Imazalil resistance in *Penicillium digitatum* and *P. italicum* causing citrus postharvest green and blue mould: impact and options

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Abstract

Citrus green and blue mould, caused by *Penicillium digitatum* (PD) and *P. italicum* (PI), respectively, are mostly controlled by means of postharvest fungicide applications. Currently, IMZ is regarded as the most effective fungicide in use. Effective IMZ concentrations that inhibit 50% (EC_{50}) growth of nine PD and five PI isolates were assessed *in vitro* and the various isolates categorized according to their resistance (R) factors. Effective residue levels that provided 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of these isolates were determined *in vivo*. All the PI isolates were sensitive, having EC_{50} values of 0.005 - 0.050 µg.mL^{-1}. Three PD isolates were sensitive (0.027 – 0.038 µg.mL^{-1}), while one resistant isolate was categorized as low resistant (R-factor of 19), one as moderately resistant (R-factor of 33.2), three as resistant (R-factor of 50 - 57.6) and one as highly resistant (R-factor of 70.7). Sensitive PD isolates had mean $ER_{50}C$ and $ER_{50}P$ values on Valencia orange fruit of 0.29 and 0.20 µg.g^{-1}, and 0.33 and 0.32 µg.g^{-1} on navel fruit, respectively. ER_{50} values for resistant isolates did not always correlate with EC_{50} values and ranged from 1.22 – 4.56 µg.g^{-1} for $ER_{50}C$ and 1.00 – 6.62 µg.g^{-1} for $ER_{50}P$ values. $ER_{50}P$ values for resistant isolates could not be obtained on navel orange fruit, but $ER_{50}C$ values (1.42 – 1.65 µg.g^{-1}) were similar to those obtained on Valencia fruit. The PI isolates all behaved similar to the sensitive PD isolates with $ER_{50}C$ and $ER_{50}P$ values on navel and Valencia fruit < 0.38 µg.g^{-1}. Alternative fungicides were assessed for the control of an IMZ sensitive, resistant and highly resistant PD isolate; these included sodium *ortho*-phenylpenate (SOPP), thiabendazole (TBZ), guazatine (GZT), imazalil (IMZ), pyrimethanil (PYR) and Philabuster® (PLB; a combination of IMZ and PYR), fludioxonil (FLU), azoxystrobin (AZO), Graduate®A^{+} (GRA; a combination of FLU and AZO) and propiconazole (PPZ). Multiple fungicide

resistance was shown to IMZ, GZT, TBZ and PPZ in both resistant isolates. For the sensitive isolates, IMZ, SOPP, TBZ, GZT and PLB provided best curative control, while IMZ, GZT and PLB provided best protective control. For the IMZ-resistant isolates, SOPP, PYR and PLB gave the best curative control, while none of the fungicides provided adequate protective control.

Key words: fungicide, residue, azoxystrobin, fludioxonil, guazatine, propiconazole, pyrimethanil, thiabendazole

1. Introduction

South Africa is the largest exporter of shipped fresh citrus fruit worldwide (Edmonds, 2013). Due to harvest and postharvest handling processes fruit can incur injuries and these make the fruit susceptible to green and blue mould (Rose et al., 1951). Green mould is caused by the pathogen *Penicillium digitatum* (Pers.:Fr.) Sacc (PD) (Smith, 1897; Eckert and Eaks, 1989). It is regarded as one of the major causes of losses due to decay on South African export fruit (Pelser, 1977). *Penicillium italicum* Wehmer (PI), which causes blue mould, is often neglected in literature due to the main focus mostly being on PD. *Penicillium italicum* is of economic importance due to being more adapted for growth and development at lower temperatures (< 4°C) than PD (Wyatt and Parish, 1995; Plaza et al., 2003) Shipping and storage temperature protocols require temperatures <10°C for the majority of exported citrus cultivars. Both these pathogens require a fresh wound as shallow as 0.25 mm for successful infection (Kavanagh and Wood, 1971). It takes as short as 4 h from germination to establishment of infection (Plaza et al., 2003) and a water-soaked lesion should be visible around the infection site 3 days after infection. Seven to 10 days after inoculation the whole fruit rind could be covered with sporulating green or blue mould (Smilanick et al., 2006). Except for the losses due to the actual decayed fruit, the sporulating infections also soil the neighbouring fruit and this has a further economic impact.

Due to long-distance export routes, the South African citrus industry has to rely on fungicides for the control of postharvest diseases. Currently there are six registered postharvest fungicides for the control of green mould in the South African citrus industry. Prochloraz is not favoured commercially, due to apparent reduced efficacy (Keith Lesar, pers. comm.). Sodium ortho-phenyl-phenol (SOPP) has been in use for > 50 years (Johnson et al., 2001), but is not commonly used in South Africa, mostly due to the possible risk of phytotoxicity if not managed well. Resistance to SOPP has already been reported in the early 1960s (Harding, 1962). Thiabendazole is widely used in South Africa and world-wide, especially in the drench and wax applications. It

has been in use since the late 1960s, soon after which resistance development in green and blue mould populations were reported (Harding, 1972). Guazatine (GZT) is the only green mould fungicide that also has good curative action against sour rot (*Geotricum citri-aurantii*), but is only allowed in member countries of Codex Alimentarius (www.codexalimentarius.org). Guazatine has been in use since the early 1980s (Brown, 1988). Wild (1983) reported GZT resistance already in 1983. Imazalil (IMZ) is the most effective and reliable green mould fungicide currently in use. Laville (1977) reported first on IMZ's efficacy in the late 1970s and registered use started in the early 1980s (Bus et al., 1991); soon after implementation the first case of IMZ resistance was reported (Eckert, 1987). Pyrimethanil (PYR) has more recently been introduced as green mould fungicide. It became available for citrus use in California more than two decades after the registration of IMZ (Kanetis et al., 2007; 2008). The combination product, Philabuster® (PLB, which contains IMZ and PYR; Janssen PMP, Belgium) was developed and registered for use on citrus against green mould. Resistance to PYR has already been reported in field populations, but not yet from the packhouse environment (Kinay et al., 2007; Kanetis et al., 2008). Other active ingredients being evaluated for postharvest citrus use are fludioxonil (FLU) and azoxystrobin (AZO), and were found to have some activity against green mould (Kanetis et al., 2007; 2008). These researchers also showed that the combination of the two actives (FLU and AZO) had potential. Graduate®A^{+} [GRA); Syngenta, USA] has been developed and may have some potential for the control of green mould. Fludioxonil is still new and being registered in certain countries (D'Aquino et al., 2013); no resistance have been reported yet to our knowledge. Finally, propiconazole (PPZ) was reported to have an action against green mould (McKay et al., 2012a). The fungicides FLU, AZO, GRA and PPZ are not registered in South Africa for postharvest use on fresh citrus fruit.

The majority (> 78%) of South African packhouses apply IMZ in a sulphate formulation by means of a fungicide dip tank (Erasmus et al., 2011). This application gives variable results in terms of residue loading due to differences in exposure time, solution pH and concentration. A survey conducted by Erasmus et al. (2011) indicated that the median residue level loaded was 1.02 $\mu g.g^{-1}$ with the lowest level at 0.24 $\mu g.g^{-1}$ and the highest level at 3.85 $\mu g.g^{-1}$. The ideal IMZ residue level for control and sporulation inhibition is regarded as 2 $\mu g.g^{-1}$ (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997; Erasmus et al., 2011). The maximum residue level (MRL) is 5 $\mu g.g^{-1}$, but certain markets demand even lower levels (Cranney, 2012). In previous studies, an IMZ residue level of ≈ 1 $\mu g.mg^{-1}$ was shown to give adequate control of an IMZ sensitive (S) isolate of PD and ≥ 2 $\mu g.mg^{-1}$ was needed to control 80 - 95% infections caused by an IMZ resistant (R) isolate (Erasmus et al., 2011; 2013). However, the previous studies were conducted using one S

and one R isolate only and with 4 – 6 h incubation time. To our knowledge information on effective IMZ residue levels for control of PI is not documented.

The mechanism of IMZ resistance development is described as the over-expression of the gene P*d*CYP51, which will increase the amount of P405-dependant sterol 14α-demethylase, which will in its turn affect IMZ-sensitivity (Hamamoto et al., 2000b; Ghosoph et al., 2007; Kiralj and Ferreira, 2008). Resistance to IMZ is polygenic and involves 21 genes on 8 loci and is linked with 6 groups; hence, it is theoretically more difficult for *Penicillium* to develop resistance to this fungicide (Laville et al., 1977). IMZ-resistant isolates of *P. digitatum* are generally less fit on fruit not treated with IMZ when compared to IMZ-sensitive isolates (Dave, Sales and Walia 1989; Holmes and Eckert 1995; Kinay et al. 2007). This loss of fitness was only evident when the IMZ-resistant isolate is in competition with the IMZ-sensitive isolate and is not well understood (Holmes and Eckert 1995).

In this study, a number of PD and PI isolates with various levels of sensitivity to IMZ were used to determine effective IMZ residue levels for the curative and protective control of both *Penicillium* species following IMZ application in fungicide dip tanks. Additionally, this study determined the efficacy of 9 active ingredients or combinations thereof as alternatives for IMZ, specifically against resistant isolates to understand their potential role in an anti-resistance fungicide program.

2. Materials and methods

2.1. Penicillium *isolates and inoculum preparation*

Nine PD and five PI isolates were used in trials done for this project. Seven PD and all five PI isolates were obtained from University Pretoria (UP; South Africa). The sensitive and resistant PD isolates used in previous work (Erasmus et al., 2011; 2013) were included in the study and were coded PD3 and PD5, respectively. The UP isolates were coded PD1, PD2, PD4, PD6, PD7, PD8 and PD9. The five PI isolates were coded PI1, PI2, PI3, PI4 and PI7.

In order to obtain inoculum for biological efficacy tests, the isolates were grown at ambient temperature on potato dextrose agar medium (PDA; Difco™ Potato dextrose agar, Becton, Dickinson and company, Sparks, USA) in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 18 hours before trials commenced and stored at 4°C. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA) at a concentration of 0.01 $mL.L^{-1}$. Spore suspensions were adjusted with a spectrophotometer (Cecil 1011, Cecil

Instruments Limited, Peterborough, UK) to an absorbance of 0.1 at 420 nm, which correlates with a concentration of 1×10^6 spores.ml^{-1} (Morris and Nicholls, 1978; Eckert and Brown, 1986). The conidial suspensions were placed on magnetic stirrers to maintain a uniform suspension of spores during inoculation.

2.2. IMZ sensitivity of the nine PD and five PI isolates

The effective concentration that inhibits 50% mycelial growth (EC_{50}) of the various isolates was determined *in vitro*. Potato dextrose agar was amended with IMZ (Imazacure, 750 g.kg^{-1} SG, ICA International Chemicals, Stellenbosch, South Africa), which ranged in specific concentrations levels. Isolates were pre-screened for sensitivity and depending on the results, a specific range was used for each isolate. The ranges were 0, 0.005, 0.01, 0.015, 0.02, 0.03, 0.05, 0.1, 0.5 and 1 µg.mL^{-1} or 0, 1, 1.5, 2, 2.5, 3, 4 and 5 µg.mL^{-1}. Spore suspensions with a concentration of 1×10^6 spores.mL^{-1} were prepared for each isolate. A droplet of 20 µL spore suspension was placed in the middle of each specific amended PDA plate. The plates were incubated at 22°C for 5 days after which the colony diameters were measured twice perpendicularly. The percentage inhibition was calculated relative to colony diameters on unamended control plates for each specific isolate. Five replicate plates were prepared for each concentration per isolate and the trial was repeated three times. Percentage inhibition data of each isolate was regressed against IMZ concentration using non-linear regression with the function, Y = C/(1+Exp(-A-B×X)). The coefficient of determination (R^2) was used to demonstrate goodness of fit. EC_{50} value was calculated from the model for each isolate. This was done for each trial. The resistance (R) factor for resistance isolates was determined by dividing the EC_{50} value of a resistant isolate by the mean EC_{50} value of the sensitive PD isolates. From these results the isolates were arbitrarily categorised in terms of IMZ sensitivity as sensitive, low resistant, moderate resistant, resistant and highly resistant.

2.3. Effective IMZ residue levels for curative and protective control

2.3.1. Fruit

Untreated export quality Valencia sweet orange ('McClean' and 'Valencia Late') and navel orange ('Palmer' and 'Washington') fruit were obtained from various citrus packhouses in the Mpumalanga and Limpopo provinces of South Africa during the seasons of 2011 and 2012. Fruit were washed with an aqueous solution of 125 µg.mL^{-1} didecyl dimethyl ammonium chloride (Sprorekill, ICA International Chemicals, Stellenbosch, South Africa) and left to dry before it was 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 condensation to evaporate.

2.3.2. Imazalil treatment

Fruit were treated with an IMZ sulphate formulation (Imazacure, 750 $g.kg^{-1}$ SG). A pilot trial was conducted first to determine the various IMZ concentrations needed to load a range of residue levels from very low (< 0.1 $\mu g.g^{-1}$) to very high (> 10 $\mu g.g^{-1}$) on the fruit following a 60-s dip at 22°C. From the pilot trial IMZ concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 $\mu g.mL^{-1}$ were selected as the range that was used for treatments. The trial was repeated twice with all the PD isolates (PD1 – PD9) and three times with the PI isolates (PI1 – PI4 and PI7) on Valencia orange fruit. For comparative purposes, two isolates of PD (PD5 and PD9) were included with the PI isolates. Two similar trials were conducted on navel oranges with four PD isolates (PD3, PD5, PD6 and PD7) and two PI isolates (PI2 and PI4). Within each trial three replicate groups consisting of 12 fruit (six for curative control and six for protective control) were dipped at a time. Six extra fruit were added to two replicates for each treatment concentration per trial for IMZ residue analyses.

2.3.3. Residue analyses

Within 48 hours after each trial the fruit destined for residue analyses were prepared. For each replicate treatment, 6 fruit were cut (from stem- to stylar-end) into 4 equally sized pieces of which 3 were discarded and the rest weighed and macerated to a fine pulp by using a blender (Salton Elite Blender, Almalgamated Appliance Holdings Limited, Reuven, South Africa). The samples were frozen until analysis. Imazalil (chloramizol) residue analyses were conducted 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 tandem mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, California, USA).

2.3.4. Inoculation, incubation and evaluation

Fruit were treated protectively and curatively. Those destined for curative treatments were inoculated with the various isolates 24 h before treatment and those destined for protective treatment were first dip-treated, left to dry and then inoculated within ± 4 h after treatment. Fruit were simultaneously wounded and inoculated by dipping a stainless steel rod with a narrow, concave tip (2 mm long; 1mm diameter) into the spore suspension

and then wounding the fruit rind through the flavedo into the top layer of the albedo. Four inoculated wounds were induced equal distances apart surrounding the calyx. After treatment and inoculation the fruit were incubated at 22°C. The fruit were incubated in lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa) and enclosed in transparent polyethylene bags.

Fruit were rated for infection and sporulation 5 and 10 days after inoculation, respectively. The number of infected wounds per fruit were evaluated using an ultra-violet light source (UV-A at 365 nm, Labino Mid-light; www.labino.com). Infected wounds could be identified as yellow fluorescence under UV light (Erasmus et al., 2011). Infection data were normalised to percentage control in relation to the untreated control. Sporulation was evaluated for each fruit as described by Erasmus et al. (2011). Infected fruit were given a rating from 1 to 6 relating to the fruit area covered with green sporulation. Where 1 = infection with no sporulation, 2 = sporulation area less than 100 mm^2, 3 = sporulation area less than 50% of the fruit and more than 100 mm^2, 4 = sporulation area more than 50% of fruit and less than 75%, 5 = sporulation area more than 75% of the fruit and less 100%; and 6 = 100% covered with sporulating green mould. Infected fruit rated ≥ 4 were regarded as sporulating. Sporulation incidence per replicate (%) was calculated.

2.3.5. Calculating the effective residues for curative and protective control

Percentage control data for each replicate were regressed against residue levels of each specific trial using the non-linear function, Y = C/ (1+Exp (-A-B*X)), in XLSTAT®. The coefficient of determination (R^2) was used to demonstrate goodness of fit. The effective residue levels for 50% curative or protective control ($ER_{50}C$ and $ER_{50}P$, respectively) were calculated from the model for each isolate.

2.4. Efficacy of alternative fungicides against IMZ resistant isolates of P. digitatum

Three PD isolates, an IMZ sensitive, resistant and highly resistant (as determined in 2.2.), were used and inoculated as described in 2.3.4. Fruit were treated curatively and protectively with 9 fungicides: SOPP (SOPP Super 20%, Advantage Agri Products, Paarl, South Africa), TBZ (ICA TBZ, ICA International Chemicals), GZT (Citricure, ICA International Chemicals), IMZ (Imazacure SG, ICA International Chemicals), PYR (Protector, ICA International Chemicals), FLU (Scholar, Syngenta, Midrand, South Africa), AZO (Ortiva, Syngenta), PLB (Janssen PMP, Belgium), GRA (Syngenta) and PPZ (Tilt, Syngenta) were applied at concentrations registered in South Africa or at experimental concentrations, as 60 s dip treatments (Supplementary Table 3). Fruit were

incubated and evaluated as described in 2.3.4. Trials consisted of three replicates with six fruit and each trial was repeated three times each on navel and Valencia oranges.

2.5. Statistical analyses

Data for EC_{50} levels, residue levels, percentage control, sporulation incidence and ER_{50} levels from the respective trials were subjected to analysis of variance using XLSTAT® (Addinsoft, www.xlstat.com) and Fisher's test to compare differences at a 95% confidence interval.

Table 1. Effective imazalil (IMZ) concentration that inhibits 50% mycelia growth (EC_{50}) and resistance factors (R factor) of nine *Penicillium digitatum* (PD) and five *P. italicum* (PI) isolates that were determined following mycelium growth measurements on potato dextrose agar amended with IMZ at concentrations ranging from 0 to 5 µg.mL^{-1} from which percentage growth inhibition data for each isolate and IMZ concentrations were subjected to non-linear regression using the function, Y = C/ (1+Exp (-A-B*X).

Sensitivity category[a]	Function variables and standard deviations[b]				EC_{50} (µg.mL^{-1})[c]	R factor[d]
and isolate	A	B	C	R^2		
Sensitive						
PI4	-4.5 (2.0)	1132.7 (602.4)	84.5 (5.2)	> 0.85	0.005 e	
PI1	-4.8 (2.5)	1167.9 (801.2)	93.2 (1.6)	> 0.95	0.005 e	
PI7	-6.7 (4.4)	3338.9 (4124.6)	87.1 (2.8)	> 0.90	0.007 e	
PI2	-4.0 (1.8)	174.1 (124.2)	98.0 (1.3)	> 0.88	0.027 e	
PD6	-23.7 (13.9)	991.7 (643.3)	100.9 (1.2)	> 0.99	0.027 e	
PD3	-5.1 (1.1)	135.1 (28.9)	87.6 (36.6)	> 0.98	0.032 e	
PD9	-7.8 (4.5)	192.2 (74.7)	101.4 (1.5)	> 0.84	0.038 e	
PI3	-4.6 (1.6)	91.2 (30.6)	100.8 (1.0)	> 0.99	0.050 e	
Low resistant						
PD2	-4.2 (1.0)	6.7 (1.5)	101.3 (1.4)	> 0.97	0.615 d	19.0
Moderately resistant						
PD8	-17.6 (10.2)	17.4 (10.5)	100.5 (0.7)	> 0.98	1.075 c	33.2
Resistant						
PD7	-7.6 (0.7)	4.7 (0.1)	99.9 (0.1)	> 0.95	1.618 b	50.0
PD1	-15.4 (1.3)	9.5 (1.2)	100.0 (0.2)	> 0.99	1.634 b	50.5
PD4	-6.6 (1.2)	3.5 (0.5)	101.4 (0.2)	> 0.90	1.865 b	57.6
Highly resistant						
PD5	-26.2 (25.2)	9.5 (7.0)	100.8 (0.6)	> 0.99	2.290 a	70.7

[a]The resistant isolates were categorized using the R factor and the Fisher's LSD test.

[b]Mean values of three trials, where lines were fitted on the data from 5 replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

[d]The R factor was determined by dividing the EC_{50} value of a resistant isolate by the mean EC_{50} value of the three sensitive PD isolates (PD3, PD6 and PD9).

3. Results

3.1. IMZ sensitivity of nine PD and five PI isolates

Non-linear regression with the function Y = C/ (1+Exp (-A-B*X)) resulted in very good fits for all isolates; R^2 values of > 0.84 (Table 1). There were significant differences between EC_{50} values of the various isolates (P< 0.0001; ANOVA not shown). PD5 had the highest level of resistance with an EC_{50} of 2.29 µg.mL^{-1}. PD1, PD4 and PD7 all had similar EC_{50} values (1.63, 1.87 and 1.62 µg.mL^{-1}, respectively; Table 1), but significantly lower than PD5. PD8 and PD2 had significantly lower EC_{50} levels than PD1, PD4 and PD7 and also differed significantly from each other (1.08 and 0.62 µg.mL^{-1}, respectively). PD3, PD6, PD9 and all the PI isolates had similar and significantly lower EC_{50} levels compared to the resistant isolates which ranged from 0.05 to 0.01 µg.mL^{-1}. Based on the Fisher's LSD test on EC_{50} values and their R factors the isolates were characterised as sensitive (PD3, PD6, PD9 and all the PI isolates), low resistant (PD2), moderately resistant (PD8), resistant (PD1, PD4 and PD7) and highly resistant (PD5).

3.2. Effective IMZ residue levels for curative and protective control

3.2.1. Valencia orange fruit – Penicillium digitatum

3.2.1.1. IMZ residue levels

Statistically similar residues were loaded following the 5 – 640 µg.mL^{-1} treatments (0.18 – 1.26 µg.g^{-1}; results not shown), and significantly higher residue levels loaded following dips in 1280 µg.mL^{-1} (5.43 µg.g^{-1}), and 2560 treatment (10.60 µg.g^{-1}).

3.2.1.2. ER_{50} levels

The R^2 values for the function, Y = C/ (1+Exp (-A-B*X)), were > 0.75 indicating very good fits (Table 2). Analysis of variance for the IMZ ER_{50}C and ER_{50}P values for the nine isolates showed a meaningful action × isolate interaction (P = 0.069; ANOVA not shown). The three sensitive isolates clustered together having the lowest ER_{50}C and ER_{50}P values (ranging from 0.26 – 0.29 µg.g^{-1} and 0.13 – 0.27 µg.g^{-1}, respectively; Table 2). For curative control, the low resistant, moderately resistant, one resistant (PD1) and the highly resistant isolates had similar ER_{50}C values (ranging from 1.22 – 1.91 µg.g^{-1}); these values were 4- to 7-fold higher than those for

the sensitive isolates but were not significantly different. The other two resistant isolates (PD4 and PD7) had significantly higher $EC_{50}C$ values (4.37 and 4.56 µg.g^{-1}, respectively) than all the isolates, but similar to the lower level of the highly resistant isolate (1.91 µg.g^{-1}). Protectively, the low resistant isolate clustered with two resistant isolates (PD4 and PD7) having significantly higher $ER_{50}P$ values (2.94 – 4.46 µg.g^{-1}) compared to the moderately resistant (PD8) and one of the resistant isolates (PD1) with values of 1.08 and 1.00 µg.g^{-1}, respectively. The highly resistant isolate (PD5) had the highest $ER_{50}P$ value (6.62 µg.g^{-1}), but not significantly higher than PD2 and PD7.

3.2.1.3. Sporulation

As certain treatments had zero infected fruit, data for the various sensitive and resistant isolates were pooled for statistical analyses. Analysis of variance for percentage sporulating fruit showed a significant isolate × concentration interaction (P = 0.032; ANOVA not shown). For the sensitive isolates there was generally no significant difference in sporulation levels on infected fruit between the untreated fruit (83.1%) and the majority of IMZ treated fruit (80.0 – 100.0%) regardless of residue level with the 0.18 µg.mL^{-1} treatment being the only exception (66.2%; Supplementary data, Table 1). Similarly, there was generally no statistical difference for the resistant isolates between the untreated (70.8%) and IMZ treated fruit (27.8 – 80.6). Interestingly, on fruit loaded with 0.18 and 0.24 µg.g^{-1} significantly lower sporulation incidence (33.3 and 27.8%, respectively) was observed than on fruit loaded with 5.43 and 10.60 µg.g^{-1} (80.6 and 79.6, respectively). A meaningful effect for curative or protective action was also observed (P = 0.112). The curative treatments had generally higher levels (> 15% overall) of sporulating fruit compared to the protective treatments (results not shown).

3.2.2. Valencia orange fruit – Penicillium italicum *and two comparative PD isolates*

3.2.2.1. Residue levels

IMZ residue levels loaded following dips in concentrations of 5 – 320 µg.mL^{-1} increased from 0.07 – 0.61 µg.g^{-1} (results not shown). The highest three dip concentrations loaded significantly higher residue levels and differed significantly from each other (1.15, 2.31 and 5.53 µg.g^{-1} for 640, 1280 and 2560 µg.mL^{-1}, respectively).

Table 2. Effective imazalil (IMZ) residue values for predicted 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of green mould caused by nine *Penicilium digitatum* (PD) isolates respectively inoculated 24 h prior to or ± 4 h after treatment on Valencia orange fruit dipped in a range of IMZ concentrations (0 – 2560 µg.mL^{-1}) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Isolate[a]	Function variables and standard deviances[b]			R^2	ER_{50} (µg.g^{-1})[c]
	A	B	C		
Curative					
Sensitive					
PD3	-4.8 (0.9)	19.4 (1.5)	88.5 (1.7)	> 0.92	0.26 ab
PD6	-3.0 (0.8)	11.5 (4.6)	94.8 (0.8)	> 0.89	0.29 ab
PD9	-4.5 (0.2)	11.8 (8.4)	100.6 (10.9)	> 0.90	0.26 ab
Low resistant					
PD2	-4.4 (2.3)	4.8 (3.8)	88.8 (3.5)	> 0.81	1.65 abc
Moderately resistant					
PD8	-4.7 (0.7)	9.4 (4.3)	94.3 (3.5)	> 0.92	1.22 ab
Resistant					
PD1	-4.0 (0.7)	3.7 (2.0)	99.6 (0.4)	> 0.94	1.40 abc
PD4	-2.5 (0.1)	0.7 (0.2)	85.3 (14.8)	> 0.82	4.37 de
PD7	-2.5 (0.2)	0.8 (0.4)	84.7 (7.0)	> 0.79	4.56 de
Highly resistant					
PD5	-5.5 (2.5)	2.2 (0.7)	1252.9 (1173.9)	> 0.87	1.91 abcd
Protective					
Sensitive					
PD3	-3.7 (1.6)	19.8 (4.7)	93.7 (2.4)	> 0.84	0.19 a
PD6	-3.4 (0.5)	14.2 (3.4)	95.2 (6.4)	> 0.91	0.27 ab
PD9	-7.7 (4.2)	91.7 (71.6)	92.3 (3.6)	> 0.82	0.13 a
Low resistant					
PD2	-2.2 (0.2)	0.7 (0.4)	85.9 (0.2)	> 0.75	4.46 de
Moderately resistant					
PD8	-4.7 (0.5)	6.0 (1.6)	65.8 (8.9)	> 0.81	1.08 ab
Resistant					
PD1	-8.0 (0.9)	8.1 (1.4)	98.8 (0.0)	> 0.95	1.00 ab
PD4	-5.5 (2.9)	2.2 (0.9)	69.6 (2.5)	> 0.90	2.94 bcd
PD7	-2.7 (0.3)	0.8 (0.3)	85.0 (0.9)	> 0.84	4.00 cde
Highly resistant					
PD5	18.4 (20.8)	-6.5 (7.0)	52.3 (47.6)	> 0.79	6.62 e

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of two trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

3.2.2.2. ER_{50} levels

Data fitted the function, Y = C/ (1+Exp (-A-B*X)), relatively well ($R^2 > 0.68$; Table 3). Analysis of variance for the IMZ ER_{50} values showed a significant action × isolate interaction ($P = 0.0001$; ANOVA not

shown). The $ER_{50}P$ level for the highly resistant isolate, PD5 (4.38 µg.g^{-1}; Table 3), was significantly higher than its $ER_{50}C$ level (1.47 µg.g^{-1}). Both these values were significantly higher than the rest of the sensitive PD and PI isolates' $ER_{50}P$ and $ER_{50}C$ levels (0.09 - 0.20 µg.g^{-1} and 0.11 – 0.26 µg.g^{-1}, respectively).

Table 3. Effective imazalil (IMZ) residue values for predicted 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of green mould caused by two *Penicilium digitatum* (PD) and five *P. italicum* (PI) isolates respectively inoculated 24 h prior to or ± 4 h after treatment on Valencia orange fruit dipped in a range of IMZ concentrations (0 – 2560 µg.mL^{-1}) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Isolate[a]	Function variables and standard deviances[b]			R^2	ER_{50} (µg.g^{-1})[c]
	A	B	C		
Curative					
Sensitive					
PD9	-2.6 (1.2)	22.3 (15.1)	92.7 (3.5)	> 0.88	0.15 a
PI1	-2.0 (0.2)	12.3 (2.4)	96.4 (2.1)	> 0.83	0.18 a
PI2	-2.8 (0.4)	11.3 (1.0)	93.2 (1.6)	> 0.87	0.26 a
PI3	-3.1 (0.9)	23.9 (9.9)	95.8 (2.0)	> 0.81	0.14 a
PI4	-3.0 (1.8)	31.2 (19.3)	95.5 (3.8)	> 0.76	0.11 a
PI7	-13.5 (15.8)	137.1 (176.7)	89.6 (1.7)	> 0.77	0.17 a
Highly resistant					
PD5	-4.0 (0.6)	5.2 (3.4)	75.8 (9.5)	> 0.78	1.47 b
Protective					
Sensitive					
PD9	-3.0 (1.4)	41.0 (26.3)	93.0 (2.0)	> 0.81	0.11 a
PI1	-3.0 (0.7)	31.5 (19.8)	93.1 (0.6)	> 0.84	0.12 a
PI2	-3.1 (0.6)	17.1 (3.2)	88.0 (4.6)	> 0.88	0.20 a
PI3	-2.2 (0.4)	13.8 (3.9)	97.3 (1.8)	> 0.90	0.17 a
PI4	-3.4 (0.7)	36.2 (8.2)	95.8 (1.1)	> 0.69	0.10 a
PI7	-7.3 (4.3)	86.7 (57.0)	96.1 (1.3)	> 0.85	0.09 a
Highly resistant					
PD5	-4.2 (1.3)	1.4 (0.2)	47.6 (14.0)	> 0.68	4.38 c

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of three trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

3.2.3. Navel orange fruit – Penicillium digitatum *and* P. italicum

3.2.3.1. Residue levels

IMZ residue levels loaded on navel orange fruit increased with treatment concentration: means of 0.13, 0.19, 0.22, 0.36, 0.56, 0.87, 1.37, 1.72, 3.09 and 5.64 $\mu g.g^{-1}$ were loaded for the 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 $\mu g.mL^{-1}$ treatments, respectively (results not shown).

3.2.3.2. ER_{50} levels

The $ER_{50}P$ values for PD5 and PD7 could not be determined, due to low levels of control (≈ 4%; result not shown). Therefore, ER_{50} data were analysed separately for curative and protective action. The R^2 values were ≥ 0.74 for lines that were fitted by means of the function, Y = C/ (1+Exp (-A-B*X)) (Table 4 and 5). Analysis of variance for the $ER_{50}C$ levels for green and blue mould caused by the 4 PD and 2 PI isolates indicated

Table 4. Effective imazalil (IMZ) residue values for predicted 50% curative ($ER_{50}C$) control of green mould caused by four *Penicilium digitatum* (PD) and two *P. italicum* (PI) isolates inoculated 24 h prior to treatment on navel orange fruit dipped in a range of IMZ concentrations (0 – 2560 $\mu g.mL^{-1}$) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Sensitivity category[a]	Isolate	Function variables and standard deviances[b]			R^2	$ER_{50}C$ ($\mu g.g^{-1}$)[c]
		A	B	C		
Sensitive	PD3	-3.9 (0.7)	12.3 (3.3)	94.2 (3.8)	> 0.89	0.37 a
	PD6	-2.2 (0.7)	9.3 (4.8)	98.7 (0.5)	> 0.85	0.28 a
	PI2	-3.5 (0.9)	12.0 (3.9)	91.3 (4.2)	> 0.93	0.37 a
	PI4	-17.6 (15.3)	106.1 (97.5)	97.5 (0.0)	> 0.94	0.21 a
Resistant	PD7	-25.9 (22.2)	15.3 (12.5)	68.1 (12.6)	> 0.93	1.65 b
Highly resistant	PD5	-5.1 (0.4)	4.0 (0.6)	78.6 (3.5)	> 0.88	1.42 b

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of two trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

significant differences between isolates ($P = 0.001$; ANOVA not shown). The highly resistant and resistant isolate, PD5 and PD7, had significantly higher $ER_{50}C$ levels (1.42 and 1.65 $\mu g.g^{-1}$, respectively) than the sensitive PD and PI isolates (0.21 – 0.37 $\mu g.g^{-1}$; Table 4); $ER_{50}C$ values for the sensitive PD and PI isolates did not differ significantly. Analyses of variance for the $ER_{50}P$ levels showed no significant difference ($P = 0.213$; ANOVA's not shown) between the $ER_{50}P$ levels for IMZ sensitive PD and PI isolates, which were 0.28 - 0.35 and 0.15 – 0.29 $\mu g.g^{-1}$, respectively (Table 5).

Table 5. Effective imazalil (IMZ) residue values for predicted 50% protective ($ER_{50}P$) control of green mould caused by two *Penicilium digitatum* (PD) and two *P. italicum* (PI) isolates inoculated ± 4 h after treatment on navel orange fruit dipped in a range of IMZ concentrations (0 – 2560 µg.mL^{-1}) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Sensitivity category[a]	Isolate	Function variables and standard deviances[b]			R^2	$ER_{50}P$ (µg.g^{-1})[c]
		A	B	C		
Sensitive	PD3	-3.1 (1.0)	12.3 (5.5)	96.3 (2.1)	> 0.90	0.28 a
	PD6	-2.3 (0.2)	6.7 (0.4)	96.9 (0.4)	> 0.91	0.35 a
	PI2	-7.7 (5.3)	23.5 (10.3)	94.1 (2.6)	> 0.74	0.29 a
	PI4	-5.8 (2.2)	42.2 (19.0)	96.3 (1.2)	> 0.85	0.15 a

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of two trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

3.2.3.3. Sporulation inhibition

Similar to section 3.2.1.3. data for the sensitive PD, resistant PD and sensitive PI isolates were pooled for statistical analyses. Analyses of variance for percentage sporulation incidence data showed a significant isolate × concentration interaction ($P < 0.0001$; ANOVA not shown). For sensitive PD isolates, there was no significant difference in sporulation incidence between the untreated fruit and IMZ treated fruit with residues of 0.13 – 0.56 µg.g^{-1}, where these levels ranged from 94.4 - 100.0% (Supplementary data, Table 2). Treatments that loaded higher residue levels (0.87 – 5.64 µg.g^{-1}) had significantly lower levels of sporulating fruit (76.9 – 26.4%). For the resistant PD isolates, no significant differences were found regardless of residue level and the sporulation incidence was > 90.0%. The sensitive PI isolates infected fruit showed a similar trend to the sensitive PD infected fruit, and significant reduction in sporulation incidence was observed from 0.19 – 1.72 µg.g^{-1} (63.7 – 16.9%) compared with the untreated control (95.8%). The ANOVA showed a significant isolate × action (curative and protective) interaction ($P = 0.006$; ANOVA not shown). Sporulation incidence on infected fruit levels did not differ between the curative and protective treatments when inoculated with resistant PD isolates (> 95.0%; results not shown), but curative and protective treatments of the sensitive PD isolates showed significantly lower sporulation levels (85.6 and 74.6%, respectively) and differed significantly from each other. The sensitive PI isolates showed significantly lower sporulation incidence for curative and protective treatments (46.9 and 42.3%, respectively).

3.3. Efficacy of alternative fungicides against IMZ resistant isolates of P. digitatum

3.3.1. Green mould control

Analysis of variance for percentage green mould control data showed a significant four factor interaction for citrus kind (navel and Valencia) × action (curative and protective) × isolate [sensitive (PD3), resistant (PD4) and highly resistant (PD5)] × fungicide (AZO, GRA, FLU, GZT, PLB, IMZ, PPZ, PYR, SOPP and TBZ) (P = 0.0002; ANOVA not shown). This difference could be mostly ascribed to Valencia trials having lower control levels compared to the navel trials. To simplify the interpretation the significant action x isolate x fungicide interaction was discussed ($P < 0.0001$) Curatively, SOPP, TBZ, GZT and PLB gave similar control levels to IMZ (> 90.0%; Figure 1) of the sensitive isolate. Pyrimethanil gave weaker control (78.0%) than these fungicides, but

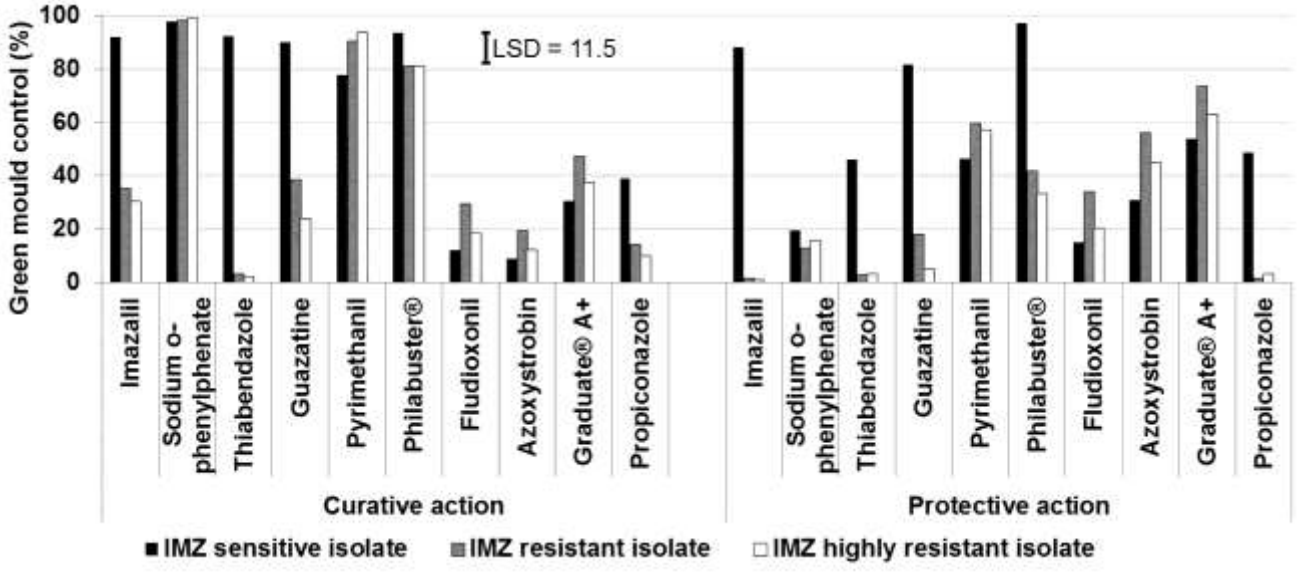

Figure 1. Mean percentage green mould control on orange fruit inoculated with an imazalil (IMZ) sensitive, resistant or highly resistant *Pencillium digitatum* isolate 24 h prior to (curative) or ± 4 h after (protective) 60 s aqueous dip treatment with 10 different fungicides at registered or experimental concentrations. The LSD value (11.5) was determined by means of Fisher's test ($P \leq 0.05$).

significantly higher than FLU, AZO, GRA and PPZ (< 39.0%). Imazalil exhibited poor control of the two IMZ resistant isolates (35.3 and 30.3% for the resistant and highly resistant isolate, respectively). Propiconazole, TBZ and GZT treatment also resulted in very low control levels of these two isolates (< 39.0%). Sodium ortho-phenyl-phenol and PYR controlled the resistant isolates the best (> 90%). Philabuster® gave ≈ 81.0% control of the resistant isolates, which was markedly lower than PYR and SOPP control of the resistant isolates, as well as PLB control of the sensitive isolate. The rest of the fungicides showed low control levels of < 48.0%.

Protectively, IMZ gave slightly poorer control (88.2%), but not significant poorer than its curative control of the IMZ sensitive isolate. Guazatine gave similar protective control (81.6%), while PLB gave the best protective control at 97.5%. Sodium ortho-phenyl-phenol gave much weaker protective control (19.6%) than curative, which could be ascribed to the rinsing of fruit immediately after SOPP treatment. Thiabendazole and PYR gave significantly lower protective control (46.0 and 46.4%, respectively), compared to its curative treatments. Fludioxonil, AZO, GRA and PPZ gave slightly better protective control levels than curative, but even though it was significant in some cases the control levels were relatively low (< 54.0%). Imazalil, PPZ, TBZ and GZT failed to protectively control the IMZ resistant isolates (< 18.1%), while PLB also gave relative poor protective control (33.3 – 41.9%). Graduate®A^{+}, PYR and AZO showed some protective control potential against the IMZ resistant isolates, but at varying levels (45.1 - 73.6%). Interestingly, control of the IMZ resistant isolates by these fungicides was markedly to significantly better than their control of the sensitive isolate (30.8 - 53.9%).

3.3.2. Sporulation

Sporulation data of one navel and three Valencia trials were combined. Analyses of variance for percentage sporulation incidence of infected orange fruit showed a significant action × fungicide and isolate × fungicide interaction (P = 0.015 and < 0.0001, respectively; ANOVA not shown). However, the three factor isolate × action × fungicide interaction (P = 0.571) will be discussed. For the sensitive isolate, the levels of sporulation incidence on infected fruit were generally high (> 80.0; Supplementary data, Table 3), regardless of curative or protective treatment with most fungicides. However, IMZ treatment resulted in significant lower levels (62.8 and 69.4%, for curative and protective treatments, respectively), as well as curative and protective PLB treatment (23.6 and 2.8%, respectively). IMZ and PLB did not inhibit sporulation of the resistant isolates (>74.3%) and low levels of sporulation incidence were recorded only for SOPP (33.3%), but only in the curative treatments. For the rest of the fungicides the majority of levels were > 72.9 %, regardless of curative or protective treatment.

4. Discussion

Fungicide resistance monitoring and characterisation is generally conducted by means of *in vitro* growth studies (Staub and Sozzi, 1984; Russell, 2004). From these studies the baseline sensitivity for the given fungicide can be determined that forms the basis from where resistance development can be followed. In many

citrus production regions including California (Holmes and Eckert, 1999), Florida (Brown, 1989), Uruguay (Pérez et al., 2011) and Morocco (Boubaker et al., 2009) the baseline sensitivity for IMZ has been established. All these studied determined the baseline sensitivity of IMZ to be < 0.05 $\mu g.g^{-1}$. To our knowledge no IMZ baseline has been determined for South Africa. *In* vitro fungicide sensitivity characterisation is rarely corroborated with *in vivo* characterisation (Wild, 1994; Kinay et al., 2007). Peréz et al. (2011) showed that isolates that would grow on 1 $\mu g.mL^{-1}$ IMZ *in vitro* would be able to overcome an IMZ residue of 3 $\mu g.g^{-1}$ loaded on citrus fruit; this was an attempt to establish the *in vitro* concentration by which practical resistance can be detected. This study is a first attempt to quantitatively categorise different PD and PI isolates based on *in vitro* as well as *in vivo* studies. It is clearly shown that the practical impact of a specific resistant isolate may differ significantly from what could be predicted or expected from *in vitro* categorisation.

Only one of the sensitive PD isolates (PD3) originated from a secluded orchard that has never been exposed to any postharvest fungicides. More isolates that have never been exposed to imazalil are required to establish a proper IMZ baseline sensitivity level (Russell, 2004; Kinay et al., 2007). Nonetheless, the average EC_{50} value of the sensitive PD isolates in this study (0.03 $\mu g.g^{-1}$) relates well to baselines determined in other work (Brown, 1989; Holmes and Eckert, 1999; Kinay et al., 2007; Pérez et al., 2011).

Hamamoto et al. (2000) suggested that the *PdCYP51* gene can be expressed in increasing levels that will render higher levels of DMI resistance. Ghosoph et al. (2007) confirmed these findings and alluded to the same conclusion that the level of *PdCYP51* expression could be related to the level of IMZ resistance. So far three *CYP51* genes that contributes to IMZ resistance have been characterised: IMZ-R1 (Hamamoto et al., 2000), IMZ-R2 (Ghosoph et al., 2007) and IMZ-R3 (Sun et al., 2013). Through molecular characterisation, all the resistant isolates in this study were characterised as the IMZ-R3 genotype (Mareli Kellerman, pers. comm.). From the EC_{50} values it was shown that the IMZ resistant isolates in our study had different levels of IMZ resistance with R-factors of 19 to 71. Theoretically, an isolate with an R-factor of >2 can be considered resistant (Delp and Dekker, 1985). R-factors typically vary from as low as 5 to 100 (Brent and Hollomon, 2007). R-factor values obtained in our study may be considered to be small when compared to those found with other fungicides, where the differences in terms of sensitivity levels are much wider (Staub, 1991). Even though the difference between the sensitive and resistant PD isolates in this study could be considered relatively low, the effect on disease control on fruit was quite substantial. Furthermore, the ER_{50} values, which were determined *in vivo* on fruit, did not fall into these distinct categories as determined *in vitro*.

The sensitive PD isolates had similar ER_{50} values regardless of action (curative of protective) treatment or citrus type (< 0.37 $\mu g.g^{-1}$). However, the $ER_{50}C$ and $ER_{50}P$ values of the resistant isolates could not be predicted from their EC_{50} values. On Valencia, two resistant isolates, PD4 and PD7, showed the highest level of resistance in curative treatment ($ER_{50}C$ levels of 4.37- 4.56 $\mu g.g^{-1}$), while the highly resistant isolate, PD5, had a significantly lower $ER_{50}C$ level of 1.91 $\mu g.g^{-1}$, which was similar to the low and moderately resistant isolates. For protective control, however, this highly resistant isolate, as well as the low resistant isolate had the highest $ER_{50}P$ values. The low resistant and highly resistant isolates behaved similarly on Valencia with lower $ER_{50}C$ (1.65 and 1.91 $\mu g.g^{-1}$, respectively) and higher $ER_{50}P$ values (4.46 and 6.62 $\mu g.g^{-1}$, respectively). The other resistant isolates had similar respective values for curative and protective ER_{50} values.

In vitro EC_{50} values did not correlate with *in vivo* ER_{50} values, and this was further complicated when different ER_{50} values were obtained from different citrus types. Infection levels of the resistant isolates on navel oranges were so severe that $ER_{50}P$ values could not be determined. The highly resistant isolate behaved similarly in curative treatments on navel and Valencia ($ER_{50}C$ values of 1.91 and 1.42 $\mu g.g^{-1}$, respectively), but the resistant isolate had lower ER_{50} values on navel compared with Valencia (1.65 and 4.56 $\mu g.g^{-1}$, respectively). In cases with high ER_{50} values, it could be assumed that a residue level of > 5 $\mu g.g^{-1}$(exceeding the IMZ MRL) would be required to completely control the resistant isolates. Also the isolate that were categorised as low resistant proved to be “highly resistant” with an $ER_{50}P$ value of 4.46 $\mu g.g^{-1}$. If isolates with this level of resistance prevail in an environment where fruit are stored in a long term protocol and repacked; failure of control can be expected (Holmes and Eckert, 1999).

In vitro categorisation of fungal populations is commonly used as a diagnostic measure of fungicide resistance. For PD, a discriminatory concentration of 1.0 $\mu g.mL^{-1}$ IMZ amended PDA was suggested for commercial resistance monitoring (Pérez et al., 2011). Our work indicates that this level could be reduced to 0.5 $\mu g.mL^{-1}$. While none of the sensitive isolates in our study was able to grow at 0.5 $\mu g.mL^{-1}$, the low resistant PD isolate had an EC_{50} of 0.62 $\mu g.mL^{-1}$and EC_{95} of 1.05 $\mu g.mL^{-1}$ (results not shown). As this isolate will be substantially inhibited at 1.0 $\mu g.mL^{-1}$, it might be regarded as sensitive in a discriminatory *in vitro* assay. Importantly, this isolate had ER_{50} values of 1.65 and 4.46 $\mu g.g^{-1}$ (for curative and protective treatment, respectively), which will cause loss of control in a packhouse as demonstrated in this study.

At the onset of the study, a collection of sensitive and resistant PI isolates was sourced for inclusion in the study. However, following *in vitro* and *in vivo* characterisation, all the PI isolates proved to be sensitive. Imazalil resistant isolates of PI are usually less prevalent than PD (Eckert, 1990; Bus et al., 1991; Holmes and

Eckert, 1999). One reason for this could be due to the fact that PD grows much faster than PI at temperatures 15°C - 28°C (Eckert and Eaks, 1989). These temperatures are predominant during harvest time in most citrus regions. It was shown that resistant PD isolates were not less virulent compared to sensitive PD isolates, but less competitive (Holmes and Eckert, 1995).

Residue levels varied between the different trials. Imazalil residue levels on Valencia orange fruit were approximately double in the trial where all nine PD isolates were used compared to the trial where the PI isolates were involved (0.24 -10.6 $\mu g.g^{-1}$ compared to 0.07 – 5.53 $\mu g.g^{-1}$ in dip treatments that ranged from 5 – 2560 $\mu g.mL^{-1}$ for the respective trials). The IMZ residue levels on navel oranges were similar to those on the second Valencia orange trial mentioned above (0.13 – 5.64 $\mu g.g^{-1}$). It is suspected that the fluctuation in residue loading can be ascribed to fluctuating municipal water quality, where an increase in the pH level of water can have an increasing effect on residue loading in an IMZ sulphate solution (Erasmus et al., 2011; 2013). Regrettably, the pH levels of IMZ sulphate solutions used in this study were not monitored. Despite variation in residue loading between fruit types and batches, the sensitive and highly resistant isolates (PD9 and PD5, respectively) were used as reference isolates and relatively comparable ER_{50} values were determined in the different trials.

This project was not specifically designed to study sporulation or its inhibition as was done in previous studies (Brown et al., 1983; Brown and Dezman, 1990); however, trends in sporulation inhibition were observed between treatments. On Valencia, no differences were found between curative and protective treatments, but there was a tendency for the resistant PD isolates to have lower sporulation incidences on infected fruit with lower residue levels (27.8 – 67.6% on 0.18 – 0.89 $\mu g.g^{-1}$), compared to the sensitive PD isolates (all > 66.2%). In contrast, sensitive PD infections showed lower levels of sporulation on navel fruit with higher residue levels (26.4 – 68.1% on 1.37 – 5.64 $\mu g.g^{-1}$), while resistant PD isolates had levels of > 90% sporulation regardless of residue level. The sensitive PI isolates had the lowest level of sporulation; on fruit with residues of 1.37 and 1.72 $\mu g.g^{-1}$, sporulation incidences were 12.0 and 16.9%, respectively. This apparently enhanced sporulation inhibition effect by IMZ could in part explain why resistant PI isolates are less prevalent than those of PD. The poor inhibition of sporulation on fruit infected with resistant isolates, regardless of residue level is problematic and shows that increasing the IMZ residue level in order to combat resistance is not an effective practice. This was also shown in previous studies where a residue of ≈ 5 $\mu g.g^{-1}$ could not lead to sporulation inhibition of a resistant isolate (Eckert, 1990; Erasmus et al., 2011; 2013).

The conventional green mould fungicides (IMZ, SOPP, TBZ, GZT, PYR and PLB) gave excellent curative control (≥ 90.0%) against IMZ sensitive PD isolates. Pyrimethanil and SOPP equalled this level of

curative control of the IMZ resistant isolates. Philabuster® also performed well with > 80% curative control of the resistant isolates. Protectively, only PLB, IMZ and GZT stood out giving levels of > 80.0% control of the sensitive isolate infections, while none of the fungicides were as effective against the resistant isolate infections. The highest protective control levels on these isolates resulted from PYR and GRA (≈ 60 and ≈ 70%, respectively). The protective action of green mould fungicides can possibly be improved by an alternative application method. This was the case for IMZ, where application in wax coatings resulted in better protective control compared to dip treatments (Njombolwana et al., 2013a; 2013b).

Multiple resistance was observed in the two IMZ resistant isolates (PD4 and PD5). GZT, TBZ and PPZ were ineffective in controlling the IMZ resistant isolates, while providing significantly better control of the sensitive isolate. These fungicides have been in use longer than IMZ and resistance in the PD population is well known. Double resistance to GZT and TBZ was reported in 1983 in Australia (Wild, 1983). To our knowledge, this is the first report of multiple resistance to IMZ, GZT, PPZ and TBZ in PD. Multiple resistance have been reported for IMZ, TBZ and SOPP (Holmes and Eckert, 1999). Imazalil and PPZ fall in the same DMI class, and cross resistance has been shown between these two fungicides (Mckay et al., 2012b). Some indication of potential negative cross resistance was also observed where the IMZ resistant isolates were markedly better controlled with GRA, PYR and AZO than their respective control of the sensitive isolates. This will, however, have to be investigated further.

The poor control of the IMZ sensitive isolate with PPZ in our study could be due to the 24 h incubation period being too long. Good results were obtained on infection of up to16 h (McKay et al., 2012a).

The older fungicide SOPP proved to be a very good alternative for curative control, but cannot be considered for protective control as fruit needs to be rinsed after treatment to prevent phytotoxicity. If the pH is controlled the risk of phytotoxicity may be reduced. The pH should be managed at a level of 12, and lowering the pH will increase residue loading (Dezman et al., 1986). Protective control by SOPP in our trials was relatively poor, but the pH was not adjusted to 12.

The excellent curative control by PYR reported in this work confirm other work showing this fungicide as a favourable IMZ alternative (Adaskaveg et al., 2005; D'Aquino et al., 2006; Smilanick et al., 2006). Unfortunately, relatively poor protective control was achieved.

Philabuster® provided excellent curative and protective control of IMZ sensitive isolates and relatively good curative control of IMZ resistant isolates. However, the significantly weaker control of IMZ resistant isolates, especially protectively, was most probably due to IMZ resistance, as well as the lower PYR

concentration in the formulated product (500 µg.mL^{-1}), which is 50% lower when PYR is recommended as stand-alone product. Moreover, Lado et al. (2010) recommended 750 µg.mL^{-1} PLB as an effective concentration, while 500 µg.mL^{-1} (the registered concentration) was evaluated in this study. Schirra et al. (2010) got very good results with a combination of 600 µg.g^{-1} each of IMZ and PYR.

Azoxystrobin, FLU and GRA gave relatively poor curative control (< 62%). Kanetis et al. (2007) showed that these actives showed very good potential on infections of 21 h and younger. In our work the infections was 24 h old, which is realistically comparable to industry situations where fruit can stand for longer than a day after harvest before the first fungicide application. D'Aquino et al. (2013) showed excellent curative control (≤ 15% infection) with 600 µg.mL^{-1} FLU on 24 h old green mould infections. The differences between our work and that of D'Aquino et al. (2013) might be ascribed to different inoculation methods, as they induced 2 mm wide by 2 mm deep wounds before dipping the fruit in a spore suspension of 1 x 10^5 spores.mL^{-1}. In our study, fruit were wound-inoculated simultaneously by dipping the wound-inducer in a suspension of 1 x 10^6 spores.mL^{-1} prior to wounding; this resulted in approximately 4 x 10^4 spores deposited at each wound site, which might be more severe than found in the field. Schirra et al. (2010) used a similar inoculation method to D'Aquino et al. (2013), but their spore suspension was 1 x 10^6 spores.mL^{-1}. They could only achieve ≈ 12 – 13% control with 30 and 60 s dip treatments in 600 µg.g^{-1} FLU and AZO. Other fungicides, however, were able to control infections that originate from this type of inoculation. Protectively these actives showed some potential, especially GRA giving 73.6 and 63.1% control of infections from the two respective IMZ resistant isolates in this study. Interestingly, protective control of the sensitive isolate was markedly to significantly poorer than that of the IMZ resistant isolates following treatment with GRA, FLU, AZO and PYR. One of the attributes of FLU and AZO is the inhibition of conidium germination (Bushong and Timmer, 2000; Rosslenbroich and Stuebler, 2000; Kanetis et al., 2007), which might explain the protective ability of these fungicides.

The sporulation inhibition effect of IMZ is better expressed when it is applied in wax coatings than when applied in an aqueous solution (Erasmus et al., 2011; 2013; Njombolwana et al., 2013a; 2013b). Brown and Dezman (1990) showed that fruit with an intact wax layer and treated with IMZ had lower levels of sporulation compared to fruit with the wax layer removed. Residues levels of > 2 µg.g^{-1} are required for the inhibition of sporulation where the IMZ EC formulation was used (Brown and Dezman, 1990; Smilanick et al., 1997). So far no consistent trends could be observed in terms of sporulation control with aqueous IMZ sulphate treatments. PYR has also been shown to have the ability to inhibit or reduce green mould sporulation, but not as well as IMZ (Smilanick et al., 2006). In their study, the combination of IMZ and PYR showed sporulation inhibition of an IMZ

sensitive isolate of *P. digitatum* applied within wax or aqueous solution. Our work confirms this as PLB was able to control sporulation the best on the sensitive isolate infections (23.6 and 2.8% sporulation for curative and protective, respectively). This might be ascribed to a synergistic effect between IMZ and PYR, as sporulation inhibition for these actives individually was relatively poor. However, sporulation inhibition by PLB was relatively poor for the resistant isolate infections, and comparable to PYR alone. Kanetis et al. (2007) also found that PLB was unable to inhibit sporulation on green mould caused by IMZ resistant isolates. The only fungicide that showed some level of sporulation inhibition of the resistant isolates was SOPP, but for curative treatment only (33.3%).

Erasmus et al. (2015) have shown that post dip-treatment brushing can reduce > 90% of the potential residue to levels of < 0.5 $\mu g.g^{-1}$. This level gave good curative control of the sensitive isolate (PD3), but poor inhibition of sporulation due to the reduced residue levels. The protective ability of the reduced residue loads was also questioned. In this study, the $ER_{50}C$ and $ER_{50}P$ values for sensitive PD isolates from 0.20 - 0.33 $\mu g.g^{-1}$ confirms the good curative control at low residue levels, but also indicate that good protective control of IMZ sensitive isolates could also be achieved. In the absence of IMZ resistance, green mould can therefore be effectively controlled if fruit is dip-treated within 24 h after harvest with relatively low residue levels. However, it can be anticipated that older infections will escape control (Erasmus et al., 2015) and their sporulation will not be inhibited.

This work shows the importance of loading an effective IMZ residue to combat green and blue mould. Although sensitive isolates could be controlled with < 0.5 $\mu g.g^{-1}$, sporulation inhibition was mostly observed at higher residue levels (> 2 $\mu g.g^{-1}$). The ideal IMZ residue level of 2 – 3 $\mu g.g^{-1}$ was, however, not effective against all resistant isolates, but should reduce infection and inoculum build-up in packhouse environments. Our work showed that further increasing IMZ residue levels will not be effective in improving control of resistance PD isolates, especially considering the MRL restriction. In packhouses where IMZ resistance is prevalent, fungicides with alternative modes of action could be applied; although none of these fungicides equalled IMZ in its combined curative, protective and sporulation control abilities of IMZ sensitive PD isolates, they would be effective when applied in an integrated programme. Each fungicide's optimal application needs to be specifically determined, as the profound effect of application on residue loading and control has been demonstrated in previous studies (Erasmus et al., 2011; 2013; Njombolwana et al., 2013a; 2013b).

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Supplementary data, Table 1. Mean sporulation incidence (%) on infected Valencia orange fruit that were inoculated with three imazalil (IMZ) sensitive and six IMZ resistant *Pencillium digitatum* isolates, and curatively and protectively dip-treated in IMZ solutions ranging from 0 to 2560 µg.mL^{-1} to effect a range of IMZ residues on fruit.

IMZ residue (µg.g^{-1})	Sporulation incidence on infected fruit (%)[a]	
	Sensitive isolates	Resistant isolates
0.00	83.1 abc	70.8 bcde
0.18	66.2 cde	33.3 de
0.20	92.5 ab	63.8 cde
0.24	88.1 abc	27.8 e
0.30	85.2 abc	57.4 cde
0.57	100.0 a	62.3 cde
0.89	98.6 a	67.6 cde
1.20	89.6 abc	72.3 bcd
1.26	80.0 abc	71.5 bcde
5.43		80.6 abc
10.60		79.6 abc

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Supplementary data, Table 2. Mean sporulation incidence (%) on infected Navel orange fruit that were inoculated with 2 imazalil (IMZ) sensitive *Penicillium digitatum* (PD), two IMZ resistant PD and two IMZ sensitive *P. italicum* (PI) isolates, and curatively and protectively dip-treated in IMZ solutions ranging from 0 to 2560 µg.mL^{-1} to affect a range of IMZ residues on fruit.

IMZ residue (µg.g^{-1})	Sporulation incidence on infected fruit (%)[a]		
	Sensitive PD isolates	Resistant PD isolates	Sensitive PI isolates
0.00	100.0 a	100.0 a	95.8 ab
0.13	100.0 a	99.3 a	89.5 ab
0.19	99.3 a	100.0 a	63.7 c
0.22	99.3 a	100.0 a	60.3 cd
0.36	95.0 ab	94.4 ab	42.7 de
0.56	94.4 ab	91.7 ab	36.7 ef
0.87	76.9 bc	99.3 a	24.0 efg
1.37	68.1 c	90.1 ab	12.0 g
1.72	57.4 cd	98.3 a	16.9 fg
3.09	26.4 efg	96.9 a	
5.64	64.4 c	95.4 ab	

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Supplementary data, Table 3. Mean sporulation incidence (%) on infected orange fruit inoculated with an imazalil (IMZ) sensitive, resistant or highly resistant *Pencillium digitatum* isolate 24 h prior to (curative) or ± 4 h after (protective) 60 s aqueous dip treatment with 10 different fungicides at registered or experimental concentrations.

Action	Product	Concentration ($\mu g.mL^{-1}$)	Sporulation incidence on infected fruit (%)[a]		
			IMZ sensitive isolate	IMZ resistant isolate	IMZ highly resistant isolate
Curative	Imazalil	500	62.8 def	98.6 l	100.0 l
	Sodium o-phenylphenate	20000	100.0 l	33.3 cd	33.3 abc
	Thiabendazole	1000	80.7 fghijkl	98.6 l	100.0 l
	Guazatine	1000	83.3 fghijkl	91.4 ghijkl	100.0 l
	Pyrimethanil	1000	84.7 fghijkl	53.9 cde	72.9 defghi
	Philabuster®	500	23.6 ab	74.3 defghij	93.3 hijkl
	Fludioxonil	500	100.0 l	88.9 ghijkl	100.0 l
	Azoxystrobin	500	100.0 l	84.4 fghijkl	100.0 l
	Graduate®A^{+}	500	84.0 fghijkl	71.5 defgh	98.6 l
	Propiconazole	500	94.4 hijkl	98.6 l	100.0 l
	Water control		100.0 l	100.0 l	100.0 l
	Untreated control		100.0 l	100.0 l	100.0 l
Protective	Imazalil	500	69.4 defg	100.0 l	100.0 l
	Sodium o-phenylphenate	20000	93.7 hijkl	79.2 fghijkl	89.4 ghijkl
	Thiabendazole	1000	93.3 hijkl	100.0 l	100.0 l
	Guazatine	1000	92.8 ghijkl	98.3 kl	100.0 l
	Pyrimethanil	1000	98.6 l	85.7 fghijkl	96.9 jkl
	Philabuster®	500	2.8 a	95.8 ijkl	100.0 l
	Fludioxonil	500	87.5 ghijkl	74.7 efghijk	100.0 l
	Azoxystrobin	500	100.0 l	79.2 fghijkl	95.3 hijkl
	Graduate®A^{+}	500	89.4 ghijkl	50.6 cd	92.1 ghijkl
	Propiconazole	500	95.8 ijkl	98.3 kl	100.0 l
	Water control		100.0 l	100.0 l	100.0 l
	Untreated control		100.0 l	100.0 l	100.0 l

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)