}^{-1}$. Behandlings by pH 6 het hoër residue by 55 en 65°C gelaai, met die maksimum residulimiet (MRL) van 5 $\mu\text{g}\cdot\text{g}^{-1}$ omtrent deurgaans oorskry. Residulading deur die vloedtoediener was genoegsaam en het goeie genesende, sowel as beskermende beskerming verleen. Sporulasie inhibisie van groenskimmel was ook gekoppel aan residulading, met volledige inhibisie teen hoër residuladings. Die vloedtoediener gee effektiewe toediening van IMZ.

Die swamdoderbad is die mees algemene IMZ toedieningsmetode in Suid Afrika. Die vermoë van IMZ om groenskimmel te beheer in 'n koue bad teen 10°C is ondersoek en vergelyk met baddens teen omgewingstemperatuur en 35°C. Die oplossingstemperatuur het geen noemenswaardige effek gehad op die vermoë van IMZ om 24 uur-oue groenskimmel infeksies te beheer nie, met alle temperature wat tot meer as 80% beheer gelei het. Sporulasie inhibisie en residulading het toegeneem met toenemende pH en temperatuur van die oplossing, asook blootstellingstyd. Sporulasie inhibisie in pH 3 baddens was < 50%, ongeag die temperatuur, met volledig inhibisie behaal teen 35°C en pH 6, alhoewel die IMZ MRL oorskry is teen langer blootstellingstye (> 45 s).

Die *in vitro* oorlewing van *Rhizopus stolonifer* is bestudeer teen verskeie watertemperature (10°C to 65°C) en vir blootstellingstye van 1 of 60 minute, asook nà 'n pasteurisasie stap. Tussenbehandelings het die byvoeging van of IMZ of groenskimmelspore ingesluit, met IMZ wat oënskynlik 'n noemenswaardige effek het op spooroorlewing gehad het. Dit het nie gegeld vir oplossings onder 35°C nie, maar temperature van 45, 55 en 65°C met veral 60 min blootstelling het 'n noemenswaardige verlaging in *Rhizopus* spooroorlewing tot gevolg gehad het. Volledige uitwissing van *Rhizopus* is behaal met 65°C en die pasteurisasie stap. Ten einde swamkontaminasie in

die swamdoderbad te beheer, behoort pakhuis IMZ in verhitte oplossings (*circa* 45°C) aan te wend en/of moet hulle oornag pasteuriseer.

Imazalil residuvlakke op sitrus kan verhoog word met verhoging van die oplossing se pH, temperatuur, konsentrasie of verlenging van blootstellingstyd. Die meeste behandelings gee uitstekende infeksiebeheer en slegs 'n lae residuwaarde is voldoende om 'n groenskimmelinfeksie te genees of voorkom. Sporulasie inhibisie, daarteenoor, is nòù gekoppel aan die residuvlakke. Beide die verhitte vloedtoediener en die swamdoderbad het goeie groenskimmelbeheer gegee. Die opbou van kontaminante in die oplossing kan uitgewis word by hoë temperature.

ACKNOWLEDGEMENTS

Thank you to everyone who helped and encouraged me along the way of this thesis.

To **Arno**, who managed to convince me with sheer passion and enthusiasm to spend my time playing with rotten fruit. Thank you for the doors you opened for me and the friendship gained along the way.

Thank you to all my supervisors, **Prof. Paul Fourie**, **Dr. Cheryl Lennox**, **Dr. Wilma du Plooy** and **Dr. Arno Erasmus**, for making your years of experience and knowledge available to me.

To **CRI** for making this project a possibility.

Thank you to **Sue Peall** and **Hearshaw and Kinnes** for all the invaluable residue analysis work, **ICA International Chemicals** for providing chemicals free of charge and **JBT South Africa** for the experimental flooder.

Special thanks to **Piet Englebrecht Trust** for the use of their commercial flooder and to **Dr. John Mildenhall** for his pioneering work on pasteurisation of the fungicide bath.

Thank you to **Citrus Academy** for the financial support, the industry exposure but more importantly, for the inclusion in the Citrus Academy family. And thank you to the students who, for their holiday work, helped out with some of the trials.

To the **CRI staff and friends** who physically helped with my projects (**Themba**, **Thabang**, and **Lindokhule**) and particularly to **Charmaine O.**, who started this whole journey with me, and **Wouter Jnr.**, who ended it with me. And to practically everyone else at CRI Nelspruit for all the friendship, encouragement, coffee, chocolate, flowers and food.

And finally, thank you to my **family**, who are always there for me.

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CHAPTER 1

A review of in-line aqueous applications of the fungicide imazalil to control green mould (*Penicillium digitatum*) in South African citrus packhouses

CITRUS IN SOUTH AFRICA

The origin of citrus is not completely certain, although many believe it to be China and India (Sippel, 2006). The first recorded introduction of citrus into South Africa coincided with the arrival of the Dutch East India Company and the establishment of the Dutch in the Cape. Jan van Riebeeck planted orange and lemon trees in his personal garden in June 1654. In the following decades, citrus tree imports increased dramatically - such that in a mere 250 years, citrus fruit was being grown commercially and exported in exponentially increasing volumes (Davis, 1928; Sippel, 2006).

Of greatest commercial interest are four groups of citrus, namely oranges (*Citrus sinensis*), various species of soft citrus (mandarins and Clementines (*Citrus reticulata*) and Satsumas (*Citrus unshi*)), grapefruit (*Citrus paradisi*), and lemons (*Citrus limon*). Other citrus types include citrons, pummelos, limes and bitter Sevilles (Saunt, 1990). The four main groups of citrus are economically important crops in South Africa, with the citrus market contributing approximately R8.3 billion to the gross value of South African agriculture (DAFF, 2014). Citrus is grown across the country in nearly every province. Along with producers in Swaziland and Zimbabwe, all citrus production is organised under the Citrus Growers' Association of Southern Africa (CGA, 2015). By the early 1900's, citrus production in South Africa had grown so much that most of the commodity was being exported. The industry has grown from 3000 boxes being exported to Britain in 1907 to 72 million cartons exported worldwide a century later, in 2006 (CGA, 2007). In 2015, 67% of the total production (115 million cartons) was exported; 27% was processed and only 6% went to the local market (Edmonds, 2016; PPECB, 2016). This represents a decline from the 2012 export figures of 74% exported and 18% processed, but an increase from 63% exported and 31% processed in 2014 (Edmonds, 2013, 2015). The change in numbers may be due to the market access restrictions pertaining to citrus black spot (*Phyllosticta citricarpa* (McAlp.) van der Aa). This pathogen has hindered some of South Africa's citrus export by the enforcement of trade restrictions which South Africa is opposing as demonstrated by the 2015 figures (Kotze, 1981; Edmonds, 2013, 2015, 2016). The main export markets for South African citrus are the following countries or areas: Northern Europe (22%), Middle East (21%), Asia (11%), United Kingdom (10%), Far East (9%), Russia

(9%), Southern Europe (7%), United States (4%), Canada (3%), and others (4%) (Edmonds, 2016). South Africa is currently the second largest exporter of fresh citrus fruit (after Spain) (Edmonds, 2016), however, in terms of volumes of citrus being shipped long distances and over an extended time period, South Africa is the largest exporter (Paul Fourie, personal communication, 2015).

Citrus is successfully produced in South Africa because many regions in the country have favourably warm climates, with frost-free winter months, which support the successful establishment of this subtropical crop (DAFF, 2009). South Africa's location in the Southern hemisphere means that citrus is often produced and supplied to markets in the Northern hemisphere who have opposite seasonality. This, in turn, means that the markets are geographically very far from the production areas and citrus spend weeks travelling to get to them (Hough, 1969; Pelser, 1977). The fruit not only need to arrive in a satisfactory condition, but should remain so during further storage; either in supermarkets or in the customer's home before consumption (Roth, 1967; Eckert and Eaks, 1989). A number of postharvest conditions can befall a shipment of fruit, and there are varieties of ways to combat these problems.

POSTHARVEST DISEASES

Citrus fruit can be unacceptable to a market for reasons that can be either physiological or pathological in origin (Eckert and Eaks, 1989). However, the majority of the losses experienced are due to fungal attack, and in particular, green mould caused by *Penicillium digitatum* Pers. Sacc. (Christ, 1965; Pelser, 1973, 1977).

Citrus postharvest diseases can initiate both in the orchard and in the packhouse (Smoot and Melvin, 1961; Eckert and Brown, 1986; Eckert and Eaks, 1989). Diseases such as stem-end rots (caused by: *Diplodia natalensis*; *Phomopsis citri*; *Alternaria citri*), black rot (caused by: *Alternaria* spp.), brown rot (caused by: *Phytophthora* spp.), grey rot (caused by: *Botrytis* spp.), and anthracnose (caused by: *Colletotrichum gloeosporioides*) are all diseases that originate in the orchard, often infecting the flower or young fruit. Diseases such as green mould (caused by: *Penicillium digitatum*), blue mould (caused by: *P. italicum*), sour rot (caused by: *Galactomyces citri-aurantii*), and trichoderma rot (caused by: *Trichoderma* spp.) are all obligate wound pathogens which mean that they need wounds as a point of entry for infection to be successful (Christ, 1964). These wounds may be due to injuries that are inflicted by insects (Bates, 1936; Roth, 1967), but more often they are as a result of poor handling during harvest (Smoot and Melvin, 1961; Christ, 1966; Eckert, 1977; Pelser, 1977; Eckert and Brown, 1986; Eckert and Eaks, 1989).

Green mould - *Penicillium digitatum*

Etiology

There are hundreds of *Penicillium* spp. known (Pitt, 1979). Only two species of *Penicillium* are, however, important pathogens on citrus fruit, namely *P. digitatum* Sacc. and *P. italicum* Wehmer causing green and blue mould, respectively (Eckert and Eaks, 1989). These pathogens are found naturally as saprophytes on plant debris in the soil of orchards (Pitt, 1979). Of the two, *P. digitatum* is the more economically important pathogen as it causes the majority ($\approx 90\%$) of postharvest citrus losses in South Africa (Fawcett, 1927; Christ, 1964, 1965; Roth, 1967; Hough, 1970; Pelser, 1977; Eckert and Eaks, 1989).

There are three other *Penicillium* spp. reported on citrus namely, *P. ulaiense* Hsieh, Su & Tzean (causing whisker mould); *P. crustosum* Thom; and *P. expansum* Link (Holmes and Eckert, 1993; Youssef *et al.*, 2010; Louw and Korsten, 2015). Although *P. ulaiense* has been reported in the USA to be resistant to commonly used postharvest chemicals that effectively manage *P. italicum* and *P. digitatum*, it has not been reported so far for other parts of the world (Holmes *et al.*, 1994). *Penicillium crustosum* has been isolated from decayed citrus fruit (Jacobs and Korsten, 2010) but has not developed to be a problem in South Africa. All three lesser known *Penicillium* pathogens generally have slower growth and are less aggressive than *P. digitatum* and *P. italicum* (Louw and Korsten, 2015). None have been reported to be of any economic significance to the Southern African citrus industry, despite some sporadic incidences of *P. ulaiense* in the late 1990's (Keith Lesar, personal communication, 2016; Lesar, 2001).

Penicillium digitatum is unique in the presence of very large metulae (secondary branches) producing conidia, collectively making up the *penicillus* or 'brush', when compared to other *Penicillium* species (Pitt, 1979). This species grows at different rates on different substrates and at different temperatures; nonetheless the unifying characteristics of the fungus is the production of 6 – 8 μm long, usually elliptical, conidia (spores) that are yellow green to olive green, giving rise to its common name, green mould (Fawcett and Lee, 1926; Hess *et al.*, 1968). A further seemingly trivial characteristic of both *P. digitatum* and *P. italicum* is their predominant ability to rot citrus fruit (Fawcett and Lee, 1926; Pitt, 1979; Louw and Korsten, 2015). Green mould will grow at temperatures between 5 and 30°C, with optimal growth at around 23 - 25°C (Fawcett, 1927; Plaza *et al.*, 2003). Growth is inhibited at storage and transport temperatures of 3 – 5°C. However, once in a favourable environment, the mould will continue to grow and can rot an infected fruit in a matter of 2 – 3 days (Eckert and Eaks, 1989). *Penicillium digitatum* will also be inhibited at temperatures above 30°C (Fawcett, 1927; Plaza *et al.*, 2003; Nunes *et al.*, 2007). Furthermore, *Penicillium digitatum* is sensitive to low oxygen levels caused by low atmospheric pressure and spore germination will be decreased

at a pressure of 100 mm Hg (at 23°C), although sporulation and colony growth is not inhibited until pressure is lowered to about 50 mm Hg (Apelbaum and Barkai-Golan, 1977). Standard atmospheric pressure is 760 mm Hg, at which 100% of *P. digitatum* grows normally (Apelbaum and Barkai-Golan, 1977). The air in citrus packed cartons often has a high relative humidity (RH) at room temperature (approx. 90%), but once the cartons are put into cold storage (approx. 4°C), the relative humidity in the cartons drops (Harding Jr., 1959). The reduction of RH is important for controlling green mould as *Penicillium digitatum* grows best at a RH of \approx 90%, but will not germinate at RH levels of between 55 and 75% (Brown, 1973). *Penicillium* spp. were seen to have higher infection levels at 100 % RH compared to 80% (Nadel-Schiffmann and Littauer, 1956).

Epidemiology (Pathogen and Host)

Penicillium digitatum, being a wound obligate pathogen, means that a wound is essential before rot can occur (Kavanagh and Wood, 1967; Barmore and Brown, 1982; Eckert and Eaks, 1989). *In vitro* studies showed that the volatile compounds released when citrus is wounded are limonene, α -pinene, β -pinene and myrcene (Droby *et al.*, 2008). These volatiles act as elicitors for the germination and growth of *P. digitatum* (Eckert *et al.*, 1992; Eckert and Ratnayake, 1994). Careful handling of the fruit, particularly thin-rind fruit, during picking and packing can significantly reduce wounds created and therefore reduce infection (Christ, 1966; Pelsler, 1977). However, once infection occurs at favourable conditions (within 48 hr at temperatures of 20 – 25°C; relative humidity of 90 – 96%), the damaged tissue becomes soft and a water soaked lesion appears within 5 to 7 days (Christ, 1964, 1965; Brown, 1973; Eckert and Brown, 1986; Eckert and Eaks, 1989). As the infection continues, white mycelia grow outwards from the initial point of infection and is followed by sporulation, also originating at the infection point 5 – 7 days later with complete fruit coverage 10 - 14 days later (Fawcett and Lee, 1926; Eckert and Brown, 1986; Eckert and Eaks, 1989).

Smoot and Melvin (1961) showed that puncture wounds gave the most consistent results in artificial inoculation, and that a depth of 3 mm will always result in decay. Additionally, Kavanagh and Wood (1967) demonstrated consistently high infection levels in wounds deeper than 2 mm, or into oil vesicles. Similarly, earlier work by Nadel-Schiffmann and Littauer (1956), demonstrated that wounds 2 mm or deeper gave 100% infection, and Bates (1936) noted that the amount of infection positively correlates to the depth of the wound. A 2 mm deep wound could penetrate to the albedo (mesocarp; white part) of the fruit, which is ideal for green mould spores to germinate and cause infection (Nadel-Schiffmann and Littauer, 1956; Kavanagh and Wood, 1967; Eckert and Eaks, 1989).

Several factors influence the rate and incidence of green mould decay. For example, wounded citrus will lignify around the wound site after around 3 days at a high relative humidity

(90 - 96%) and high temperature (30 – 33°C). Although spore germination is not inhibited, no *P. digitatum* growth is seen further than the lignified cells (Brown, 1973; Brown *et al.*, 1978). Lignin production in wounds is beneficial to preventing green mould infection, however, if during injury oil glands in the peel is ruptured and oil secretions are absorbed by the damaged flavedo (orange part of the rind) cells, these cells are killed and so defensive lignification is impeded. Furthermore, albedo cells are unable to lignify and offer no resistance to wound-related infection (Brown, 1973). Sufficient moisture is necessary for spores to infect citrus with dry inoculum, with infection only resulting when oil vesicles are ruptured, or the spores penetrate to the pulp (Bates, 1936). In an alternate study, Strange *et al.* (1993) found evidence that wound gum is responsible for the resistance instead of lignin. The resistance of cells (lignified or otherwise) to *P. digitatum* infection may be attributed to the lack of pectic substances. Yellow and white parts of cell walls of citrus rinds contain pectic substances which consist of, among others, sugars, hemicellulose, and oils (Green, 1932). Without access through a wound to the specific pectic substances, *Penicillium* spp. are not induced to produce the appropriate pectin enzymes (Kavanagh and Wood, 1971; Barmore and Brown, 1980). Another line of defence is preformed antifungal compounds in the flavedo of the citrus fruit. Ben-Yehoshua *et al.* (1992) reported that compounds such as limettin and citral were seen to inhibit germ tube elongation, however, this partially refutes earlier work done by French *et al.* (1978) who claimed that nonanal and citral stimulates germination of *Penicillium digitatum* conidia. There are several other reports of various citrus compounds that stimulate *P. digitatum* germination on fruit (Eckert and Ratnayake, 1994; Arimoto *et al.*, 1995; Droby *et al.*, 2010).

Fawcett (1927) observed that decay is more severe on the stem end of the fruit compared to the styler end, with the rate of decay more rapid on stem end infections. This difference was predominant for *Penicillium digitatum* growth under sub-optimal air temperatures. During trials done with green mould, inoculation usually takes place around the stem end and this ensures that control achieved is for the worst possible scenario (Achilea *et al.*, 1985; Erasmus *et al.*, 2011, 2013). It has also been noted that the susceptibility of fruit to green mould, increases as fruit matures (Smoot and Melvin, 1961).

Control

To reduce the incidence of green mould decay, sufficient postharvest control of the disease is required. Several such control options are available and are discussed in detail below. However, an integrated approach using sanitation and fungicides (Hough, 1970), as well as handling practices is always the best way to manage green mould in the packhouse (Christ, 1966; Pelsler, 1977). Taverner (2014) invented the acronym IPHM connecting the idea of Integrated Pest Management (IPM) to Integrated Postharvest Management (IPHM), and to

encourage packhouse managers to rotate fungicides as an avoidance strategy against resistant pathogenic cultures. The concept of an integrated management system using chemicals, sanitation, biological agents and other management options is not new, as others have suggested similar systems over the years (Bancroft *et al.*, 1984; Gardner *et al.*, 1986; Jacobsen and Backman, 1993; Bower *et al.*, 2003).

Harvesting practices, wound prevention and sanitation

There are two main methods of preventing green mould infection. The first is by wound prevention, and the second by effective sanitation. Since *Penicillium* spp. need a wound to infect, a reduction of fresh wounds will result in a reduction of decayed fruit. Wounds are most likely to be induced during harvesting and handling; these present the greatest risk, as the spore load of *Penicillium* spp. is often highest in citrus orchards (Pelser, 1980; Eckert, 1995). Wounds can be created when the stem is cut, torn or damaged during harvesting. Examples include: harvesters mishandling the fruit (Pelser, 1973) particularly if fingernails are long; woody stems that protrude from the fruit and fruit is then packed tightly in bags or bins; the thorns on some citrus cultivars; and even weed thorns blown about in the orchard. Physiological injuries such as sun burn or chilling injury can also result in wounding (Eckert, 1990).

In addition to preventing wounds, inoculum must be kept to a minimum by, for instance, regular orchard sanitation, as well as disinfecting fruit and the environment. This includes, but is not limited to, packing bins, conveyors, packhouse floors and equipment, and workers' hands (Eckert, 1990; Boyette *et al.*, 1993; Lesar and Pretorius, 2010). Other cultural pre-harvest practices such as skirting the trees, removing weeds and having a well-managed irrigation system aids in the control of postharvest diseases (Pelser, 1980; Monselise and Goren, 1987; Jacobsen and Backman, 1993).

Biological control methods

Biological control is an important control method by organic producers and consumers alike (Chalutz *et al.*, 1988; Jacobsen and Backman, 1993; Adaskaveg and Föster, 2009). Several naturally occurring antagonistic yeasts, harvested from the surface of citrus fruits, have measurably been able to reduce green mould incidence (Chalutz and Wilson, 1990; Droby *et al.*, 1999a; Zhang *et al.*, 2005; Taqarort *et al.*, 2008). It appears that yeast is effective at controlling green mould mostly because it is a better competitor for space and nutrients (Janisiewicz *et al.*, 2000). While colonising the hyphae of *Penicillium digitatum*, yeasts were not noted to alter the hyphae's structure (Arras *et al.*, 1999). Arras *et al.* (1999) suggested that if the yeast is resistant to a green mould controlling fungicide, then that yeast could be used in conjunction with the fungicide to offer improved green mould control. However, despite biological control agents offering some control, they often fall short when compared to

the control offered by commercial fungicides (Brown and Chambers, 1996). Two commercial biological agents, Aspire (a yeast) (Droby *et al.*, 1998) and Biosave 10 (a bacterium) (Bull *et al.*, 1998), proved to have some control in the trials performed by Brown and Chambers (1996), although are inferior to the control offered thiabendazole and imazalil.

Physical and alternative control methods

More reliable control methods involve that of physical, as well as alternative chemical treatments. As with biological control, these treatments are used for their organic and environmentally friendly status, but also because fungicide use is being banned or at least reduced in many markets (Erkan *et al.*, 2005). Heat treatments can be used to control fungal rots, and can be applied as hot water, vapour or air (Lurie, 1998; Zhang and Swingle, 2005). Postharvest hot water dips to cure fungal infections can occur at relatively high temperatures, and for shorter periods of time, since the organism resides only on the outer few layers of the fruit. Thus a shorter, more intense heat (50 - 70°C for no more than 60s) is suitable to kill the fungus as opposed to insect treatments which require the heating of the inner area (Lurie, 1998). An important point is that the use of fungicides in combination with hot water can lead to an increased effectiveness of the fungicide (Lurie, 1998), and this will be discussed in detail further on. Opposite to heat, cold storage is another method of preserving fruit quality and preventing onset of rot. *Penicillium digitatum* growth is arrested at temperatures below 5°C (Eckert and Eaks, 1989) although some citrus cultivars develop chilling injury at these temperatures.

GRAS (Generally Regarded As Safe) chemicals are acceptable food additives that have a variety of uses, such as their ability to modify pH, and antimicrobial capabilities (Corral *et al.*, 1988). GRAS chemicals are often bicarbonates and carbonates. In trials by Smilanick *et al.* (1999) they found that such GRAS chemicals are fungistatic towards *Penicillium digitatum*, and can reduce green mould incidence on fruit in varying degrees. They demonstrated in various papers (Smilanick *et al.*, 1995, 1999, 2008), that GRAS chemicals are compatible with many citrus postharvest chemicals such as imazalil (EC formulation), thiabendazole, pyrimethanil and fludioxonil, and that when combined with a fungicide they offered superior green mould control than either applied alone. The use of heat or GRAS chemicals is always more effective when applied in combination with commercial fungicides (Smilanick *et al.*, 2006a, b), or even a biological control agent (Droby *et al.*, 1997; Teixido *et al.*, 2001). Alternative treatments increase the control achieved with commercial fungicides; however, compared to the fungicides as a stand-alone treatment, they are not as effective, and so chemical control of green mould is still the most relied upon method (Palou *et al.*, 2008) except in cases where production follows more organic guidelines.

Other alternate control methods include the use of essential oils (du Plooy *et al.*, 2009; Tripathi *et al.*, 2004) or plant growth regulators (Chitzandis *et al.*, 1988; Droby *et al.*, 1999b). Current evaluation of alternatives relies on protocols designed for synthetic chemicals and need to be revised if an equitable comparison is to be made. Under the current practices, for both oils and plant regulators, their efficacy seems limited and so their use is restricted to organic producers and not yet feasible for use in a large-scale commercial setting.

Chemical control methods

As is seen in the previous discussions, fungicides are usually more effective than alternative methods of postharvest decay control (Winston, 1935). The sooner an appropriate fungicide is applied to the fruit, the better the disease management achieved (McCornack, 1970). Trials conducted in 1924 were the first investigation into chemical action against *Penicillium* spp. specifically. Boric acid and borax were found to control over 90% decay compared to hot water treatments (Barger and Hawkins, 1925) and, together with sodium carbonate (Winston, 1935), formed the earliest known postharvest chemical control methods of *Penicillium* rots on citrus (Smith, 1962). The use of these compounds have been largely discontinued in South Africa due to their inferior action compared to more modern fungicides (McCornack, 1970).

For a considerable time imazalil, thiabendazole and sodium *o*-phenylphenate (SOPP) were the most important compounds for chemical control of green mould (McOnie, 1969; Pelsler and La Grange, 1981). However imazalil, the newest fungicide of the three, is the only one still commonly used in South Africa, due to resistance build up in *Penicillium* populations against the older thiabendazole and SOPP fungicides (Harding Jr., 1962; Holmes and Eckert, 1999; Pelsler and La Grange, 1981; Kanetis *et al.*, 2008a). SOPP's also have other complicating considerations that have contributed to their reduced use (Wild and Rippon, 1975). The active has to be carefully managed to prevent rind injuries, and therefore, the most important aspect in its use is that the SOPP solution is rinsed off after treatment (Smith, 1962; McCornack, 1970). Thiabendazole, though still actively used in various applications in the citrus packing line, needs constant agitation to remain in solution (McCornack, 1970; Kellerman *et al.*, 2014).

The use of all fungicides is constantly being questioned and scrutinised for safety and effectiveness (Staub and Stozzi, 1984). Reduced effectiveness due to resistance has raised the need for new fungicides, so azoxystrobin, fludioxonil, and pyrimethanil were introduced in the last decade (Smilanick *et al.*, 2006a; Kanetis *et al.*, 2007, 2008a; Horuz, 2010). As far as imazalil is concerned, it appears that resistant or less-sensitive strains of *Penicillium digitatum* cannot compete as effectively as the sensitive strains on untreated fruit (Erasmus *et al.*, 2015b; Holmes and Eckert, 1995; van Gestel, 1988), which may aid in imazalil staying an effective fungicide for longer. There is evidence, though, that as time passes, the fitness of

the resistant populations may improve which emphasises the need for resistance management (Kinay *et al.*, 2007). The exact nature of the competitive interaction between resistance and sensitive strains is still unknown

Citrus postharvest fungicides may be evaluated according to several factors, as detailed by Eckert and Brown (1986). These factors are the eradication of latent pathogens, the prevention of pathogenic spores germinating in a wound; fungicide persistence to protect the fruit from infection after treatment, prevention of disease spread due to contact, and prevention of sporulation (particularly for *Penicillium* spp.). Furthermore, fungicides may be evaluated for their volatile or fumigant properties, as well as their compatibility with other formulations (Eckert and Brown, 1986). In the case of *Penicillium* spp. a fungicide needs to be able to achieve three outcomes. Firstly, it has to eradicate an infection that occurred prior to treatment (curative action); secondly, it must protect the fruit from infections that occur after treatment (protective action); finally, the fungicide should be able to inhibit the formation of *Penicillium* spp. spores (sporulation inhibition) (Eckert and Brown, 1986). Imazalil is such a fungicide and thus is still highly valuable in citrus packhouses (Erasmus *et al.*, 2011).

IMAZALIL

Imazalil (IMZ; 1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl)-1H imidazole) (Siegel *et al.*, 1977; FAO, 2001) is a postharvest fungicide with the ability to control *Penicillium digitatum* decay and sporulation on citrus (Smilanick *et al.*, 1997b). The compound was discovered in 1969 (Tuset *et al.*, 1981) and was commercialised by Janssen Pharmaceutica (United States Environmental Protection Agency, 2003). It is classified as a systemic fungicide, with registration on bananas, citrus, and barley and wheat seeds, as well as for use in chicken hatcheries (United States Environmental Protection Agency, 2003). It has activity against *Penicillium italicum* and *P. expansum*, but in studies on pome fruit infected with *P. crustosum* it had very little activity against the latter pathogen (Prusky and Ben-Arie, 1985). Imazalil is registered for use in South Africa under Act 36 (1947) and its introduction to South African postharvest treatments resulted in 47% reduction in the costs associated with losses during export between 1979 and 1980 (Pelser and La Grange, 1981). As the most widely used fungicide, it is also the most effective against green mould (Tuset *et al.*, 1981; Schirra *et al.*, 1992, 2010; Altieri *et al.*, 2005; Erasmus *et al.*, 2011; Pérez *et al.*, 2011; Youssef *et al.*, 2014).

Before the use of imazalil, thiabendazole was the most important fungicide in postharvest green mould management. Unfortunately, the introduction of benomyl as an orchard spray to control citrus black spot aided in resistance against thiabendazole, since the two fungicides are both benzimidazoles (Harding Jr., 1962; Pelser and La Grange, 1981; Taverner, 2004). Imazalil was therefore introduced into the citrus disease management programme to counter this resistance (Brown, 1977; McCornack and Brown, 1977). Pelser and La Grange (1981)

reported that a 1979 survey indicated serious thiabendazole resistance, but that resistance in South Africa had come about more slowly compared to other citrus producing areas around the world. They contribute part of the reason to good sanitation practices both in the orchard and in the packhouse. The slower resistance build-up may equally be contributed to South Africa exporting nearly all treated fruit, thus reducing the population pressure to adapt. Additionally, reduced exposure to benomyl due to spray programmes, and black spot free production areas may also have contributed (Pelser and La Grange, 1981; Erasmus, Personal communication, 2015). Whatever the reason, it must be noted that sanitation is crucial to reduce green mould incidence (Hough, 1970; Smilanick and Mansour, 2007).

Imazalil was revolutionary for three reasons: 1) as already stated, it was effective against benzimidazole resistant strains; 2) it controlled *Penicillium* rots extremely well; and 3) it had activity against several other postharvest pathogens (Laville *et al.*, 1977; McCornack and Brown, 1977). The sporulation inhibition ability of IMZ is very important to combat the problem of soilage (McCornack and Brown, 1977). Soilage decreases the value of healthy, sound fruit, because it is cosmetically contaminated with spores from infected fruit (Pelser, 1977; Eckert and Kolbezen, 1978; Barmore and Brown, 1982; Eckert and Eaks, 1989). Although healthy, such fruit is unmarketable until it has been cleaned and repackaged, thus adding cost to the value chain. Previously soilage was controlled using biphenyl infused packaging which would inhibit *Penicillium* sporulation; the drawback was that it would leave a slight chemical odour on the fruit (Smith, 1962). Resistance development in *P. digitatum* contributed to the discontinued use of biphenyl (Harding and Savage, 1961).

Imazalil is available in two formulations, namely an emulsifiable concentrate (EC), and a sulphate salt form in either soluble powder (SP) or soluble granules (SG) (Pelser, 1980; Sepulveda *et al.*, 2015). South Africa uses the sulphate form in all aqueous applications at a registered concentration of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ (Pelser and La Grange, 1981; Fourie and Lesar, 2008; Erasmus *et al.*, 2011). Some of the reasons South Africa favours the sulphate formulations are that, unlike the EC formulation, it is more stable, needs less agitation and is less likely to adhere to the sides of the container, which subsequently results in the active being unavailable as a fungicide (Eckert, 1977; Altieri *et al.*, 2005). A true solution is one that does not need to be mixed in order to remain homogenous (Eckert, 1977). The sulphate form consists of the IMZ base molecule with an added sulphuric acid group (Erasmus *et al.*, 2011). The EC formulation is also used in South Africa, although it is solely utilised as a wax augmentation. An aqueous application of imazalil is more effective than applying it in wax, with the reason being speculated that water as a fungicide carrier is less viscose than wax and can therefore enter small spaces and wounds, offering more effective coverage and control (Brown, 1984). Wax applications typically need a much higher concentration of IMZ than an aqueous application in order to offer a similar level of control (Brown, 1984). A recent

study comparing EC and sulphate formulations of IMZ found that the two formulations offer comparable levels of protective control, however the sulphate form was more chemically available to bind to the citrus rind since it is more soluble in aqueous applications (Sepulveda *et al.*, 2015). Another SP/SG formulation exists as a nitrate salt instead of a sulphate salt (Laville *et al.*, 1977; Dezman *et al.*, 1986), but it has never been known to be used in South Africa (Keith Lesar, personal communication, 2016; Wouter Schreuder Snr, personal communication, 2016).

Imazalil has a widespread use globally and is used extensively in citrus packhouses (Erasmus *et al.*, 2011; Njombolwana *et al.*, 2013). The dependency on imazalil to control *Penicillium* spp., is often coupled with a market-demand for under-application of the fungicide (Erasmus *et al.*, 2011). This, together with a high disease pressure, has led to resistance build-up (Wild, 1994; Holmes and Eckert, 1999). Imazalil resistance is wide spread and, within resistant populations, not even the highest registered dose of IMZ will prevent sporulation (Eckert *et al.*, 1994). South African producers are aware of the risk of resistance and tries to implement good IMZ management and sanitation to avoid the loss of IMZ fungicide use (Erasmus *et al.*, 2014). Resistance has led to the need for new postharvest chemicals with alternative modes of actions. Pyrimethanil, fludioxonil, and azoxystrobin are more recent fungicides available on the market for the control of green mould and other citrus postharvest diseases (Kanetis *et al.*, 2007, 2008a; Taverner, 2014).

Mode of action

Imazalil is a demethylation inhibitor (DMI). The fungicide genetically alters the Sterol 14- α -demethylase gene (CYP51), which results in the P450_{14DM} enzyme being produced (Hamamoto *et al.*, 2000). This enzyme demethylates the C-14 methyl group (Siegel and Ragsdale, 1978) on sterols in *Penicillium* spp. membranes. This in turn interferes with cellular permeability of the membrane but more importantly, prevents further synthesis of membranes and thus prevents the fungi's growth and survival (Siegel, 1981; Dezman *et al.*, 1986; FAO, 2001). In experiments with *P. italicum*, Siegel and Ragsdale (1978) showed that initial growth of the fungus is not affected. Although IMZ was found to inhibit protein and nucleic acid synthesis, dry weight increase and energy production amongst other biological processes are not hindered at first (10 – 12 hr); this is because of a store of sterols which can be used for membrane synthesis. However, once the reserve is depleted, membranes lose functionality and the ability to synthesise, thus resulting in either fungistasis or death of the *Penicillium* spp. (Siegel and Ragsdale, 1978). The particular targeted action of imazalil to fungi is not toxic to man or the environment, and will not accumulate in mammals. It has a DT₅₀ in river water of 18.15 hr due to photolytic degradation (European Commission, 2007). Due to IMZ's particular mode of action, one of the most common ways *Penicillium digitatum* is able to become

resistance is when there is a mutation in the transcriptional enhancer unit leading to an over-expression of the CYP51 gene (Hamamoto *et al.*, 2000; Ghosop *et al.*, 2007). Resistance development is a very complex process and there are several other mechanisms that have been investigated (Waard and Nistelrooy, 1990; Deising *et al.*, 2008).

Imazalil is lipophilic and its long lasting protection on citrus fruit is due to its ability to adhere to, and translocate into the citrus rind (Brown and Dezman, 1990), which is in itself affected by many different factors, for instance application method, solution temperature, solution pH, IMZ formulation, which will be discussed later.

Imazalil application

Postharvest fungicides can all be added at various points before or during the packline. In South African packhouses, Erasmus *et al.* (2011) reported that 78.4% of all surveyed packhouses applies IMZ in the fungicide bath (dip tank). Wax application was another common application point (62.2% of all surveyed packhouses). Other points of applications included pre-packline points such as a drench (3%) or in different applicators such as sprays (3%). Imazalil is often used in double application (dip and wax; 49% of packhouses surveyed) or even triple application (drench, dip and wax; 5% of packhouses surveyed). However, a single application was common (46%) too (Erasmus *et al.*, 2011). Njombolwana *et al.* (2013), found that IMZ was most effective when used in a double application: first in the fungicide bath and then in the wax. This combination appears have the best green mould control and sporulation inhibition when compared to either treatment alone. This is partly due to the fact that the different application methods provide different types of control. Dip application provides good curative control (Dore *et al.*, 2009), while the wax provides increased protective control (Njombolwana *et al.*, 2013). Such combination treatments provide adequate coverage of the fruit, loading residue levels that offer green mould control, yet do not exceed the MRL of $5 \mu\text{g}\cdot\text{g}^{-1}$ (Njombolwana *et al.*, 2013; Sepulveda *et al.*, 2015). Brown *et al.* (1983) demonstrated that different methods of applying IMZ (EC; water or wax) gave similar levels of control when fungicide concentrations were adjusted (about three times higher necessary in wax applications compared to water). They attested that the non-recovery spray treatment with a lower concentration, was more effective than a wax application as the fungicide penetrated deeper into the exocarp and thus the fruit were less prone to infection post treatment (Brown *et al.*, 1983). Schirra *et al.* (1992), also noted that IMZ applied in water was more effective as opposed to wax application. Smilanick *et al.* (1997b) showed that the efficacy of IMZ (EC) is better in a heated aqueous application compared to a wax application. Lower residues were loaded with the aqueous application, but the efficacy was greater. The aqueous application contained $500 \mu\text{g}\cdot\text{mL}^{-1}$ IMZ, while the wax contained $4000 \mu\text{g}\cdot\text{mL}^{-1}$, yet control was superior (95 compared to 60%) with the heated aqueous application. The reasons

attributed to this effect were that the aqueous application was heated, and that the fruit were immersed rather than being sprayed (Smilanick *et al.*, 1997a). They postulated that a double application is not necessary if you achieve desired residues by correct and efficient aqueous dip application (IMZ EC of 350 – 500 $\mu\text{g}\cdot\text{mL}^{-1}$, heated to $\approx 35^\circ\text{C}$, with an exposure time of 30 s or longer) (Smilanick *et al.*, 1997b). The application of wax has many other benefits such as moisture retention and shine, and it is unlikely to be removed from packhouse lines. Despite these studies showing the low efficiency of wax as a fungicide application, new product development is constantly making improvements, which include its ability to be an effective fungicide carrier.

An abandoned practice of fungicide application in South Africa involved moving citrus on a conveyer that allowed the fruit to pass under and through an overhead brush saturated with the fungicide (Eckert, 1977; Pelsler, 1977). Following that, the main application of IMZ was by dripping or spraying the solution onto fruit passing over brushes. This application method was very brief (2 - 3 s) and resulted in poor coverage of the fruit, which is why the fungicide bath application gained popularity (Brown and Dezman, 1990; Bronkhorst *et al.*, 1993). A more recent application method has been investigated described as Imazalil Thin Film Treatment (ITFT), which reduced *Penicillium* decay. Similarly to the older application methods, it did not perform as well as the dip application (Altieri *et al.*, 2013). Despite the fungicide baths performing more adequately than other aqueous applications tried, there is still room for improvement. New applications such as the cascade (Besil *et al.*, 2016) and the heated flooder offers that superior coverage for IMZ aqueous application (Erasmus *et al.*, unpublished).

The fungicide bath

The dip tank or fungicide bath is the primary method of IMZ sulphate fungicide application in South Africa (Erasmus *et al.*, 2011) and has been for quite some time (Pelsler and La Grange, 1981), however, the finer details of this application method are extremely varied. In 2008, Erasmus *et al.* (2011) conducted a survey of South African packhouses and found that fungicide baths significantly differed in their volumetric capacity, and length, resulting in fruit treatment that was highly varied. Treatment differences included solution temperature (12 to 45°C), solution pH (3 – 8), exposure time of the fruit (16 – 107 s) and concentration of the IMZ in the tank (131 – 2175 $\mu\text{g}\cdot\text{mL}^{-1}$) (Erasmus *et al.*, 2011). Regardless of the physical parameters of the tank, one of the primary management points is maintaining the correct concentration of IMZ (Pelsler and La Grange, 1981). As tonnes of fruit go through the bath, the IMZ is stripped out of the bath, decreasing the overall concentration to below that of the recommended 500 $\mu\text{g}\cdot\text{mL}^{-1}$ IMZ. For this reason the concentration needs to be measured and managed accordingly (Eckert, 1977; Altieri *et al.*, 2005; Lesar, 2008). Erasmus *et al.* (2011)

clarified some further points regarding fungicide bath use in South Africa. They determined that a temperature of 35°C is most commonly used and recommended, and that the fungicide bath provides better curative control (infections present before treatment) than that of protective (infections originating after treatment). The fungicide bath is very effective if fruit can be fully submerged during treatment, however, that is often not the case (Eckert, 1977). In addition, the fungicide bath has a few other disadvantages, such as the large volume of water, implying large quantities of fungicide needed to treat fruit. The cost of these consumables compel packhouses want to keep the same tank for several weeks as well as the problem of disposal of used fungicide solution, these factors, in turn, necessitate the need to have a sanitising agent in the bath to keep it clean of contaminants. The most popular sanitiser used in packhouses, chlorine, is incompatible with IMZ. Regardless, the low pH (3 – 5) often used in IMZ sulphate treatment is below the necessary pH (6.5 – 7.5) for effective chlorine use. Other factors such as the concentration of fungicide and pH need to be maintained too (Eckert, 1977). A trial looking at the effectiveness of pre-prepared IMZ (EC) solutions showed that after 19 days the solution offered 6% less decay control compared to freshly prepared solutions (Hall, 1991). These disadvantages have led to the application of fungicide solution in a spray or flood mechanism (Eckert, 1977).

The heated flooder

In terms of in-line aqueous fungicide application, the alternative to a bath is an overhead spray or flood of solution (McCornack, 1970; Smoot and Melvin, 1970; Wild *et al.*, 1975; Brown, 1977). These systems can either be closed, where the solution is retained and recirculated (recirculating systems) or open, where solution that has come into contact with the fruit is discarded (total loss systems). In either case a low or high volume of solution can be used. For disease management, a high volume is generally more effective than a low one (Förster *et al.*, 2007; Kanetis *et al.*, 2008b). Unfortunately, high volume, total loss systems are usually impractical in terms of cost and water consumption. The ideal situation is therefore a recirculating system that uses an overall smaller volume of water, but delivers a higher volume of solution at any one point to the fruit, resulting in increased decay control (Brown and Dezman, 1990; Kanetis *et al.*, 2008b). Additionally to the conservation of fungicides and water, other benefits of a recirculating system are that heating the system is more manageable, and any fungicides in solution are more thoroughly agitated and mixed, although some chemicals such as thiabendazole, precipitate very easily regardless (Smoot and Melvin, 1970). Recirculating systems are closed systems like the fungicide baths, so problems of fungicide stripping and contamination build up are the same (Eckert, 1977; Pelsler, 1980). However these issues can be mitigated with proper packhouse management (Lesar, 2008; Lesar and Erasmus, 2014). In 2003 Smilanick *et al.* published a study looking at a heated

drench application that applied fungicide solution onto fruit in a high volume, low pressure drench over rotating brushes. The elevated temperature (35°C) was found to significantly reduce green mould incidence at both treatment times examined (15 s and 30 s). This experimental unit probably initialised the development and use of the heated flooder in the USA. The heated flooder differs from the fungicide bath in that water is enclosed in a tank, and constantly circulated and heated. Fruit going through the flooder are rolled over rotating brushes, and pass through several high volume, smooth laminar waterfalls or weirs that deliver the solution. This ensures complete solution coverage of the fruit compared to the fungicide bath where floating fruit may not have all sides exposed to the fungicide for a long enough exposure time. The JBT Heated Flooder is a new concept in South African packhouses. Work done by Erasmus *et al.* (unpublished) shows that it has the potential to be an alternative to the fungicide bath, as well as offering superior green mould control. Compared to the fungicide bath, the flooder has a smaller tank which, being closed, retains the solution temperature very effectively. The application weirs are covered too, aiding in the retention of temperature and protecting the solution from outside contamination by dirt and debris which is important in terms of packhouse sanitation (Bancroft *et al.*, 1984). Since the flooder is newly introduced to the South African citrus industry, the correct use of IMZ still needs to be investigated. Similar applications that are also known as in-line drenchers have been reported, such as the high-volume recirculating drench consisting of pumped fungicide solution falling through a metal sheet perforated with many 5 mm, evenly distributed holes. This meant that the solution is poured over the fruit moving along a roller bed (Kanetis *et al.*, 2008b). In a study using this application method, Kanetis *et al.* (2008b) determined that for three different fungicides tested, results from the in-line drench was significantly improved compared to the controlled droplet application over either brushes or rollers. The same high volume in-line drench applicator was tested on stone fruit, and showed increased levels of disease control compared to the commonly used low-volume spray (Förster *et al.*, 2007). Although these applications proved successful, the use of brushes may be very important. In hot water trials over brushes (56°C; 20 s) it was seen that not only was *Penicillium digitatum* decay reduced, but that the epicuticular wax had been smoothed to cover stomata and cracks and thus further protecting the fruit (Porat *et al.*, 2000).

Residue loading

The persistence (in the form of a residue) of a fungicide or pesticide remaining on a consumable product is strictly laid down by the CODEX Alimentarius and/or each country's regulatory bodies (FAO, 2013). The MRL (Maximum Residue Limit) for IMZ on citrus is 5 µg.g⁻¹ for the European Union and 10 µg.g⁻¹ for the USA (DAFF, 2008; AgriIntel, 2015). Despite concerns, MRL's are very rarely exceeded (Fernández *et al.*, 2001; Blasco *et al.*,

2006; Erasmus *et al.*, 2011). Blasco *et al.* (2006) sampled over 100 citrus fruit for 10 different chemicals and in the case of IMZ, it was detected on only 15% of fruits sampled in a concentration range of 0.02 – 1.2 $\mu\text{g}\cdot\text{g}^{-1}$. Fernández *et al.* (2001) found IMZ residues on 112 of 115 fruit sampled with an average residue of 1.2 $\mu\text{g}\cdot\text{g}^{-1}$. In South Africa, an external body, PPECB (Perishable Products Export Control Board), inspects residue levels on citrus before it leaves the packhouse, to ensure that a consignment has not exceeded the MRL (Wilma du Plooy, personal communication, 2016). South African packhouses have been shown to regularly load $\approx 1 \mu\text{g}\cdot\text{g}^{-1}$, half of the recommended necessary residue to control green mould, and certainly below the MRL of 5 $\mu\text{g}\cdot\text{g}^{-1}$ (Erasmus *et al.*, 2011).

Imazalil (applied in a 15 s aqueous dip) will slowly migrate deeper into the fruit but after a week no more than 20% of the original residue will have moved from the exocarp to the mesocarp (Brown and Dezman, 1990). The persistence of IMZ on the surface of the fruit is what allows the excellent sporulation inhibition properties of IMZ (Brown and Dezman, 1990). Schirra *et al.* (1996) did trials on IMZ (EC) residue persistence over a very long period (14 weeks), and found that the residue did decrease over that time period; however, the maximum decrease was around 60%, and in some cases only a 10% decrease was recorded. In another study, citrus fruit treated with the EC formulation of IMZ showed an average 15% decrease in fungicide residue after 9 weeks in storage (Cabras *et al.*, 1999). It is important to note that oil gland distribution and migration of IMZ into citrus rinds may be different for citrus types and cultivars (Obenland *et al.*, 1997). Dore *et al.* (2009) found that as concentration and temperature increased concurrently, residue loaded increased and residue persistence remained much more stable. Brown and Dezman (1990) showed that the overall concentration of IMZ remains at 100%: at the time of treatment 99.1% was in the exocarp and after 7 days of storage, the exocarp residue was reduced to 77.5 %, however, the remaining 22.5% was translocated to the mesocarp. A more recent study took into account the differences between the IMZ formulations, and demonstrated that IMZ sulphate has a half-life of approximately 17 days, slightly shorter than that of the EC formulation applied in wax, which was closer to 19 days (Besil *et al.*, 2016).

Residue loading can be influenced significantly by solution temperature, exposure time, solution pH, IMZ formulation and concentration amongst others. Dip trials with IMZ (EC) on oranges and lemons showed increased residues when any combination of temperature, exposure time and concentration were increased (Smilanick *et al.*, 1997b). The IMZ residue loading on citrus fruit can therefore be readily manipulated using these variables (Erasmus *et al.*, 2011, 2013).

It is possible that IMZ residues may have a maximum loading point. Treatments involving cascade aqueous application (an application similar to the flooder) showed that very similar residues were loaded at 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ treatment and at 2000 $\mu\text{g}\cdot\text{mL}^{-1}$ treatment (0.48 and 0.43

$\mu\text{g.g}^{-1}$ respectively) (Besil *et al.*, 2016). This similarity may be due more to the fact that the pH (3) and exposure times were the same in both treatments, and that those variables have a stronger effect in residue loading, than the actual solution concentration. Erasmus *et al.* (2011) noted that at pH 3, increased exposure time in IMZ sulphate dips did not result in an increased residue loading as in a pH 8 solution.

While it is commercially important that residue levels remain below the MRL specifications, it is proven that residues within the MRL restrictions offer sufficient control in most cases. For example, Smilanick *et al.* (1997b) showed that to achieve 95% control of green mould (IMZ sensitive strain), an IMZ residue of between 1 and 3 $\mu\text{g.g}^{-1}$ is needed; while for sporulation inhibition, residues of 2 – 3.5 $\mu\text{g.g}^{-1}$ are required. Erasmus *et al.* (2011) confirmed these results, and went on to specify similar residues (2 - 3 $\mu\text{g.mL}^{-1}$ to inhibit sporulation) for the sulphate form of IMZ. Eckert and Kolbezen (1978) recommended a slightly higher residue of between 2 and 6 $\mu\text{g.g}^{-1}$ for benzimidazole fungicides to successfully control *P. digitatum* sporulation. A study by Kaplan and Dave (1979) on effective residue levels against *Penicillium* spp. showed that IMZ residues between 0.6 and 2.0 $\mu\text{g.mL}^{-1}$ gave acceptable levels of disease control and sporulation inhibition on citrus. Overall, a minimum limit of 3 $\mu\text{g.g}^{-1}$ IMZ residue was recommended by Schirra *et al.* (1996) to control pathogen development. Furthermore, Schirra *et al.* (1996) noticed more decay on fruit that loaded high levels of IMZ (EC). They postulated that as IMZ degrades, it produces structurally similar compounds which lack any inhibitory properties. This degradation lowers the effective compound concentration, with an increase in degradation products. It is thought that these by-products then compete with the remaining active ingredient and thus reduce efficacy of the fungicide as a whole (Schirra *et al.*, 1996).

Factors influencing residue loading and green mould control

There are several factors which play a role in the effectiveness of a fungicide applied aqueously. These include concentration, temperature and pH of the solution, as well as other factors such as surfactants and exposure time (Eckert, 1977). The application method plays a role and is linked to coverage, and it was found that a dip treatment could load about 25% more IMZ than a non-recovery spray (Brown and Dezman, 1990).

Fruit

There are several kinds of citrus fruit (soft citrus, lemons, grapefruit, oranges etc.) and within each of these kinds are various cultivars. Postharvest research is only possible with the availability of fruit, and different fruit kinds become available at different times throughout the season. This leads to a naturally occurring variation among seasons and, within a production area, between harvest batches of the same kind, or even the same cultivar. Erasmus *et al.* (2015a, b) discerned significant fruit type and harvest batch interaction, as did Kellerman *et*

al. (2016). These differences can be attributed to fruit quality and maturity, which differ as explained. It is important to keep these factors in mind for future postharvest research.

Fruit maturity plays a role on the susceptibility of the fruit. As mentioned earlier, Smoot and Melvin (1961) identified that susceptibility of fruit to green mould increased as the fruit matured. A 1956 study by Nadel-Schiffmann and Littauer noted that different citrus types differed in their susceptibility and maturity relationship. Grapefruit and lemons showed little difference in susceptibility regardless of maturity, however, Shamouti oranges increased in susceptibility with increased maturity, yet decreased again when over-ripe (Nadel-Schiffmann and Littauer, 1956). In lemons that were dark green and therefore not very mature, Smilanick *et al.* (2005) found a maximum pH of 5.6 for the albedo tissue. Very mature lemons, those with yellow colour, had a minimum pH of 5.1. Although this difference is not vast, it does show a linear relationship of decreasing pH as fruit matures, and this may have an influence on green mould infections and fungicide control (Smilanick *et al.*, 2005). It has been noticed that a turgid yellow lemon will be more likely to decay compared to a green lemon (Eckert, 1995). The age of fruit may play a role in residue uptake through the rind as this concept has been seen in other plant functions such as the fungicide diffusion across the plant leaf cuticle which decreases as fruit maturity increases (Riederer and Schreiber, 1995).

Different citrus kinds do not all react the same in terms of disease susceptibility and management. Nova mandarins inoculated with green mould were dipped in 20°C water and suffered 100% rot. However, when the same treatment was applied to Valencia oranges, 93 - 97% rot was observed. Although this difference is not profound, increases in solution temperature emphasised the difference. At 40°C, the results were variable, with the virulence of the sensitive or resistant strain of *P. digitatum* playing a role. The most prominent difference was seen at 50°C treatments, with the infection on Valencia's averaging 50%, while Nova's had above 95% infection (Schirra *et al.*, 2008). The level of disease management may be attributed to the level at which fungicides are loaded onto the fruit rind. A recent study noted that soft citrus (Clementine) loaded significantly higher residues in some cases compared to lemons and navel oranges, which loaded similar levels (Kellerman *et al.*, 2016). Despite the differences seen using different fruit kinds, it is often clear that the overall trends in reaction to treatments are consistent (Kellerman *et al.*, 2016).

Solution pH

It has been found that IMZ's solubility in water is pH sensitive: since it is a weak base, it is most soluble with a low solution pH when the molecule is ionised (FAO, 2001). For 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ of IMZ EC in 30°C water, at a pH of 4.9, all of the IMZ was dissolved. At a pH of 7.6 (above the pKa value of between 5.85 – 6.5 (FAO, 2001) the solubility was drastically reduced when the molecule was more neutral in charge, so that only 180 of the 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ IMZ was

dissolved. At pH 10, when the charge is neutral, the solubility dropped to $1.8 \mu\text{g}\cdot\text{mL}^{-1}$ (Altieri *et al.*, 2005; European Commission, 2007).

The pKa of a solution is the equilibrium point where 50% will be dissociated and protonated and the other 50% will be undissociated and unprotonated, and is a measure of the strength of an acid (the smaller the value, the stronger the acid (Campbell and Farrell, 2008)). The pKa of IMZ is 6.53 (this can vary slightly depending on the manufacturer of IMZ (Siegel *et al.*, 1977; FAO, 2001). At a pH below the pKa value, nearly all of the IMZ molecule is in a dissociated form, with a charged or protonated nitrogen group (Siegel *et al.*, 1977). At a pH above the pKa, such as pH 7, the IMZ loses protons and exists in an undissociated, oily form, becoming more lipophilic (Siegel *et al.*, 1977; Brown and Dezman, 1990; Campbell and Farrell, 2008). It is understood that undissociated or uncharged forms of fungicides more readily permeate fungal membranes (Lukens, 1971). This chemistry explains why there was a marked difference in efficacy of IMZ (EC) between pH 7.5 and pH 4, with improvement at the higher pH level. The loaded residues from IMZ (EC) solutions are also affected by pH, and at pH 7 and 9 loaded almost double that of a pH 3 or 5 solution (Smilanick *et al.*, 2005). It was found that similar levels of residues of imazalil sulphate and the emulsifiable concentrate loaded in solutions of pH 3 to 5; however, increases to pH 7, and even more dramatically at pH 10, resulted in the sulphate form loading approximately double and quadruple amounts respectively (Sepulveda *et al.*, 2015). The Food and Agriculture Organisation's 2001 report on IMZ states that "Precipitation from solution might occur if pH was such as to change the free base into the salt or vice versa". However, in the same report, Janssen Pharmaceutica (the first manufacturer of IMZ), concluded that in normal water (neutral pH and moderate hardness) IMZ acted satisfactorily and that there was no need to include pH specifications to the users. Nonetheless, Erasmus *et al.* (2011, 2013, 2015a) has since showed that there is often great variability in the pH of IMZ solutions, and this infers that the water conditions are not always satisfactory. South African water specifications are often outside of the ideal values, because municipal water varies in pH. Additionally, packhouses often source their water from rivers or dams, boreholes and rainwater (Savage *et al.*, unpublished). Therefore, there is a need to adjust solution pH in order to maintain the IMZ formulation to achieve the maximum activity from the product through careful management of both pH and temperature (Erasmus *et al.*, 2011, 2013, 2015a). The pH of the IMZ solution in a fungicide bath could potentially be adjusted to any value, however, research to date on the sulphate form of imazalil has shown some limitations and interesting aspects in regards to the solution pH. The sulphuric acid molecule of the sulphate formulation is what will initially drop the pH of the solution when added to water. An IMZ sulphate solution of $500 \mu\text{g}\cdot\text{mL}^{-1}$ with a pH of 3 will load sufficient ($1.00 - 2.00 \mu\text{g}\cdot\text{g}^{-1}$) residues to control green mould on dipped citrus fruit (Erasmus *et al.*, 2011; Kellerman *et al.*, 2016), while similar solutions at pH 6 and pH 8 loaded higher

residues as pH is increased (Erasmus *et al.*, 2013; Kellerman *et al.*, 2016) These studies demonstrated that pH plays an influence on the availability of IMZ from the sulphate formulation and that if pH is not monitored, residues could be loaded onto citrus higher than that of the MRL of 5 $\mu\text{g}\cdot\text{mL}^{-1}$. Kellerman *et al.* (2016) recorded that at pH 6, residue loading increased compared to pH 3 (averaging 1.30 and 5.66 $\mu\text{g}\cdot\text{g}^{-1}$ respectively) and that correspondingly better green mould infection control and sporulation inhibition was seen at the pH 6 treatments. Residue levels and the resultant disease management levels can be linked to the pH of the treatment as Hall (1991) noticed that IMZ (EC) solutions had better anti-sporulation activity at higher pH levels (8, 9, 10) than lower ones (5, 6, 7), unfortunately the study did not expand on these results, since focus was on stored solutions compared to freshly made solutions. In contrast, later work by Altieri *et al.* (2005) observed that IMZ EC concentration in solution had no correlation with pH. The pH of commercial wax coating is around 9.5, and could attribute to some of the variation we see between wax and aqueous applications (Brown *et al.*, 1983).

In addition to the influence that pH has on an IMZ solution by increasing the amount of fungicide taken up by the tissue, it is thought that pH may have an influence on the action against the pathogen (Eckert, 1977). Siegel *et al.* (1977) investigated IMZ's antifungal abilities against *P. italicum* and found a pH dependency. At pH 5.2 growth of *P. italicum* on IMZ amended media was more vigorous than at pH 7.0 (Siegel *et al.*, 1977). Imazalil was more effective against *P. digitatum* in media with a pH of 5.9 compared to pH 5.1 indicating that there was a "greater concentration of dissociated imazalil at the higher pH value" (Holmes and Eckert, 1999).

A further aspect is that *Penicillium digitatum* itself is influenced by pH. The fungus can germinate at pH levels as low as 3.5 and as high as 8.0, however, it grows most effectively at a pH range of between 4.0 and 5.5 (Pelser and Eckert, 1997). Smilanick *et al.* (2005) observed that 98% of *P. digitatum* spores germinated between pH 3 and 7, however, that number decreased to 70% at pH 8, and down to 10% at pH 9. They also saw that lower concentrations of IMZ were needed to control green mould *in vitro* as the pH of the medium was increased (pH 4, $\text{ED}_{50} = 0.16 \mu\text{g}\cdot\text{mL}^{-1}$; pH 7, $\text{ED}_{50} = 0.006 \mu\text{g}\cdot\text{mL}^{-1}$).

In the absence of a chemical control, *Penicillium* spp. can often overcome the host's defence system. Some explanations of how it achieves this includes that an infection on citrus leads to an increase in gluconic and citric acids, which lower the pH; subsequent colonisation was found to be faster in tissues with lowered pH (Prusky *et al.*, 2004). Related results by Achilea *et al.* (1985) showed that *Penicillium digitatum* causes a drop in the pH of citrus peel as the infection results in an average 10-fold increase in galacturonic acid compared to healthy tissue. pH levels of the albedo and flavedo drop from around pH 5 in healthy tissue to around pH 4 in infected tissue. The reduction in pH was found to have an effect on water soluble

proteins in the fruit. Tests done showed that reducing pH from 5.4 to 4.5 reduced the proteins by 20%. Further pH decrease to 3.5 had no further effect on the protein content. The decrease of water soluble proteins (4.11 mg.g^{-1} to 0.73 mg.g^{-1}) seen in *P. digitatum* infected tissue may therefore be partly attributed to green mould infection, and partly to the pH of the fruit peel (Achilea *et al.*, 1985). Whether solution pH has an effect on total water soluble proteins in fruit, infected or otherwise, is not certain.

Solution temperature

Heat treatments can be a method of disease control in their own right (Erkan *et al.*, 2005; Şen *et al.*, 2010). Treatments of water at 48 and 53°C gave around 20% less decay compared to untreated control fruit (Erkan *et al.*, 2005). This physical treatment is an alternative to chemical treatments, and while successful against fungal infections, it is more commonly used against insect infestations (Couey, 1989; Lurie, 1998). The study by Nafussi *et al.* (2001) looking into the mode of action of IMZ has shown that with hot water treatments (53°C), lignin accumulation around the wound site is prolonged, contributing to prevent green mould development. Furthermore, the study showed the increase of phytoalexin concentrations (scoparone and scopoletin) which aid in *P. digitatum* inhibition (Nafussi *et al.*, 2001). Subtropical fruit such as citrus can also be affected by disorders such as chilling injury during storage (Eckert and Eaks, 1989), and there is some evidence that hot water treatments can beneficially reduce or prevent chilling injury (Erkan *et al.*, 2005). McDonald *et al.* (1991) showed that hot water (53°C) in conjunction with a fungicide (such as IMZ or thiabendazole) can have a positive effect, not only by reducing decay, but also reducing physiological storage disorders such as chilling injury. However, the reduction of chilling injury when using IMZ (EC), could simply be due to the effect of the hot water and not the fungicide (Schirra *et al.*, 2000).

Untreated fruit surfaces (as seen with SEM) has rough or granular surfaces with deep cracks. A comparison of hot water dip treatments (50 – 58°C) found that after treatment at dip temperatures of 50; 52; or 54°C, the surface did not exhibit cracking, and was relatively smooth (Schirra and D'hallewin, 1997). It is important to note that some of the cracking seen, including the severity of the cracks, may be effects of sample preparing for visualisation by SEM, and some of the variation noted may be due to inherent properties of natural wax during SEM preparation and not due to the heat treatments. Regardless, it was also noticed that these treatments resulted in reduced decay and chilling injury. Increasing the temperature to 56°C resulted in an apparent movement of the epicuticular wax with zones of heavy deposits and zones of no determinable wax. Increasing the dip temperature to 58°C it was observed that the wax apparently melted and that any trace of surface wax was removed (Schirra and D'hallewin, 1997). The changes in epicuticular wax with the 56 and 58°C treatments resulted in rind browning and peel water loss, which in turn resulted in a negative appearance of the

fruit. None of the fruit in these trials were brushed, but simply left to air dry for 6 h after treatment, implying that the wax movement is definitely due to the heat of the solution (Schirra and D'hallewin, 1997). Porat *et al.* (2000) also noted the smoothing of epicuticular wax with heated water at 56°C and brushing treatment. A later study by Schirra *et al.* (2005) confirmed that with dip temperatures of 50, 55, and 60°C the epicuticular wax had melted and appeared homogenous on the fruit's surface. This could account for the higher residues experienced with IMZ at 50°C compared to 20°C, despite a reduction in concentration (100 µg.mL⁻¹ to 400 µg.mL⁻¹), as well as the longer persistence of the fungicide seen in fruit treated at 50°C. The phenomenon did not occur with dip temperatures of 30 or 40°C (Schirra *et al.*, 2005).

Penicillium digitatum conidia are heat sensitive, which may explain some of the beneficial effects seen from heated solutions. Heated dips (47 – 53°C) were shown to decrease *Penicillium* spp. populations while not negatively affecting beneficial organisms such as yeast which could aid green mould management (Şen *et al.*, 2010). Heated drench trials demonstrated that the lethal temperature for *P. digitatum* conidia was ≈ 60°C after a short exposure time < 30 s (Smilanick *et al.*, 2003). Smilanick and Mansour (2007) furthermore found that the conidia will not germinate at air temperatures above 50°C (95% RH; 5 hr exposure time).

Hot water treatments alone can be effective at high temperatures, although the addition of a fungicide always improves that control. At 56°C treatment without a fungicide, just more than 40% control was noted, however, with the addition of IMZ, this went up to 100% (Puawongphat *et al.*, 2008). However, it is hardly a reliable source of control as seen in experiments where 45 and 50°C water (for both 60 s and 150 s) did not control *P. digitatum* or *P. italicum* (Palou *et al.*, 2002). Disease reduction due to warm water treatments is often not significant (≈ 10%) (Scherrer Montero *et al.*, 2010). Schirra *et al.* (2010) did a dip trial involving the use of azoxystrobin, fludioxonil, pyrimethanil and IMZ (EC). According to this study, increasing the exposure time and/or temperature could have some influence over the efficacy of the fungicide. Fludioxonil at 50°C increased its efficacy to that of pyrimethanil at 20°C. Imazalil (EC) outperformed all the other postharvest fungicides, and enabled very high levels of control, especially at 50°C dip (concentration of either 300 or 600 µg.mL⁻¹, and exposure times of 30 or 60 s). To the contrary, a study using the EC formulation of IMZ showed little to no improved effect of disease control (*P. digitatum*) when solution temperatures were increased from 20°C to 45°C. This study was conducted with an exposure time of 30 s and in combination with temperatures below 50°C, which may explain the contradiction found with many other heated solution studies (Cunningham and Taverner, 2007). These studies all demonstrate the necessity of fungicide use, but also highlight that the concentration of IMZ can be drastically reduced if supplemented with synergistic temperature use. No significant differences were seen in the control achieved from a range

of IMZ concentrations from 50 $\mu\text{g}\cdot\text{mL}^{-1}$ to 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ as long as higher temperatures were used at lower concentrations (Schirra *et al.*, 1997). Some heat damage was observed at treatments with high concentrations of IMZ (1000 $\mu\text{g}\cdot\text{mL}^{-1}$), which was not seen with 50°C treatment alone, therefore it is deduced to be due to the addition of the fungicide (Schirra *et al.*, 1997). Furthermore, juice analysis identified an increase ($\approx 10\%$) in ethanol with treatments at 200 and 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ above that of the lower concentration treatments, and that was also above the level seen in untreated control fruit. The ethanol present resulted in an off taste, and fruit were unacceptable for consumption after 14 weeks. To the best of our knowledge there has been no reports of imazalil causing any sort of decrease to fruit quality in South Africa (Paul Cronje, personal communication, 2016).

As stated earlier, the IMZ concentration of a heated solution is often important. Heat can increase the concentration of soluble particles, increase movement of molecules into infection sites and increase diffusion through fruit cuticles (Eckert, 1977). With increasing fungicide concentration, an increase in residue loading is often seen; however, this phenomenon is exponentially increased when solution temperature is increased too. Imazalil (EC) loaded $< 5 \mu\text{g}\cdot\text{g}^{-1}$ at 400 $\mu\text{g}\cdot\text{mL}^{-1}$ treatment at 20°C and $\approx 20 \mu\text{g}\cdot\text{g}^{-1}$ at the same concentration and a solution temperature of 50°C (D'Aquino *et al.*, 2006). Imazalil penetrates easier and persists longer in citrus rind when applied in a heated solution. A study comparing the effects of solution temperature and concentration showed that higher concentrations always loaded higher residues of IMZ, although that trend was significant at 50°C, but not at 25°C. At 50°C there was an average 5 fold increase of the residue loaded onto the flavedo when compared to the 25°C immersion of lemons into an IMZ (EC) solution (Dore *et al.*, 2009). In 1996, Schirra *et al.* looked at IMZ EC residues loaded at different temperatures and concentrations. A range of 250 to 1500 $\mu\text{g}\cdot\text{g}^{-1}$ showed a linear increase of residues on lemons dipped at 20°C for 3 min. The same treatments applied at 50°C loaded consistently higher levels (≈ 4.5 fold) (Schirra *et al.*, 1996). Trials done looking at dip application of IMZ EC formulation demonstrated that by increasing the solution temperature, the residue loaded onto the fruit increased steadily (temperatures of 20; 30; 40; and 50°C loaded 0.7; 1.4; 3.4; and 5.9 $\mu\text{g}\cdot\text{g}^{-1}$ of IMZ respectively) (Cabras *et al.*, 1999). Schirra *et al.* (2010) found that at a concentration of 300 $\mu\text{g}\cdot\text{mL}^{-1}$ at 50°C loaded higher residues ($\approx 6 \mu\text{g}\cdot\text{g}^{-1}$) than that of a 600 $\mu\text{g}\cdot\text{mL}^{-1}$ solution at 20°C ($\approx 3 \mu\text{g}\cdot\text{g}^{-1}$). The paper concluded that by increasing the temperature of the solution one can use significantly less fungicide to achieve the same residue loading result. In contrast Erasmus *et al.* (2011) observed no correlation between increased solution temperature and residue loading when working with the sulphate formulation of IMZ. The study showed that with a solution temperature between 20 and 35°C, the sulphate form did not react to the increase in temperature. However, other researchers have found a distinct increase in residues as temperature increases when using the EC formulation of IMZ (Schirra *et al.*, 1996;

Smilanick *et al.*, 1997b). It is suggested that the sulphate form of IMZ is somehow influenced by solution temperature, but perhaps only at higher temperatures than those studied by Erasmus *et al.* (2011) looked at (max 35°C) in comparison to Schirra *et al.* (1996) at 50°C. Since then, it has been proven that the IMZ sulphate solution is influenced by temperature under certain conditions of pH and exposure time (Kellerman *et al.*, 2016).

Another interesting aspect of solution temperature is that, although the trend of increased residues with increasing temperature remains true, residues were higher in the albedo of wounds than in the unwounded albedo (Dore *et al.*, 2009). Higher residues in wounds was similarly reported a year later by Dore *et al.* (2010) showing that as concentration increases, wound loading becomes significant compared to unwounded albedo tissue. Erasmus *et al.* (2015a) also reported that wounds load higher residues than surrounding tissue. Larger wounds were seen to load a higher residue than smaller wounds. In addition, the study reaffirmed the principle that heated IMZ solutions control green mould decay more than unheated ones (Dore *et al.*, 2009).

Another benefit of solution temperature is the removal of not only *Penicillium* spp. spores, but also other saprophytic or contaminant pathogens. *Rhizopus stolonifer* (Fr.) Lind is one of the prevalent contaminant pathogens in South African packhouses (Lesar, 2013; Mildenhall *et al.*, unpublished). The rots caused are soft and infection usually occurs through a wound, often in conjunction with other postharvest diseases. *Rhizopus* will decay an entire fruit within 48 hr under warm and moist conditions, and is able to spread to fruit that it has direct contact with (Lesar, 2013). *Rhizopus stolonifer* is very hardy, surviving easily and causes postharvest decay for many crops as well as surviving on any decaying matter (Goos *et al.*, 1967; Yuan *et al.*, 1985). The fungus grows optimally between 20 and 25°C while no growth was observed at 37°C (Yuan *et al.*, 1985). Fungicide solutions are often pasteurized when fruit is absent in order to remove contaminants that have built up during treatment hours (Mildenhall *et al.*, unpublished; Smilanick *et al.*, 1997a; Morris, 1980), particularly in application methods that retain the solution for long periods of time. Morris (1980) demonstrated that the fungicides in the bath did not deteriorate even after extended periods at a temperature of 70°C. Wells and Harvey (1970) showed that hot water of 51 – 55°C could control lesion development in stone fruit inoculated with *Rhizopus stolonifer* and that the effectiveness improved as exposure time increased (0.5 – 3 min). Since *Rhizopus stolonifer* is a contaminant pathogen in South African packhouses (Lesar, 2013), heated solution may help keeping it under control though this has never been investigated thoroughly. The only fungicides reported to control rhizopus rots are SOPP formulations (Smith, 1962), which are no longer used as part of a decay management scheme in South Africa. Packhouse sanitation and careful handling of the fruit, as well as maintaining the cold chain are the best ways to prevent *R. stolonifer* infections (Lesar, 2013). There is some evidence that *Rhizopus* spp. may be resistant to high temperatures. Curing

trials at 35°C for 70 hr saw that *P. digitatum* was reduced, but that *R. stolonifer* infections increased (Del Rio *et al.*, 1992). A slight increase in heat may promote germination as Del Rio and co-workers observed but germination is inhibited at higher temperatures (> 35°C) (Miller *et al.*, 1959). Further work observed detrimental cellular changes to *Rhizopus* spores at 52°C (Baker and Smith, 1970). A different study focusing on hot water treatments observed a reduction of rhizopus rot with a 2.5 min dip at 50°C (Margosan *et al.*, 1997).

A final advantage of using hot water being alluded to by packhouse managers in South Africa, as well as some research claims, is that fruit coming from a warm fungicide bath will dry more readily in the subsequent drying tunnel (Pelser, 1977; Smilanick *et al.*, 1997a; Erasmus, personal communication, 2014). It is also commonly believed that warm water is better at replacing air in wounds, ensuring more effective fungicide delivery. Finally, a warm, dry fruit appears to receive a more uniform wax coating than cold or wet fruit (Smilanick *et al.*, 1997a).

Regardless of the benefits of heated solution, it must always be kept in mind that heat can damage fruit. The temperature of the water and/or the exposure time of the fruit must be carefully managed to prevent rind heat damage (McDonald *et al.*, 1991). Solution temperature can result in physiological damage as seen on Satsuma mandarins at high temperature dips (56°C), which in turn resulted in increased levels of decay (Şen *et al.*, 2010). In another study, heated solutions of 65°C and 75°C effectively controlled blue mould on oranges, yet this resulted in severe fruit injury and discolouration. Lower temperatures of 55°C saw 30% of fruit experience moderate heat damage in the form of rind blemishes (Palou *et al.*, 2001). Another trial looking at solution temperatures from 50 - 58°C gave decidedly lower levels of infection for each 2°C increase. At 58°C just over 94% of green mould was controlled, however heat damage occurred (Puawongphat *et al.*, 2008).

Exposure time

Exposure time is important when using a fungicide. For example, pyrimethanil and fludioxonil loaded higher residues when exposure time was increased from 30 s to 60 s, however, this effect was not noticed with IMZ (EC) dip trials (Schirra *et al.*, 2010). This contradicts dip application trials of IMZ (EC), which distinguished that by increasing the exposure time of the fruit to the solution, residue loading onto the fruit was increased. Times of 30; 90; and 180 s loaded 2.8; 4.1; and 5.6 µg.g⁻¹ of IMZ respectively at a solution concentration of 1200 µg.mL⁻¹ (20°C) (Cabras *et al.*, 1999). The EC formulation of IMZ is much more affected by exposure time than the sulphate form. Both increased as time was increased, however, residue loading by the EC formulation was double after the initial 100 s (Sepulveda *et al.*, 2015). Looking at the sulphate formulation, exposure time of fruit to the IMZ solution had an influence on residue loaded on the fruit, and the subsequent level of green mould curative

control and sporulation inhibition achieved. The longer exposure time results in higher residues (Erasmus *et al.*, 2015a; Kellerman *et al.*, 2016). Increased contact time allows for more of the fungicide to be incorporated by the rind, and residues began to increase as exposure time was lengthened from 2 s to 15 s (Brown and Dezman, 1990). However, it seems there may be a saturation point, because during a 3 min wetting period, less than 1% of the IMZ solution had moved through the cuticle, and this adsorption was not significantly improved by removing the cuticle of all citrus kinds except lemons (Brown and Dezman, 1990). Solution pH plays an important role in conjunction with exposure time and adsorption of IMZ (sulphate) and Erasmus *et al.* (2015a) noted that at pH 3 (20 and 35°C), exposure time of 15 to 540 s showed no increase of residue, however at pH 8, residues loaded rose exponentially as exposure time increased. Additionally, with pH 6 (a pH treatment below the pKa and acceptable for use in solution), exposure time needed to be carefully managed (no more than 45 s) to avoid exceeding the MRL. Kellerman *et al.* (2016) found the same, adding that pH 3 solutions remained very stable regardless of exposure time, but that at pH 6, anything above a 15 s dip at a temperature of 35°C resulted in high levels of IMZ (max 13.10 µg.g⁻¹), exceeding that of the allowed MRL. In packhouses, there are often times when the packline is halted. It can happen that fruit is held in the fungicide bath for extended periods of time, and if the pH is around 6 the MRL could be exceeded, resulting in a rejection during export inspections (Erasmus *et al.*, 2013).

Post-treatment brushing

The majority of work done on IMZ dip application to control *P. digitatum* has involved treating fruit, and then allowing them to air dry before being packed, stored or incubated prior to being evaluated for disease incidence or fungicide residue (Erasmus *et al.*, 2015a). The results gained from this methodology ignores the effect of brushing which is standard procedure in all citrus packhouses following IMZ aqueous application, with anything from 8 to 52 brushes post treatment (Erasmus *et al.*, 2011). Erasmus *et al.* (2015a) examined various factors affecting curative control of green mould, and how brushing affected residue loading and green mould control. The study showed that brushing significantly reduced the amount of residue on the fruit. Fruit treated in a pH 3 solution showed an approximate 30% decrease in residue after brushing; pH 6 treatments loaded higher residues and brushing removed an approximate 60% of residue. However, fruit treated at pH 6 and then brushed still had higher residues than unbrushed pH 3 fruit (Erasmus *et al.*, 2015a). Furthermore, Erasmus *et al.* (2015a) looked at green mould control and noted that brushing did not have a significant influence on the control in pH 6 treated fruit, but at pH 3 the much lower levels of residue loaded resulted in a decrease in curative control. Trials on the effect of washing fruit directly after aqueous application of IMZ EC indicated that washing using tumbler brushes only, decreased the residue by 3%.

With a wax application, washing over brushes led to a significant 31% decrease (Brown *et al.*, 1983). These authors concluded that post treatment brushing reduce residues of other fungicides. The effect may be less with IMZ because of this particular fungicide's ability to penetrate the rind quickly and easily (Brown *et al.*, 1983). Nonetheless, researchers agree that post-treatment brushing is an important factor to consider when analysing experimental residues (Erasmus *et al.*, 2015a; Kellerman *et al.*, 2016).

CONCLUSION

Green mould caused by *Penicillium digitatum*, and the fungicide that most effectively controls it has been studied in detail over the years. Regardless, discernible losses in the South African citrus industry are still recorded. Due to the ever increasing need to improve pack-out and export fruit quality, finding the optimal application method and treatment parameters (temperature, pH, exposure time and concentration) is paramount. It is clear from the literature that all the potential parameters in an imazalil solution play a role in residue loading and influence each other, implying that one factor never ever acts in isolation. By understanding the interactions among treatment variables, a potential precision application of the fungicide IMZ sulphate can be achieved. Imazalil residues on citrus fruit and the resultant green mould control achieved can be influenced and possibly even predicted. Application methods are constantly evolving, and in order to be pro-active in the large and successful South African citrus industry, it is important to evaluate these new applications (such as the heated flooder) timeously.

Additionally, there may be unnecessary action steps in citrus packhouses, such as heating the solution or long exposure times, and it is important to establish the optimal parameters for these aspects to minimise costs and other detrimental issues such as residue exceedances. Conversely, unforeseen benefits to the current treatment methodology may be going unnoticed, such as control of contaminant organisms, and it is important to be aware of any such aspects.

Aim

The aim of this study is to further the knowledge already in place about aqueous IMZ application, and to answer questions about precision application of this fungicide to aid the South African citrus industry in better green mould control. Parallel to the precision application of imazalil in a cold bath, the effect of solution temperature on eradicating contaminants was investigated.

Objectives

From this literature study the following objectives were outlined for this study:

- Investigate the residue loaded from IMZ sulphate with respect to pH in conjunction with temperature and exposure time in aqueous treatments, particularly at pH 3 and 4, which have not yet been studied.
- Explore the use of the heated flooder as an alternative aqueous application to that of the fungicide bath, in conjunction with solution temperature and pH.
- Investigate the effect of solution temperature, and in particular the action of IMZ in cold water.
- Determine the survival of *Rhizopus stolonifer* spores at various water temperatures as an indication of contaminants that could build up in the fungicide bath.

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CHAPTER 2

Influence of pH and temperature in a heated flooder application on imazalil residue loading, citrus green mould control and sporulation inhibition

ABSTRACT

Green mould (caused by *Penicillium digitatum*) is the largest contributor of loss due to postharvest decay of citrus fruit. Imazalil (IMZ) in the IMZ sulphate formulation is the most important fungicide in green mould management in South African citrus packhouses. Recent studies highlighted the importance of pH and temperature of IMZ sulphate solutions, as well as exposure time of fruit to the solution, but were focussed on dip application as the common IMZ application method in South Africa. Heated flooder (in-line drench or cascade) application is a novel means of IMZ application and studies on its optimal use and efficacy were needed. Variables that were studied included the effects of pH (3, 4, 5 and 6), temperature of the solution (45, 55 and 65°C at pH 6), and concentration (250 or 500 µg.mL⁻¹) in a time of 8 s. Imazalil residue loading models were developed for the effects of pH and temperature. Levels loaded on Satsuma mandarin, lemon, navel and Valencia orange fruit increased with increasing pH range, increasing temperature range and increasing concentration, and were generally in the range between 0.4 and 3.0 µg.g⁻¹. The Maximum Residue Limit (MRL) of 5.0 µg.g⁻¹ was often exceeded at pH of 6 and temperatures of 55 and 65°C. At residue levels within the MRL, IMZ application by means of the heated flooder offered excellent curative control of 24-hour old infections, excellent protective control as well as sporulation inhibition. Sporulation inhibition of *P. digitatum* from green mould lesions was modelled on residue levels, and 90% inhibition was obtained at residue levels of 1.84 to 2.47 µg.g⁻¹.

INTRODUCTION

South Africa has a successful citrus industry and exported 115 million cartons during the 2015 season (Edmonds, 2016; PPECB, 2016). With production being situated in the Southern hemisphere, citrus is often supplied to markets in the Northern hemisphere who have opposite seasonality, with South Africa's main markets being Europe and Asia. Unfortunately, with markets geographically very far from the production areas, the fruit spend weeks being shipped, and not only need to arrive in a satisfactory condition, but should remain so during further storage and marketing (Hough, 1969; Pelsler, 1977).

The largest contributor to loss in a shipment of citrus is fungal decay, with *Penicillium digitatum* Pers. Sacc. being the foremost cause (Eckert and Eaks, 1989). *Penicillium digitatum* is an obligate wound pathogen that turns damaged tissue into a soft, water soaked lesion within 5 to 7 days. As the infection continues, white mycelia grow outwards from the initial point of infection and are followed by sporulation, also originating at the infection point (10 - 14 days later). The fungus is characterised by olive green spores, hence the disease's common name 'green mould' (Fawcett and Lee, 1926; Eckert and Brown, 1986; Eckert and Eaks, 1989). Growth is inhibited at storage and transport temperatures of 3 – 5°C. However, once in a favourable environment, the mould will continue to proliferate, with infected fruit decayed in a matter of 2 – 3 days (Eckert and Eaks, 1989).

Imazalil (IMZ; 1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl)-1H imidazole) (Siegel *et al.*, 1977; FAO, 2001) is the most effective fungicide against green mould, and is still widely used in the international citrus industry (Smilanick *et al.*, 1997; Erasmus *et al.*, 2011; Besil *et al.*, 2016). Imazalil is able to eradicate an infection that occurred prior to treatment (curative action), protect the fruit from infections that occur after treatment (protective action), and inhibit the formation of *P. digitatum* spores (sporulation inhibition) (Erasmus *et al.*, 2011; Sepulveda *et al.*, 2015). Sporulation inhibition is very important in combating the problem of soilage, a cosmetic problem whereby spores from infected fruit dusts the surface of healthy, sound fruit (McCornack and Brown, 1977; Eckert and Kolbezen, 1978; Eckert and Eaks, 1989). Such fruit is unmarketable until it has been cleaned and repackaged, thus adding cost to the value chain. The Maximum Residue Limit (MRL) for IMZ on citrus is 5 µg.g⁻¹ for the European Union and 10 µg.g⁻¹ for the USA (DAFF, 2008; AgrIntel, 2015). Despite concerns, MRL's are very rarely exceeded (Fernández *et al.*, 2001; Blasco *et al.*, 2006; Erasmus *et al.*, 2011). South African packhouses actually load much lower levels (≈ 1.0 µg.g⁻¹) (Erasmus *et al.*, 2011). An IMZ residue level of between 1.0 and 3.0 µg.g⁻¹ can offer adequate levels of control, but may fail to completely inhibit sporulation (Kaplan and Dave, 1979; Schirra *et al.*, 1996; Smilanick *et al.*, 1997; Erasmus *et al.*, 2011). Imazalil is available in two formulations, namely an emulsifiable concentrate (EC), and a sulphate salt form in either soluble powder (SP) or soluble granules (SG) (Pelser, 1980; Sepulveda *et al.*, 2015). The local industry uses the sulphate form in all aqueous applications at a registered concentration of 500 µg.mL⁻¹ (Pelser and La Grange, 1981; Fourie and Lesar, 2008; Erasmus *et al.*, 2011). The EC formulation is solely used in the wax application (Erasmus *et al.*, 2011; Njombolwana *et al.*, 2013a, b). South Africa favours the sulphate formulations because, unlike the EC formulation, it is more stable, needs less agitation and does not settle out of suspension, making it unavailable as a fungicide (Eckert, 1977; Altieri *et al.*, 2005; Erasmus *et al.*, 2011). While IMZ blends well into commercial fruit wax, such wax applications typically need a much higher concentration of IMZ than an

aqueous application in order to offer a similar level of control (Brown, 1984; Njombolwana *et al.*, 2013a).

The most popular method of applying IMZ in South Africa is by means of a fungicide bath (dip tank) (Pelser and La Grange, 1981; Erasmus *et al.*, 2011). Imazalil sulphate solutions in these tanks differ widely in terms of temperature (12 - 45°C), solution pH (3 – 8), exposure time of the fruit (16 – 107 s) and concentration of the IMZ (typically 250 – 615 µg.mL⁻¹) (Erasmus *et al.*, 2011). Despite variations, the application method is very effective if fruit can be fully submerged during treatment (Eckert, 1977) and offers excellent curative control (Dore *et al.*, 2009; Erasmus *et al.*, 2011, 2013, 2015a, b). It is superior to application methods such as sprays, overhead brushes saturated with solution (Eckert, 1977; Pelser, 1977; Brown and Dezman, 1990; Bronkhorst *et al.*, 1993; Erasmus *et al.*, 2011) or Imazalil Thin Film Treatment (ITFT) (Altieri *et al.*, 2013).

Since the management of a fungicide bath can be problematic, especially considering the variation in dip application parameters (Erasmus *et al.*, 2011), there has been a need in the South African industry for a more manageable application method. An innovative approach used in some citrus producing regions, often referred to as a flooder or cascade, is an in-line drench of fruit with a heated and recirculated IMZ solution. The concept of fungicide application using recirculating sprays and cascades over rollers or brushes is not new (McCornack, 1970; Smoot and Melvin, 1970; Wild *et al.*, 1975; Brown, 1977). Recirculating systems are advantageous in comparison to total loss systems as they retain the solution, thereby conserving fungicides and water. Furthermore, the solution temperature is more easily managed in a closed system. Higher volume application is often much more effective than low volume (Förster *et al.*, 2007; Kanetis *et al.*, 2008), but high volume applications cannot readily be considered in total loss systems, due to the vast volume of solution needed, and the economic and ecological footprints associated with that. In a recirculating system a smaller overall volume is used, but a greater volume of solution comes in contact with fruit, leading to better coverage, which results in increased decay control (Brown and Dezman, 1990; Kanetis *et al.*, 2008). One disadvantage of recirculating systems is that, because the solution is kept, contaminants can build up and the fungicide concentrations reduced (Eckert, 1977); however, these are the same issues seen in the fungicide bath and can be easily mitigated with proper packhouse management (Lesar, 2008; Lesar and Erasmus, 2014).

Smilanick *et al.* (2003) studied a heated drench application that applied fungicide solution onto fruit in a high volume, low pressure drench over rotating brushes. The elevated temperature (35°C) was found to significantly reduce green mould incidence at both treatment times examined (15 s and 30 s). The heated in-line drench, cascade or flooder differs from other drench applications, as well as the fungicide bath, in that the water is contained in a closed tank, and constantly circulated and heated. The weirs are covered aiding in heat

retention and protection from outside contamination by dirt and debris. The fruit going through the in-line drench are rolled over rotating brushes, and pass through several laminar flows of high volume waterfalls of fungicide solution. This ensures complete coverage of the fruit compared to the fungicide bath where floating fruit may not have all sides suitably exposed to the fungicide. The JBT Heated Flooder (JBT FoodTech, Brakenfell, Cape Town, South Africa), as well as other similar in-line drenchers are becoming more common place in South Africa. Work done by Erasmus *et al.* (unpublished) shows that it has the potential to be an alternative to the fungicide bath application of IMZ, also offering superior green mould control. However, since the flooder is new to the South African citrus industry, various parameters still need to be investigated to ensure optimal application of IMZ. It is important to look at solution parameters such as IMZ concentration, solution temperature, and solution pH in conjunction with the application method, as these factors influence IMZ's ability to adhere to the fruit exocarp in the form of a residue (Brown and Dezman, 1990; Erasmus *et al.*, 2011, 2013).

It has been found that IMZ's solubility in water is pH sensitive: since it is a weak base, it is most soluble at a low solution pH (FAO, 2001). Erasmus *et al.* (2011, 2013, 2015a) and Kellerman *et al.* (2016) have shown that solution pH and temperature play a crucial role in residue loading of IMZ sulphate. Both researchers have demonstrated that at pH 3, IMZ residue loading is low and stable regardless of other solution parameters but that at pH 6 and above, residue loading increased linearly to levels far exceeding the MRL. Historically, the fact that IMZ is pH sensitive was not considered, but addition of the IMZ sulphate form to neutral pH water drops the solution pH to around 3 due to the presence of the sulphuric acid molecule (FAO, 2001). South African water specifications are often outside of the ideal values and pH levels in packhouses are often higher, because different municipal water supplies vary in pH, and packhouses often source their water from rivers or dams, boreholes and rainwater (Savage *et al.*, unpublished). Effective management of IMZ application therefore depends on adjustment of the solution pH in order to maintain availability of the active, creating the potential to achieve the maximum activity from the product through simultaneous regulation of temperature (Erasmus *et al.*, 2011, 2013, 2015a).

Although hot water treatments by itself can be effective at high temperatures (Erkan *et al.*, 2005; Şen *et al.*, 2010), the addition of a fungicide always improves that control (Schirra *et al.*, 1997; Puawongphat *et al.*, 2008). Imazalil penetrates easier and persists longer in citrus rind when applied in a heated solution (Schirra *et al.*, 1996, 2010; Cabras *et al.*, 1999; Dore *et al.*, 2009). Dore *et al.* (2009) found that as concentration and temperature increased concurrently, residue loading increased and residue persistence remained much more stable. Finally longer exposure times also resulted in higher residues (Erasmus *et al.*, 2015a; Kellerman *et al.*, 2016).

The aim of this study was to determine the influence of pH and temperature in a heated floodler application on IMZ residue loading and subsequent green mould control and sporulation inhibition.

MATERIALS AND METHODS

Fruit

Export quality, freshly harvested lemon, Satsuma mandarin, navel orange and Valencia orange citrus fruit were collected from various packhouses in the northern citrus producing regions of South Africa (Mpumalanga and Limpopo provinces) during the 2014 season. Eight trials were conducted, twice on each citrus variety. Fruit were hand selected for uniformity in size and quality. Upon arrival at the Citrus Research International laboratories in Nelspruit, South Africa, the fruit were washed with a 75 $\mu\text{g}\cdot\text{mL}^{-1}$ chlorine high pressure spray (2 bar and a flow rate of 2 $\text{L}\cdot\text{min}^{-1}$) over rotating brushes. This was done to clean the fruit, reduce spore load on the fruit coming from the orchard and to simulate normal packhouse procedure, where fruit is washed before being treated. Fruit were stored at 4°C for no more than 4 days before trials were conducted. Fruit were removed from cold storage 1 day before the trials commenced in order to reach ambient temperature ($\approx 22^\circ\text{C}$) and to evaporate condensation.

***Penicillium digitatum* isolate and inoculation**

For all trials an IMZ sensitive isolate (STE-U 6560, Department of Plant Pathology, University Stellenbosch, South Africa) of *P. digitatum* was cultured on chloramphenicol amended potato dextrose agar (PDA+; Difco Potato Dextrose Agar, Becton, Dickinson and Company, Sparks, MD, USA; Chloramphenicol, Chlorcol, 250 mg CAP 500, Adcock Ingram, Midrand, Gauteng, South Africa) at 25°C for 10 – 14 days. Cultures were flooded with sterile water amended with one drop of Tween 20 to a concentration of $\approx 0.01 \mu\text{L}\cdot\text{mL}^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA). Conidia were dislodged from cultures using a sterilized bent glass rod spreader and filtered through a double layer of cheesecloth into 200 mL of the Tween 20 amended sterile water. Concentration of the spore suspension was adjusted to 1×10^6 spores. mL^{-1} by means of a spectrophotometer (absorbance of 0.100 at 425 nm; Cecil CE1011, Lasec, Midrand, Gauteng, South Africa) (Morris and Nicholls, 1978; Eckert and Brown, 1986). Spores were maintained in a uniform suspension by use of magnetic stirrers throughout inoculation.

Two methods of inoculation were used during trials. In order to test curative and protective ability of imazalil against infections, a custom made wounding tool was used. This tool had a flattened cylindrical tip that mimicked the cut stem of a citrus fruit and delivered a wound 1 mm in diameter and 2 mm deep through the flavedo into the albedo of the fruit. Wounds were made around the stem end of the fruit except in the case of lemons, which

typically have a more oblong shape, when the wounds were made on the sides of the fruit. Four wounds were made on each fruit, in a square pattern with a distance of approximately 4 cm between each. There were 12 fruit in each replicate, with 3 replicates per treatment during a trial. Curative inoculations occurred 24 hours prior to treatment, while protective inoculations occurred 24 hours after treatment.

The second method of inoculation was to test the sporulation inhibition of imazalil. A sterile 0.60 x 25 mm gauge needle (NN*2325R, Terumo Corporation, Tokyo, Japan) was used to inject 0.2 mL of spore suspension between 1 and 2 cm deep into the shoulder on the stem end of a fruit. Only one point of entry was made per fruit. The sporulation inoculation method was chosen to ensure rot while still enabling the effects of the IMZ residue to be seen clearly on the surface of the fruit (Brown *et al.*, 1983; Brown and Dezman, 1990). There were 12 fruit in each replicate with 3 replicates per treatment during a trial. Sporulation inoculations were done approximately 30 min before treatment.

Residue analysis

Fruit samples for residue analysis consisted of two replicates of six uninoculated fruit added to the first and last replicate of each treatment. After treatment, they were stored in plastic bags at 4°C for no more than a week before being chopped and blended (Salton Elite Blenders, Amalgamated Appliance Holdings Limited, Reuven, South Africa) into pulp using distilled water to dilute the fruit into a soft, paste-like consistency. The dilution factor for each sample was recorded. The pulp was frozen into sub-samples that were couriered to an accredited analytical laboratory (Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa) for IMZ (chloramizol) residue analyses. Acetonitrile was used for extraction, which was followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography tandem mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA) according to an accredited procedure. Residue data received from the analytical laboratory were recalculated using the dilution factor recorded when the pulp was blended.

Treatment conditions

All eight trials conducted had the same parameters, and fruit was treated using an experimental packline, which simulated commercial packlines. A custom-built, experimental unit of the heated flooder (JBT FoodTech, Brakenfell, Cape Town, South Africa) was used in all cases (Fig. 1). The unit was 185 cm in length and 88 cm wide. Treatment involved fruit being moved along 14 rotating brushes (tufted polypropylene brushes at 85 rpm), passing through five laminar waterfalls of the heated fungicide solution. The solution flow rate was 380 L.min⁻¹. The fruit were pushed at a controlled speed by several rotating metal sweepers for a total contact time of 8 s. The experimental flooder has a tank capacity of 375 L.

Fruit batches for the curative, protective and sporulation inhibition treatments were treated with the IMZ solution applied using the flooder at 250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ IMZ at pH 3, 4, 5 and 6. At pH 6, the effect of solution temperature at 45, 55 and 65°C was evaluated. All treatments had an 8 s contact time. Imazalil sulphate (Imzacure, 750 $\text{g}\cdot\text{kg}^{-1}$ SG, ICA International Chemicals, Stellenbosch, South Africa) was added to a concentration of 250 $\mu\text{g}\cdot\text{mL}^{-1}$ and the temperature was raised to 45°C. The pH of the solution was measured with a portable pH meter (HI 98121 Waterproof pH/ORP & Temperature meter, Hanna Instruments, Morninghill, Johannesburg, South Africa) and the pH was adjusted with hydrochloric acid (32%; Merck (Pty.) Ltd., Modderfontein, Gauteng, South Africa) to a pH of 3. After treatment of the respective fruit batches for curative, protective and sporulation inhibition treatments, the pH of the IMZ solution was increased using sodium bicarbonate at $\approx 0.01\%$ increments (NaHCO_3 ; Saarchem uniLAB, Merck Chemicals (Pty) Ltd., Wadeville, Gauteng, South Africa) to 4, and another set of fruit batches was treated. The protocol was repeated twice more, at an IMZ solution of pH 5 and 6. After the pH 6 treatment at 45°C, the temperature was raised to 55°C and 65°C for subsequent treatments. The flooder was then completely drained, rinsed out twice with clean water, and refilled with water. The water was adjusted to an IMZ concentration of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ and the same processes as explained with the 250 $\mu\text{g}\cdot\text{mL}^{-1}$ treatments were repeated. Throughout treatment, temperature and pH were measured and maintained. Untreated but inoculated fruit acted as controls for the treatments.

Incubation and treatment evaluation

After IMZ treatment with the flooder, the fruit followed a commercial packline set up by moving over 14 rotating brushes under an airknife (± 100 nozzels supplying a stream of air onto the fruit to remove moisture) and then moving through an unheated forced air drying tunnel. No wax coating was applied to the fruit. The dry fruit were packaged stem end facing upward on SFT13 nectarine trays (Huhta-maki South Africa (Pty) Ltd., Atlantis, South Africa) placed in lock-back stonefruit cartons (115 mm; Mpact, Epping, South Africa).

The cartons were then covered in clear polyethylene bags (50 MIC, Lanpack Manufacturers C.C., Woodstock, Cape Town, South Africa) with the end not sealed but folded under the carton. Four to six small (≈ 5 mm diameter) holes were made to reduce humidity and moisture build-up, which could modify the atmosphere and in turn, inhibit sporulation. The cartons were stacked and stored at ambient temperature ($\approx 22^\circ\text{C}$) for 4 days for curative and protective inoculations, and 10 days for sporulation inoculations.

Evaluations for curative and protective treatments were done by recording the number of infected wounds on each fruit. Early infection was visualised using a near-UV light (UV-A at 365 nm, Labino Mid-light, www.labino.com), which caused infected wounds to fluoresce bright yellow (Njombolwana *et al.*, 2013a). In the sporulation inhibition fruit batches,

sporulation was evaluated for each individual fruit using a rating index of 1 – 6: 1 = complete sporulation inhibition i.e. completely white fruit; 2 = sporulating area was small ($\approx 20\%$); 3 = sporulating area larger than a quarter of the fruit, but smaller than half of the fruit ($\approx 40\%$); 4 = sporulating area larger than half of the fruit, but smaller than three quarters of the fruit ($\approx 60\%$); 5 = sporulating area larger than three quarters of the fruit, but smaller than the whole fruit ($\approx 80\%$); and 6 = sporulating area covering the whole fruit (= 100%). Fruit that showed no sign of infection was regarded as missing data points.

Residue loading in a commercial flooder

Residue loading following IMZ application with a commercial flooder was assessed in an operational packhouse in the northern part of South Africa. The flooder had very similar parameters to the experimental unit described above. The commercial unit differed in that it was slightly wider and had 16 nylon brushes rotating at 100 rpm. Immediately following the flooder, the packline had 16 donut sponge rollers, a drying tunnel and wax applicator. IMZ at $500 \mu\text{g}\cdot\text{mL}^{-1}$ was applied to lemon (cv. Eureka) and early navel oranges (cv. Navelina) at temperatures of 25, 35 and 45°C . The pH was managed and adjusted to 3 or 6, and fruit samples were collected in various treatment combinations, namely after the flooder, after the donuts and after a wax coating application with a commercial citrus wax coating (PolyOrange polyethylene coating, 18% solids, Citrashine (Pty) Ltd., Johannesburg, South Africa) incorporated with either $1000 \mu\text{g}\cdot\text{mL}^{-1}$ or $2000 \mu\text{g}\cdot\text{mL}^{-1}$ IMZ (EC). Fruit were collected for residue analysis and prepared as described above.

Statistical analysis

For curative and protective infection data (percentage wounds infected per fruit) were normalised relative to the data for untreated control treatments; percentage control data were subsequently used. All data were subjected to analyses of variance (ANOVA) and Fisher's least significant difference test at 95% confidence interval to compare means. To demonstrate trends between residues loaded from treatments, or the levels of control achieved, values were regressed using an appropriate non-linear regression model. The coefficient of determination (R^2) was used to demonstrate goodness of fit. Where possible, the model was used to predict either residue results or percentage control that would be achieved through treatment. XLSTAT (version 2016.7.01, Addinsoft, www.xlstat.com) was used for the analysis described. Regression models with reliable fits were used to determine residue levels that would be indicative of 50% or 90% control (Erasmus *et al.*, 2015b).

RESULTS

Statistical analyses for the 250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ solutions were done separately. Results are presented as 'effect of pH' and 'effect of temperature'. The former is to determine the effect of pH at the range of 3, 4, 5 and 6 at a temperature of 45°C, while the latter determines the effect of temperature at pH 6, using a temperature range of 45, 55, and 65°C. To increase the robustness of the data, and because no meaningful interpretation could be drawn from batch differences, despite frequent statistical differences between them, batches of the same citrus types were combined. Citrus types were analysed separately as the type effect was always significant in combined analyses. Analysis of variance results are described below.

Imazalil residue loading

The effect of pH at a solution temperature of 45°C

Analysis of variance for imazalil (IMZ) residue data for all four citrus types treated at a concentration of either 250 $\mu\text{g}\cdot\text{mL}^{-1}$ or 500 $\mu\text{g}\cdot\text{mL}^{-1}$ showed that pH was a significant effect ($P < 0.026$). Therefore, data were regressed using a Three Parametric Logistic non-linear regression model with the function $Y = \text{pr}3/(1+\text{Exp}(-\text{pr}1-\text{pr}2*X1))$ and fitted with good R^2 values (0.635 – 0.865 for the 250 $\mu\text{g}\cdot\text{mL}^{-1}$ treatment and 0.549 – 0.966 for 500 $\mu\text{g}\cdot\text{mL}^{-1}$; Table 1). Residue levels increased as pH increased for both concentrations. Residue loading was greater and steeper for 500 $\mu\text{g}\cdot\text{mL}^{-1}$ concentrations than 250 $\mu\text{g}\cdot\text{mL}^{-1}$. For both concentrations, the highest residues levels were recorded on Valencia (0.83 and 4.04 $\mu\text{g}\cdot\text{g}^{-1}$), the lowest on navel oranges (0.29 and 2.06 $\mu\text{g}\cdot\text{g}^{-1}$), while intermediate and similar levels were recorded on lemon (0.48 and 3.18 $\mu\text{g}\cdot\text{g}^{-1}$) and Satsuma mandarin (0.43 and 3.35 $\mu\text{g}\cdot\text{g}^{-1}$; Fig. 2A and B).

The effect of temperature at a solution pH of 6

Analysis of variance for IMZ residue data for all four citrus types for either concentration, showed a significant effect for the temperature range ($P < 0.008$), except lemon at 250 $\mu\text{g}\cdot\text{mL}^{-1}$, which presented a significant effect at a 90% confidence level ($P = 0.080$). Data were regressed using a Three Parametric Logistic non-linear regression model with the function $Y = \text{pr}3/(1+\text{Exp}(-\text{pr}1-\text{pr}2*X1))$ and fitted with good R^2 values (0.426 – 0.954 for the 250 $\mu\text{g}\cdot\text{mL}^{-1}$ treatment and 0.722 – 0.952 for 500 $\mu\text{g}\cdot\text{mL}^{-1}$; Table 2). Residue levels increased as temperature increased for both concentrations. Residue loading was greater and steeper for 500 $\mu\text{g}\cdot\text{mL}^{-1}$ concentrations than 250 $\mu\text{g}\cdot\text{mL}^{-1}$. For both concentrations, progressive residue loading for all citrus types was shown. Valencia once again loaded the highest (2.84 – 12.50 $\mu\text{g}\cdot\text{g}^{-1}$), navel the lowest (1.10 – 5.23 $\mu\text{g}\cdot\text{g}^{-1}$), and lemon and Satsuma mandarin acting more moderately and similarly (1.48 – 9.95 and 1.68 – 15.85 $\mu\text{g}\cdot\text{g}^{-1}$ respectively), except where

Satsuma mandarin loaded very high levels with the 500 $\mu\text{g}\cdot\text{mL}^{-1}$ dose at 65°C, pH 6 (Fig. 3A and B).

Green mould control

The effect of pH at a solution temperature of 45°C

Curative control

The pH was not a significant effect for Satsuma mandarin and navel at 250 $\mu\text{g}\cdot\text{mL}^{-1}$ and for lemon at 500 $\mu\text{g}\cdot\text{mL}^{-1}$ ($P > 0.206$), but were significant at a 90% confidence level ($P < 0.091$) for the remainder of the treatments. The mean curative control achieved was consistently high $> 89\%$, except for some lower pH treatments on Satsuma mandarin with control levels of 71.55 – 82.36% (Table 3). Regression analysis gave very poor fits (results not shown) and models could not be used to predict residue levels required to achieve 90% control.

Protective control

Analysis of variance for protective control data showed that pH was a significant effect for all citrus types ($P < 0.001$). All treatments expressed a level of control above 75% (Table 4), but on all citrus types protective control was generally significantly better at pH 5 and 6 (93.99 – 100%), compared with pH 3 and 4 (74.89 – 98.31%). Regression analysis gave poor fits and, residue levels required to achieve 90% protective control could not be modelled (results not shown).

Sporulation inhibition

Analysis of variance for sporulation inhibition data indicated that pH had a significant effect at both tested concentrations for all citrus types ($P < 0.0001$). Regression analysis was conducted on the combined residue data from 250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ treatments and sporulation inhibition data. The non-linear regression using the afore-mentioned three parameter logistic model presented excellent fits with $R^2 > 0.906$ for all but Satsuma mandarin ($R^2 = 0.573$; Table 5). Sporulation inhibition increased very quickly relative to IMZ residue levels (Fig. 4). The model predicted that IMZ residue levels between 1.29 and 2.47 $\mu\text{g}\cdot\text{g}^{-1}$ were required to achieve 90% sporulation inhibition. Between 0.47 and 1.49 $\mu\text{g}\cdot\text{g}^{-1}$ were predicted necessary for only 50% inhibition. Higher residue levels were required for lemon and Valencia orange (2.47 and 2.34 $\mu\text{g}\cdot\text{g}^{-1}$ for 90% inhibition, respectively) compared with Satsuma mandarin and navel orange (1.84 and 1.29 $\mu\text{g}\cdot\text{g}^{-1}$, respectively; Table 5).

The effect of temperature at a solution pH of 6

Curative control

Analysis of variance showed a significant temperature effect for lemon and navel in the 250 $\mu\text{g}\cdot\text{mL}^{-1}$ solution ($P = 0.036$ and $P = 0.019$, respectively) while Valencia citrus was the only fruit type where temperature played a significant role at 500 $\mu\text{g}\cdot\text{mL}^{-1}$ ($P = 0.006$). For all other

treatments the temperature effect was insignificant ($P > 0.168$). The mean curative control achieved was high $> 77.69\%$ (Table 6), with more consistent control at $500 \mu\text{g.mL}^{-1}$ ($93.38 - 100\%$); Satsuma mandarin expressing the worst levels of control and only lemon demonstrating almost 100% control. At $500 \mu\text{g.mL}^{-1}$ the best control levels were observed on lemon at 100% , but $> 90\%$ control was observed on the other citrus types. No clear trend could be seen of an increasing relationship between curative control and these high temperatures at pH 6.

Protective control

Analysis of variance indicated that temperature was not a significant effect, and in some cases ANOVA could not be performed due to the uniform 100% control achieved (ANOVA not shown). Protective control was never lower than 97.94% which was for Satsuma mandarin at 45°C , while consistently close to 100% protective control was observed on other citrus types (results not shown).

Sporulation inhibition

Analysis of variance for sporulation inhibition data indicated that temperature was a significant effect for the $250 \mu\text{g.mL}^{-1}$ solutions ($P < 0.0001$). A trend of sporulation inhibition increasing as temperature increased was observed for the $250 \mu\text{g.mL}^{-1}$ concentration. Very high levels of sporulation inhibition ($> 89.58\%$) were observed on all citrus types at 55°C , with levels close to 100% sporulation inhibition observed at 65°C . All types had the most inconsistent sporulation inhibition at 45°C ($25.93 - 100\%$). At $500 \mu\text{g.mL}^{-1}$, inhibition values ranged from 95.21 to 100% at $500 \mu\text{g.mL}^{-1}$ (results not shown). Rind damage in the form of browning was noted on both lemon and Satsuma mandarin citrus types treated at 55 and 65°C at either concentration (Fig. 5). Damage was higher at 65°C , but was not quantified due to the mycelial growth on the fruit, which, although decreased and inconsistent, was still present and obscured the brown discolouration.

Commercial flooder

Analysis of variance for IMZ residue levels on lemon fruit showed that there were significant temperature x treatment ($P = 0.001$) and pH x treatment ($P = 0.031$) interactions, however, for simplicity, the 3-way interaction of pH x temperature x treatment ($P = 0.217$) is discussed. Imazalil residue data on navel fruit showed a 3-way interaction of pH x temperature x treatment ($P = 0.000$). From the mean values in Table 7 it can be seen that residue loading increased with increasing temperature and increasing pH. The use of donuts after the flooder treatment reduced the residue by an average factor of 1.5. The addition of a wax coating augmented with IMZ increased the residue in almost all cases, with the $2000 \mu\text{g.mL}^{-1}$ IMZ wax application often resulting in higher levels of IMZ residue than the $1000 \mu\text{g.mL}^{-1}$ IMZ wax application.

For lemon treated at 45°C in a 500 µg.mL⁻¹ solution, the commercial flooder loaded an average 0.86 µg.g⁻¹ after the flooder alone, and 0.37 µg.g⁻¹ after fruit had rolled over donuts at pH 3. At pH 6, the commercial flooder loaded 2.45 µg.g⁻¹, which decreased to 1.70 µg.g⁻¹ after donuts were used. For navel treated under the same conditions, pH 3 solutions loaded 0.74 µg.g⁻¹ and 0.34 µg.g⁻¹ after the flooder and after donuts, respectively. At pH 6, the residue on navel after the flooder was 2.09 µg.g⁻¹ and after donuts, 1.63 µg.g⁻¹ (Table 7).

The residue loading model developed in this study predicted 0.71 µg.g⁻¹ residue on lemon that had been brushed at pH 3 and 45°C, while the commercial flooder loaded 0.86 µg.g⁻¹ before the donuts and 0.37 µg.g⁻¹ after the donuts. For the pH 6 and 45°C solution on lemon, the model predicted a residue of 3.20 µg.g⁻¹. The commercial flooder loaded 2.45 µg.g⁻¹ before the donuts and 1.70 µg.g⁻¹ after the donuts. The prediction for navel from a pH 3 and 45°C solution predicted 0.52 µg.g⁻¹ residue while the commercial flooder loaded 0.74 µg.g⁻¹ before the donuts and 0.34 µg.g⁻¹ after the donuts. For the pH 6 and 45°C solution on navel, the model predicted a residue of 2.04 µg.g⁻¹. The commercial flooder loaded 2.09 µg.g⁻¹ before the donuts and 1.63 µg.g⁻¹ after the donuts (Table 7; Table 8).

DISCUSSION

The influence of heated flooder solution parameters on IMZ residue loading on citrus fruit was clearly determined in this study: higher levels of IMZ residue were loaded by increasing the solution temperature, pH and/or concentration of the IMZ sulphate solution. Based on residue levels, sporulation inhibition could successfully be modelled, but similar models could not be developed for curative and protective control due to the very high levels of control observed in this study. Maximum Residue Limits (MRL) were exceeded at high temperatures (55 and 65°C) at pH 6, and heat damage was also a concern on lemon and Satsuma mandarin.

The results in this study demonstrate clear trends, but also confirm previous studies that found that different citrus types react differently in their susceptibility to green mould, as well as the amount of IMZ residue loaded (Nadel-Schiffmann and Littauer, 1956; Schirra *et al.*, 2008; Kellerman *et al.*, 2016). Our study also indicated significant differences between different batches of the same citrus type, but these were regarded as a statistical block-effect in order to increase the robustness of the findings. Batch differences, due to variations in fruit maturity, climate and cultivar, are often observed in postharvest citrus pathology research (Njombolwana *et al.*, 2013a; Erasmus *et al.*, 2015a, b; Kellerman *et al.*, 2016). These are near impossible to avoid as postharvest research is altogether dependent on fruit that becomes available throughout a season. Citrus susceptibility to green mould increases as fruit matures (Smoot and Melvin, 1961). The susceptibility and maturity relationship may in part be due to

the pH of the albedo tissue that has shown to decrease in lemons (Smilanick *et al.*, 2005), and where yellow lemons are more likely to decay compared to green ones (Eckert, 1995).

In this study, it was noted that residue levels increased significantly with an increase in solution pH. It is well documented that IMZ loads more residues with increasing pH, and more so when temperature and exposure time of the solution are increased too (Brown and Dezman, 1990; Schirra *et al.*, 1996; Smilanick *et al.*, 1997; Cabras *et al.*, 1999; D'Aquino *et al.*, 2006; Erasmus *et al.*, 2011, 2013, 2015a; Kellerman *et al.*, 2016). Imazalil solutions at pH 3 have been shown to be very stable with low residue loading, but at pH 6, residues are increased and easily exceeded the MRL of $5 \mu\text{g}\cdot\text{g}^{-1}$ after a 45 s exposure time (Erasmus *et al.*, 2011; Kellerman *et al.*, 2016). A short exposure time (8 s) was used in this study and therefore at $500 \mu\text{g}\cdot\text{mL}^{-1}$ the pH 6 treatment at 45°C gave a maximum residue of below the MRL at $\approx 4.04 \mu\text{g}\cdot\text{g}^{-1}$. Generally the $250 \mu\text{g}\cdot\text{mL}^{-1}$ dose loaded lower residue than the $500 \mu\text{g}\cdot\text{mL}^{-1}$ solution; however, at pH 3 the average residues loaded were comparable (≈ 0.51 and $0.85 \mu\text{g}\cdot\text{g}^{-1}$, respectively). It is possible that at a solution of pH 3, concentration, like exposure time, may not be a major influencing factor. A similar observation was made with treatments involving the cascade application (an application similar to the flooders) of IMZ sulphate that showed that very similar residues were loaded at $1000 \mu\text{g}\cdot\text{mL}^{-1}$ treatment and at $2000 \mu\text{g}\cdot\text{mL}^{-1}$ treatment (0.5 and $0.4 \mu\text{g}\cdot\text{g}^{-1}$, respectively). In the cascade, IMZ was applied at ambient temperature and at pH 3 for both concentrations (Besil *et al.*, 2016).

The effects of concentration are more noticeable at high temperatures. In our study, the $500 \mu\text{g}\cdot\text{mL}^{-1}$ treatment loaded much higher levels ($\approx 6.32 \mu\text{g}\cdot\text{g}^{-1}$) than the $250 \mu\text{g}\cdot\text{mL}^{-1}$ ($\approx 3.52 \mu\text{g}\cdot\text{g}^{-1}$) solution, such that at 65°C the MRL was always exceeded regardless of citrus type. The effects of higher temperatures loading higher residues, especially in conjunction with an increase in concentration has been noted previously (Schirra *et al.*, 1996; Smilanick *et al.*, 1997; Cabras *et al.*, 1999; D'Aquino *et al.*, 2006; Kellerman *et al.*, 2016). It is important to note that the temperature range was conducted at a solution pH of 6 where it is known that exposure time is important to avoid exceeding the MRL as already discussed. We found that the MRL of $5.0 \mu\text{g}\cdot\text{g}^{-1}$ was exceeded at 65°C and pH 6 despite a short treatment time of 8 s, suggesting that temperature has a more compelling effect than time in terms of residue loading at high temperatures. It is feasible to use a lower IMZ concentration by increasing the solution temperature to achieve the same level of control as demonstrated by Schirra *et al.* (2010). They used the EC formulation of IMZ and observed a similar result as obtained in our study with the sulphate formulation in the flooders. However, further research on maintenance of fungicide mixtures must be conducted to determine the effects of large quantities of fruit moving through the fungicide solution and stripping the fungicide from the solution, as well as the reduction of available fungicide as organic matter builds up from the fruit coming in from the orchard.

Post treatment brushing removes excess fungicide solution to assist drying of fruit prior to wax coating application and packing (Pelser, 1977; Smilanick *et al.*, 1997; Erasmus *et al.*, 2015a). However, it also removes some of the IMZ active and this has been shown by lower residues in the commercial flooder evaluation. Our findings in the commercial packhouse using donuts, confirm reports following post-dip brushing (Erasmus *et al.*, 2015a). How much residue is removed was linked to the pH of the solution. The reaction of IMZ to the pH is due to the lipophilic nature of undissociated IMZ at the higher pH, with this compound then binding more strongly to the oily constituents in the citrus rind when in a pH 6 solution (Siegel *et al.*, 1977). Despite reductions ($\approx 75\%$ at pH 3 and $\approx 44\%$ at pH 6), good infection control was still observed in green mould management trials by Erasmus *et al.* (2015a).

The commercial flooder was not entirely comparable to the experimental unit in terms of the drying mechanisms. On the commercial unit fruit were moved over donuts, while the experimental unit had brushes and with an air-knife after treatment. In the commercial flooder trials, it was seen that fruit taken after the donuts, compared to fruit treated with the flooder alone, had significantly less IMZ residue. Regardless, the residues loaded after treatment were still usefully close to those predicted by the model. The differences in results can in part be attributed to batch differences as explained above, but more likely due to the difference in effect between brushes and donuts. Whether donuts or brushes are more acceptable in terms of disease control would need to be investigated in a future study.

At the recommended IMZ application regimes evaluated, very good control was observed (average curative control of 90.8% and protective control of 93.3%), despite harsh inoculation methods and ideal incubation conditions provided. In terms of disease management in a commercial packhouse, where conditions are less favourable and pathogen/disease incidence is kept as low as possible through sanitation practices, the control achieved here is considered very good.

Good curative control ($> 71.6\%$) was achieved across a wide range of IMZ residues ($> 0.40 \mu\text{g}\cdot\text{g}^{-1}$). Whole fruit residue data was poorly correlated with curative control, and can be attributed to the fact that residue loading is more concentrated in the wounded tissue where the curative fungicidal action is required (Dore *et al.*, 2009, 2010; Erasmus *et al.*, 2015a). Previous studies have indicated that dip application resulted in excellent curative control (Dore *et al.*, 2009; Erasmus *et al.*, 2011, 2015a; Njombolwana *et al.*, 2013a; Kellerman *et al.*, 2016), and was generally better than other aqueous applications such as drench or spray (Erasmus *et al.*, 2011; Kellerman *et al.*, 2014). This efficacy was attributed to more effective residue loading in wound sites by dip application (Erasmus *et al.*, 2015a), and is supported by reports that curative control improved with longer exposure time in fungicide dip-treatments (Erasmus *et al.*, 2011, 2013, 2015a; Kellerman *et al.*, 2016). Exposure time (8 s) in the flooder is markedly shorter than typical dip treatments (16 – 107 s) (Erasmus *et al.*, 2011). Excellent

curative control despite this brief exposure time can be attributed to the flooder's effective and uniform means of fungicide deposition by means of five water films over rotating brushes. A possible explanation could be that, due to the high volume of heated solution in contact with the fruit, control is improved (Kanetis *et al.*, 2008). Erasmus *et al.* (unpublished) found that the higher volume and longer exposure time of solution gained from 3 to 5 weirs resulted in more effective residue loading and subsequent green mould control. Additionally, the increased mechanical action of solution being brushed onto the fruit by the rotational action of the brushes, may be enabling improved coverage and penetration of wound sites.

Protective control in dip applications was previously found to be relatively poor when compared to curative control following dip treatments, or to protective control following IMZ application in wax coatings (Njombolwana *et al.*, 2013a). The excellent protective control that was seen following IMZ application using the heated flooder was therefore a surprising result. This attribute of the flooder was first reported by Erasmus *et al.* (unpublished) in comparison studies between the dip and flooder application. Improved protective control following heated flooder applications can be attributed to better and more uniform residue loading, as discussed above. The residue levels corresponding to effective green mould control presented in this study correlate with various other studies that has shown that a residue of between 0.6 and 3.0 $\mu\text{g}\cdot\text{g}^{-1}$ is necessary to effectively control green mould infections (Kaplan and Dave, 1979; Schirra *et al.*, 1996; Smilanick *et al.*, 1997; Erasmus *et al.*, 2011).

Sporulation inhibition was good where residue levels were high, which, as seen in this study and others, is strongly correlated to pH with solutions of pH 6 giving better sporulation inhibition results (Hall, 1991; Erasmus *et al.*, 2011, 2013, 2015a; Kellerman *et al.*, 2016). Using the sporulation inhibition model developed, sporulation inhibition could be predicted from residue levels and a residue of between 1.29 and 2.47 $\mu\text{g}\cdot\text{g}^{-1}$ was predicted to inhibit sporulation at 90%. These values, are similar to previous research indicating that a minimum residue of 2.0 $\mu\text{g}\cdot\text{g}^{-1}$, but not necessarily higher than 3.5 $\mu\text{g}\cdot\text{g}^{-1}$, is sufficient to control sporulation (Kaplan and Dave, 1979; Schirra *et al.*, 1996; Smilanick *et al.*, 1997; Erasmus *et al.*, 2011).

Previous studies have shown that heat treatments alone can be useful in disease control, with a reduction in decay seen with hot water dips only (McDonald *et al.*, 1991; Erkan *et al.*, 2005; Şen *et al.*, 2010). This may in part be due to heated dips (47 – 53°C) decreasing *Penicillium* spp. inoculum (Şen *et al.*, 2010). The addition of a fungicide invariably increases the control achieved to more than what is possible with heated water or ambient fungicide solution alone (Schirra *et al.*, 1997; Puawongphat *et al.*, 2008; Dore *et al.*, 2009). Our study consistently demonstrated very good levels of control, with improved residue loading and sporulation inhibition at higher pH and temperatures. A heated solution only was not included as treatment, and conclusions cannot be drawn whether improved sporulation inhibition could

also be attributed to some synergistic effect of heat treatment. Despite reports that heat can have a beneficial effect on reducing decay, we observed rind injury, blemishes and discolouration on lemon and Satsuma mandarin fruit even after very brief exposure to heated imazalil sulphate solutions in the flooder at 55 and 65°C. This is an unacceptable risk, particularly with less hardy fruit such as lemons or soft citrus types. Temperatures above 55°C were seen to injure fruit in various studies (McDonald *et al.*, 1991; Palou *et al.*, 2001; Puawongphat *et al.*, 2008) and the damages inflicted resulted in higher incidence of decay (Şen *et al.*, 2010).

A sensitive strain of *P. digitatum* was used in this study. Erasmus *et al.* (2015b) demonstrated practical resistance, *i.e.* significantly diminished levels of control and loss of sporulation inhibition, when attempting to control IMZ resistant strains by means of IMZ dip treatments. The heated flooder application has demonstrated some superior benefits to dip application and further research is required to investigate whether improved IMZ application by means of the heated flooder will improve control of IMZ resistant strains.

The addition of the IMZ in wax treatment in the commercial trials, apart from the heated flooder treatments, was for practical industry purposes. It was important to confirm that the MRL would not be exceeded with the double application of IMZ, which is commonplace in South African packhouses (Erasmus *et al.*, 2011). It was also necessary to determine if the second application of IMZ would be necessary if adequate residue levels could be achieved with a heated solution as was seen by Smilanick *et al.* (1997). Njombolwana *et al.* (2013a, b) found that, whilst IMZ application via wax coatings gave relatively poor curative control, it supported IMZ dip application which gave excellent curative control but moderate protective control. Based on the results presented here, the flooder offers both curative and protective control, negating the need for an additional application of IMZ in the wax. The flooder will load sufficient IMZ residues for both infection and sporulation control if the right solution parameters are implemented. Nonetheless, should packhouses choose to remain with the double IMZ application strategy, it was seen from these initial tests that the MRL was not exceeded, even when applying the higher 2000 µg.mL⁻¹ IMZ in the wax coating.

Most research on IMZ application has shown increased residues were loaded when applied at increased temperature, pH, exposure time and concentration variables (Smilanick *et al.*, 1997; Erasmus *et al.*, 2011, 2013; Kellerman *et al.*, 2016). To date, all the work done on the sulphate form has been in dip trials and with limited attention paid to the effect of aqueous treatment over rotating brushes, as in the heated flooder. The effect of brushing during treatment seems to aid protective control of infections, while post treatment brushing was shown to decrease residues without compromising control of green mould. The flooder has been shown to offer excellent green mould control both in terms of curing established infections and protecting from infections occurring post treatment, as well as effective

sporulation inhibition. This is a major advantage over the fungicide bath which lacks adequate protective action. The flooder is easier to manage and loads residues more precisely than fungicide dip applications. This is an important characteristic to be aware of as there is a current demand from supermarkets for fruit with residues up to 60% below the MRL. Whilst this requirement is driven by market forces, rather than scientifically based food safety concerns, producers and packhouses are pressured to abide by these demands (Wilma du Plooy, personal communication, 2016). From a sustainable green mould control perspective, our results show that infection control should not be affected by markedly reduced IMZ residue levels relative to the MRL; however, it is not practically feasible to treat fruit in a packhouse within 24 hours after infection/harvest. Moreover, sporulation control will be compromised, leading to further problems of soilage, increased spore load and the risk of fungicide resistance development. In conclusion, we have demonstrated that heated flooder solution parameters can be manipulated to precisely load imazalil fungicide on citrus rinds to ensure adequate green mould control while maintaining residues below the MRL.

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Table 1. Parameters and goodness of fit of non-linear regression using the function $Y = \text{pr3}/(1+\text{Exp}(-\text{pr1}-\text{pr2}*\text{X1}))$ of imazalil residue data determined on lemon, Satsuma mandarin, navel orange and Valencia orange treated with a heated flooder with imazalil concentrations of 250 and 500 $\mu\text{g}.\text{mL}^{-1}$ and a pH range of 3, 4, 5, and 6 at a solution temperature of 45°C.

Citrus Kinds	Model parameter values and goodness of fit			
	Pr1	Pr2	Pr3	R^2
250 $\mu\text{g}.\text{mL}^{-1}$				
Lemon	-4.417	0.574	4.581	0.834
Satsuma mandarin	-5.470	0.509	20.480	0.685
Navel	-4.315	0.512	4.940	0.865
Valencia	-3.572	0.562	6.299	0.635
500 $\mu\text{g}.\text{mL}^{-1}$				
Lemon	-7.904	0.561	293.462	0.966
Satsuma mandarin	-7.376	0.549	199.420	0.549
Navel	-7.145	0.487	139.047	0.963
Valencia	-6.149	0.425	150.564	0.696

Table 2. Parameters and goodness of fit of non-linear regression using the function $Y = \frac{pr3}{(1+Exp(-pr1-pr2*X1))}$ of imazalil residue data determined on lemon, Satsuma mandarin, navel orange and Valencia orange treated with a heated flooder with imazalil concentrations of 250 and 500 $\mu\text{g.mL}^{-1}$ and a temperature range of 45, 55 and 65°C at a solution pH of 6.

Citrus Kinds	Model parameter values and goodness of fit			
	Pr1	Pr2	Pr3	R^2
250 $\mu\text{g.mL}^{-1}$				
Lemon	-9.740	0.087	412.627	0.426
Satsuma mandarin	-7.606	0.069	146.821	0.731
Navel	-6.442	0.050	70.544	0.954
Valencia	-5.395	0.051	66.965	0.661
500 $\mu\text{g.mL}^{-1}$				
Lemon	-7.634	0.060	426.051	0.722
Satsuma mandarin	-10.306	0.095	997.916	0.842
Navel	-7.249	0.052	248.176	0.952
Valencia	-8.124	0.064	679.667	0.747

Table 3. Mean curative control levels (%) determined on lemon, Satsuma mandarin, navel orange and Valencia orange treated with a heated flooders with imazalil concentrations of 250 and 500 $\mu\text{g.mL}^{-1}$ and a pH range of 3, 4, 5, and 6 at a solution temperature of 45°C.

pH	Green mould control (%) ^a			
	Lemon	Satsuma mandarin	Navel	Valencia
250 $\mu\text{g.mL}^{-1}$				
3	93.33 b	72.18 b	89.17 a	91.26 b
4	97.18 ab	78.01 ab	93.25 a	92.53 ab
5	98.91 a	82.36 a	90.43 a	95.98 a
6	97.21 ab	79.17 ab	88.28 a	93.87 ab
500 $\mu\text{g.mL}^{-1}$				
3	100.0 a	75.17 b	89.51 b	93.55 b
4	99.63 a	71.55 b	91.44 ab	94.19 b
5	99.62 a	80.93 b	90.16 b	97.84 a
6	99.63 a	94.23 a	95.38 a	97.89 a

^a For each citrus type and IMZ concentration separately, means followed by the same letter do not differ significantly ($P > 0.05$)

Table 4. Mean protective control levels (%) determined on lemon, Satsuma mandarin, navel orange and Valencia orange treated with a heated flooder with imazalil concentrations of 250 and 500 $\mu\text{g.mL}^{-1}$ and a pH range of 3, 4, 5, and 6 at a solution temperature of 45°C.

pH	Green mould control (%) ^a			
	Lemon	Satsuma mandarin	Navel	Valencia
250 $\mu\text{g.mL}^{-1}$				
3	74.91 b	83.14 b	78.17 b	92.08 b
4	74.89 b	87.67 b	82.19 b	94.77 b
5	93.99 a	95.85 a	94.54 a	99.23 a
6	99.55 a	97.94 a	98.59 a	100.0 a
500 $\mu\text{g.mL}^{-1}$				
3	80.62 c	93.74 b	92.03 c	94.58 b
4	92.77 b	95.69 ab	95.25 bc	98.31 a
5	100.0 a	98.04 a	97.89 ba	99.65 a
6	100.0 a	99.31 a	99.63 a	100.0 a

^a For each citrus type and IMZ concentration separately, means followed by the same letter do not differ significantly ($P > 0.05$)

Table 5. Parameters, goodness of fit and effective IMZ residue levels for predicted 50 and 90% sporulation inhibition of *Penicillium digitatum* of non-linear regression using the function $Y = pr3/(1+Exp(-pr1-pr2*X1))$ of imazalil residue data measured on lemon, Satsuma mandarin, navel orange and Valencia orange after treatment with either 250 or 500 $\mu\text{g.mL}^{-1}$ imazalil sulphate at pH levels of 3, 4, 5, and 6 and temperatures of 45, 55 and 65°C.

Citrus type	Model parameter values and goodness of fit				Residue level ($\mu\text{g.g}^{-1}$)	
	Pr1	Pr2	Pr3	R^2	90% control	50% control
Lemon	-3.925	2.686	96.021	0.943	2.468	1.492
Satsuma mandarin	-0.764	1.658	99.149	0.573	1.84	0.471
Navel	-4.385	5.217	98.482	0.966	1.2932	0.8464
Valencia	-3.173	2.326	99.265	0.906	2.3426	1.371

Table 6. Mean curative control levels (%) determined on lemon, Satsuma mandarin, navel and Valencia orange treated with the heated floodler at a concentration of 250 and 500 $\mu\text{g.mL}^{-1}$ for the temperature range of 45, 55 and 65°C at a solution pH of 6.

Temperature (°C)	Green mould control (%) ^a			
	Lemon	Satsuma mandarin	Navel	Valencia
250 $\mu\text{g.mL}^{-1}$				
45	97.21 b	79.17 a	88.28 ab	93.87 a
55	98.90 ab	77.69 a	84.97 b	96.36 a
65	100.0 a	82.21 a	93.31 a	96.69 a
500 $\mu\text{g.mL}^{-1}$				
45	99.63 a	94.23 a	95.38 a	97.89 a
55	100.0 a	94.32 a	95.36 a	93.78 b
65	100.0 a	95.28 a	97.36 a	97.80 a

^a For each citrus type and IMZ concentration separately, means followed by the same letter do not differ significantly ($P > 0.05$)

Table 7. Mean imazalil residue levels determined on lemon and navel fruit treated with a commercial heated flooder at 500 µg.mL⁻¹ at a solution temperature of 25, 35 or 45°C and a solution pH of either 3 or 6.

pH	Temperature (°C)	IMZ residues (µg.g ⁻¹) ^a			
		Flooder alone ^b	Donuts ^b	Wax + 1000 µg.mL ⁻¹ IMZ ^b	Wax + 2000 µg.mL ⁻¹ IMZ ^b
Lemon					
3	25	0.92 ghijkl	0.38 n	0.72 jklmn	0.55 lmn
	35	0.98 ghijk	0.52 mn	0.88 hijklm	1.47 bcde
	45	0.86 ijklm	0.37 n	0.68 klmn	1.45 bcdef
6	25	1.29 cdefg	1.08 fghij	1.18 defghi	1.11 efghi
	35	1.51 bcd	1.10 efghi	1.25 cdefgh	1.46 bcdef
	45	2.45 a	1.70 b	1.56 bc	2.32 a
Navel					
3	25	0.66 jkl	0.41 lm	0.71 ijk	0.79 ghijk
	35	0.74 hijk	0.52 klm	0.85 ghij	1.86 abc
	45	0.74 hijk	0.34 m	0.64 jkl	1.86 abc
6	25	1.35 ef	1.04 g	1.05 fg	1.52 de
	35	1.37 e	1.02 gh	1.01 ghi	1.90 ab
	45	2.09 a	1.63 bcde	1.59 cde	1.79 bcd

^a For each citrus type treatment separately, means followed by the same letter do not differ significantly ($P > 0.05$)

^b Fruit were sampled after each of the flooder alone, over donut sponges, a 1000 µg.mL⁻¹ IMZ wax and a 2000 µg.mL⁻¹ IMZ wax treatment

Table 8. Parameters, goodness of fit and effective IMZ residue levels predicted at pH levels of 3, 4, 5 or 6 of non-linear regression using the function $Y = pr3/(1+Exp(-pr1-pr2*X1))$ of imazalil residue data measured on lemon, Satsuma mandarin, navel and Valencia oranges after treatment with IMZ at $500 \mu\text{g.mL}^{-1}$ in a heated flooder at a temperature of 45°C .

Citrus type	Model parameter values and goodness of fit					IMZ residue ($\mu\text{g.g}^{-1}$)			
	Pr1	Pr2	Pr3	SSE	R^2	pH 3	pH 4	pH 5	pH 6
Lemon	-1.329	1.854	6.063	0.668	0.966	0.709	1.075	1.557	3.200
Satsuma	-0.297	0.949	6.029	6.976	0.604	0.309	1.030	1.990	6.000
Navel	-1.236	2.284	6.205	0.755	0.962	0.515	0.805	1.165	2.040
Valencia	-0.686	0.732	6.362	5.869	0.707	0.781	1.660	2.720	4.800



Figure 1. Photograph of the experimental flooder used to apply imazalil sulphate solution to citrus fruit at different heated temperatures and pHs. The solution was applied through 5 weirs, creating 5 sheets of smooth laminar flow at a rate of $580 \text{ L}\cdot\text{min}^{-1}$, with fruit moving over rotating brushes (85 rpm).

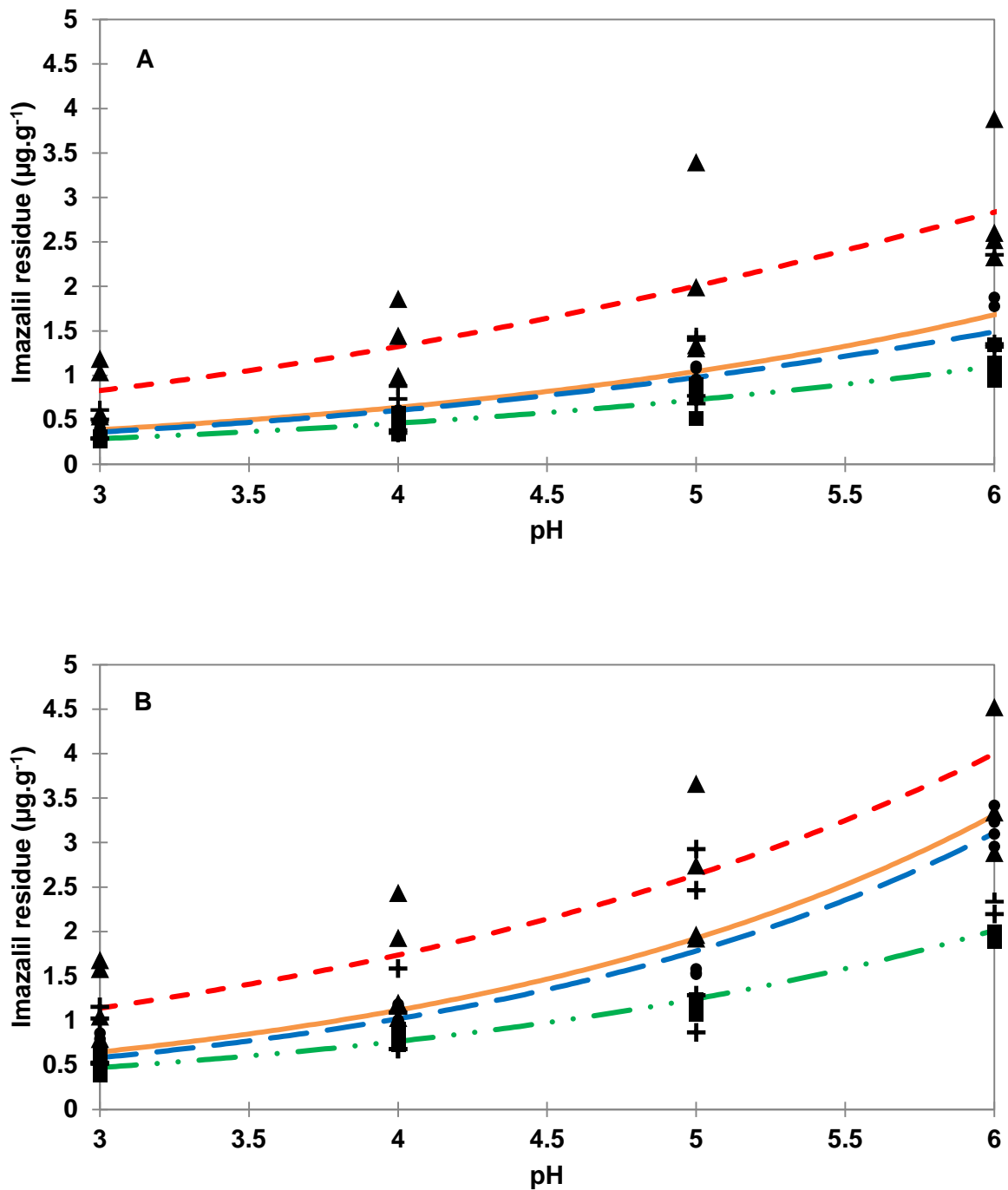


Figure 2. Mean imazalil residues values analysed on citrus fruit after treatment in imazalil solutions at 250 (A) and 500 $\mu\text{g.mL}^{-1}$ (B) at a pH range of 3, 4, 5, or 6 at a temperature of 45°C and non-linear regression models [$Y = pr3/(1+Exp(-pr1-pr2*X1))$] fitted indicating residue loading trends for lemon (●, blue long dash line, $R^2 = 0.834$ (A); 0.966 (B)), Satsuma mandarin (+, orange solid line, $R^2 = 0.685$ (A); 0.549 (B)), navel (■, green dash dot line, $R^2 = 0.865$ (A); 0.963 (B)), Valencia (▲, red short dash line; $R^2 = 0.635$ (A); 0.696 (B)) as influenced by pH.

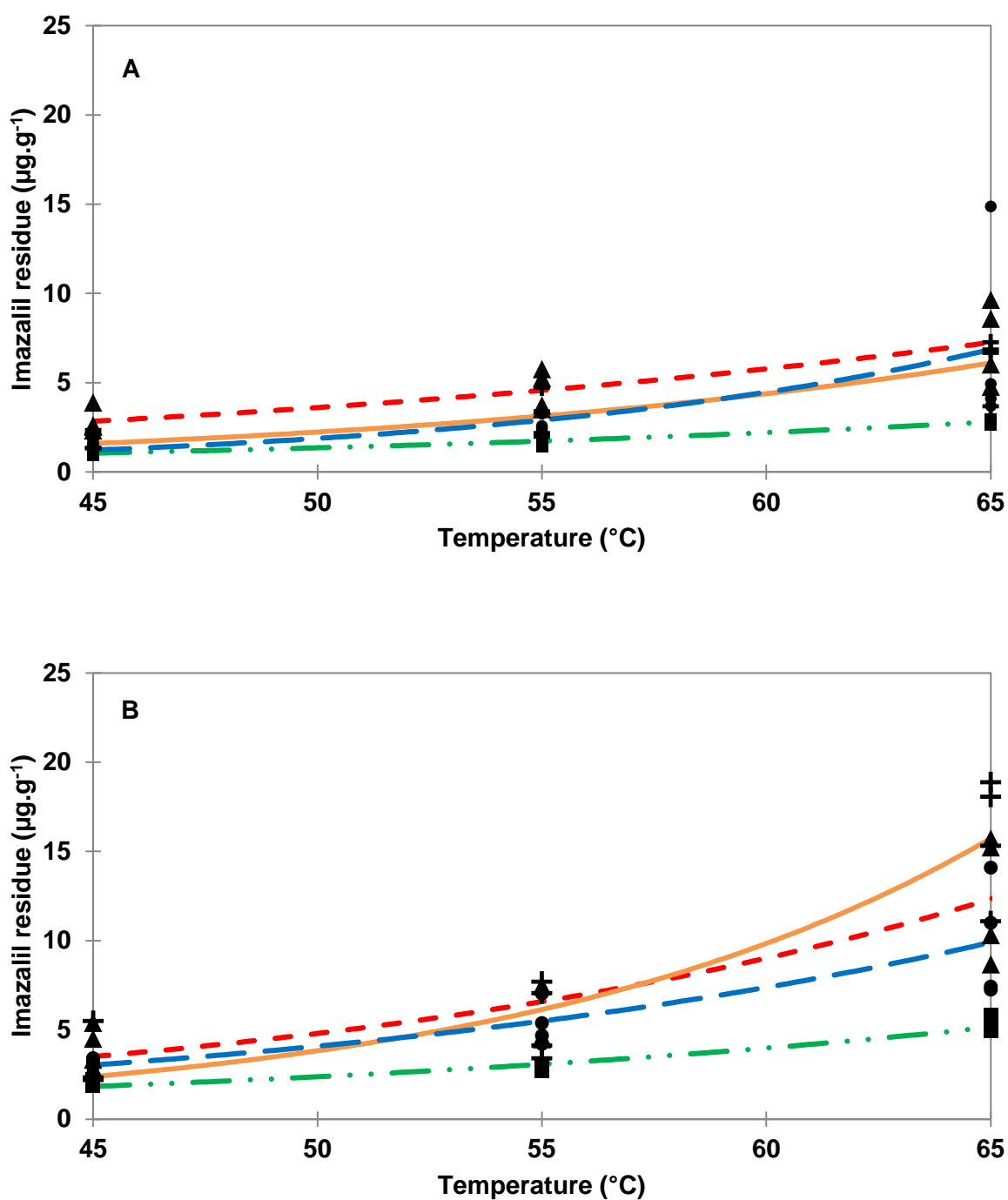


Figure 3. Mean imazalil residues values analysed on citrus fruit after treatment in imazalil solutions at 250 (A) and 500 µg.mL⁻¹ (B) at a temperature range of 45, 55, or 65 at a pH of 6 and non-linear regression models [$Y = pr3/(1+Exp(-pr1-pr2*X1))$] fitted indicating residue loading trends for lemon (●, blue long dash line, $R^2 = 0.426$ (A); 0.722 (B)), Satsuma mandarin (+, orange solid line, $R^2 = 0.731$ (A); 0.842 (B)), navel (■, green dash dot line, $R^2 = 0.954$ (A); 0.952 (B)), Valencia (▲, red short dash line; $R^2 = 0.661$ (A); 0.747 (B)) as influenced by temperature.

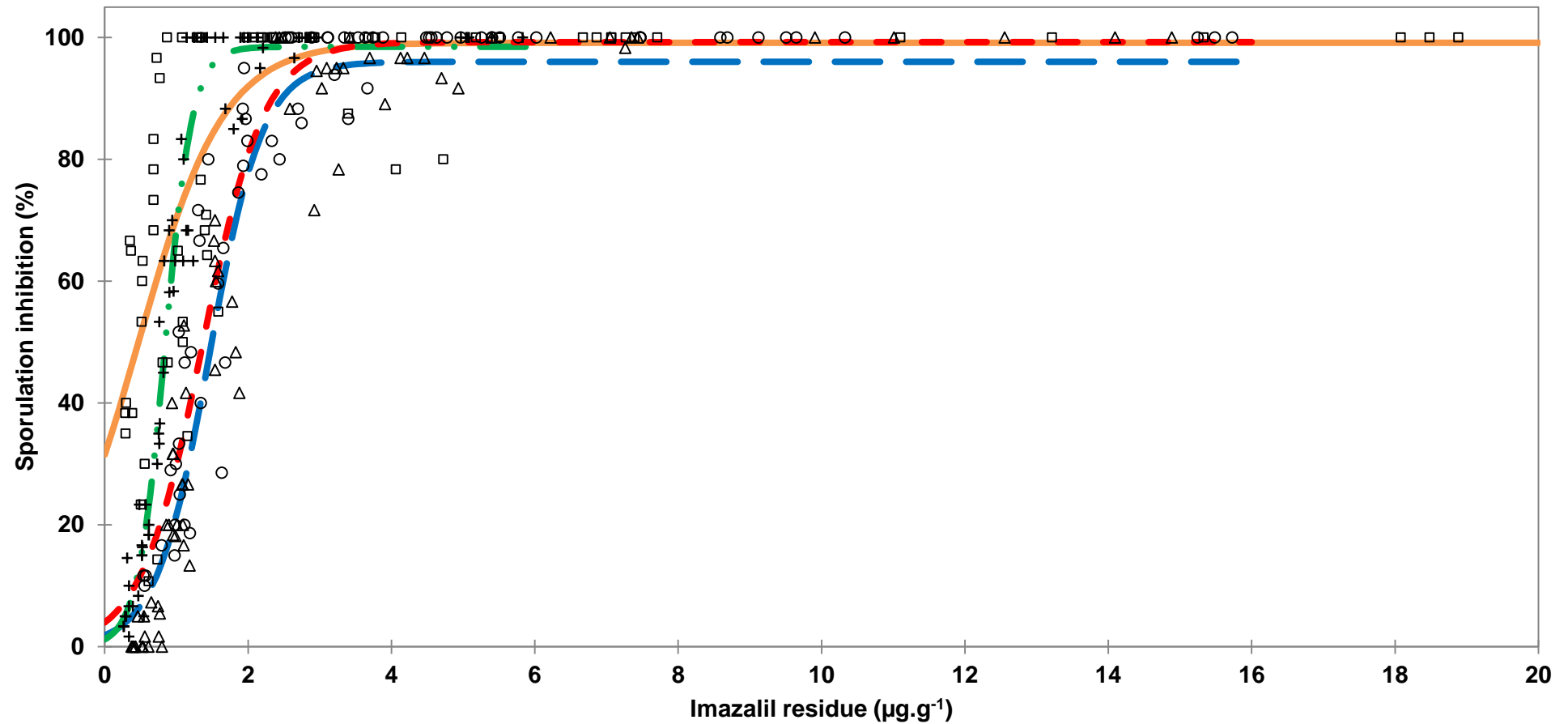


Figure 4. Mean percentage green mould sporulation inhibition on citrus fruit and non-linear regression models [$Y = pr3/(1+Exp(-pr1-pr2*X1))$] indicating sporulation inhibition trends for lemon (\circ , blue long dash line, $R^2 = 0.943$), Satsuma mandarin ($+$, orange solid line, $R^2 = 0.573$), navel (\square , green dash dot line, $R^2 = 0.966$), Valencia (Δ , red short dash line; $R^2 = 0.906$) as influenced by imazalil residue.



Figure 5. Photographs of heat damage on lemons (left) and Satsuma mandarins (right) after treatment with the flooder at temperatures $>55^{\circ}\text{C}$.

CHAPTER 3

The effect of pH, temperature and exposure time in imazalil dip applications on postharvest citrus green mould control and survival of *Rhizopus stolonifer*

ABSTRACT

Citrus green mould (caused by *Penicillium digitatum*) is commonly controlled using imazalil applied by means of dip treatments in a fungicide bath. In South Africa, the fungicide bath is often heated to 35°C, as this has several benefits. However, heated solutions require high energy costs, and, the cost of replacing the bath water and fungicides is high. Chemicals are therefore topped up to maintain concentration, and the bath is kept for extended periods of time. However, research has shown that adequate disease control can be obtained with fungicide dip treatments at lower temperatures. The aims of this study were to study the effects of pH, temperature and exposure time in imazalil dip applications on postharvest citrus green mould control, specifically curative control and sporulation inhibition, as well as to study the risk of contaminant build-up, in particular *Rhizopus stolonifer* spores. The ability of imazalil to control green mould was investigated in a cold bath of 10°C and compared to baths at ambient temperature and 35°C. In conjunction with these efficacy trials, the survival of *Rhizopus stolonifer* spores was studied *in vitro* at various water temperatures (10°C to 65°C) for exposure times of 1 and 60 minutes, as well as a simulated 1-hour pasteurisation step at 65°C followed by an overnight cool-down period. Sub-treatments included the addition of imazalil fungicide or green mould spores, as would commonly be found in a citrus fungicide bath. The efficacy trials on navel, Valencia and mandarin fruit demonstrated that solution temperature had no significant effect on the ability of imazalil to cure 24 hr old green mould infections with all temperatures providing control > 84% on oranges, and good but variable control on mandarin (52-76%). However, IMZ residue loading and sporulation inhibition increased as solution pH, temperature, and exposure time increased. Whilst sporulation inhibition was < 50% in pH 3 baths, irrespective of temperature and exposure time, complete inhibition was obtained at 35°C and pH 6, but maximum residue limits for IMZ were exceeded at longer exposure times (> 45 s). Solution temperature had no significant effect on *Rhizopus* spore survival at temperatures below 35°C, but temperatures of 45, 55 and 65°C, particularly after a 60-minute exposure, caused a significant reduction in *Rhizopus* spore viability (> 90%). Complete *Rhizopus* spore control was achieved following the 65°C pasteurisation step.

INTRODUCTION

Citrus is an economically important crop in South Africa and a large volume of the produce is exported (DAFF, 2014; Edmonds, 2016; PPECB, 2016). The importance of maintaining fruit quality during shipping is crucial (Roth, 1967; Eckert and Eaks, 1989). The largest decay threat to fruit quality is green mould, a fungal disease caused by *Penicillium digitatum* Pers. Sacc. (Fawcett, 1927; Eckert and Eaks, 1989), an obligate wound pathogen (Kavanagh and Wood, 1967; Eckert and Eaks, 1989). Once infection occurs, the damaged tissue becomes soft and a water soaked lesion appears within 5 to 7 days, followed by white mycelial growth outwards from the infection point. Matured infection results in distinctive olive green sporulation 10 – 14 days later (Eckert and Brown, 1986; Eckert and Eaks, 1989).

Imazalil (IMZ; 1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl)-1H imidazole) (Siegel *et al.*, 1977; FAO, 2001) is the most effective fungicide against green mould and used globally in the citrus industry (Smilanick *et al.*, 1997b; Erasmus *et al.*, 2011; Besil *et al.*, 2016). Imazalil has action against both infections and spore formation (Erasmus *et al.*, 2011). Additionally, the active's anti-sporulation properties are of high value as it decreases the problem of soilage (McCornack and Brown, 1977). Soilage occurs when green mould spores rubs onto and stick to the surface of an otherwise healthy fruit (Pelser, 1977; Eckert and Kolbezen, 1978; Barmore and Brown, 1982; Eckert and Eaks, 1989). Although the healthy fruit is still marketable it has to be cleaned prior to repacking, and has a high risk of rot if the fruit becomes wounded. The Maximum Residue Limit (MRL) for IMZ on citrus is 5 $\mu\text{g.g}^{-1}$ for the European Union and 10 $\mu\text{g.g}^{-1}$ for the USA (DAFF, 2008; AgrilIntel, 2015). South African packhouses tend to load much lower levels ($\approx 1 \mu\text{g.g}^{-1}$) (Erasmus *et al.*, 2011). An IMZ residue level of between 1 and 3 $\mu\text{g.g}^{-1}$ is necessary to achieve adequate levels of control (Kaplan and Dave, 1979; Schirra *et al.*, 1996; Smilanick *et al.*, 1997b; Erasmus *et al.*, 2011).

South Africa uses the sulphate form of IMZ in all aqueous applications at a registered concentration of 500 $\mu\text{g.mL}^{-1}$ (Pelser and La Grange, 1981; Erasmus *et al.*, 2011). The other available formulation, an emulsifiable concentrate (EC), is also used in South Africa, although it is solely applied via the commercial wax coating application (Erasmus *et al.*, 2011; Njombolwana *et al.*, 2013a, b). South African packhouses prefer the sulphate formulations in dip applications because, unlike the EC formulation: it is more stable, needs less agitation and is less likely to precipitate, in which case it then becomes unavailable (Eckert, 1977; Altieri *et al.*, 2005).

Treatment of citrus with IMZ sulphate occurs primarily in the fungicide bath (also known as the dip tank) (Pelser and La Grange, 1981; Erasmus *et al.*, 2011). Due to the individual nature of packhouses in South Africa, IMZ solutions differ widely in terms of concentration (usually 250 - 615 $\mu\text{g.mL}^{-1}$), temperature (12 - 45°C), solution pH (3 – 8), and exposure time of the fruit (16 – 107 s) in the tank (Erasmus *et al.*, 2011). Despite variations, the fungicide

bath is very effective if fruit can be fully submerged during treatment (Eckert, 1977) and offers excellent curative control (Dore *et al.*, 2009; Erasmus *et al.*, 2011, 2013, 2015a). The current recommendation for citrus packinghouses in South Africa is a solution temperature of 35°C, a pH of 3 (where exposure time is negligible) and the concentration maintained at 500 µg.mL⁻¹ (Pelser, 1980; Erasmus *et al.*, 2011, 2013, 2015a; Lesar and Erasmus, 2014). Top-up procedures are in place for the industry so it is generally found that the concentration is satisfactory in most packhouses (Erasmus *et al.*, 2011; Lesar and Erasmus, 2014). In terms of recommendations for the other factors regarding IMZ sulphate solution (temperature, pH and exposure time) there has been a great advance in knowledge in recent years (Erasmus *et al.*, 2011, 2013, 2015a; Kellerman *et al.*, 2016; Chapter 2).

The temperature recommendation of 35°C comes partly from work done with the EC formulation (Smilanick *et al.*, 1997b, 2003) as well as some other advantages of a heated solution: fruit coming from a warm fungicide bath will dry more quickly and easily in the subsequent drying tunnel (Pelser, 1977; Smilanick *et al.*, 1997a; Erasmus, personal communication, 2014); warm water is believed to be better at replacing air in wounds ensuring more effective fungicide delivery; and finally, that a warm, dry fruit appears to receive a more uniform wax coating than cold or wet fruit (Smilanick *et al.*, 1997a). Temperature is difficult to maintain adequately in the fungicide dip tank as these tanks typically do not have good circulation and colder fruit is constantly entering the solution. This difficulty as well as the high energy cost associated with heated fungicide bath, have led to the need to investigate the influence of temperature in fungicide dip tanks on green mould control. Research to date on the sulphate formulation has looked at temperatures of 25°C and up (Erasmus *et al.*, 2011; Kellerman *et al.*, 2016). This research, as well as work on the EC formulation, has highlighted the benefits of a heated IMZ solution where hot water not only offers some level of control (Erkan *et al.*, 2005; Şen *et al.*, 2010), but that the addition of a fungicide always improves that control (Schirra *et al.*, 1997; Puawongphat *et al.*, 2008). With regards to IMZ, it has been seen that the fungicide enters the citrus rind more easily and maintains a residue for longer when applied in a heated solution (Schirra *et al.*, 1996, 2010; Cabras *et al.*, 1999; Dore *et al.*, 2009).

Apart from temperature, Erasmus *et al.* (2011, 2013, 2015a) and Kellerman *et al.* (2016) have demonstrated that solution pH plays an immense role in residue loading of IMZ sulphate. This is due to the pH sensitive nature of IMZ's solubility in water: since it is a weak base, it is most soluble with a low solution pH (FAO, 2001). Exposure time is also very important when using a fungicide and residues usually increase as exposure time is lengthened (Brown and Dezman, 1990; Cabras *et al.*, 1999; Erasmus *et al.*, 2015a; Kellerman *et al.*, 2016). As with all IMZ research, formulation is important and it has been illustrated that the EC formulation of IMZ is much more affected by exposure time than the sulphate form. Both increased as time was increased, however, the EC formulation was recorded to load

double the residues particularly after the initial 100 s (Sepulveda *et al.*, 2015). The pH is an important factor in this aspect, as the EC and sulphate formulations will once again act similarly at high pH solutions (Erasmus *et al.*, 2011, 2013).

An important concern about cooler fungicide dip tanks is the build-up of bacterial and fungal contaminants in the solution. Moreover, because of the cost involved when replacing both fungicides and water, the fungicide bath solution is often kept for extended period of time (Erasmus *et al.*, 2011) during which contaminants can build up (Eckert, 1977). It is a recommended practice to pasteurize fungicide solutions when fruit is absent in order to remove contaminants that have built up during treatment hours (Mildenhall *et al.*, unpublished; Morris, 1980; Smilanick *et al.*, 1997a), particularly in application methods where the solution is retained for long periods of time.

Rhizopus stolonifer (Fr.) Lind is a contaminant pathogen in South African packhouses (Lesar, 2013) that is very prevalent in fungicide baths (Mildenhall *et al.*, unpublished). The rots caused are soft and infection usually occurs through a wound, often in conjunction with other postharvest diseases. *Rhizopus* will decay an entire fruit within 48 hr under warm and moist conditions and is able to spread to fruit that it has had direct contact with (Lesar, 2013). *Rhizopus stolonifer* is a very hardy survivor on any decaying matter, and causes postharvest decay on many crops (Goos *et al.*, 1967; Yuan *et al.*, 1985). Due to its prevalence among citrus and its rapid, destructive nature, it was chosen as a model organism to study contamination in the bath. *Rhizopus stolonifer* grows optimally between 20 and 25°C while no growth was observed at 37°C (Yuan *et al.*, 1985). Pasteurisation may eliminate the spores in a fungicide bath, and a heated solution may help keeping it under control, though these assumptions have never been investigated thoroughly. Morris (1980) demonstrated that the fungicides in the bath did not deteriorate even after extended periods at a temperature of 70°C.

The research done by Erasmus *et al.* (2011, 2013, 2015a) and Kellerman *et al.* (2016) have very clearly defined the improvement in IMZ residue loading and green mould control gained from a heated bath, as well as the linked effects of temperature to exposure time and solution pH. Despite this, the question remains if the same is true for a cool fungicide bath and if the elements of exposure time and pH play the same synergistic roles. Additionally, many of the presumed benefits of a heated bath have never been adequately studied, particularly that of the reduction or elimination of contaminants. The aim of this research was therefore to clarify the synergistic relationship of lower temperatures and all ancillary variables, as well as the efficacy of pasteurisation in contaminant control.

MATERIALS AND METHODS

Effect of temperature, pH and exposure time in imazalil dip application on control of green mould

Fruit

Export quality, freshly harvested navel orange, Valencia orange and mandarin (cv. Nadorcott) fruit were collected from various packhouses in the northern citrus producing regions of South Africa (Mpumalanga and Limpopo provinces) during the 2015 and 2016 seasons. Five trials were conducted, two each on navel and Valencia, and one on mandarin (due to fruit availability). Fruit were hand selected for uniformity in size and quality. Upon arrival at the Citrus Research International laboratories in Nelspruit, South Africa, the fruit were washed with a total loss ozone applicator that doses water with ozone at the spray nozzle (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 using four nozzles; ArcAqua (Pty) Ltd., Westlake Business Park, 7945, Cape Town, South Africa). During washing, fruit were pushed over six rotating brushes and into an ambient air drying tunnel. The fruit were stored at 4°C for no more than 4 days before trials were conducted. Fruit were removed from cold storage 1 day before the trials were initiated in order to reach ambient temperature ($\approx 22^{\circ}\text{C}$) and to evaporate condensation. Trials were conducted simultaneously for the first navel and Valencia fruit, although treated in separate solutions. The second rounds for each of navel and Valencia oranges, and the mandarin fruit, were all treated individually.

Penicillium digitatum isolation and inoculation

For all trials, an imazalil sensitive isolate (STE-U 6560, Department of Plant Pathology, University Stellenbosch, South Africa) of *P. digitatum* was cultured on streptomycin amended potato dextrose agar (PDA+; Difco Potato Dextrose Agar, Becon, Dickinson and Company, Sparks, USA; Steptomycin Sulphate, Ultrapure USBioAnalyzed, 725 $\mu\text{g}.\text{mg}^{-1}$, USB Corporation, Cleveland, OH USA) at 25°C for 10 – 14 days. Cultures were flooded with sterile water amended with one drop of Tween 20 to a concentration of $\approx 0.01 \mu\text{L}.\text{mL}^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA). Conidia were dislodged from cultures using a sterilized hockey stick and filtered through a double layer of cheesecloth into 200 mL of the Tween 20 amended sterile water. Concentration of the spore suspension was adjusted to 1×10^6 spores.mL⁻¹ by means of a spectrophotometer (absorbance of 0.100 at 425 nm; Cecil CE1011, Lasec, Midrand, Gauteng, South Africa) (Morris and Nicholls, 1978; Eckert and Brown, 1986). Spores were maintained in a uniform suspension by use of magnetic stirrers throughout inoculation.

Two methods of inoculation were utilised during trials. In order to test the curative ability of imazalil against infections, a custom made wounding tool was used. This tool had a flattened cylindrical tip that mimicked the cut stem of a citrus fruit and delivered a wound 1 mm

in diameter and 2 mm deep through the flavedo into the albedo of the fruit. Wounds were made on the shoulder area around the stem end of each fruit, in a square pattern with a distance of approximately 4 cm between each. There were 12 fruit in each replicate with 3 replicates per treatment during a trial. Curative inoculations were done 24 hours before treatment. The second method of inoculation was to test the sporulation inhibition of imazalil. A sterile 0.60 x 25 mm gauge needle (NN*2325R, Terumo corporation, Tokyo, Japan) was used to inject 0.2 mL of spore suspension between 1 and 2 cm deep into the shoulder on stem end of a fruit. Only one point of entry was made per fruit. There were 12 fruit in each replicate with 3 replicates per treatment during a trial. Sporulation inhibition inoculations were done approximately 30 min before treatment.

Residue analysis

Fruit samples for residue analysis consisted of two replicates of six uninoculated fruit added to the first and last replicate of each treatment. After treatment, they were stored in plastic bags at 4°C for no more than a week before being chopped and blended (2.9 L Robot Coupe R2 Bowl Cutter Mixers, Bonanza Shop and Catering Equipment, Nelspruit, South Africa) into pulp using distilled water until the pulp was reduced to a pulp-like consistency. The dilution factor for each sample was recorded and the pulp was frozen into sub-samples that were couriered to an accredited analytical laboratory (Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa) for IMZ (chloramizol) residue analyses. Acetonitrile was used for extraction which was followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography tandem mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA) according to an accredited procedure. Residue data received from the analytical laboratory were recalculated using the dilution factor recorded when the pulp was blended.

Treatment and incubation

The five trials conducted all had the same parameters unless stated otherwise. For each trial, six water baths (50 L each) containing 500 µg.mL⁻¹ imazalil sulphate (Imzacure, 750 g.kg⁻¹ SG, ICA International Chemicals, Stellenbosch, South Africa) were prepared. Three temperatures were investigated, namely 10°C, ambient (≈ 19 - 22°C) and 35°C. For each temperature, there were two baths, one containing a solution at pH 3 and the other at pH 6. The pH of the solution was measured with a portable pH meter (HI 98121 Waterproof pH/ORP & Temperature meter, Hanna Instruments, Morninghill, Johannesburg, South Africa) and was decreased using hydrochloric acid (32%; Merck (Pty.) Ltd., Modderfontein, Gauteng, South Africa) or increased using sodium hydrogen carbonate (sodium bicarbonate; NaHCO₃; Saarchem uniLAB, Merck Chemicals (Pty) Ltd., Wadeville, Gauteng, South Africa). Fruit were dipped for each treatment for 15 s, 45 s, 90 s or 180 s. In each case there were 3 replicates

of each inoculation method. Uninoculated fruit were treated simultaneously for residue analysis.

After the fungicide treatments, the fruit followed a simulated commercial packline set up by moving over 14 rotating brushes passing under an airknife (± 100 nozzels supplying a stream of forced air onto the fruit to remove moisture) and then moving through an unheated forced air drying tunnel. No commercial wax coating was added to the fruit at any point. The dry fruit were packaged stem end facing upward on SFT13 nectarine trays (Huhta-maki South Africa (Pty) Ltd., Atlantis, South Africa) placed in lock-back stonefruit cartons (115 mm; Mpact, Epping, South Africa). The cartons were then covered in clear polyethylene bags (50 μm , Lanpack Manufacturers C.C., Woodstock, Cape Town, South Africa) with the end folded under the carton but not sealed tightly. Four to six small holes (≈ 5 mm in diameter) were made to reduce humidity and moisture build-up. The cartons were stacked and incubated at ambient temperature ($\approx 22^\circ\text{C}$) for 4 days before evaluating the curative and protective inoculations and 10 days for sporulation inoculations.

In the case of the first trials that were conducted on navel and Valencia (described as ‘delayed brushing’), the fruit were dipped in the solution, thereafter they remained in the crate for approximately 10 minutes before progressing through the rest of the packline. i.e. these fruit had a delayed brushing. In the case of the second trials on navel and Valencia (described as ‘immediate brushing’), as well as that on the mandarin, the trial had been optimised so that fruit followed the full packline sequence without any delays. i.e. the fruit were subjected to immediate brushing.

Treatment evaluation

Evaluations for curative treatments were done by counting infected wounds out of four on each fruit. Early infection was visualised using a near-UV light (UV-A at 365 nm, Labino Mid-light, www.labino.com) which caused infected wounds to fluoresce bright yellow (Njombolwana *et al.*, 2013a). Sporulation fruit were evaluated for each fruit using a rating index of 1 – 6, where fruit that showed no sign of disease was taken as missing data points: 1 = complete sporulation inhibition i.e. completely white fruit; 2 = sporulating area was small ($\approx 20\%$), 3 = sporulating area larger than a quarter of the fruit, but smaller than half of the fruit ($\approx 40\%$), 4 = sporulating area larger than half of the fruit, but smaller than three quarters of the fruit ($\approx 60\%$); 5 = sporulating area larger than three quarters of the fruit, but smaller than the whole fruit ($\approx 80\%$); and 6 = sporulating area covering the whole fruit (= 100%). Fruit that showed no sign of infection were regarded as missing data points.

Statistical analysis

For curative infection data (percentage wounds infected per fruit) and sporulation inhibition percentage data, were normalised relative to the data for untreated control treatments; percentage control data were subsequently used. Data were subjected to analyses of variance (ANOVA) and Fisher's least significant difference test at 95% confidence interval to compare means. To demonstrate trends between residues loaded from treatments, or the levels of control achieved, values were regressed using an appropriate non-linear regression model. The coefficient of determination (R^2) was used to demonstrate goodness of fit. Where possible, the model was used to predict either residue results or percentage control that would be achieved through treatment. XLSTAT (version 2016.7.01, Addinsoft, www.xlstat.com) was used for the analysis described. Regression models with reliable fits were used to determine residue levels that would be indicative of 50% or 90% control (Erasmus *et al.*, 2015b).

***In vitro* effect of solution temperature on *Rhizopus* spores in the fungicide bath**

Treatment

A *Rhizopus stolonifer* isolate was obtained from an infected citrus fruit and identified based on its morphological characteristics (Yuan *et al.*, 1985; Lesar, 2013). It was plated onto Rose Bengal (Rose Bengal Chloramphenicol Agar, Neogen, Michigan, USA) and PDA+ media to observe its growth. After initial assessment of the media, Rose Bengal media, offering slightly retarded growth, was used for all the cultures. *Rhizopus* spore suspension was created by flooding the lid of a petri dish with 0.01 $\mu\text{L}\cdot\text{mL}^{-1}$ Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) amended water. A scalpel was used to gently remove the sporangia of the *R. stolonifer* culture from the mycelia on the pink coloured Rose Bengal media. The spore mass was placed in the water and gently agitated to release the sporangiospores. The suspension was prepared as described for the *P. digitatum* above using the spectrophotometer. The solution was then diluted through a dilution series to 10^{-3} spores. mL^{-1} . Based on a previous investigation which evaluated dilutions from 10^{-0} to 10^{-20} , the 10^{-3} spores. mL^{-1} dilution factor was determined to be the best for colony counts and used in all following trials.

The trials were repeated three times. In each case, 15 mL of *R. stolonifer* spore suspension was dispensed into test tubes as follows: *R. stolonifer* spore suspension only; *R. stolonifer* spore suspension and a 10^3 spores. mL^{-1} green mould spore suspension; *R. stolonifer* spore suspension and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ imazalil sulphate. Each of these suspensions in test tubes was held in water baths for 1 minute and then 60 minutes at temperatures of 10, 15, 20, 25, 35, 45, 55, and 65°C. The suspensions treated at 65°C for 1 hour were kept in the bath overnight as the bath cooled down (± 12 hr); this simulated a pasteurisation step as would occur in a packhouse. After each time period, 300 μL was plated out onto Rose Bengal media. There were five replicates for each treatment.

Statistical analysis

Rhizopus stolonifer data consisted of counts of the colony forming units (CFU). The data were normalised relative to the untreated control counts and percentage control data were subsequently used. Data were subjected to analyses of variance (ANOVA) and Fisher's least significant difference test at 95% confidence interval to compare means. To demonstrate trends between the control achieved at the treatment temperatures, where possible, values were regressed using an appropriate non-linear regression model. The coefficient of determination (R^2) was used to demonstrate goodness of fit. XLSTAT (version 2016.7.01, Addinsoft, www.xlstat.com) was used for the analysis described.

RESULTS

Effect of temperature, pH and exposure time in imazalil dip application on control of green mould

Imazalil residue loading

Analysis of variance showed that there was a significant citrus type interaction ($P < 0.0001$) when all the citrus types were included; this interaction was ascribed to the delayed brushing in the first trials, and the data were analysed separately. No type interaction was observed for the delayed brushing trials with navel and Valencia oranges ($P = 0.989$). A significant interaction between temperature x pH x exposure time ($P < 0.0001$) was observed. The second set trials with navel and Valencia fruit (immediate brushing) also demonstrated no citrus type interaction between the batches ($P = 0.743$), as well as a significant interaction between temperature x pH x exposure time ($P = 0.005$; Table 1). The mandarin trial was analysed separately.

Navel and Valencia

At pH 3, there was no significant differences between fruit treated at any of the temperatures (10°C, ambient or 35°C) and for any period of time (15 – 180 s) in the experiment. A range of 0.76 – 1.36 $\mu\text{g}\cdot\text{g}^{-1}$ for the delayed brushing and 0.57 – 0.96 for the immediate brushing trial were determined (Table 2), although a trend toward higher residue levels at higher temperatures could be observed. At pH 6, residue levels were significantly higher as temperature and exposure time increased, except for 10°C where the effect of exposure time was not significant, and residue levels were not significantly different from the pH 3 treatments in most cases. Residue levels in the delayed brushing trial were generally higher than those in the immediate brushing trial, especially at higher pH and temperatures and longer exposure times. In fact, residue levels on fruit in the delayed brushing trial at pH 6, 35°C and exposure times 45, 90 and 180 s exceeded the MRL.

Mandarin

Analysis of variance of the residue data from mandarin fruit showed that there was a temperature x pH x exposure time interaction ($P < 0.0001$; Table 3). Similar to the oranges, residue levels following treatment at pH 3 did not often differ significantly and ranged from 0.38 – 0.66 $\mu\text{g.g}^{-1}$, with trends indicating marginally higher residue loading at higher temperatures and longer exposure times (Table 2). At pH 6, residue levels were generally significantly higher than those measured following pH 3 treatments, and ranged from 0.48 – 4.74 $\mu\text{g.g}^{-1}$. Residue levels increased significantly as temperature and exposure time increased. Residue levels were generally comparable to those of the immediate brushing navel and Valencia trial. Residue analysis also unexpectedly revealed that the mandarin fruit used in this trial had been drenched prior to collection for the trial. Average drench fungicides present were 2,4-D (0.01 $\mu\text{g.g}^{-1}$), pyrimethanil (1.25 $\mu\text{g.g}^{-1}$), and thiabendazole (0.13 $\mu\text{g.g}^{-1}$). Importantly, these residue levels did not appear to be affected by the IMZ treatments in any way (results not shown).

Green mould control

Curative control

Analysis of variance of the combined data set showed significant interactions with citrus type (and batch), and these were subsequently analysed separately. ANOVA for data from navel (delayed brushing) indicated a significant effect for temperature only ($P = 0.004$); for Valencia (delayed brushing) and for navel (immediate brushing) exposure time was significant ($P = 0.009$ and 0.004 , respectively); for Valencia (immediate brushing) a significant interaction for temperature x pH x exposure time was observed ($P = 0.0026$); and for mandarin a significant interaction for temperature x pH was observed ($P = 0.034$; Table 4). Despite these significant effects, average control levels were > 99% for navel and Valencia in the delayed brushing trial, and > 97 and > 84% respectively for navel and Valencia in the immediate brushing trial (results not shown). Mandarin control was variable across treatments and generally lower (52 - 76%) with only the lowest value (52% at pH 3 at 35°C) differing significantly from other treatments (results not shown).

Sporulation inhibition

Analysis of variance of the combined data set showed significant interactions with citrus type (and batch), which was ascribed to differences between the delayed and immediate brushing and mandarin trials. These were therefore analysed separately.

Navel and Valencia

Analysis of variance for data from the two trials using navel and Valencia fruit showed that there was a significant temperature x pH x exposure time interaction ($P < 0.0001$ and < 0.0001 , respectively; Table 5). Sporulation inhibition levels varied significantly, but generally increased as temperature, pH or exposure time increased (Table 6). Higher levels of sporulation inhibition were generally observed in the delayed brushing trial when compared to the immediate brushing trial; in the latter, 100% sporulation inhibition was only at the maximum of all parameters, whereas fruit in the delayed brushing trial exhibited 100% sporulation inhibition for exposure times at 35°C and with a solution pH of 6 (Table 6). Non-linear regression for sporulation inhibition data against IMZ residue data using a Three Parametric Logistic model with the function $Y = pr3/(1+Exp(-pr1-pr2*X1))$ gave good fits at an R^2 value of 0.682 for navel and 0.693 for Valencia. The model predicted that IMZ residue levels between 1.91 – 2.26 $\mu\text{g.g}^{-1}$ were required to achieve 90% sporulation inhibition, while between 1.06 and 1.24 $\mu\text{g.g}^{-1}$ were predicted necessary for 50% inhibition (Table 7). Sporulation inhibition increased steeply relative to the residue level, with 100% inhibition possible below the MRL (Fig. 1).

Mandarin

Analysis of variance of sporulation inhibition data from mandarin showed a significant temperature x pH x exposure time interaction ($P < 0.0001$; Table 5). Treatments at pH 3 did not differ significantly, but sporulation inhibition levels were very low (0.1 – 3.1%; Table 6). At pH 6, sporulation inhibition levels ranged from 3.2 to 79.5%, and significantly increased as temperature and exposure time increased (Table 6). Non-linear regression for sporulation inhibition data against IMZ residue data using the model described previously gave very good fits at an R^2 value of 0.919. The highest level of sporulation inhibition predicted was 77% at a residue of 2.40 $\mu\text{g.g}^{-1}$. For 50% sporulation inhibition, an IMZ residue level of 1.06 $\mu\text{g.g}^{-1}$ was predicted (Table 7; Fig. 1).

***In vitro* effect of solution temperature on *Rhizopus* spores in the fungicide bath**

Analysis of variance of percentage control data indicated significant trial effects (results not shown), but since all treatments were conducted identically, the trials were regarded as a statistical block effect to increase the robustness of the analysis.

The pasteurisation step at 65°C resulted in 100% control (except for one replicate with 99.7% control), regardless of treatment (results not shown).

Analysis of variance of percentage control data obtained using the solution containing *Rhizopus* spores indicated a significant temperature x exposure time interaction ($P < 0.0001$; Table 8). The data allowed for non-linear regression using the model described above, which

gave good fits with R^2 values of 0.767 and 0.656 for 1 and 60 minutes respectively (Table 9). The model predicted greater and faster control from a 60 minute exposure time than a 1 minute exposure time. Means indicated that 100% control was achieved at temperatures $> 45^\circ\text{C}$ for 60 minutes but the model predicted that only at 65°C would 100% control be seen at either exposure time (Fig. 2).

Analysis of variance of percentage control data obtained using the solution containing *Rhizopus* spores and *P. digitatum* spores indicated a significant temperature x exposure time interaction ($P < 0.0001$; Table 8). Non-linear regression gave poor fits for the data ($R^2 < 0.400$) and regression models were not presented. Treatments for a minute, ranging from 10 to 45°C showed minimal *Rhizopus* spore control with percentages between 9 and 31%. The trend was not clear between these temperatures, but at 55°C the control increased to 59% and 100% of the spores were controlled at 65°C . Similar to the *Rhizopus* solution alone, the 60-minute exposure time provided significantly higher levels of control in most cases. Finally, 93% control was achieved at 45°C and 100% at both 55 and 65°C (Table 10).

Analysis of variance of percentage control data obtained using the solution containing *Rhizopus* spores and IMZ fungicide indicated a significant temperature x exposure time interaction ($P = 0.0038$; Table 8). Non-linear regression gave poor fits for the data ($R^2 < 0.4$) and regression models were not presented. Control levels were generally markedly higher ($> 77\%$) than observed for other suspensions, especially at cooler temperatures. Complete control was achieved at 65°C for 1 minute and at 45, 55, and 65°C for 60 minutes (Table 10).

DISCUSSION

Previous studies highlighted the importance and effect of solution temperature in conjunction with solution pH and exposure time on IMZ residue loading and subsequent green mould control (Erasmus *et al.*, 2011, 2013, 2015a; Kellerman *et al.*, 2016; Chapter 2). Findings from this study confirm results from these studies, and contribute to our understanding of IMZ residue loading, green mould control and particularly sporulation inhibition following dip-treatment in cooler IMZ solutions. Additionally, this study demonstrated the pasteurisation effects of heated solutions and highlights the risk of contaminant build-up in cooler solutions.

Imazalil residues levels generally increased as either pH, temperature, or exposure time increased. At pH 3 solutions, neither the exposure time of the fruit to the solution, nor the temperature of the solution had any significant effect on IMZ residue loading. These results are a confirmation of work by Erasmus *et al.* (2011, 2013, 2015a) and Kellerman *et al.* (2016) who have shown that a pH 3 solution confer very stable loading with relatively low levels of IMZ that are below the MRL of $5 \mu\text{g}\cdot\text{g}^{-1}$. In their studies, they demonstrated that solutions with a pH of 6 or higher, increased residues to levels above the MRL with exposure times longer than 45 s in warm baths (*circa* 35°C , as recommended for the South African citrus

industry). The same was noticed with our study, but an interesting difference was that at the cooler temperatures, particularly of 10°C, pH 6 acted very similarly to the pH 3 treatments, and demonstrated lower and more stable residue loading trends.

The effect of brushing on IMZ sulphate residues on citrus has only recently been demonstrated by Erasmus *et al.* (2015a). Brushing of citrus is standard procedure in all citrus packhouses following IMZ aqueous application. In commercial packhouses, anything from 8 to 52 brushes can be operational post IMZ treatment (Erasmus *et al.*, 2011). In this study, fruit were always brushed, but the time between dip-treatment and brushing differed. The effect of this can be seen with the residues loaded where fruit subjected to delayed brushing loaded higher residues than fruit exposed to immediate brushing. Navel and Valencia oranges loaded similar residues as was also seen in Erasmus *et al.* (2011, 2013), but not in Erasmus *et al.* (2015a) or in Chapter 2. Mandarin fruit loaded lower residues, but followed the same trends as the oranges in the immediate brushing trial, as this fruit kind was also immediately brushed. The implications of the brushing effect on residues were highlighted at 35°C as residues in the delayed brushing trial exceeded the MRL of 5 µg.g⁻¹, while fruit with immediate brushing, treated identically otherwise, loaded about 70% less residue (maximum of 3.13 µg.g⁻¹ for delayed brushing compared to a maximum of 11.56 µg.g⁻¹ for immediate brushing). The reduction in residues is likely because of the removal of the excess solution carrying the active, removing the lipophilic IMZ before it has time to adhere and permeate the rind. At similar treatment conditions, pH 6 solutions loaded higher residues. This is due to the lipophilic nature of undissociated IMZ, where this compound then binds more strongly to oily constituents in the citrus rind (Siegel *et al.*, 1977; Brown and Dezman, 1990).

The effect of increased residue loading with increasing exposure time and higher temperature is apparent both here and in previous studies (Brown and Dezman, 1990; Schirra *et al.*, 1996, 2010; Smilanick *et al.*, 1997b; Cabras *et al.*, 1999; Puawongphat *et al.*, 2008; Sepulveda *et al.*, 2015) with the effect of the conjunction of a high solution pH even more so (Erasmus *et al.*, 2015a; Kellerman *et al.*, 2016; Chapter 2).

Delayed or immediate brushing did not seem to have an effect on curative control, as no clear differences were noted between fruit with different brushing treatments, and overall high levels (> 84%) of curative control were achieved regardless of citrus type or treatment parameters. Fairly low residue levels (≈ 0.5 µg.g⁻¹) will still exert curative control if treatment is applied timeously. The observation is explained by loading of residues in wound tissue, where higher residue levels were loaded in wounds than surrounding unwounded tissue (Dore *et al.*, 2009, 2010; Erasmus *et al.*, 2015a). Whole fruit residue levels are therefore poor predictors of curative control. However, sporulation inhibition was clearly linked to residue levels as was also observed previously (Erasmus *et al.*, 2011, 2013, 2015a; Kellerman *et al.*, 2016; Chapter 2). From the analysis of the results it was seen that overall an increase in

sporulation inhibition was achieved with an increase in treatment parameters and this was linked to the residues loaded onto the fruit. This relationship is explained by a logistic curve, with a residue of around $2.0 \mu\text{g}\cdot\text{g}^{-1}$ required to achieve 90% sporulation inhibition, and a residue of approximately $1.0 \mu\text{g}\cdot\text{g}^{-1}$, will achieve 50% sporulation inhibition, and still be sufficient for good curative control. These findings correlate strongly with those of Erasmus *et al.* (2011, 2013, 2015a) and Kellerman *et al.* (2016). Compared to the findings in Chapter 2 using the heated flooder, Valencia residues were very similar, needing just above 2.0 and $1.0 \mu\text{g}\cdot\text{g}^{-1}$ for 90 and 50% sporulation inhibition, respectively. Navel fruit indicated that lower residue levels were necessary in the heated flooder application with 1.29 and $0.85 \mu\text{g}\cdot\text{g}^{-1}$ for the respective control. This difference might be attributed to batch effects, and direct comparisons will have to be made to elucidate whether it can be ascribed to superior IMZ application in the heated flooder and/or a synergistic effect of temperature.

Drench chemicals such as thiabendazole and pyrimethanil convey some anti-sporulation activity, but markedly less than IMZ, where the sporulation inhibition is patchy, comprised of several smaller zones of inhibition (Kellerman *et al.*, 2014; Christie, 2016). Residue analysis revealed that the mandarin fruit had been drenched before collection. These residues must have exerted some protective control against the curative inoculation prior to treatment as the decay levels in the untreated control fruit decay were lower (average 81%) than near 100%, as is commonly expected following artificial inoculations. Despite the lowered decay influencing this efficacy trial negatively, the putative 20% control conferred from drench chemicals indicates how important the IMZ application in the packhouse is for effective green mould management (Smilanick *et al.*, 2006; Kellerman *et al.*, 2014).

In this study, it was observed that the control of *Rhizopus* spores were possible in solutions $> 45^\circ\text{C}$ given sufficient exposure time (1 hour). For a shorter exposure time, higher temperatures are required, pointing to the importance of carefully considered sanitation protocols. Packhouse sanitation and careful handling of the fruit, as well as maintaining the cold chain are the best ways to prevent *R. stolonifer* infections (Lesar, 2013). Heat ($> 35^\circ\text{C}$) has been demonstrated to be detrimental to *Rhizopus* (Miller *et al.*, 1959; Baker and Smith, 1970; Margosan *et al.*, 1997), and pasteurisation of a fungicide bath is a useful step to decrease contamination in the fungicide bath. The addition of IMZ drastically decreased the presence of *Rhizopus* spores. This study demonstrated that complete eradication of *Rhizopus* spores occurred at 55 and 65°C for the hour long exposure time. If exposure time is short (one minute), only a temperature of 65°C , with either green mould spores or IMZ present in the solution, will offer 100% control. It is important to note that sensitive citrus types such as lemon could experience heat damage at these high temperatures, therefore a good alternative is to pasteurize the bath each night after packing. The results of this study showed 100% control at 65°C for the pasteurisation step.

Residue levels and the subsequent sporulation inhibition of green mould can be optimized by increasing the pH, temperature or exposure time of an IMZ bath solution. Care should be taken to not exceed the MRL, particularly with longer exposure times in pH 6 solutions; however, it was apparent from this study that if sufficient and timely brushing is applied, infection control is not compromised yet residues remain below the MRL. It is known that the fungicide bath offers excellent curative control (Dore *et al.*, 2009; Erasmus *et al.*, 2011, 2015a; Kellerman *et al.*, 2016), and this was confirmed for cold solutions. A cold temperature bath could be considered a viable treatment option; however, residues and other benefits may be compromised in this case, particularly sporulation inhibition as a result of reduced residue loading. Heating the bath to more than 45°C may be necessary to achieve a greater level of benefits such as the eradication of *Rhizopus* spores. Additionally, hot water treatments may have other benefits such as a reduction of chilling injury (McDonald *et al.*, 1991; Schirra *et al.*, 2000; Erkan *et al.*, 2005), increasing lignin accumulation around wound sites to help prevent green mould development, or an increase in phytoalexin concentrations that aid in *P. digitatum* inhibition (Nafussi *et al.*, 2001). Further benefits are that warm fruit assists in drying off excess water before the wax application, which in turn leads to a more uniform wax coating (Pelser, 1977; Smilanick *et al.*, 1997a; Erasmus, personal communication, 2014). For these reasons, it is not recommended to treat fruit in a cold fungicide bath. Brushing after aqueous treatments in a packhouse is crucial to reduce the risk of exceeding the MRL. However, brushing also needs to be considered in postharvest research where misleading residue results and subsequent observations in residue loading and disease control could be skewed due to the lack of brushing in experimental designs. A final recommendation from this study is that, in order to control fungal contaminants in the fungicide bath, packhouses need apply imazalil in heated solutions (*circa* 45°C) and/or pasteurize fungicide baths overnight.

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Table 1. Analysis of variance for imazalil residue data as analysed from navel and Valencia fruit (delayed and immediate brushing) after dip application in imazalil solutions of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ IMZ at temperatures of 10, ambient ($\approx 19 - 22$) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

Source	Navel & Valencia (delayed brushing)			Navel & Valencia (immediate brushing)		
	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c
Model	47	14.762	<0.0001	47	0.961	<0.0001
Temperature	2	108.737	<0.0001	2	5.635	<0.0001
pH	1	138.705	<0.0001	1	18.708	<0.0001
Exposure time	3	10.254	<0.0001	3	0.878	<0.0001
Fruit kind	1	1.814	0.016	1	0.382	0.013
Temperature*pH	2	91.483	<0.0001	2	2.903	<0.0001
Temperature*Exposure time	6	7.934	<0.0001	6	0.167	0.016
Temperature*Fruit kind	2	0.396	0.269	2	0.227	0.025
pH*Exposure time	3	7.936	<0.0001	3	0.792	<0.0001
pH*Fruit kind	1	0.862	0.093	1	0.028	0.488
Exposure time*Fruit kind	3	0.100	0.796	3	0.014	0.860
Temperature*pH*Exp.time	6	7.550	<0.0001	6	0.328	0.000
Temperature*pH*Fruit kind	2	0.556	0.161	2	0.029	0.604
Temp.*Exp.time*Fruit kind	6	0.203	0.657	6	0.009	0.986
pH*Exposure time*Fruit kind	3	0.279	0.424	3	0.026	0.710
Temp.*pH*Exp.time*Fruit kind	6	0.043	0.989	6	0.053	0.480
Error	48	0.293		48	0.057	
Corrected Total	95			95		

^aDF = Degrees of freedom

^bMS = Mean sum of squares

^cP = Probability

Table 2. Mean imazalil residue data as analysed from navel and Valencia fruit (delayed and immediate brushing) and mandarin fruit after dip application in imazalil solutions of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ IMZ at temperatures of 10, ambient ($\approx 19 - 22$) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

		Imazalil residues ($\mu\text{g}\cdot\text{g}^{-1}$) ^a					
		Exposure time					
pH	Temp. (°C)	15 s	45 s	90 s	180 s		
Navel & Valencia (delayed brushing)							
3	10	0.76 h	0.97 efgh	0.77 gh	0.87 gh		
	Ambient	0.88 fgh	1.03 efgh	1.17 efgh	1.13 efgh		
	35	1.10 efgh	1.16 efgh	1.19 efgh	1.36 efgh		
6	10	1.09 efgh	1.21 efgh	1.24 efgh	1.33 efgh		
	Ambient	1.47 efgh	1.54 efg	1.68 e	1.65 ef		
	35	3.57 d	5.91 c	9.00 b	11.56 a		
Navel & Valencia (immediate brushing)							
3	10	0.57 j	0.61 j	0.57 j	0.68 hij		
	Ambient	0.58 j	0.62 ij	0.68 hij	0.72 hij		
	35	0.96 gh	0.73 hij	0.88 ghij	0.75 hij		
6	10	0.89 ghij	0.85 ghij	0.95 ghi	1.17 efg		
	Ambient	1.09 fg	1.35 ef	1.44 de	1.72 d		
	35	1.40 def	2.22 c	2.75 b	3.13 a		
Mandarin							
3	10	0.38 kl	0.43 jkl	0.49 hijkl	0.48 hijkl		
	Ambient	0.37 l	0.43 jkl	0.45 jkl	0.51 hijkl		
	35	0.49 hijkl	0.46 ijkl	0.66 ghij	0.61 ghijk		
6	10	0.48 hijkl	0.70 ghi	0.62 ghijkl	0.75 efg		
	Ambient	0.58 e	0.72 fgh	0.94 ef	0.99 e		
	35	1.54 d	2.39 c	2.99 b	4.74 a		

^a For delayed and immediate brushing and mandarins separately, means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 0.770 and 0.339, 0.238 respectively)

Table 3. Analysis of variance for imazalil residue data as analysed from mandarin fruit (immediate brushing) after dip application in imazalil solutions of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ IMZ at temperatures of 10, ambient ($\approx 19 - 22$) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

Source	Mandarin		
	DF ^a	MS ^b	P ^c
Model	23	2.110	<0.0001
Temperature	2	7.124	<0.0001
pH	1	11.337	<0.0001
Exposure time	3	1.072	<0.0001
Temperature*pH	2	5.789	<0.0001
Temperature*Exposure time	6	0.508	<0.0001
pH*Exposure time	3	0.715	<0.0001
Temperature*pH*Exposure time	6	0.494	<0.0001
Error	24	0.013	
Corrected Total	47		

^a DF = Degrees of freedom ^b MS = Mean sum of squares ^c P = Probability

Table 4. Analysis of variance for curative infection control data as analysed from navel and Valencia fruit (delayed and immediate brushing) and mandarin fruit after dip application in imazalil solutions of 500 $\mu\text{g.mL}^{-1}$ IMZ at temperatures of 10, ambient ($\approx 19 - 22$) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

Source	Navel (delayed brushing)			Valencia (delayed brushing)			Navel (immediate brushing)			Valencia (immediate brushing)			Mandarin		
	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c
Model	23	14.971	0.059	23	8.950	0.187	23	52.994	0.006	23	885.976	<0.0001	23	4830.265	<0.0001
Temp.	2	54.885	0.004	2	13.724	0.146	2	277.470	<0.0001	2	5478.615	<0.0001	2	14574.652	<0.0001
pH	1	26.346	0.103	1	2.973	0.518	1	31.032	0.290	1	997.746	0.013	1	13507.013	0.001
Exp. time	3	4.824	0.691	3	27.879	0.009	3	123.735	0.004	3	522.801	0.022	3	15519.612	<0.0001
Temp.*pH	2	8.740	0.414	2	5.084	0.490	2	6.150	0.801	2	66.111	0.665	2	4208.101	0.034
Temp.*Exp. time	6	1.915	0.979	6	7.043	0.431	6	18.085	0.688	6	351.552	0.043	6	1270.608	0.405
pH*Exp. time	3	20.065	0.109	3	0.991	0.936	3	12.472	0.717	3	766.000	0.003	3	550.891	0.720
Temp.*pH*Exp. time	6	17.079	0.112	6	6.070	0.529	6	15.349	0.767	6	388.404	0.026	6	667.984	0.777
Error	834	9.908		839	7.118		830	27.707		837	161.676		838	1234.225	
Corrected Total	857			862			853			860			861		

^a DF = Degrees of freedom

^b MS = Mean sum of squares

^c P = Probability

Table 5. Analysis of variance for green mould sporulation inhibition data as analysed from navel and Valencia fruit (delayed and immediate brushing) and mandarin fruit after dip application in imazalil solutions of 500 µg.mL⁻¹ IMZ at temperatures of 10, ambient (≈ 19 - 22) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

Source	Navel & Valencia (delayed brushing)			Navel & Valencia (immediate brushing)			Mandarin		
	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c
Model	23	64178.755	<0.0001	23	53681.888	<0.0001	23	30446.225	<0.0001
Temperature	2	144397.890	<0.0001	2	129500.943	<0.0001	2	88017.518	<0.0001
pH	1	1129038.928	<0.0001	1	810263.301	<0.0001	1	301624.971	<0.0001
Exposure time	3	2300.934	<0.0001	3	8443.770	<0.0001	3	6713.469	<0.0001
Temperature*pH	2	15877.533	<0.0001	2	47085.884	<0.0001	2	82695.719	<0.0001
Temperature*Exposure time	6	509.146	0.018	6	1808.749	<0.0001	6	1609.802	<0.0001
pH*Exposure time	3	925.982	0.003	3	6571.842	<0.0001	3	5241.858	<0.0001
Temperature*pH*Exposure time	6	939.800	<0.0001	6	2280.992	<0.0001	6	1597.466	<0.0001
Error	1677	198.946		1700	349.009		835	72.661	
Corrected Total	1700			1723			858		

^a DF = Degrees of freedom

^b MS = Mean sum of squares

^c P = Probability

Table 6. Mean green mould sporulation inhibition data as analysed from navel and Valencia fruit (delayed and immediate brushing) and mandarin fruit after dip application in imazalil solutions of 500 µg.mL⁻¹ IMZ at temperatures of 10, ambient (≈ 19 - 22) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

pH	Temp. (°C)	Sporulation inhibition (%) ^a							
		Exposure time							
		15 s	45 s	90 s	180 s				
Navel & Valencia (delayed brushing)									
3	10	26.67	hi	24.06	hij	28.33	h	23.61	ij
	Ambient	16.94	kl	20.83	jk	16.11	l	22.00	ij
	35	50.56	g	56.00	f	53.14	fg	50.06	f
6	10	67.25	e	68.41	e	66.29	e	80.00	cd
	Ambient	78.87	d	84.17	c	78.59	d	89.14	b
	35	100.0	a	100.0	a	100.0	a	100.0	a
Navel & Valencia (immediate brushing)									
3	10	18.06	l	24.23	jk	24.17	jk	16.62	l
	Ambient	21.94	jkl	38.89	gh	20.56	jkl	18.89	kl
	35	25.59	ghi	33.89	hi	32.78	i	38.61	ghi
6	10	41.94	fg	41.71	fg	52.50	e	45.56	f
	Ambient	58.06	e	64.72	d	75.83	c	80.00	bc
	35	81.94	b	94.72	a	97.50	a	100.0	a
Mandarin fruit									
3	10	0.10	j	0.19	ij	0.53	ij	0.41	ij
	Ambient	0.09	j	2.67	hij	1.57	hij	1.35	hij
	35	0.14	j	1.58	hij	1.34	hij	3.10	hij
6	10	3.23	hij	4.65	gh	8.40	g	20.21	f
	Ambient	4.17	hi	39.34	d	24.96	e	51.66	c
	35	67.67	b	79.52	a	79.52	a	79.52	a

^a For delayed and immediate brushing and mandarins separately, means followed by the same letter do not differ significantly ($P > 0.05$; LSD = 4.647, 6.114 and 3.955 respectively)

Table 7. Parameters, goodness of fit and effective IMZ residue levels for predicted 50 and 90% sporulation inhibition of non-linear regression using the function $Y = pr3/(1+Exp(-pr1-pr2*X1))$ of imazalil residue data measured on navel, Valencia and mandarin fruit after treatment with 500 $\mu\text{g.mL}^{-1}$ IMZ at temperatures of 10, ambient ($\approx 19 - 22$) or 35°C and pH levels of 3 or 6, at exposure times of 15, 45, 90 or 180 s.

Citrus type	Model parameter values and goodness of fit				IMZ residue $\mu\text{g.g}^{-1}$	
	Pr1	Pr2	Pr3	R^2	90%	50%
Navel	-2.126	1.791	102.459	0.682	2.292	1.1607
Valencia	-3.071	2.935	95.203	0.693	2.018	1.0807
Mandarin	-6.272	6.511	77.225	0.919	2.4 for 77%	1.05669

Table 8. Analysis of variance for *Rhizopus stolonifer* control data as analysed from colony forming unit counts for solutions containing spore suspensions of *Rhizopus* only, *Rhizopus* and green mould, and *Rhizopus* and imazalil (500 µg.mL⁻¹) held in water baths of temperatures of 10, 15, 20, 25, 35, 45, 55 or 65°C at exposure times of 1 minute or 60 minutes.

Source	<i>Rhizopus</i> only			<i>Rhizopus</i> and green mould			<i>Rhizopus</i> and imazalil		
	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c	DF ^a	MS ^b	P ^c
Model	15	18279.351	<0.0001	15	15506.228	<0.0001	15	576.024	<0.0001
Temp.	7	22934.014	<0.0001	7	20928.412	<0.0001	7	1064.093	<0.0001
Exp. time	1	79662.661	<0.0001	1	65550.402	<0.0001	1	160.518	0.126
Temp.*Exp. time	7	4855.644	<0.0001	7	2934.877	<0.0001	7	147.314	0.0038
Error	224	228.917		224	454.411		224	67.971	
Corrected Total	239			239			239		

^a DF = Degrees of freedom

^b MS = Mean sum of squares

^c P = Probability

Table 9. Parameters and goodness of fit of non-linear regression using the function $Y = pr3/(1+Exp(-pr1-pr2*X1))$ of spore suspensions of *Rhizopus* only, held in water baths of 10, 15, 20, 25, 35, 45, 55 or 65°C for either 1 minute or 60 minutes.

Exposure time	Model parameter values and goodness of fit			
	Pr1	Pr2	Pr3	R ²
1 minute	-10.852	0.090	13603.132	0.767
60 minute	-1.981	0.042	157.153	0.656

Table 10. Mean percentage *Rhizopus* spore control for spore suspensions of *Rhizopus* only, *Rhizopus* and green mould, *Rhizopus* and imazalil (500 µg.mL⁻¹) held in water baths of 10, 15, 20, 25, 35, 45, 55 or 65°C for either 1 minute or 60 minutes.

Exposure time (s)	Temperature (°C)	Control (%) ^a		
		<i>Rhizopus</i> only	<i>Rhizopus</i> and green mould	<i>Rhizopus</i> and imazalil
1 minute	10	7.40 e	12.65 gh	90.93 cde
	15	6.60 e	23.18 fgh	85.06 e
	20	12.19 e	31.79 ef	94.06 bcd
	25	15.03 e	24.87 fg	94.35 abcd
	35	9.15 e	9.50 h	90.14 de
	45	14.02 e	26.52 fg	96.53 abc
	55	30.08 cd	59.19 bc	96.94 ab
	65	94.25 a	100.0 a	100.0 a
60 minutes	10	43.03 b	49.20 cd	93.01 bcd
	15	35.59 bcd	67.51 b	77.11 f
	20	37.96 bc	65.13 b	96.14 abc
	25	26.46 d	43.46 de	97.07 ab
	35	45.69 b	33.30 ef	97.77 ab
	45	91.38 a	93.51 a	100.0 a
	55	100.0 a	100.0 a	100.0 a
	65	100.0 a	100.0 a	100.0 a

^a Means in each column followed by the same letter do not differ significantly ($P > 0.05$; LSD = 10.887, 15.339 and 5.932, respectively)

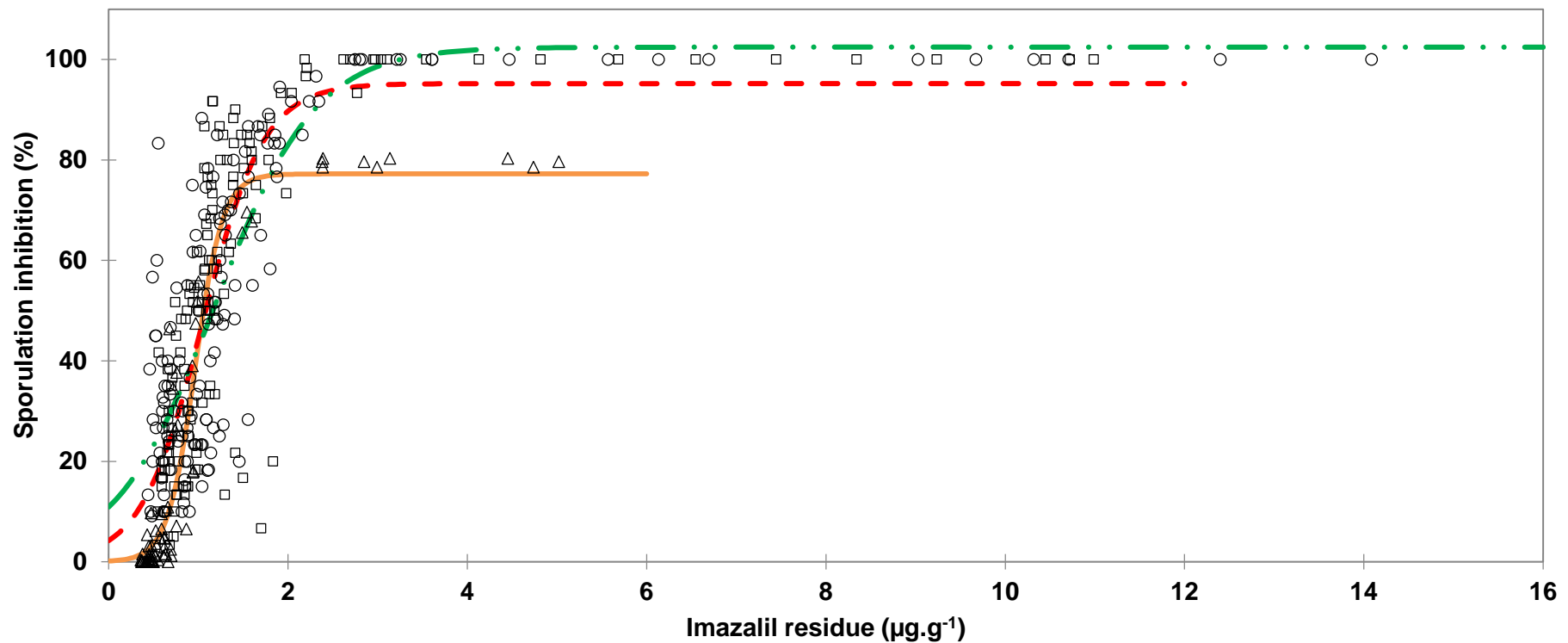


Figure 1. Mean imazalil residues values analysed on citrus fruit after treatment in imazalil solutions at 500 µg.mL⁻¹ at a pH of 3 or 6 at a temperature range of 10, ambient (≈ 19 – 22°C) or 45°C and non-linear regression models [$Y = pr3/(1+Exp(-pr1-pr2*X1))$] fitted indicating residue loading trends for navel (○, green dash-dot-dot line, $R^2 = 0.682$), Valencia (□, red dashed line, $R^2 = 0.693$), and mandarin (△, orange solid line, $R^2 = 0.919$), for sporulation inhibition.

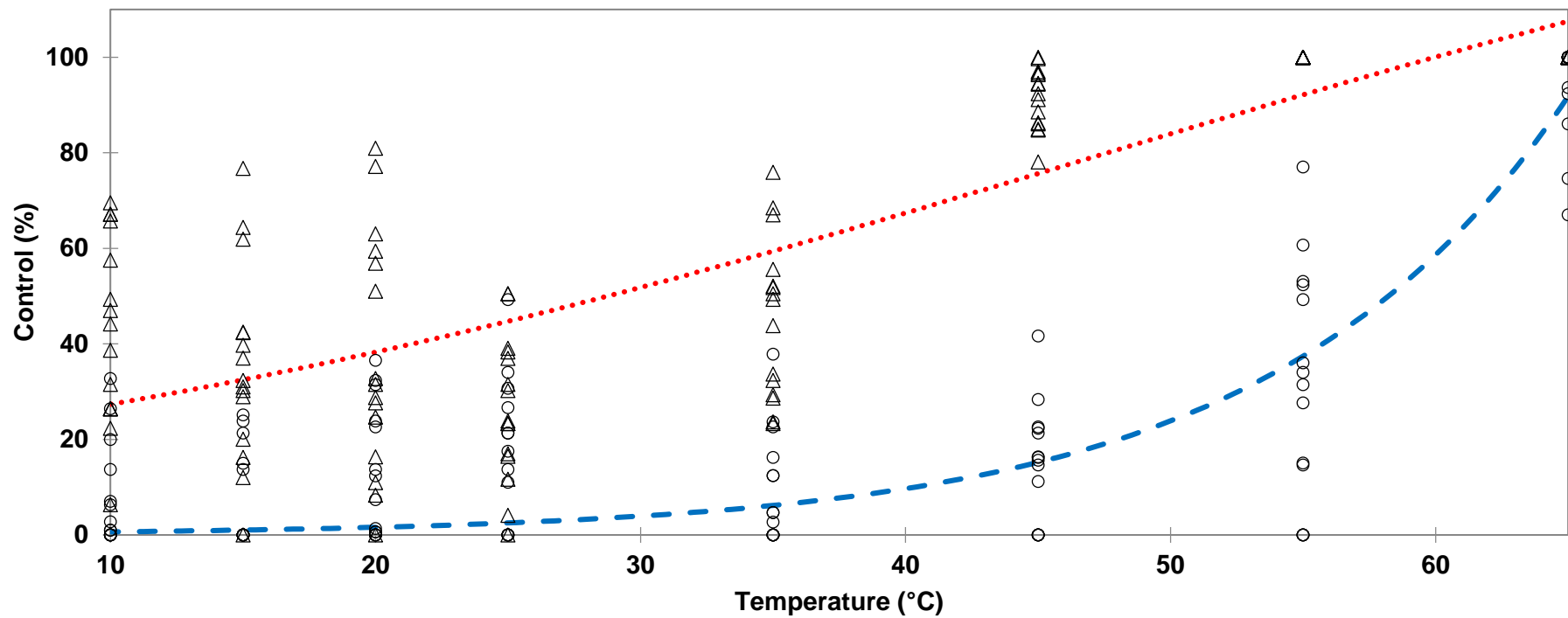


Figure 2. Mean percentage control of *Rhizopus stolonifer* colony forming units (CFU) at a temperature range of 10, 15, 20, 25, 35, 45, 55, 65°C for either 1 or 60 minutes' exposure time, and non-linear regression models [$Y = pr3/(1+Exp(-pr1-pr2*X1))$] fitted indicating control for 1 minute (○, blue dashed line, $R^2 = 0.767$) and 60 minutes (△, red dotted line, $R^2 = 0.656$).