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Imazalil residue loading and green mould control in citrus packhouses

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ABSTRACT

Imazalil (IMZ) is commonly applied in South African citrus packhouses for the control of green mould, caused by *Penicillium digitatum*, yet the disease still causes significant postharvest losses. The maximum residue limit (MRL) for IMZ on citrus fruit is 5 $\mu g\,g^{-1}$, whereas 2–3 $\mu g\,g^{-1}$ is a biologically effective residue level that should at least inhibit green mould sporulation. Standard compliance auditing of residue levels of citrus fruit, however, indicate that fruit from the majority of packhouses have residues of $\approx$1 $\mu g\,g^{-1}$. Poor disease control from insufficient residue loading might further be compounded by the presence of IMZ-resistant isolates of *P. digitatum* in packhouses. This study was conducted to assess the current status of IMZ application in South African packhouses, to determine the adequate residue levels needed to control green mould and inhibit its sporulation using both IMZ sensitive and resistant isolates, to investigate IMZ application methods and resultant residue levels in commercial citrus packhouses, and to study optimisation of modes of IMZ application in citrus packhouses. Factors studied were IMZ concentration, application type (spray *vs.* dip and drench), exposure time, solution temperature and pH, as well as curative and protective control of *P. digitatum*. The packhouse survey showed that the majority of packhouses applied IMZ in a sulphate salt formulation through a fungicide dip tank, and loaded an IMZ residue of $\approx$1 $\mu g\,g^{-1}$. In dip applications, IMZ had excellent curative and protective activity against *Penicillium* isolates sensitive to IMZ. However, curative control of IMZ resistant isolates was substantially reduced and protective control was lost, even at twice the recommended concentration, nor was sporulation inhibited. The use of sodium bicarbonate (2%) buffered imazalil sulphate solutions at pH $\pm$8, compared with pH $\pm$3 of the unbuffered solutions, markedly increased IMZ residue loading on Navel and Valencia oranges and improved curative and protective control of IMZ resistant isolates. Exposure time did not affect IMZ residue loading in IMZ sulphate solutions at pH 3, although the MRL was exceeded after 45 s exposure in pH 8 solutions. Imazalil applied through spray or drench application improved residue loading, but green mould control was less effective than after dip application.

1. Introduction

Imazalil (IMZ) is applied in citrus packhouses for the control of *Penicillium digitatum* (Pers.:Fr.) Sacc. (Laville et al., 1977; Eckert, 1995), which causes green mould (Smith, 1897). This postharvest disease is a major cause of decay on citrus fruit globally (Eckert and Eaks, 1989). Various application methods of IMZ are possible, one possibility being pre-packline drenching of fruit bins shortly after harvest. In the packline it can be applied in several ways: a fungicide dip tank, spray, drench or mixed with wax in wax application systems (Laville et al., 1977; McCornack et al., 1977; Kaplan and Dave, 1979). Combinations of these methods can also be done, where double or even triple application of IMZ is possible. In the early 1980s, a dip treatment for 1–3 min in 250–500 $\mu g\,mL^{-1}$ IMZ was recommended as the most effective application method and IMZ sulphate was recommended as the preferred formulation due to its water solubility (Pelser and La Grange, 1981). Currently IMZ is available in two different formulations. Imazalil sulphate, which is formulated as soluble granules or powders and has a very good ability to dissolve in water. It is therefore recommended in aque-

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ous application at 500 μg mL^{-1}. The IMZ emulsifiable concentrate (EC) is an oily emulsion of IMZ and is mostly recommended for incorporation in wax applications at 3000 μg mL^{-1}.

Smilanick et al. (2005) showed that the effectiveness of IMZ to control green mould was positively correlated with the pH level of the IMZ solution. They found that IMZ residues measured on orange fruit dipped for 30 s in a 500 μg mL^{-1} IMZ EC solution at pH 7 or 9 were 2-fold those measured on fruit dipped in solutions at pH 3 or 5. In another test they found that amending a 500 μg mL^{-1} IMZ solution with 3% $NaHCO_3$ did not significantly affect residue loading on the fruit, although the addition of the $NaHCO_3$ markedly improved the effectiveness of the IMZ to control green mould and affected partial control of IMZ resistant isolates of the pathogen. In Australia, efficacy of IMZ has been shown to be improved when mixed with either 0.5% or 3% $NaHCO_3$ (Cunningham and Taverner, 2006). Recently, Dore et al. (2010) confirmed these findings showing that $NaHCO_3$ increased IMZ residues in wounds, but not intact fruit. Prior work employed the IMZ EC formulation, which has a relatively neutral pH and limited water solubility, and not that of IMZ sulphate, which has a low pH and is very water soluble. It is not known how IMZ sulphate would perform in similar trials. It is particularly important to know the influence of $NaHCO_3$ on fruit IMZ residues, since compliance with regulator tolerances has become increasingly important, and its influence on IMZ performance, since IMZ resistant isolates of *P. digitatum* are common.

Smilanick et al. (1997) found that IMZ solution temperature, concentration and exposure time all have an effect on the subsequent loading of IMZ residues on fruit. Warmer temperatures, higher concentrations and longer exposure times increased the IMZ residue on treated fruit. Using the EC formulation of IMZ, they found the ideal combination was a solution temperature of 37.8 °C, at a concentration of 350–400 μg mL^{-1} and an exposure time of 30 s. An IMZ residue of 2–4 μg g^{-1} on fruit can be expected. Schirra et al. (1997) found that dipping lemon fruit in a much lower IMZ concentration, *i.e.* 50 μg g^{-1}, but at longer exposure (3 min) and higher solution temperature (50 °C) loaded residue levels of ≥2 μg g^{-1} and gave significant protection against citrus green mould.

In earlier studies, atomisers were used to apply IMZ, either in water or in water-based wax solutions over rotating brushes, and adequate residue levels were obtained; however, IMZ applied in water gave better results in terms of residue loading and green mould control (Brown et al., 1983; Brown, 1984). Application of IMZ in water will also give more protection against infections occurring after treatment (Brown, 1984).

The potential to develop fungicide resistance was shown in a group of fungi, which included *P. digitatum* (van Tuyl, 1977). Resistance to IMZ is polygenic and involves 21 genes on 8 loci and is linked with six groups; this means that it is theoretically more difficult for *Penicillium* to develop resistance due to all the different genes involved (Laville et al., 1977). However, resistance did eventually develop (Holmes and Eckert, 1995). Imazalil is a demethylation inhibitor of the biosynthesis of ergosterol, and resistance was affected primarily by overexpression of cytochrome P450-dependent sterol 14 alpha-demethylase (Ghosoph et al., 2007). Infection caused by resistant isolates cannot be controlled or its sporulation inhibited with normal packhouse IMZ treatments (Eckert et al., 1994). A higher IMZ residue will improve green mould control and combat IMZ resistance development (Dore et al., 2009). IMZ resistance has been reported in South Africa (Jacobs and Korsten, 2010), but the extent and level is not known.

The purpose of this study was firstly to conduct a survey in South African packhouses in order to determine the most commonly used IMZ application methods and their efficacy in terms of IMZ residue loading. Secondly, the most commonly used IMZ application method was studied and compared to other methods in terms of residue loading and control of resistant and sensitive isolates of *P. digitatum*.

2. Materials and methods

2.1. Survey of commercial IMZ application in South African citrus packhouses

Current fungicide application methods were evaluated by a survey of South African packhouses. Detailed surveys of the various pack lines included IMZ concentration, solution pH and temperature, fruit exposure time and IMZ fruit residue analyses.

The temperatures of the dip tank solutions in the operating packhouses were measured by means of a thermo probe connected to an infrared thermometer (Sentry ST642; Sentry Optronics Corporation, Shanghai, China). The exposure time of the fruit to the dip tank solution was measured by wrapping some of the fruit in aluminium foil and measuring the exposure time of these marked fruit in the dip tank; this was replicated 12 times. A 250 mL sample of the dip tank solution was taken if IMZ was part of the solution. The samples were stored at −20 °C until the IMZ concentration of each was determined by a two-phase titration method (Janssen Pharmaceutica, Beerse, Belgium). Sodium lauryl sulphate (90–91%, UniLab, Krugersdorp, South Africa) was titrated into 25 mL of the IMZ solution to which 10 mL sulphuric acid (Synthon, Nijmegen, Netherlands), 25 mL dichloromethane (99.8%, Sigma–Aldrich, St. Louis, MO, USA) and 12 drops of indophenol blue indicator (Sigma–Aldrich, St. Louis, MO, USA) had been added. When the end point was reached the amount of sodium lauryl sulphate (mL) used was noted as "A". The same procedure was followed with a blank solution of water and the end point (mL) was noted as "B". The IMZ concentration (μg mL^{-1}) was calculated as (A mL − B mL) × 0.1 × 40 × 297.18. The pH of the solutions were measured by means of a pH meter (Jenway Model 3310; Bibby Scientific Limited, Staffordshire, UK).

Six fruit were sampled before and after each IMZ application on a specific packline. The sampled fruit were deep frozen (−20 °C) until prepared for IMZ residue analysis. The fruit were defrosted, measured and weighed and macerated to a fine pulp using a blender (Salton Elite, Almalgamated Appliance Holdings Limited, Reuven, South Africa) and re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (chloramizol) residue analyses by Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa. The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography mass spectrometry mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA). The data collected were subjected to descriptive and Pearson correlation statistics using SAS (SAS Institute Inc. Cary, NC, USA).

2.2. IMZ residue loading and biological efficacy trials

IMZ residue loading after simulated dip, spray and drench application systems and curative and protective application for the control of green mould was studied in several experiments.

2.2.1. Isolates, IMZ sensitivity and storage

An IMZ-resistant (R) isolate of *P. digitatum* was obtained from Citrus Research International (CRI) in Nelspruit, South Africa and an IMZ-sensitive (S) isolate was obtained from a Satsuma orchard on the Stellenbosch University experimental farm, Welgevallen, Stellenbosch, South Africa. The species identity of the two isolates was confirmed as being *P. digitatum*, since BLAST analyses in GenBank showed that (1) the ITS sequence of both isolates had 100% similarity to the *P. digitatum* sequence (AY373910) of (Haugland

et al., 2004) and (2) the β-tubulin sequence of both isolates had 99.8% similarity to the *P. digitatum* sequence (AY674405) of Samson et al. (2004). Sensitivity to IMZ was determined by Janssen Pharmaceutica, Beerse, Belgium. In brief, potato dextrose agar was amended with an IMZ concentration range of 10–0.001 $\mu g\,g^{-1}$ on which spore suspensions of the tested isolates were inoculated. Cultures were incubated for 7 days at 22 °C before growth was measured and compared to the control (no IMZ). EC50 and EC95 values were calculated by linear interpolation in a *XY*-plot with a logarithmic concentration scale using the growth diameter values of cultures with 50% and 5% growth of the control. The respective EC50 and EC95 values for the S-isolate (STEU6560) were 0.07 and 0.10 $\mu g\,mL^{-1}$ and for the R-isolate (STEU6590) they were 1.83 and 4.52 $\mu g\,mL^{-1}$. The isolates were stored in the culture collection of the Department Plant Pathology, University Stellenbosch, South Africa.

In order to obtain inoculum for biological efficacy tests, the isolates were grown at ambient temperature on potato dextrose agar (PDA) medium in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 1 h before trials commenced. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma–Aldrich, St. Louis, MO, USA) added at a concentration of 0.01 $mL\,L^{-1}$ into a 500 mL Schotts bottle to a volume of 100 mL. The conidial suspensions were amended to a concentration of 1×10^6 spores mL^{-1} by means of a haemocytometer. The conidial suspensions were placed on magnetic stirrers to maintain a homogenised suspension of spores.

2.2.2. Fruit

Untreated export quality citrus fruit were collected from various citrus packhouses in the Western Cape province of South Africa. Citrus type and cultivar used for specific trials varied in accordance to seasonal availability. Before the trials commenced fruit were stored at 3.5–7 °C for ± 3 days. A day before a trial, fruit were transferred from cold storage to ambient in order for fruit temperature to reach ambient and to allow any possible condensation to evaporate.

2.2.3. Inoculation

Fruit were treated before inoculation (protectively) or after inoculation (curatively) to evaluate the efficacy of IMZ treatment dosages to control S and R isolates of *P. digitatum*. The fruit for the curative treatment were inoculated 4–6 h before treatment with IMZ. Fruit were wounded with a triple wound inducer, which consisted of three insect needles placed in a needle clamp to create three small wounds of 0.5 mm wide and 2 mm deep at a triangular distance of 1.5 mm apart. Wounding and inoculation were conducted simultaneously by dipping the wound inducer into a spore suspension of *P. digitatum* (1×10^6 spores mL^{-1}) immediately prior to wounding. Four wounds were induced on each fruit at equal distances around the stem-end; 12 fruit were inoculated per treatment with three replications. Fruit for protective treatment were first treated with IMZ, left to dry and inoculated as above before placed with the curatively treated fruit in the specific storage regimes.

2.2.4. Imazalil and residue analysis

In the various experiments conducted, fruit were treated with the imazalil sulphate formulation (Imazacure, 750 $g\,kg^{-1}$ SG, ICA International Chemicals, Stellenbosch, South Africa), unless stated differently. IMZ sulphate dissolved in municipal water and at concentrations of 250–1000 $\mu g\,mL^{-1}$ resulted in a solution pH of ≈3. For certain treatments, 2% sodium bicarbonate ($NaHCO_3$; Alkalinity Plus, Pool Perfect, Bellville, South Africa) was applied to IMZ sulphate solutions to increase the pH to ≈8 in an attempt to improve residue loading (Smilanick et al., 2005). A fresh treatment solution was prepared for each treatment. Six fruit per treatment were sampled and frozen (−20 °C) until prepared for IMZ residue analysis as described previously. Two separate samples were analysed for each treatment.

2.2.5. Fruit storage and evaluation

The 12 treated and inoculated fruit from each treatment combination were packed in lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd., Atlantis, South Africa). Each carton was covered with a transparent polyethylene bag and sealed with a cable tie. Different storage regimes were used in the various experiments and will be discussed. At the end of storage, the percentage of infected fruit was determined and sporulation was evaluated for each fruit by means of a rating index of 0–6, where 0 = no sign of disease, 1 = lesion visible but no sporulation, 2 = sporulating area on lesion smaller than a quarter of the fruit, 3 = sporulating area larger than a quarter of the fruit, but smaller than half of the fruit, 4 = sporulating area larger than half of the fruit, but smaller than three quarters of the fruit, 5 = sporulating area larger than three quarters of the fruit, but smaller than the whole fruit and 6 = sporulating area covering the whole fruit. Sporulation incidence (%) was determined from infected fruit with a sporulation index of 2 and higher. The incidence of infection (%) was derived from the sporulation ratings, where fruit were regarded as infected if they had a sporulation index of higher than 0.

2.2.6. Statistical analysis

Imazalil residue data were continuous and analysed by an analysis of variance appropriate to the experimental layout. Binomial infection incidence and sporulation data were transformed to percentages and working logits before subjected to an appropriate analysis of variance using statistical software (SAS version 9.2, SAS Institute Inc. Cary, NC, USA). Shapiro–Wilk test was performed to test for non-normality (Shapiro and Wilk, 1965). In cases where non-normality was due to high kurtosis, analysis was conducted on percentages (Glass et al., 1972). In the case of non-normality due to skewness, logit transformed data were used. Student's *t*-Least Significant Difference was calculated at the 5% significance level to compare treatment means. The experiments (on the different citrus types) were analysed separately, but if variances between citrus types were of the same magnitude as determined by Levene's test, the data were combined (John and Quenouille, 1977). If heterogeneity of variances were found a weighted analysis of variances was done. Weights were calculated using the reciprocal of the experimental variance (mean square error) for each experiment. In the combined analysis this led to a mean square error of 1 (MSE = 1).

2.3. IMZ application in a dip tank

2.3.1. Effect of IMZ concentration

Oranges (*Citrus sinensis* (L.) Osbeck) were immersed for 60 s in IMZ concentrations 0, 250, 500 and 1000 $\mu g\,mL^{-1}$ at pH ≈3 or 8 (amended with 2% $NaHCO_3$) at a solution temperature of ±20 °C and left to dry at ambient temperature. A plastic container (330 mm × 620 mm × 285 mm) with 20 L of each specific solution was used as IMZ dip tank. Fruit were treated before inoculation (protectively) or after inoculation (curatively) with the S or R isolate of *P. digitatum* as described previously. Two storage regimes were followed after treatment; one at 7 °C for 21 days plus a subsequent 7 days at 23 °C (simulating the cold chain for export fruit) and another at 23 °C for 14 days. Evaluations were conducted 11, 14, 18 and 21 days after treatment for fruit stored at 7 °C, and after the additional 7 days at 23 °C. For fruit stored at 23 °C, evaluations were conducted 2, 4, 7, 11 and 14 days after treatment. Sporulation and infection incidences were determined as described earlier.

Three replications of 12 fruit per treatment were used. For each treatment, two of the three replications had six additional fruit for residue analysis. The trial was done twice, once with Navel and once with Valencia oranges.

2.3.2. Effect of exposure time and solution temperature

Valencia oranges (2 replications of 6 fruit per treatment combination) were immersed for 15, 45, 90, 180 or 540 s in the registered concentration of 500 μg mL^{-1} IMZ solution that was not amended or amended with 2% $NaHCO_3$ at either 20 or 35 °C. After treatment fruit were left to dry at ambient before it was deep frozen and prepared for residue analysis as previously described. A temperature-controlled stainless steel water bath (Unitemp Water Bath, Baird and Tatlock (London) Ltd., Essex, UK) was used as an IMZ dip tank.

2.3.3. IMZ dip application compared to spray application

By measuring deposition of a fluorescent pigment, Fourie et al. (2009) demonstrated significantly higher deposition values with spray volumes to the point of run-off, compared with sprays past run-off and dip applications. In order to ascertain whether point-of-run-off IMZ sprays to citrus fruit will result in better residue loading than dip treatments, an experiment was conducted to compare spray application with dip treatment. Eureka lemons (*Citrus limon*) inoculated with the IMZ S and R isolates were treated curatively in this experiment. For the dip tank application, fruit were immersed for 60 s in a 0, 250, 500 and 1000 μg mL^{-1} IMZ solution (20 °C), which was not amended (pH 3) or amended with 2% $NaHCO_3$ (pH 8), or in a IMZ EC (Imazacure 500 EC, ICA International Chemicals, Stellenbosch, South Africa) solution at the same concentrations. For spray application, the point of run-off was determined using the methods described by Fourie et al. (2009). A gravity-fed mist spray gun (ITW DEVILBISS Spray Equipment Products, Glendale Heights, IL 60139, USA) was used to apply the spray volumes to fruit at a pressure of 1 bar. Spraying was done in a spray chamber [660 mm × 1410 mm × 880 mm ($h \times l \times w$)] with individual fruit positioned directly below the spray gun, which was mounted onto the spray chamber at a distance of 60 cm from the fruit. A spray volume of 3 mL of IMZ sulphate, alone (pH ≈3) or amended with 2% $NaHCO_3$ (pH ≈8), was applied at concentrations of 0, 1000, 2000 and 4000 μg mL^{-1} and IMZ EC at the same concentrations. Three replications of 12 fruit per treatment combination were treated. An additional 6 fruit (not inoculated) were treated in each of two replications for residue analysis. After each dip or spray treatment, the fruit were left to dry at ambient before fruit were sampled for residue analysis. The remaining fruit were stored at 20 °C for 14 days to evaluate infection and sporulation. The trial was repeated on Valencia oranges.

2.4. IMZ application in a drench

Mineola (Tangelo Mineola) fruit inoculated with the IMZ S and R isolates were treated curatively in this experiment. Commercial bin-drenching systems were simulated using plastic fruit crates (280 mm × 480 mm × 310 mm) with 20 mm × 20 mm diameter holes drilled in the bottom of the plastic crates to simulate the floor-openings in a commercial bin. Three crates were stacked on top of each other to represent a commercial fruit bin stack. The sides of the crates were closed with plastic sheets to ensure that the solutions used for drench treatment would flow from the top, through the middle and bottom crates into an empty collection crate in the bottom. Non-inoculated fruit were placed at the bottom and sides of each experimental crate. In three replications, 24 inoculated fruit, 12 × R and 12 × S, plus six non-inoculated fruit for IMZ residue analyses were placed in the middle of each crate. The inoculated fruit were placed at different angles in relation to the position of the inoculated sites around the stem end: four of the 12 fruit inoculated with a specific isolate were placed on their sides, 4 with the stem end facing upward and 4 with the stem end downward. The 3-crate stacks were drenched with 6.25, 12.5, or 25.0 L of a 500 μg mL^{-1} IMZ EC solution at ambient temperature. These three volumes related, respectively, to 125, 250, or 500 L of the IMZ solution running through a stack comprising three commercial fruit bins. The control was treated with 12.5 L water containing no IMZ. Approximately 15 min after each application the experimental stack was disassembled and left overnight to dry. The inoculated fruit were packed in cartons as described previously and stored for 14 days at 20 °C before being evaluated for green mould decay and sporulation as described previously. The trial was repeated on Eureka lemons and Valencia oranges.

3. Results

3.1. Survey of commercial IMZ application in South African citrus packhouses

During the 2008 season, 37 packhouses were visited throughout South Africa. Packhouses applied IMZ once only (46%), as a double application (49%), or even as three separate applications (5%). In the survey it was found that 78.4% of the packhouses use a dip tank to apply IMZ as a single application (38%), as a dip- and wax-application (38%), or as a pre-packline drench-, dip- and wax-application (3%). IMZ application through waxing systems was also common (62.2%), often in combination with the bath and pre-packline drench as mentioned previously, but also a single application (8%), or with inline drench (3%) and total loss spray systems (3%). Table 1 shows all the parameters measured on the dip tank during the survey. Dip tank capacity and length varied from 1000 L and 1 m to 8000 L and 10 m, respectively, with the majority containing ≈2500 L at a length of 2.5 m. Fruit exposure time in the dip tank solution was positively correlated with dip tank length ($r^2 = 0.57$) and varied from 15.5 to 106.8 s with the median at 47.1 s; only 25% of the packhouses had an exposure time longer than 60 s. Dip tank solution temperature varied from 11.5 to 44.6 °C with the median at 33.4 °C and the pH varied from 3.3 to 8.0 with the median at 5.4. The pH of the solution was negatively correlated with its IMZ concentration ($r^2 = -0.67$) and ranged from 131.0 to 2175.4 μg mL^{-1} with the median at 362.6 μg mL^{-1}; and the 25th percentile was <250.00 μg mL^{-1}. The dip tank solution age (days from starting with fresh solution) at time of assessment varied from 1 to 40 days with the median being 5 days old. After each dip tank, in every packhouse surveyed, a number of brushes were installed to reduce excess water before the fruit entered a wind tunnel for drying prior to wax application. The number of brushes after the dip tank varied from 8 to 52 with 20 brushes as the median. The IMZ residue measured on fruit that went through the different dip tanks varied from 0.2 to 3.9 μg g^{-1} with the median at 1.0 μg g^{-1}. IMZ residue level was poorly correlated with exposure time ($r^2 = -0.16$), pH ($r^2 = 0.16$), temperature ($r^2 = 0.18$) and IMZ concentration ($r^2 = 0.20$) in/of the solution.

3.2. IMZ application in a dip tank

3.2.1. Effect of IMZ concentration

Analysis of variance for IMZ residue data as determined on Navel and Valencia fruit treated in different concentrations of IMZ sulphate at either pH 3 or 8 showed no significant interaction involving citrus type ($P > 0.05$), nor was citrus type significant as main effect ($P = 0.981$); data for citrus type were therefore combined. IMZ concentration and treatment (IMZ sulphate at pH 3 or 8) showed a significant interaction ($P = 0.0006$). Mean residue levels

Table 1
Descriptive statistics of parameters measured in and around fungicide dip tank of packhouses that use this method to apply imazalil (IMZ) sulphate.

	n	Min	1st quartile	Median	3rd quartile	Max
Capacity (L)	25	1000	2000	2501	3400	8000
Length (m)	26	10	2	2.5	4.2	10
Temperature (°C)	28	11.5	26.5	33.4	37.3	44.6
pH	28	3.34	4.6	5.4	6.8	8.0
Time (s)	28	15.5	24.7	47.1	64.2	106.8
IMZ concentration ($\mu g\,mL^{-1}$)	22	131	249.6	362.6	615.2	2175.4
Age (d)	19	1	4	5	8	40
Brushes	27	8	12	20	27	52
Residue ($\mu g\,g^{-1}$)	29	0.24	0.89	1.02	1.70	3.85

loaded on Navel and Valencia fruit in 0, 250, 500 or 1000 $\mu g\,mL^{-1}$ IMZ sulphate concentration at pH 3 increased from 0.09 $\mu g\,g^{-1}$ to 0.51 $\mu g\,g^{-1}$ to 0.97 $\mu g\,g^{-1}$ to 1.45 $\mu g\,g^{-1}$ (Table 2). The concomitant increase in the same concentrations of IMZ sulphate at pH 8 was from 0.03 $\mu g\,g^{-1}$ to 1.43 $\mu g\,g^{-1}$ to 3.50 $\mu g\,g^{-1}$ to 11.20 $\mu g\,g^{-1}$, respectively.

Analysis of variance of infection incidence data obtained at the various evaluation times showed no significant and/or meaningful interaction involving evaluation time. Green mould infection data at the end of the incubation periods of the different incubation regimes, *i.e.* day 14 of fruit stored at 23 °C (ambient), day 21 of fruit stored at 7 °C (cold storage) and after the 7-day shelf period (shelf life) for the cold stored fruit, were therefore analysed. Analysis of variance of these data showed a significant interaction between IMZ concentration, isolate (S or R), treatment action (curative or protective) and solution pH ($P = 0.0006$), as well as for citrus type, IMZ concentration, isolate and incubation regime ($P = 0.0022$).

Fruit that were treated 4–6 h after inoculation, *i.e.* curatively, had lower levels of infection when compared to the protectively treated fruit (Table 3). In the control treatments (0 $\mu g\,mL^{-1}$), fruit treated curatively had significantly less infection than those treated protectively (43.6% *vs.* 67.7% for the S isolate 58.3% *vs.* 73.0% for the R isolate). The S isolate caused significantly less decay than R on the control fruit in the curative treatment, but not in the protective treatment (Table 3). The addition of 2% $NaHCO_3$ to water reduced infection significantly in the curative treatment (24.5% and 37.7% for the R and S isolate, respectively) but not in the protectively treated fruit. On fruit treated with 250 $\mu g\,mL^{-1}$ IMZ sulphate at pH 3 (residue of 0.51 $\mu g\,g^{-1}$; Table 2) the S isolate caused significantly lower levels of decay when treated curatively (2.5%; Table 3) and protectively (12.8%). This residue level on fruit also significantly reduced the R isolate infection (22.1%), but not when treated protectively (69.1%). The 0.97 $\mu g\,g^{-1}$ IMZ residue loaded with the 500 $\mu g\,mL^{-1}$ IMZ at pH 3 (Table 2) could curatively control the S isolate better than protectively (0% *vs.* 7.4%; Table 3), although infection incidences were not significantly different. On these fruit, the R isolate was significantly better controlled curatively than protectively (17.2% *vs.* 71.1%). A similar IMZ residue of 1.45 and 1.43 $\mu g\,g^{-1}$ were loaded on fruit treated with 1000 $\mu g\,mL^{-1}$ IMZ sulphate and 250 $\mu g\,mL^{-1}$ IMZ sulphate at pH 8, respectively (Table 2). These treatments had a significantly better curative than protective effect of the R isolate (8.3–11.8% *vs.* 45.6–52.0%, respectively; Table 3), which was not evident for the S isolate as it was almost completely controlled by both curative and protective treatment (<2%). The residue was increased from 0.97 $\mu g\,g^{-1}$ to 3.50 $\mu g\,g^{-1}$ when the pH of the 500 $\mu g\,mL^{-1}$ IMZ sulphate solution was increased from 3 to 8 (Table 2). The S isolate was completely controlled by this treatment, curatively and protectively, while the R isolate was controlled significantly better with the pH 8 IMZ solution in the curative (7.4%; Table 3) and protective (19.1%) treatment. The 11.20 $\mu g\,g^{-1}$ IMZ loaded with the 1000 $\mu g\,mL^{-1}$ IMZ sulphate at pH 8 (Table 2), which was more than double the MRL of 5 $\mu g\,g^{-1}$, reduced R infection levels to <7.0% when treated curatively or protectively (Table 3).

Table 4 summarises the significant interaction involving citrus type, IMZ concentration, isolate, and incubation regime. Navel oranges were generally more sensitive to green mould than Valencia oranges with significantly higher levels of infection recorded for the S and R isolate on control fruit (0 $\mu g\,mL^{-1}$) following all incubation regimes, except for the S isolate after cold storage. The highest decay levels on control fruit were observed following the ambient incubation regime (>80% on Navel fruit and >50% on Valencia fruit; Table 4). Following cold storage of Navel fruit containing no IMZ, infection levels increased significantly after the 7-day shelf life period: from 31.3% to 60.4% and 52.1% to 64.6% for the S and R isolate, respectively. This was also evident for Valencia fruit, although not at significant levels (33.3–43.1% and 38.2–47.2% for the S and R isolate, respectively). The above-mentioned differences in decay levels on control fruit that followed the various incubation regimes were also evident on IMZ treated fruit, although significant only for the R isolate on Navel fruit. Decay caused by the S isolate on IMZ treated fruit ranged from 0% to 8.3%, while significantly higher levels were recorded for the R isolate on Navel (9.7–65.5%) and Valencia fruit (6.3–34.7%). Generally, R isolate infection levels were significantly lower after IMZ treatment on Navels after cold storage plus shelf life when compared to the ambient incubation period (39.6%, 25.1% and 18.8% *vs.* 65.6%, 49.3% and 41.0%, respectively, for the 250, 500 and 1000 $\mu g\,mL^{-1}$ IMZ treatments). A similar trend was observed for Valencia, but it was in most cases not significant.

Due to the effective control of the S isolate by most IMZ treatments, sporulation incidence and severity data were too sparse for statistical analysis. The total inhibition of sporulation on infected fruit was rarely observed regardless of treatment or isolate. However, when the frequency distribution of sporulation index values was considered, a clear distinction was observed between the S and R isolate. Irrespective of IMZ treatment, sporulation index values on decayed R-inoculated fruit mostly (65%) exceeded a rating of 3 and often obtained a rating of 6. Decayed S-inoculated fruit were mostly (54%) rated at index values 1–3, while this frequency was even lower on fruit treated with higher IMZ concentrations (results not shown).

Table 2
Mean imazalil (IMZ) residue levels measured on Valencia and Navel oranges dipped for 60 s in different concentrations of IMZ sulphate at pH 3 and 8 (buffered with 2% $NaHCO_3$).

Concentration ($\mu g\,mL^{-1}$)	IMZ residue ($\mu g\,g^{-1}$)[a]	
	IMZ sulphate (pH 3)	IMZ sulphate (pH 8)
0	0.09c	0.03c
250	0.51c	1.43bc
500	0.97bc	3.50b
1000	1.45bc	11.20a

[a] Means followed by the same letter do not differ significantly ($P < 0.05$; LSD = 2.875).

Table 3
Mean green mould infection incidence (%) on Valencia and Navel oranges inoculated with either an imazalil (IMZ) sensitive or an resistant isolate of *P. digitatum*, that were dipped either curatively (after 4–6 h incubation) or protectively (inoculated after treatment) for 60 s in a range of IMZ sulphate concentrations (0–1000 μg mL^{-1}) at pH 3 and 8 (buffered with 2% $NaHCO_3$) after ambient (14 days at 23 °C) and cold-stored incubation periods (21 days at 7 °C plus an additional 7 days at 23 °C).

IMZ concentration (μg mL^{-1})	Green mould infection incidence (%)[a]			
	IMZ sulphate (pH 3)		IMZ sulphate (pH 8)	
	Sensitive	Resistant	Sensitive	Resistant
Curative				
0	43.6ef	58.3cd	24.5g	37.7f
250	2.5j	22.1g	1.0j	11.8hi
500	0.0j	17.2gh	0.0j	7.4ij
1000	0.0j	8.3ij	0.0j	5.9ij
Protective				
0	67.7ab	73.0a	61.3bc	65.2abc
250	12.8hi	69.1ab	1.0j	45.6ef
500	7.4ij	71.1a	0.0j	19.1gh
1000	2.0j	52.0de	0.0j	6.9ij

[a] Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 0.09).

3.2.2. Effect of exposure time and solution temperature

The analysis of variance for residue data on Valencia fruit dipped for various lengths of time in a 20 or 35 °C IMZ sulphate solution (500 μg mL^{-1}) at pH 3 or pH 8 showed no significant interactions among exposure time, temperature, and pH ($P = 0.6847$). Significant two-factor interactions were observed for exposure time and pH ($P = <0.0001$), while temperature showed no significant effect ($P > 0.6$). Exposure time had no significant effect on residue loading in the IMZ sulphate solutions at pH 3 and residues ranged from 1.22 (15 s) to 2.06 μg g^{-1} (540 s) (Fig. 1). Exposure time significantly affected IMZ residue loading in the IMZ sulphate solution at pH 8 where residue levels increased in a linear trend from 3.86 (15 s) to 44.41 μg g^{-1} (540 s). These levels were significantly higher than those obtained in the pH 3 IMZ solutions from an exposure time of 90 s onward. However, the MRL level of 5 μg g^{-1} was already exceeded at 45 s in the pH 8 IMZ sulphate solution.

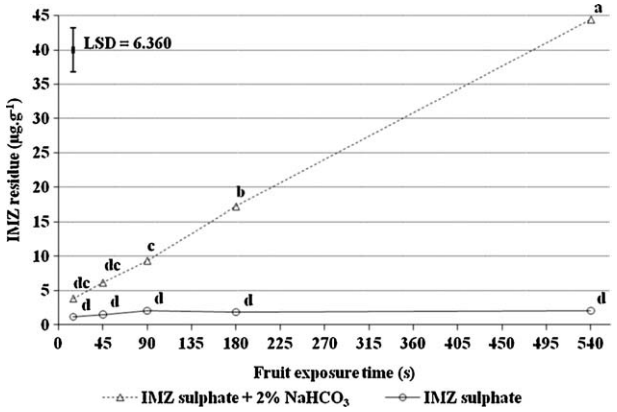

Fig. 1. Mean imazalil (IMZ) residue levels measured on Valencia oranges dipped for various exposure times in a 500 μg mL^{-1} solution (at 20 and 35 °C, mean data presented) of IMZ sulphate at pH 3 or pH 8 (buffered with 2% $NaHCO_3$). Data points followed by the same letter do not differ significantly ($P = 0.05$; LSD = 7.12).

3.2.3. IMZ dip tank application compared to spray application

Analysis of variance of the IMZ residue levels measured on Eureka lemon and Valencia fruit showed significant interactions among application method (dip or spray), IMZ concentration, and IMZ solution applied (*i.e.* IMZ sulphate at pH 3 or 8 and IMZ EC; $P = 0.0010$). Very low levels of IMZ residue (0.05–0.18 μg g^{-1}; Table 5) were detected on the fruit from the control dip or spray treatments. The registered concentration (1000 μg mL^{-1}) for IMZ sulphate sprayed loaded more than double the amount of IMZ on the fruit compared to the registered dosage (500 μg mL^{-1}) for IMZ

Table 4
Mean green mould infection incidence (%) caused by an imazalil (IMZ) sensitive or resistant isolate of *P. digitatum* on Valencia and Navel oranges dipped for 60 s in a range (0–1000 μg mL^{-1}) of IMZ sulphate concentrations at pH 3 and 8 (buffered with 2% $NaHCO_3$) after ambient (14 days at 23 °C) and cold-stored incubation periods (21 days at 7 °C plus an additional 7 days at 23 °C).

IMZ concentration (μg mL^{-1})	Green mould infection incidence (%)[a]			
	Navel		Valencia	
	Sensitive	Resistant	Sensitive	Resistant
14 days at 23 °C				
0	81.3a	84.7a	50.0cde	66.7b
250	6.9nop	65.6b	8.3mnop	34.7ghi
500	4.2op	49.3de	3.5op	35.4ghi
1000	0.7op	41.0efgh	2.1op	22.9jk
21 days at 7 °C				
0	31.3hij	52.1cd	33.3ghij	38.2fgh
250	0.7op	25.0ijk	0.0p	25.0ijk
500	0.0p	20.1kl	0.0p	15.3klmn
1000	0.0p	9.7lmnop	0.0p	6.3nop
21 days at 7 °C + 7 days at 23 °C				
0	60.4bc	64.6b	43.1defg	47.2def
250	7.3nop	39.6efgh	3.5op	33.3ghij
500	4.2op	25.1ijk	0.0p	25.7ijk
1000	0.0p	18.8klm	0.0p	11.1lmno

[a] Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 0.10).

Table 5
Mean imazalil (IMZ) residue levels measured on Valencia oranges and Eureka lemons dipped for 60 s in or sprayed with 3 mL of various concentrations (0–1000 or 4000 μg mL^{-1}, respectively) of IMZ sulphate at pH 3 or 8 (buffered with 2% $NaHCO_3$) or the IMZ EC formulation.

IMZ concentration (μg mL^{-1})	IMZ residue (μg g^{-1})[a]		
	IMZ sulphate (pH 3)	IMZ sulphate (pH 8)	IMZ EC
Dip			
0	0.05i	0.10i	0.05i
250	1.03hi	2.67ghi	2.94fghi
500	2.39ghi	8.00bcd	5.16defg
1000	4.82efg	18.34a	9.39b
Spray			
0	0.18hi	0.11i	0.18hi
1000	6.03cde	4.37efg	3.08fgh
2000	9.86b	8.69bc	5.73def
4000	19.03a	17.71a	10.78b

[a] Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 2.924).

sulphate applied in a dip tank (6.03 and 2.39 μg g^{-1}, respectively). When IMZ sulphate solution at 500 μg mL^{-1} was buffered with 2% $NaHCO_3$ at pH 8, the IMZ residue level loaded following dip application (8.00 μg g^{-1}) was comparable to that of the 1000 μg mL^{-1} spray application of IMZ sulphate (6.03 μg g^{-1}). IMZ residue loaded on fruit sprayed with or dipped in the registered concentrations of IMZ EC for the specific applications (1000 and 500 μg mL^{-1}, respectively) was statistically similar although markedly higher for the dip application (5.16 μg g^{-1} *vs.* 3.08 μg g^{-1}). Sprays with the IMZ EC formulation generally resulted in lower residue levels than the IMZ sulphate (pH 3 or 8) sprays. In most cases double the registered concentration more or less doubled the residue loaded when compared to those loaded from the registered dosages with the only exception being IMZ sulphate solution at pH 3 sprayed where the increase was just over half the residue of the registered dosage, from 6.03 to 9.86 μg g^{-1}. Furthermore, all these treatments (double the registered dosage) loaded a residue of higher than the MRL (5 μg g^{-1}) except for the 1000 μg mL^{-1} IMZ sulphate dip at pH 3, which loaded 4.82 μg g^{-1}.

Analysis of variance furthermore showed a significant citrus type × application × treatment interaction ($P = 0.0135$). Lemon fruit generally loaded more IMZ across all treatments (2.47–11.32 μg g^{-1}) compared to Valencia (1.97–8.01 μg g^{-1}; results not shown).

For infection data, analysis of variance showed significant 4-factor interactions involving citrus type ($P < 0.05$), which were largely ascribed to significantly higher mean decay levels on Valencia than on lemon fruit after dip or spray treatments with 0 μg mL^{-1} (93.1% *vs.* 19.4% and 66.7% *vs.* 94.4%, respectively; results not shown). Interactions with citrus type were therefore ignored. Significant 3-factor interactions among application method (dip or spray), treatment (IMZ sulphate at pH 3 or 8 and IMZ EC) and IMZ concentration (0, 250, 500 or 1000 μg mL^{-1}) and among application method, IMZ concentration, and isolate (S and R) were found ($P < 0.05$), but the interactions among IMZ action, IMZ solution, IMZ concentration, and isolate ($P = 0.2628$) will be discussed in order to demonstrate the effects of these four factors. As shown in Table 6, decay levels caused by the S and R isolates were generally similar after the dip or spray control treatments (0 μg mL^{-1}). Inoculated fruit dipped in water had significantly lower levels of infection (<63.0%) when compared to the water sprayed fruit (>77.0%). Addition of 2% $NaHCO_3$ alone significantly reduced infection levels in these treatments (dip and spray) to between 47.3% and 59.8%. In general, the various IMZ treatments yielded statistically similar decay levels, whether applied as dip or spray. However, residue levels loaded by spray treatments were significantly higher in the case of the IMZ sulphate solution at pH 3, and similar in the cases of IMZ sulphate at pH 8 and IMZ EC solutions, although 4 times lower concentrations were used in the dip treatments (Table 5). The 2.39, 8.00 and 5.16 μg g^{-1} loaded, respectively, by the registered IMZ concentration of 500 μg mL^{-1} dip treatment of the IMZ sulphate at pH 3, IMZ sulphate at pH 8, and IMZ EC solutions reduced decay levels to 4.2%, 0.0% and 1.4%, respectively, for S isolate infection (Table 6). These IMZ residue levels reduced the respective R isolate infection to 16.7%, 8.3% and 7.0%. The registered concentration (1000 μg mL^{-1}) to spray IMZ loaded residues of 6.03, 4.37 and 3.08 μg g^{-1} (Table 5), which reduced S isolate infection to 4.2%, 1.4% and 1.4%, respectively (Table 6). This range of IMZ residue levels reduced R isolate infection to 20.9%, 15.4% and 27.9%, respectively; this was significantly higher than the S isolate infection. Decay levels by the R isolate on IMZ treated fruit were generally higher than that of the S isolate; significantly at the lower treatment concentrations.

As mentioned previously, data for sporulation on infected fruit were sparse given the complete control of the S isolate after IMZ treatment and were therefore not statistically analysed. Total inhibition of sporulation was again rarely observed. Generally higher sporulation index values were recorded on decayed fruit inoculated with the R isolate. Sporulation index values rated for decayed S-inoculated fruit also declined with increasing IMZ concentration and similar index values was recorded on decayed dipped and sprayed fruit (results not shown).

3.3. IMZ application in a drench

Analysis of variance for IMZ residue levels measured in the drench trial showed a significant interaction between citrus type (Mineola, Lemon and Valencia) and volume ($P = <0.0001$). Crate stacking as main effect was not significant ($P = 0.6922$). Lemon fruit loaded significantly more IMZ (2.92–3.90 μg g^{-1}) across drench volumes when compared to Valencia (1.72–2.47 μg g^{-1}) and Mineola (1.56–1.99 μg g^{-1}) as shown in Table 7. When drenched with the 6.25 L, Mineola and Valencia fruit loaded similar IMZ residue levels (1.56 and 1.72 μg g^{-1}, respectively) and this was significantly lower than that loaded on lemon fruit (2.92 μg g^{-1}). When the 6.25 L volume was doubled, Mineola, Valencia and lemon fruit loaded significantly different levels of IMZ residue (1.65, 2.19 and 3.80 μg g^{-1}, respectively); except for Mineola, this was significantly higher than loaded with the 6.25 L volume. Residue levels increased, but not to significantly higher levels, when fruit was drenched with 25 L.

Analysis of variance for the green mould infection data showed a significant interaction between isolate (S and R) and solution drench volume ($P = <0.0001$). Citrus type as main effect was not significant ($P = 0.4838$), nor was crate stacking ($P = 0.1794$). As shown in Table 8, infection caused by the S isolate was significantly reduced from 91.7% (control) to 9.3%, 6.0%, or 1.9% when drenched with the IMZ EC volumes of 6.25, 12.50, or 25.00 L, respectively. Fruit inoculated with the R isolate had significant higher levels of

Table 6
Mean green mould infection incidence (%) after 14 days of storage at 20 °C rated on Valencia oranges and Eureka lemons inoculated with imazalil (IMZ) sensitive and resistant isolates of *P. digitatum*, incubated for 4–6 h and then dipped for 60 s in or sprayed with 3 mL of various concentrations (0–1000 or 4000 μg mL^{-1}, respectively) of IMZ sulphate at pH 3 or 8 (buffered with 2% $NaHCO_3$) or the IMZ EC formulation.

IMZ concentration (μg mL^{-1})	Green mould infection incidence (%)[a]					
	IMZ sulphate (pH 3)		IMZ sulphate (pH 8)		IMZ EC	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Dip						
0	62.7b	50.2bc	51.5bc	47.3c	62.7b	50.2bc
250	4.2hij	48.6c	1.4j	18.1defg	0.0j	23.7de
500	4.2hij	16.7defgh	0.0j	8.3fghij	1.4j	7.0ghij
1000	0.0j	5.6ghij	0.0j	2.8ij	1.4j	1.4j
Spray						
0	77.9a	83.4a	58.5bc	59.8bc	77.9a	83.4a
1000	4.2hij	20.9def	1.4j	15.4defghi	1.4j	27.9d
2000	0.0j	22.3de	2.8ij	20.9def	0.0j	12.6efghij
4000	1.7j	8.4fghij	0.0j	15.4defghi	0.0j	5.6ghij

[a] Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 13.0).

Table 7
Mean imazalil (IMZ) residue levels (μg mL^{-1}) measured on Mineola soft citrus, Eureka lemons and Valencia oranges drenched with different volumes (6.25–25.00 L) of 500 μg mL^{-1} IMZ EC.

Drench volume (L)	IMZ concentration (μg mL^{-1})	IMZ residue (μg g^{-1})[a]		
		Mineola	Lemon	Valencia
12.50	0	0.00g	0.06g	0.02g
6.25	500	1.56f	2.92b	1.72ef
12.50	500	1.65ef	3.80a	2.19cd
25.00	500	1.99de	3.90a	2.47c

[a] Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 0.409).

infection (56.9–69.0%) compared to those inoculated with the S isolate across the three volumes. When the 12.5 L volume was doubled to 25 L the infection decreased for the R and S isolate from 65.3% to 56.9% and 6.0% to 1.9%, respectively, although these differences were not significant.

The percentage of sporulating infected fruit showed trends similar to the infection results: fruit inoculated with the R isolate had significantly more sporulating infected fruit (74.9–80.3%, across the three volumes) compared to the infected fruit inoculated with the S isolate (16.7–39.6%, across the three volumes; results not shown). Complete sporulation inhibition could not be observed in any treatment, although there was a tendency for S isolate infected fruit to have lower sporulation index ratings when compared to the R isolate infected fruit across all treatments. The majority of the S isolate infected fruit had a sporulation rating of three or lower and no index ratings of six could be observed. The R isolate infected fruit showed the opposite results with the majority showing a sporulation rating of 3 and higher.

4. Discussion

From the packhouse survey it was evident that the majority of South African citrus packhouses apply IMZ in the sulphate formulation through fungicide dip tanks. However, considerable variation was observed in the application systems employed by the different packhouses. IMZ residue loaded to fruit was poorly correlated with any of the eight parameters measured in and around the fungicide dip tank, thus indicating a complex interaction between these factors and IMZ residue loading following dip treatment with the IMZ sulphate formulation. In research mostly using the EC formulation of IMZ, concentration, solution temperature and pH, exposure time, and brushes after aqueous treatment have been shown to have an individual or combined effect on IMZ residue loading (Eckert, 1977; Schirra et al., 1996, 1997; Smilanick et al., 1997, 2005; Cabras et al., 1999; D'Aquino et al., 2006; Dore et al., 2009, 2010).

Commercial residue results obtained during 2008 and 2009 show that South African citrus packhouses loaded a median IMZ residue of ≈1 μg g^{-1} (data not shown) on fruit and this is reflected in the survey. The ideal residue attained from aqueous treatment of IMZ is regarded to be around 2 μg g^{-1} in order to effectively control green mould or at least inhibit sporulation (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997). Inadequate residue loading will result in loss of disease control and development of IMZ resistance in packhouses, highlighting the need to study means of improving IMZ residue loading and green mould control.

Previous studies on IMZ application and the subsequent residue loading were predominantly done with the emulsifiable concen-

Table 8
Mean green mould infection incidence (%) rated on Mineola soft citrus and Valencia oranges after 14 days of storage at 20 °C inoculated with imazalil (IMZ) sensitive and resistant isolates of *P. digitatum*, incubated for 4–6 h and then drenched with different volumes (6.25–25.00 L) of 500 μg mL^{-1} IMZ EC.

Drench volume (L)	IMZ concentration (μg mL^{-1})	Green mould infection incidence (%)[a]	
		Sensitive isolate	Resistant isolate
12.50	0	91.7a	92.6a
6.25	500	9.3d	69.0b
12.50	500	6.0de	65.3bc
25.00	500	1.9e	56.9c

[a] Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 0.48).

trate formulation of IMZ (IMZ EC). Due to the extensive use of the IMZ sulphate formulation in South Africa and the fungicide dip tank it required thorough investigation. Methods to optimise IMZ residue loading were explored. Statistically, IMZ concentration had no significant effect on residue loading in fruit treated with IMZ sulphate at pH 3, although IMZ residues increased with increments of ≈0.50 $\mu g\,g^{-1}$ as treatment concentration was increased from 250 to 500 to 1000 $\mu g\,mL^{-1}$. After 60 s in a 500 $\mu g\,mL^{-1}$ IMZ sulphate dip tank (registered concentration for fungicide dip tanks in South Africa; pH 3) a residue of 0.97 $\mu g\,g^{-1}$ was loaded on oranges. These findings concur with work done by Smilanick et al. (2005) where oranges dipped for 30 s in a 500 $\mu g\,mL^{-1}$ of an IMZ EC solution at a pH of 3 loaded a residue of <1.00 $\mu g\,g^{-1}$. This residue is >1.00 $\mu g\,g^{-1}$ lower than the ideal level to sufficiently control green mould (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997), but relates with what is found on fruit treated in commercial packhouses. The suboptimal residue currently loaded on citrus through the fungicide dip tank in South African packhouses could be ascribed to the formulation and its properties, which will be discussed below.

An IMZ residue of ≈1 $\mu g\,g^{-1}$ could control 4–6 h old green mould infections of an S isolate of *P. digitatum*. Infections by an R isolate were significantly reduced, but control was not complete. The incubation time in this study for the curative treatments was short and does not simulate the worst-case scenario in industry, where fruit generally stand for ≈2 days before treatment. Overall it was clear that IMZ applied through a dip tank has better curative than protective action against green mould. Dore et al. (2009) also demonstrated better curative control on lemons dipped in IMZ EC, with better curative and protective control on fruit treated in a 50 °C compared with 25 °C solution.

IMZ residue loading was improved when the dip tank solution of IMZ sulphate was buffered with 2% $NaHCO_3$ to pH 8. Smilanick et al. (2005) showed that when the pH of a mixture of 500 $\mu g\,mL^{-1}$ IMZ EC and 3% $NaHCO_3$ were decreased from 7 to 3, the residue loaded on oranges was reduced from ≈2 $\mu g\,g^{-1}$ to ≈1 $\mu g\,g^{-1}$, but a pH increase from 7 to 9 did not influence residue loading. In our study, increasing the pH of the 500 $\mu g\,mL^{-1}$ IMZ sulphate formulation from 3 to 8 resulted in a 3-fold increase in residue level, and with the 1000 $\mu g\,mL^{-1}$ IMZ sulphate the increase was more than 7-fold. In both cases, this led to complete curative or protective control of the S isolate. The control of the R isolate infections was also improved, but not complete. Addition of 1–3% $NaHCO_3$ to an IMZ EC solution led to improved green mould control compared to IMZ EC alone and a synergy between IMZ and $NaHCO_3$ was suggested (Smilanick et al., 2005; Dore et al., 2010). In the case of IMZ sulphate combined with $NaHCO_3$ a possible synergistic effect could have been masked by the markedly increased residue loading and needs further study.

Long term cold storage (21 days at 7 °C) had an adverse effect on green mould infection, especially on the R isolate, and in the majority of cases IMZ increased this effect. The control treatments had up to 20% less infection on fruit after the cold storage plus shelf life incubation regime when compared to fruit incubated at ambient. To our knowledge, this phenomenon has not been described in the literature and our observations might be explained by the relatively short incubation time and small wounds used in our study. Curatively treated fruit were placed in cold storage 8–10 h after inoculation and treatment at 23 °C, while protectively treated fruit were incubated 2–4 h after inoculation. By this time, the majority of conidia would have germinated (Plaza et al., 2003) and the infection process started. Optimal growth and germination takes place at 25 °C and growth of *P. digitatum* is retarded at temperatures below 10 °C (Lacey, 1989; Kassim and Khan, 1996; Plaza et al., 2003). Therefore, at 7 °C the growth of germinated and the germination of conidia will be retarded and so too the inoculum potential (Garrett, 1958). Wounds induced in this study were 2 mm deep, which should be just below the flavedo. Wounds at this depth should be more resistant to infection than deeper wounds often used in other studies (Kavanagh and Wood, 1967). Proportionally more wound healing might also have taken place during cold storage, as well as the deposition of wound-induced material (Stange et al., 1993; Mulas et al., 1996; Lai et al., 2003). The effect of wound healing can be increased during the shelf-life period (Mulas et al., 1996). Nonetheless, it was clear from this study that cold storage exhibited limited levels of curative control of recent infections of *P. digitatum*. This observation provides an additional reason, other than delaying senescence, for rigorous attention to proper temperature management of the fruit after harvest.

Solution temperature at ambient (±20 °C) and 35 °C had no significant influence on residue loading for fruit dipped in either IMZ sulphate solution at pH 3 or pH 8. This is in contrast with IMZ EC formulation where temperature had a positive effect on IMZ residue loading (Schirra et al., 1996; Smilanick et al., 1997). IMZ residues loaded on Navel oranges increased more than 4-fold when a 410 $\mu g\,mL^{-1}$ IMZ sulphate solution temperature was increased from 21.1 to 40.6 °C (Smilanick et al., 1997). The effect of temperature on IMZ residue loading through the sulphate salt formulation needs to be investigated further.

Buffering of the IMZ sulphate solution with 2% $NaHCO_3$ at pH 8 significantly increased the residue loaded over time in comparison with IMZ sulphate at pH 3. Commercial IMZ sulphate is actually a bisulphate salt that consists of equimolar quantities of IMZ base (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy-ethyl)]-1 *H*-imidazole) and sulphuric acid (H_2SO_4) (Siegel and Ragsdale, 1978). Dissolution of the salt in water establishes an equilibrium mixture of different hydrated ion species; these are the protonated IMZ base and hydronium cations, as well as hydrosulphate and sulphate anions. The pK_a value of IMZ is 6.5 (Siegel et al., 1977), which is the pH in water at which 50% of dissolved IMZ molecules will be protonated and 50% will be unprotonated. Sulphuric acid is a strong diprotic acid that has both its pK_a values below 2.0. Therefore, in the equilibrium mixture of IMZ bisulphate in water the strong acid species will predominate causing the pH to be about 3 and virtually all IMZ molecules will be protonated and hydrated. On addition of a base such as $NaHCO_3$, the strong acids will be neutralised, thereby changing the composition of the equilibrium mixture and increasing the solution pH. Every change of a single pH unit above the pK_a of IMZ (6.5) will cause an order of magnitude increase in the molar concentration of IMZ base (unhydrated water insoluble) and a concomitant decrease in the protonated IMZ cations (hydrated, water soluble). Use of neutralised solutions of IMZ sulphate buffered at pH above 7 is not recommended. Based on the general principles of partition coefficients it should be expected that the hydrophobic base of IMZ will readily and preferentially dissolve in the waxy/oily cuticle of the citrus fruit. When fruit is dipped into aqueous IMZ bisulphate (pH 3) we have shown that the transfer of IMZ to the fruit rind is a slow process and may even have an upper limit (<2.10 $\mu g\,g^{-1}$ after 540 s). It is speculated that IMZ may be transferred by deprotonation of IMZ cation by basic components on the fruit surface, followed by dissolution of the liberated lipophilic IMZ base in the oils of the cuticle. In contrast, transfer of IMZ from the pH 8 IMZ sulphate solution to the rind is much faster (>3.00 $\mu g\,g^{-1}$ after 15 s). The IMZ sulphate solution at pH 3 can therefore be regarded as a safe application in terms of the MRL of 5 $\mu g\,g^{-1}$. Other results from our study support this argument. In the exposure trials, a fresh dip tank solution of 500 $\mu g\,mL^{-1}$ IMZ sulphate at pH 3 loaded an IMZ residue of 1.53 $\mu g\,g^{-1}$ after 45 s exposure time. Even under these controlled conditions, the residue level was below 2 $\mu g\,g^{-1}$. When the pH was increased to 8 by means of 2% $NaHCO_3$ the residue loading was increased significantly; after 45 s it was well above the MRL of 5 $\mu g\,g^{-1}$ and can therefore not be recommended for

commercial use. The limited solubility of IMZ sulphate at pH 8 also makes it unsuitable for practical recommendation. Further research is required to establish whether increased residue loading can be attributed to the buffer ($NaHCO_3$) or the pH level. Concomitantly, the effect of exposure time in the IMZ sulphate solution on the control of green mould also needs to be investigated.

Spray application of aqueous IMZ solutions onto fruit to just before the point of runoff (Fourie et al., 2009) improved residue loading when compared to dip application. Spray application with the registered dosage (1000 $\mu g\,mL^{-1}$) loaded a mean of 6.03 $\mu g\,g^{-1}$ and the 60 s dip application with the registered dosage (500 $\mu g\,mL^{-1}$) loaded 2.39 $\mu g\,g^{-1}$. However, this higher IMZ residue did not improve control of either the R or the S isolate of *P. digitatum* and dip application resulted in superior curative control, even at significantly lower IMZ residue levels. This could possibly be attributed to hydraulic pressure assisting IMZ residue loading into wound sites (Brown, 1984) when applied through the dip tank. The EC spray application loaded lower IMZ residue levels onto fruit, which can be attributed to increased levels of run-off observed at the spray volumes used (Fourie et al., 2009). Other researchers have also found the spray application of IMZ EC to be less effective in terms of green mould control when compared to dip applications (Kaplan and Dave, 1979; Brown and Dezman, 1990).

By drenching a simulated 3-crate stack of fruit bins with 250–500 L of 500 $\mu g\,mL^{-1}$ IMZ EC residue levels of 1.65 to >2 $\mu g\,g^{-1}$ were loaded on fruit. However, the R isolate could not be controlled in any volume or on any citrus type with the infection level >50% across treatments. The S isolate was reduced to <10% infection for the simulated 250 and 500 L drench treatments. It has been shown that field bin drenches with thiabendazole (TBZ) significantly increased control of a TBZ resistant isolate, although the infection level was close to 60% (Smilanick et al., 2006). Degreening conditions (48–72 h at high humidity and mild temperatures) are conducive to green mould development and it will be extremely risky to protect fruit with sub-lethal residue levels of IMZ and even TBZ on fruit. This will only enhance and encourage the development of resistance. The drench application system needs to be studied further with the aim to improve green mould control on fruit during the degreening process.

A very important attribute of IMZ is its ability to inhibit sporulation (McCornack and Brown, 1977). However, the ability of IMZ to inhibit sporulation was not clearly evident from this study. There were indications of this ability on the S isolate infections with sporulation ratings mostly ≈3, but sporulation inhibition of the R isolate was rarely observed with ratings mostly >3. The reason for the absence of sporulation inhibition in this study could be due to the inoculation method and incubation period of 4–6 h. In studies where sporulation inhibition was specifically examined a spore suspension of *P. digitatum* was injected into the core of the fruit before treatment with IMZ (Brown et al., 1983; Brown and Dezman, 1990).

Overall the different citrus types responded in similar trends to IMZ residue loading and green mould control. Eureka lemons consistently loaded higher IMZ residues levels compared to other citrus types, Valencia oranges and Mineola soft citrus. This tendency for lemons to load higher residues was shown with regard to thiabendazole (Schirra et al., 1997), but it was regarded not to be the case for IMZ (Cabras et al., 1999). Valencia oranges tended to be more resistant to green mould than Navel oranges, although similar IMZ residues levels were loaded. In another case Eureka lemons showed more resistance to green mould than Valencia oranges.

The IMZ sulphate formulation, about which little was known until our work, reacts rather differently in terms of residue loading following dip, drench, or spray applications compared to the well studied IMZ EC formulation. Increasing levels of postharvest green mould and/or loss of sporulation inhibition on fruit packed and exported from South Africa can be attributed to IMZ resistance development and sub-optimal IMZ treatment and residue loading in packhouses. This study shows the benefit of increased residue loading following addition of $NaHCO_3$ or increasing the pH of the dip solution. This aspect needs to be investigated further to allow its safe and practical implementation.

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References

Brown, G.E., Nagy, S., Maraulja, M., 1983. Residues from postharvest nonrecovery spray applications of imazalil to oranges and effects on green mold caused by *Penicillium digitatum*. Plant Dis. 67, 954–957.

Brown, G.E., 1984. Efficacy of citrus postharvest fungicides applied in water or resin solution water wax. Plant Dis. 68, 415–418.

Brown, G.E., Dezman, D.J., 1990. Uptake of imazalil by citrus fruit after postharvest application and the effect of residue distribution on sporulation of *Penicillium digitatum*. Plant Dis. 74, 927–930.

Cabras, P., Schirra, M., Pirisi, F.M., Garau, V.L., Angioni, A., 1999. Factors affecting imazalil and thiabendazole uptake and persistence in citrus fruits following dip treatments. J. Agric. Food Chem. 47, 3352–3354.

Cunningham, N., Taverner, P., 2006. Can carbonate salts reduce the levels of sour rot on citrus fruit? Packer Newslett. 85, 7.

D'Aquino, S., Schirra, M., Palma, A., Angioni, A., Cabras, P., Migheli, Q., 2006. Residue levels and effectiveness of pyrimethanil *vs.* imazalil when using heated postharvest dip treatments for control of *Penicillium* decay on citrus fruit. J. Agric. Food Chem. 54, 4721–4726.

Dore, A., Molinu, M.G., Venditti, T., D'Hallewin, G., 2009. Immersion of lemons into imazalil mixtures heated at 50 °C alters the cuticle and promotes permeation of imazalil into rind wounds. J. Agric. Food Chem. 57, 623–631.

Dore, A., Molinu, M.G., Venditti, T., D'Hallewin, G., 2010. Sodium bicarbonate induces crystalline wax generation, activates host-resistance, and increases imazalil level in rind wounds of oranges, improving the control of green mold during storage. J. Agric. Food Chem. 58, 7297–7304.

Eckert, J.W., 1977. Control of postharvest diseases. In: Siegel, M.R., Sisler, H.D. (Eds.), Antifungal Compounds. Marcel Dekker Inc., New York, pp. 269–352.

Eckert, J.W., Sievert, J.R., Ratnayake, M., 1994. Reduction of imazalil effectiveness against citrus green mold in California packinghouses by resistant biotypes of *Penicillium digitatum*. Plant Dis. 78, 971–974.

Eckert, J.W., 1995. Postharvest disease control: experience with citrus fruits. Tree Fruit Postharvest J. 6, 9–12.

Eckert, J.W., Eaks, I.L., 1989. Postharvest disorders and diseases of citrus fruits. In: Reuther, W., Calavan, E., Clair, Carman, G.E., Jeppson, L.R. (Eds.), The Citrus Industry. , Revised ed. University of California, Division of Agriculture and Natural Resources, Oakland, CA, pp. 179–259.

Fourie, P.H., Du Preez, M., Brink, J.C., Schutte, G.C., 2009. The effect of runoff on spray deposition and control of Alternaria brown spot of mandarins. Aust. Plant Pathol. 38, 173–182.

Garrett, S.D., 1958. Inoculum potential as a factor limiting lethal action by *Trichoderma viride* Fr. on *Armillaria mellea* (Fr.) Quél. Trans. Brit. Mycol. Soc. 41, 157–164.

Ghosoph, J.M., Schmidt, L.S., Margosan, D.A., Smilanick, J.L., 2007. Imazalil resistance linked to a unique insertion sequence in the PdCYP51 promoter region of *Penicillium digitatum*. Postharvest Biol. Technol. 44, 9–18.

Glass, G.V., Peckham, P.D., Sanders, J.R., 1972. Consequences of failure to meet assumptions underlying the fixed effects analyses of variance and covariance. Rev. Educ. Res. 42, 237–288.

Haugland, R.A., Varma, M., Wymer, L.J., Vesper, S.J., 2004. Quantitative PCR analysis of selected *Aspergillus*. Penicillium and Paecilomyces species. Syst. Appl. Microbiol. 27, 198–210.

Holmes, G.J., Eckert, J.W., 1995. Relative fitness of imazalil-resistant and -sensitive biotypes of *Penicillium digitatum*. Plant Dis. 79, 1068–1073.

Jacobs, R., Korsten, L., 2010. Screening of South African Penicillium isolates from citrus producing regions for resistance to the postharvest fungicides imazalil, guazatine and thiabendazole. In: 6th Citrus Research Symposium , Drakensberg, South Africa, 15 August, p. 59.

John, J.A., Quenouille, M.H., 1977. Experiments: Design and Analysis, second ed. Macmillan, New York.
Kaplan, H.J., Dave, B.A., 1979. The current status of imazalil: a postharvest fungicide for citrus. Proc. Fl. State Hort. Soc. 92, 37–43.
Kassim, M.Y., Khan, S., 1996. Effect of temperature on growth of *Penicillium digitatum* and *Geotrichum candidum* on agar and on citrus fruit and chemical control of post-harvest rot caused by the two fungi. J. King Saud Univ. Sci. 8, 33–37.
Kavanagh, J.A., Wood, R.K.S., 1967. The role of wounds in the infection of oranges by *Penicillium digitatum*. Sacc. Ann. Appl. Biol. 60, 375–383.
Lacey, J., 1989. Pre- and post-harvest ecology of fungi causing spoilage of foods and other stored products. J. Appl. Bacteriol. 67, 11S–25S.
Lai, S., Lai, A., Stange, R.R., McCollum, T.G., Schirra, M., 2003. Characterization of the wound-induced material in Citrus Paradisi fruit peel by carbon-13 CP-MAS solid state NMR spectroscopy. Phytochemistry 63, 177–183.
Laville, E.Y., Harding, P.R., Dagan, Y., Rafael, G., Kraght, A.J., Rippon, L.E., 1977. Studies on imazalil as potential treatment for control of citrus fruit decay. Proc. Int. Soc. Citric. 1, 269–273.
McCornack, A.A., Brown, G.E., 1977. Decay control of florida citrus fruits with imazalil. Proc. Fl. State Hort. Soc. 90, 141–144.
McCornack, A.A., Brown, G.E., Smoot, J.J., 1977. R23979, an experimental postharvest citrus fungicide with activity against benzimidazole-resistant penicilliums. Plant Dis. Rep. 61, 788–791.
Mulas, M., Lafuente, M.T., Zacarias, L., 1996. Lignin and gum deposition in wounded 'Oroval' clementines as affected by chilling and peel water content. Postharvest Biol. Technol. 7, 243–251.
Pelser, P.D.T., La Grange, J.M., 1981. Latest developments in the control of postharvest decay of citrus fruit in South Africa. Proc. Int. Soc. Citric. 2, 812–814.
Plaza, P., Usall, J., Teixidó, N., Viñas, I., 2003. Effect of water activity and temperature on germination and growth of *Penicillium digitatum*. *P. italicum* and *Geotrichum candidum*. J. Appl. Microbiol. 94, 549–554.
Samson, R.A., Seifert, K.A., Kuipers, A.F.A., Houbraken, J.A.M.P., Frisvad, J.C., 2004. Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial β-tubulin sequences. Stud. Mycol. 49, 175–200.
Schirra, M., Cabras, P., Angioni, A., Dhallewin, G., Ruggiu, R., Minelli, E.V., 1997. Effect of heated solutions on decay control and residues of imazalil in lemons. J. Agric. Food Chem. 45, 4127–4130.
Schirra, M., Cabras, P., Angioni, A., Melis, M., 1996. Residue level of imazalil fungicide in lemons following prestorage dip treatment at 20 and 50 °C. J. Agric. Food Chem. 44, 2865–2869.
Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality (complete samples). Biometrika 52, 591–611.
Siegel, M., Kerkenaar, A., Sijpesteijn, A., 1977. Antifungal activity of the systemic fungicide imazalil. Eur. J. Plant Pathol. 83, 121–133.
Siegel, M.R., Ragsdale, N.N., 1978. Antifungal mode of action of imazalil. Pestic. Biochem. Physiol. 9, 48–56.
Smilanick, J.L., Mansour, M.F., Margosan, D.A., Gabler, F.M., Goodwin, W.R., 2005. Influence of pH and $NaHCO_3$ on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold on citrus fruit. Plant Dis. 89, 640–648.
Smilanick, J.L., Mansour, M.F., Sorenson, D., 2006. Pre- and postharvest treatments to control green mold of citrus fruit during ethylene degreening. Plant Dis. 90, 89–96.
Smilanick, J.L., Michael, I.F., Mansour, M.F., Mackey, B.E., Margosan, D.A., Flores, D., Weist, C.F., 1997. Improved control of green mold of citrus with imazalil in warm water compared with its use in wax. Plant Dis. 81, 1299–1304.
Smith, R.E., 1897. The "soft spot" of oranges. Bot. Gaz. 24, 103–104.
Stange, R.R., Midland, S.A., Sims, J.J., Eckert, J.W., 1993. Evidence that wound gum, not lignin, is deposited in infection-resistant injuries of citrus peel. Acta Hortic. 343, 347–352.
van Tuyl, J.M., 1977. Genetics of Fungal Resistance to Systemic Fungicides. Mededelingen Landbouwhogeschool Wageningen, The Netherlands, p. 136.