The effectiveness of pyrimethanil to inhibit germination of *Penicillium digitatum* and to control citrus green mold after harvest

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Abstract

Pyrimethanil (PYR) has recently been approved for postharvest use on citrus fruit to control green mold, caused by *Penicillium digitatum*. The EC_{50} of PYR to inhibit germination of *P. digitatum* spores was 0.2–0.4 mg/L and was similar from pH 4 to 7. Green mold on citrus fruit was reduced more than 90% by PYR at 500 mg/L or higher applied by immersing for 30 s or drenching the fruit, while its application in wax over rotating brushes at 1000 or 2000 mg/L reduced green mold about 65%. Control of sporulation by PYR in aqueous solutions was better than the same concentration applied in wax, but it was inferior to imazalil. An imazalil-resistant *P. digitatum* isolate was controlled by PYR. The addition of sodium bicarbonate improved PYR performance.

PYR was not compatible with chlorine. An increase in the temperature of the PYR solution slightly but significantly improved its effectiveness to control green mold, although its residues on fruit were greatly increased by heat; they approximately doubled for each 5 °C increase in solution temperature above 30 °C. PYR was very effective when applied up to 24 h after inoculation, but much less effective when it was applied before inoculation. PYR effectively controls green mold and can be useful to control isolates of *P. digitatum* resistant to other fungicides.
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1. Introduction

Citrus is grown in over 100 countries on six continents (Saunt, 2000), with a worldwide crop of about 70 billion kg in 2004 (Anon, 2005), and its production exceeds that of any other fruit. It is essential to control postharvest diseases in order to maintain the quality and prolong the shelf life of citrus fruit, particularly in a market where transport from producer to consumer may take several weeks and storage in packinghouses can exceed 3 months. Green mold of citrus, caused by *Penicillium digitatum* (Pers.: Fr.) Sacc., is the most economically important postharvest disease of citrus in arid growing regions of the world. Wounds on the fruit, inflicted during harvest and subsequent handling, are infected by spores of this pathogen and these infections must be eradicated to achieve accept-

able levels of control. Applications of sodium bicarbonate, thiabendazole, sodium *o*-phenylphenate, and imazalil are used to manage postharvest decay in California (Eckert and Brown, 1986; Ismail and Zhang, 2004; Smilanick et al., 1997).

Many issues make the development of new postharvest decay control treatments for citrus fruit important, particularly the widespread occurrence of fungicide resistance in *P. digitatum*. Numerous reports document the development of resistance to imazalil, thiabendazole, and sodium *o*-phenylphenate (Harding, 1972; Holmes and Eckert, 1999; Kuramoto, 1976) in this pathogen. We have observed that resistant isolates are typically present in many packinghouses where these fungicides are used, while in groves they rarely occur. Therefore, the introduction of a new fungicide of a different mode of action class is of value. For this reason, several new fungicides have been proposed for approval for postharvest use on citrus in California (Adaskaveg et al., 2005; Kanetis, 2005).

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Pyrimethanil, an anilinopyrimidine and inhibitor of methionine biosynthesis (Fritz et al., 1997) and secretion of cell wall degrading enzymes (Daniels and Lucas, 1995), is a broad-spectrum fungicide that does not share a mode of action with any of the citrus postharvest fungicides now in use. Therefore, cross-resistance with it among the many resistant isolates of *P. digitatum* now common to thiabendazole, imazalil, or sodium *o*-phenylphenate is unlikely. Pyrimethanil, introduced in 1990 and classified by the USEPA as a "reduced-risk" fungicide, is used primarily to control gray mold, caused by *Botrytis cinerea*, on horticultural and ornamental crops. Recently, Sholberg et al. (2005) reported excellent control by pyrimethanil of postharvest blue and gray molds of stored apples, caused by *Penicillium* spp. and *B. cinerea,* respectively. Pyrimethanil completely prevented the development of these diseases during storage when it was applied after harvest at 250–1000 mg/L and did not alter the quality of the fruit. The sensitivity of spores of isolates of *Penicillium expansum* to pyrimethanil varied; the EC_{50} values were 0.054–0.566 mg/L. Li and Xiao (2005) reported higher EC_{50} values for the *P. expansum* isolates were 0.5188–2.0536 mg/L. These studies indicated pyrimethanil would be a promising candidate for the control of postharvest decay of citrus fruit caused by *P. digitatum.*

Our objectives were to quantify the influence of pyrimethanil concentration and pH on the inhibition of germination of spores of *P. digitatum,* to determine the optimum concentration, solution temperature, and method of postharvest application of pyrimethanil to citrus fruit to control citrus green mold, to study the effect of adding sodium bicarbonate on pyrimethanil effectiveness, to measure pyrimethanil residues on citrus fruit after some of these treatments, and to determine the influence of pyrimethanil to suppress sporulation of *P. digitatum* from decay lesions. We included sodium bicarbonate because, in addition to being a common buffer and food additive, it also controls many plant pathogens (Punja and Grogan, 1982; Ziv and Zitter, 1992) including postharvest green mold of citrus (Marloth, 1931; Palou et al., 2001; Smilanick et al., 1999), and improves the activity of imazalil (Smilanick et al., 2005) and thiabendazole (Smilanick et al., 2006) for the control of green mold.

2. Materials and methods

2.1. Pathogen culture

Three *P. digitatum* isolates were isolated from infected lemons in California. Isolates M6R and PD0490 were sensitive to all of the fungicides in common use in California and are typical of those that occur in citrus groves, where these fungicides are not used. Their spores would not germinate on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) containing thiabendazole (Sealbrite, 99.0%, w/v, EcoScience Corp., Orlando, FL), imazalil (Fungaflor 500 EC; 44.6%, w/w, Janssen Pharmaceutica, Beerse, Belgium), or sodium

o-phenylphenate (Freshgard 20, 23%, w/v, FMC Corp., Riverside, CA) above 0.1, 0.05, or 10 mg/L, respectively. Isolate D201 was resistant to all of the fungicides in common use in California and is typical of those that occur in citrus packinghouses where these fungicides are used. Its spores would germinate on PDA containing thiabendazole, imazalil, or sodium *o*-phenylphenate at 10, 1.0, or 40 mg/L, respectively. Isolates M6R and PD0490 are controlled by typical commercial fungicide applications in California packinghouses, while resistant isolates such as D201 commonly occur and are not controlled effectively. Isolates were stored on silica gel at −20 °C. When needed, the isolates were cultured 1–2 weeks on PDA at 25 °C. In a preliminary experiment, the level of pyrimethanil sensitivity of about 100 isolates collected from many locations was determined, and the sensitivity of M6R, PD0490, and D201 were similar to each other and typical of many other isolates. Spores were harvested by adding 5 mL of sterile, de-ionized water (diH$_2$O) containing 0.05% Triton X-100 to the Petri dish. Spores were then rubbed with a sterile glass rod, and spore suspension was passed through two layers of cheesecloth. The suspension was diluted with water to an absorbance of 0.1 at 425 nm as measured with a spectrophotometer; a density that contained about 1×10^6 spores/mL (Eckert and Brown, 1986).

2.2. Spore germination assay

Sterile dishes with 24 microwells (Nunclon Surface, Nalge Nunc Int., Roskilde, Denmark) with a capacity of 4 mL per well were used. A germination medium (PDB–Tris) was prepared by placing 96 g of potato dextrose broth (PDB, Difco Laboratories, Detroit, MI) and 12.14 g of Tris buffer (hydroxymethyl aminomethane, Polysciences Inc., Warrington, PA) in 1 L of diH$_2$O and the pH of the medium adjusted to 4, 5, 6, 7, 8, or 9, with concentrated H$_2$SO$_4$, H$_3$PO$_4$, or NaOH. In each well, 0.5 mL of PDB–Tris, 0.5 mL of D201 spore suspension, pyrimethanil (PenbotectM 400 SC, 40%, w/v, Janssen Pharmaceutica, Beerse, Belgium) at various concentrations, and diH$_2$O were added to a final volume of 2.0 mL and mixed. Actual pyrimethanil concentrations were 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2, 4, and 8 mg/L. After 24 h incubation at 24 °C, germinated and ungerminated spores in each well were counted by observation (200×) using an inverted compound microscope. Within each well, 100–150 spores of *P. digitatum* isolate D201 were examined and the percentage of germinated spores was calculated. The experiment was repeated twice.

2.3. Inoculation and treatment procedures

Commercially harvested 'Eureka' lemons, Valencia, or 'Atwood' navel oranges were randomized before use in these experiments. In most experiments, the fruit were inoculated with *P. digitatum* 24 h before treatments were applied by dipping a steel rod with a 1-mm-wide and 2-mm-long tip into the spore suspension and making a single wound per fruit with the

rod (Eckert and Brown, 1986). Fruit were immersed in 15 L of each solution contained within 22-L-capacity stainless steel tanks. The temperature of the solutions was maintained at 25–55 °C (±0.5 °C) and constantly stirred with a 5-cm-diameter propeller. After treatment, all fruit were stored for 2 weeks at 20 °C and 95% RH, after which the number of decayed fruit was counted.

2.4. Influence of concentration and method of application on pyrimethanil effectiveness

In a laboratory experiment, lemon fruit were inoculated as previously described with a mixture containing equal parts of a spore suspension containing isolates M6R and D201 24 h before pyrimethanil treatments. Lemons were immersed for 30 s in aqueous pyrimethanil solutions containing 0, 100, 250, 500, or 1000 mg/L, or sprayed with finishing wax containing pyrimethanil at 1000 mg/L. The wax (carnuba pack wax C 142, Sunkist Growers, Lindsay, CA) was applied using an air-less power sprayer and the fruit dried in air on wire racks. All treatments were at room temperature (25 °C) and each was applied to 4 replicates of 27 lemons each. The experiment was repeated twice.

In a semi-commercial experiment on lemons and navel oranges, fruit inoculated with *P. digitatum* isolate M6R were either drenched by a high volume low-pressure overhead drench for 18 s containing pyrimethanil at concentrations of 0, 250, or 500 mg/L, or waxed with a finishing wax, described previously, containing pyrimethanil at a concentration of 2000 mg/L. The wax was applied at low air pressure over 10 rotating horse hair brushes 15 cm in diameter rotating at 1.33 s^{-1} and was immediately followed by passage of the fruit through a commercial hot air fruit drier for 90 s operating at 44 °C. Each treatment was applied to 3 replicates of 75 fruit each. Treated fruit were packed into commercial fiberboard cartons. A separate sample of 20 fruit that had not been inoculated of each treatment was prepared for residue analyses.

2.5. Influence of sodium bicarbonate on pyrimethanil effectiveness

Several similar experiments were conducted to determine the influence of sodium bicarbonate on pyrimethanil effectiveness. In the first experiment, lemons were inoculated with *P. digitatum* isolate D201 24 h before treatment with aqueous solutions of pyrimethanil alone at a concentration of 500 mg/L, sodium bicarbonate (NaHCO$_3$, Sigma–Aldrich, Detroit, MI) alone at 1, 2, or 3% (w/v), or a mixture of pyrimethanil at 500 mg/L and sodium bicarbonate at 1, 2, or 3%. Fruit were immersed in each solution for 60 s at 20 °C, and then stored for 2 weeks at 20 °C. In a second experiment, lemons were inoculated with *P. digitatum* isolate M6R 24 h before treatment with pyrimethanil alone at 50 mg/L, sodium bicarbonate alone at 1%, or a mixture of both. Lower concentrations of pyrimethanil and sodium bicarbonate were

used than would be done commercially so sufficient green mold incidence would be present to determine if synergy existed between the compounds. Fruit were immersed for 60 s and stored at 20 °C for 14 days. In a third experiment, lemons were inoculated with either *P. digitatum* isolate M6R or D201. Fruit were immersed in pyrimethanil alone (200 or 500 mg/L), sodium bicarbonate alone (1 or 3%, w/v), or in all combinations of these. Immersion in aqueous imazalil at 200 mg/L was included to compare with its effectiveness to pyrimethanil. The fruit were stored as described previously.

2.6. Pyrimethanil compatibility with chlorine

To determine the stability of chlorine in pyrimethanil solutions, chlorine at concentration of 200 μL/L was added to a 1-L volume water solution of pyrimethanil at 500 mg/L, or in water only (control). The solutions were stirred constantly and the free chlorine content was determined every 15 or 30 min for a period of 4 h using colorimetric test strips (free chlorine measurement range 10–200 mg/L; model 4250-BJ; LaMotte Co., Chestertown, MD). The pH (±S.D.) of both solutions was 6.5 ± 0.1 and the temperature of both was 25 °C. The experiment was repeated twice.

2.7. Influence of heat on pyrimethanil effectiveness and residues in fruit

Two similar experiments were conducted to determine the influence of heat on pyrimethanil effectiveness and its residues in fruit. In the first experiment, lemons inoculated with *P. digitatum* isolate D201 were immersed 24 h later for 60 s in an aqueous solution containing pyrimethanil at 0, 50, or 500 mg/L, at 30, 38, or 46 °C. Each treatment included 4 replicates of 27 lemons each. Fruit were stored for 2 weeks at 20 °C before green mold incidence was recorded. For residue determinations, non-inoculated fruit (three replicates of eight lemons each) were treated similarly at each temperature with pyrimethanil at 500 mg/L. In the second experiment, Valencia oranges were inoculated with *P. digitatum* isolate D201 and immersed 24 h later for 30 s in an aqueous solution of pyrimethanil containing 0, 50, 150, or 300 mg/L, at 25, 45, 50, or 55 °C. Fruit were stored at 20 °C for 2 weeks before green mold incidence was recorded. For residue determinations, non-inoculated fruit (three replicates of seven oranges each) were treated similarly at each temperature with pyrimethanil at 300 mg/L.

2.8. Influence of the time of inoculation on pyrimethanil, imazalil, and thiabendazole effectiveness

To assess the curative and protective activities of pyrimethanil, Valencia oranges were inoculated with *P. digitatum* isolate D201 at the following periods: (1) 48 h before treatments were applied; (2) 24 h before treatments were applied; (3) about 1 h before treatments were applied; (4) 24 h after treatments were applied; and (5) 48 h after treat-

ments were applied. The treatments included 60 s immersion in: (1) water alone at 25 °C; (2) pyrimethanil at 500 mg/L at 25 °C; (3) pyrimethanil at 1000 mg/L at 25 °C; (4) imazalil at 500 mg/L plus 3% sodium bicarbonate at 50 °C; or (5) thiabendazole at 500 mg/L plus 3% sodium bicarbonate at 50 °C. The last two treatments provide some control of thiabendazole (Smilanick et al., 2006) and imazalil (Smilanick et al., 2005) resistant isolates; they are the most effective decay control treatments now in commercial use in California. Each treatment was applied to 5 replicates of 20 oranges each. Oranges were stored at 20 °C for 2 weeks before green mold incidence was recorded.

2.9. Residue analysis

Imazalil residues were determined as previously described (Smilanick et al., 2005). Pyrimethanil residues were determined by first dividing each fruit into quarters. A total of six or seven of the quarters, each from different fruit from those in a replicate, were placed in a high-speed blender with 300 mL water. The quarters were blended to a puree, and 100 g of the material was placed in a 250-mL capacity flask with 15 mL of saturated phosphate buffer (pH 7) and 50 mL of ethyl acetate. The flask was shaken for 15 min on a wrist action shaker followed by centrifugation for 5 min at $3000 \times g$. The supernatant was decanted into a paper filter with about 3 g of anhydrous sodium sulfate, then 2 mL of the filtrate were placed into an injection vial and 1 μL was injected into a gas chromatograph. The injection port, column (model DB-17, fused silica column, 15 m in length, 0.53 mm inside diameter, and 1 μm film thickness, J & W Scientific, Folsom, CA), and detector (nitrogen/phosphorous) temperatures were 290, 220, and 400 °C, respectively. The retention time of pyrimethanil was about 5 min. Both imazalil and pyrimethanil residues were reported as mg/kg of the fresh weight of the fruit.

2.10. Sporulation control assays

Pyrimethanil effectiveness to control sporulation of *P. digitatum* was determined according to the method described by Eckert and Brown (1986). Lemons were deeply inoculated by injecting 0.1 mL of spore suspension containing 1×10^6 spores/mL about 1 cm deep into the fruit with a syringe and then the fungicides were applied. Infections within the fruit inoculated this way are not stopped by the fungicides, so the effect of their residues on the production of spores from decay lesions can be observed. Sporulation was classified by index of 0–5, where 0 = a water-soaked, soft lesion present but no sporulation or mycelium, 0.5 = surface mycelium present but no sporulation present, 1 = less than 5%, 2 = 6–30%, 3 = 31–60%, 4 = 61–90%, and 5 = more than 90% of the surface of the fruit covered with spores.

In the first experiment to determine the influence of pyrimethanil residue levels on control of sporulation, pyrimethanil was applied by passage of the lemons through a drencher containing aqueous solution of the fungicide at

500 mg/L at 25, 40.6, or 49 °C. The fruit were inoculated before treatment with isolate M6R of *P. digitatum*. Four replicates of eight fruit each were not inoculated and used for residue determinations. Sporulation observations were recorded from 4 replicates of 10 lemons each after 9-day storage at 20 °C. In a second experiment, imazalil was included to compare its effectiveness to pyrimethanil. The experiment was repeated twice, once with imazalil-sensitive *P. digitatum* isolate M6R and once with imazalil-resistant *P. digitatum* isolate D201. The fungicides were applied by immersion of the lemons for 60 s in an aqueous fungicide solution or the fungicides in a storage wax (Britex 505, Brogdex Co., Pomona, CA) were sprayed on the fruit with an air-less power sprayer. Aqueous fungicide treatments included pyrimethanil at concentrations of 1000 or 2000 mg/L, imazalil at 1000 mg/L, or a combination of both fungicides at 1000 mg/L each. Fungicide in wax treatments included pyrimethanil alone at 1000 mg/L or combined with 1000 mg/L of imazalil. Sporulation observations were recorded from 2 replicates of 20 fruit each after 7 days storage at 20 °C. In a third experiment, pyrimethanil and imazalil were applied to lemons in an aqueous suspension, finishing wax, or a sequence where the aqueous suspension was applied first followed by the wax suspension. All of the solutions were at ambient temperature of about 20 °C. This experiment was conducted using commercial packinghouse equipment and simulates the typical methods of application commonly used in California packinghouses. *P. digitatum* isolate PD0490 used to inoculate the fruit was sensitive to both fungicides. The fungicides were applied: (1) by drenching the fruit for 15 s with re-circulated aqueous suspensions heated to 46 °C containing 500 mg/L pyrimethanil or 350 mg/L imazalil; (2) by spraying the fruit over rotating brushes with a finishing wax (Natural Shine 960, Pace International, Seattle, WA) containing 2000 mg/L pyrimethanil or imazalil; and (3) by drenching the fruit for 15 s with the aqueous suspensions of the fungicides followed by finishing wax application. After these treatments, the fruit passed through a drier operating at 49 °C for 2 min. Control fruit were untreated and not passed through the drier. Residues of pyrimethanil and imazalil were determined using two replicates of eight lemons each. Sporulation observations were recorded repeatedly from 4 replicates of 10 lemons each during storage for 25 days at 10 °C.

2.11. Statistical analysis

The incidence of green mold was analyzed by an analysis of variance applied to the arcsine of the square root of the proportion of infected fruit, followed by Fisher's protected least significant difference test ($P \leq 0.05$) to separate means (General Linear Modeling package, Super ANOVA Abacus Concepts, Berkeley, CA). Actual values are shown. Limpel's formula, as described by Richter (1987), was used to determine synergy among sodium bicarbonate and pyrimethanil treatments. Limpel's formula is $E_e = X + Y - (XY/100)$, in which E_e is the expected effect from additive responses of two
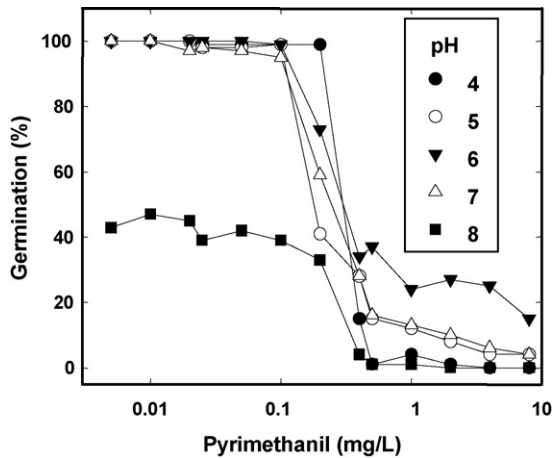
Fig. 1. Germination of spores of *Penicillium digitatum* isolate D201 after incubation for 24 h at 24 °C in solutions of buffered potato dextrose broth containing pyrimethanil at pH 4, 5, 6, 7, or 8. No spores germinated at pH 9.

treatments and *X* and *Y* are the percentages of decay reduction relative to each agent used alone. Thus, if the combination of the two agents produces any value of decay reduction greater than E_e, then synergism exists. Regressions were calculated using SPSS 14.0 (SPSS Inc. Chicago, IL). Non-linear regression was fitted to depict the relationship between pyrimethanil solution temperature and residues in lemon and orange fruit. Linear regression was used to depict the relationships between time and chlorine content in pyrimethanil solutions and to describe pyrimethanil residue content in fruit and sporulation.

3. Results

3.1. Spore germination assay

Germination of spores of *P. digitatum* isolate D201 was more than 99% in PDB–Tris from pH 4 to 7 (Fig. 1). At pH 8, germination was reduced to 50% and at pH 9 it was completely inhibited. The EC_{50} pyrimethanil concentration (that prevented 50% of spores from germinating) was between 0.2 and 0.4 mg/L. Pyrimethanil at 0.5 mg/L or higher caused short and deformed germ tubes among those spores that did germinate, particularly at pH 6. Incubation for an additional 24 h did not change these results. The pH of the solutions did not change during incubation.

3.2. Influence of concentration and method of application on pyrimethanil effectiveness

Green mold was effectively controlled on lemons and oranges after treatment with aqueous solutions of pyrimethanil at 500 mg/L or higher applied by immersing the fruit for 30 s or drenching the fruit (Fig. 2). In wax, although it was applied at very high rates, it was much less effective.
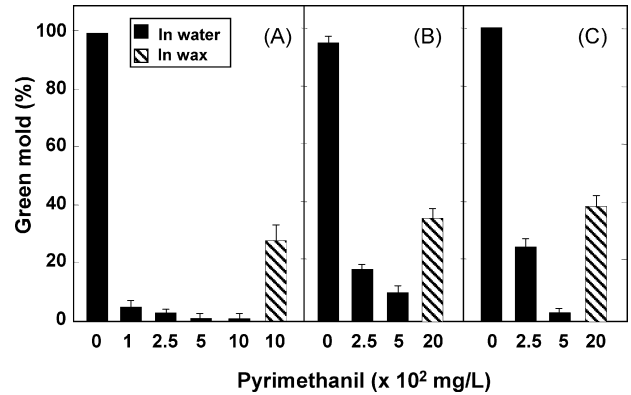

Fig. 2. Comparison of pyrimethanil effectiveness when applied in aqueous or wax suspensions to fruit inoculated 24 h before treatment. Aqueous suspensions of the fungicide were applied by immersing lemons (A) for 30 s or drenching lemons (B) or oranges (C) for 18 s with the fungicide. The fungicide was dissolved in water (lemons; A) or finishing waxes (lemons and oranges; B and C, respectively) and sprayed on the fruit over rotating brushes. In (A), the inoculum was a mixture of *Penicillium digitatum* imazalil-resistant isolate D201 and imazalil-sensitive isolate M6R. In (B and C), the inoculum was *P. digitatum* isolate M6R. All treatments were followed by storage for 14 days at 20 °C. Within each panel, unlike letters indicate significant difference according to Fisher's Protected LSD test ($P = 0.05$).

When applied in wax over rotating brushes, a concentration of 1000 or 2000 mg/L reduced the green mold by about 65%.

3.3. Influence of sodium bicarbonate on pyrimethanil effectiveness

Superior control of green mold after inoculation with either *P. digitatum* isolate was observed when mixtures of sodium bicarbonate and pyrimethanil were used (Figs. 3 and 4). In most of the treatments where this mix-
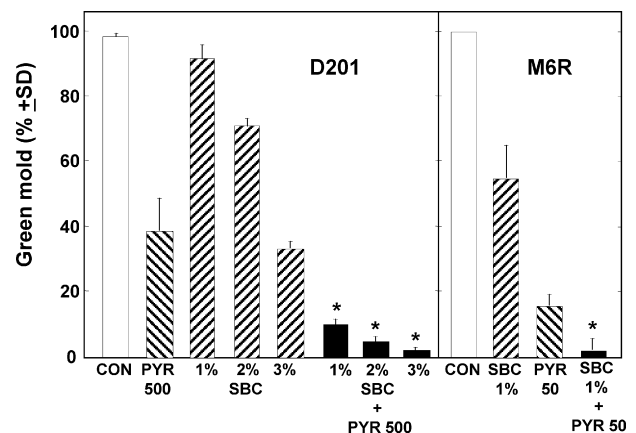

Fig. 3. Effectiveness of pyrimethanil (PYR) and sodium bicarbonate (SBC) to control green mold after inoculation of lemons with *Penicillium digitatum* imazalil-resistant isolate D201 or imazalil-sensitive isolate M6R. The fruit were immersed for 60 s in the solutions at 20 °C and stored for 14 days at 20 °C. Concentrations of PYR and SBC are in mg/L or % (w/v), respectively. Within each panel, unlike letters indicate significant difference according to Fisher's Protected LSD test ($P = 0.05$). Asterisk (*) indicates the combination was synergistic according to Limpel's formula.
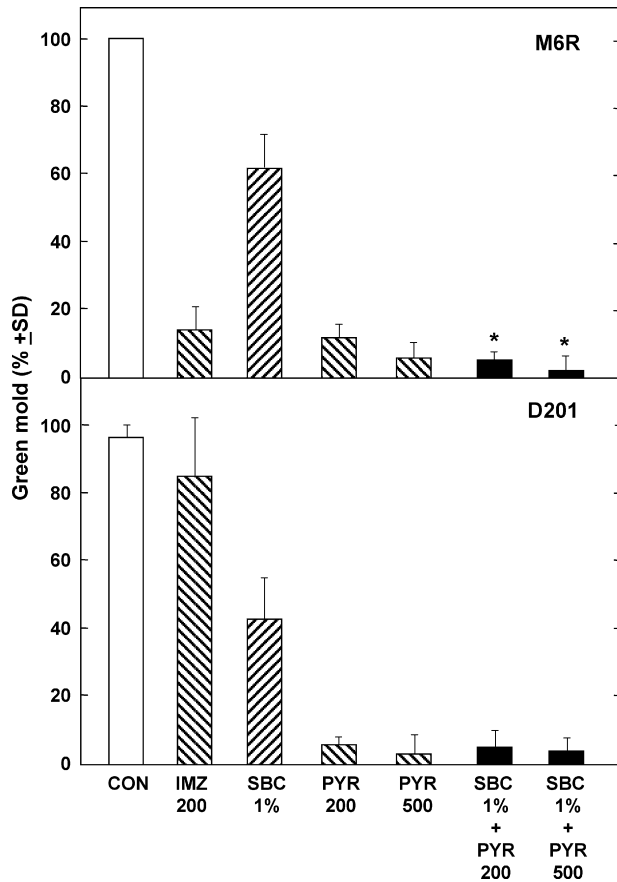
Fig. 4. Effectiveness of pyrimethanil (PYR), imazalil (IMZ) and sodium bicarbonate (SBC) to control green mold after inoculation of lemons with *Penicillium digitatum* imazalil (IMZ)-resistant isolate D201 or IMZ-sensitive isolate M6R. Lemons were immersed for 60 s in treatment solutions at 20 °C and stored for 14 days at 20 °C. Concentrations of IMZ and PYR are in mg/L, while SBC is expressed as % (w/v). Within each panel, unlike letters indicate significant difference according to Fisher's Protected LSD test (*P* = 0.05). Asterisk (*) indicates the combination was synergistic, according to Limpel's formula.

ture was used, true synergy in effectiveness occurred as determined by Limpel's formula. Control of *P. digitatum* isolate M6R by imazalil was good, while that of isolate D201 was poor (Fig. 4). Pyrimethanil controlled both isolates effectively.

3.4. Pyrimethanil compatibility with chlorine

The chlorine content in the pyrimethanil solutions declined from the initial concentration of 200 mg/L to less than 10 mg/L after 3 h (Fig. 5). A regression that depicts chlorine content was determined using measurements taken from 90 to 200 min after the chlorine was added because the decline in chlorine content began after about 90 min and continued to 200 min, when none could be detected. The chlorine content in water was unchanged over the 4 h measurement period.
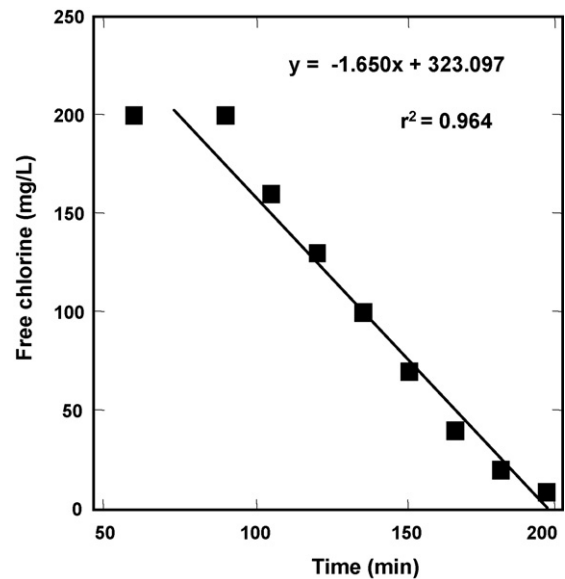

Fig. 5. Concentration of free chlorine in a 500 mg/L pyrimethanil solution during 200 min observation.

3.5. Influence of heat on pyrimethanil effectiveness and residues in fruit

An increase in the temperature of the pyrimethanil solution significantly improved its effectiveness to control green mold on lemons (*P* = 0.0127) and oranges (*P* = 0.0225), but the improvement was often irregular and small (Fig. 6). Conversely, pyrimethanil residues in both fruit were greatly increased as the solution temperature increased; they approximately doubled for each 5 °C increase in solution temperature above 30 °C (Fig. 7).
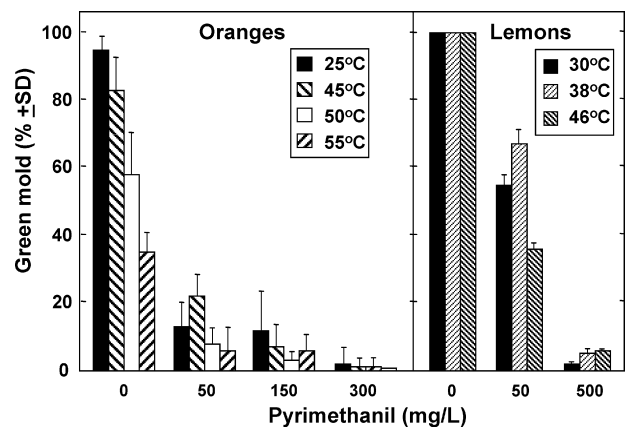

Fig. 6. Influence of pyrimethanil (PYR) solution temperature on its effectiveness to control citrus green mold. Valencia oranges or Eureka lemons were inoculated with *Penicillium digitatum* imazalil-resistant isolate D201. After 24 h, the oranges were immersed for 30 s and the lemons for 60 s in PYR solutions at different temperatures. Fruit were stored for 14 days at 20 °C. Within each panel, unlike letters indicate significant difference according to Fisher's Protected LSD test (*P* = 0.05).
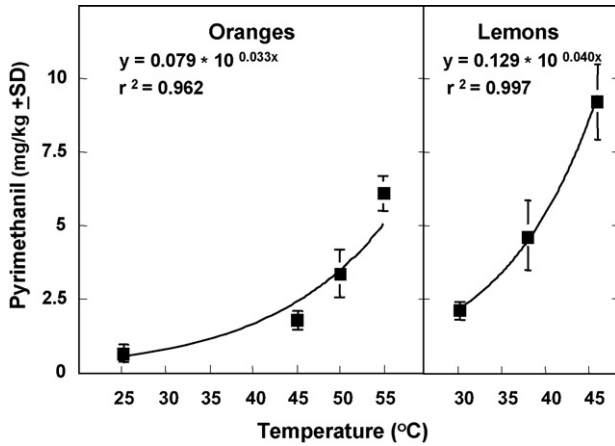
Fig. 7. Influence of pyrimethanil (PYR) solution temperature on its residues in oranges and lemons, expressed as mg/kg of the fresh weight of the fruit. Oranges were immersed for 30 s in 300 mg/L PYR and lemons for 60 s in 500 mg/L PYR solutions at various temperatures.

3.6. Influence of the time of inoculation on pyrimethanil, imazalil, and thiabendazole effectiveness

Pyrimethanil curative activity was high; it was the most effective treatment applied 24 h after inoculation ($P = 0.05$), and completely controlled green mold when applied about 1 h after inoculation (Fig. 8). Its effectiveness was very similar at concentrations of 500 or 1000 mg/L. Pyrimethanil protective activity was poor; infections from inoculations made 24 or 48 h after pyrimethanil treatment were not
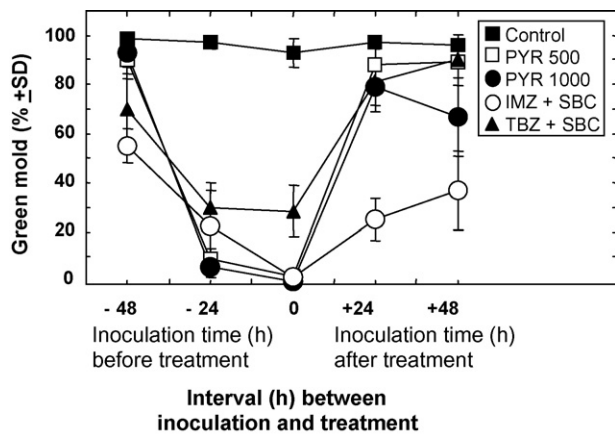

Fig. 8. Protectant and eradicant activity of several postharvest fungicide treatments to control citrus green mold on Valencia oranges. The interval between inoculation with *Penicillium digitatum* isolate D201 and treatment was varied; from inoculation 48 h before treatment (−48) to inoculation 48 h after treatment (+48). *P. digitatum* isolate D201 was resistant to both imazalil (IMZ) and thiabendazole (TBZ). Oranges were immersed for 60 s in: water alone (control) at 25 °C; 500 mg/L pyrimethanil (PYR) at 25 °C; 1000 mg/L pyrimethanil at 25 °C; 500 mg/L imazalil plus 3% sodium bicarbonate (SBC) at 50 °C; or 500 mg/L thiabendazole plus 3% sodium bicarbonate at 50 °C. Fruit were stored for 14 days at 20 °C.
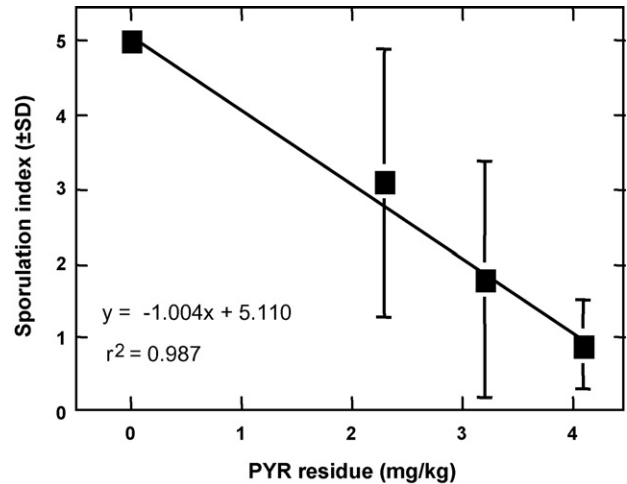

Fig. 9. Influence of pyrimethanil (PYR) residues in lemons, expressed as µg/g fresh weight, on sporulation from lesions. PYR was applied by passage of the lemons through a drencher containing aqueous solution of the fungicide at 500 mg/L at 25, 40.6, and 49 °C. The fruit were inoculated before treatment with isolate M6R of *Penicillium digitatum*. Four replicates of eight fruit each were not inoculated and used for residue determinations. Sporulation observations were recorded from 4 replicates of 10 lemons each after 9-day storage at 20 °C. A value of 5 indicates the lesions were 100% covered with spores, while a value of 0 indicates no spores were produced from decay lesions.

controlled. Even though the *P. digitatum* isolate used in this experiment was resistant to imazalil and thiabendazole, effective control was obtained when the fungicides were combined with sodium bicarbonate and heat. The combination treatment of imazalil and sodium bicarbonate applied at 50 °C had both effective curative and protective activity, although it was inferior to pyrimethanil for the control of green mold on fruit inoculated 24 h before treatment. The combination treatment of thiabendazole and sodium bicarbonate applied at 50 °C was partially effective when applied 24 h after or about the same time as inoculation.

3.7. Sporulation control assays

Increasing pyrimethanil residues were positively correlated with improved control of sporulation from lesions caused by *P. digitatum* on lemons (Fig. 9). The best control of sporulation was obtained with fruit residues of 4.1 µg/g fresh weight. Sporulation from lesions caused by imazalil-sensitive isolate M6R of *P. digitatum* was stopped by a mixture of imazalil and pyrimethanil in wax, while sporulation was reduced but not stopped by aqueous formulations of pyrimethanil and imazalil mixture or pyrimethanil alone in wax (Fig. 10). Control of sporulation of imazalil-resistant *P. digitatum* isolate D201 by either fungicide was poor; it was not reduced by treatment with either pyrimethanil or imazalil at 1000 mg/L, only slightly inhibited by pyrimethanil at higher rates or when pyrimethanil
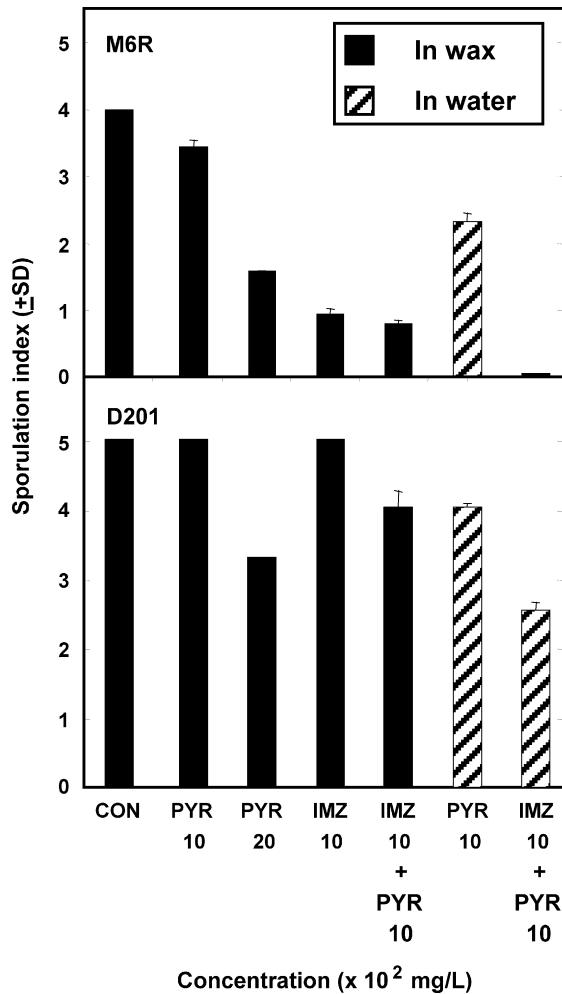
Fig. 10. Control of sporulation from lesions caused by *Penicillium digitatum* on lemons treated with water or wax formulations containing imazalil (IMZ) or pyrimethanil (PYR). The fungicides were applied by immersion of the fruit for 60 s in aqueous fungicide solutions or the fungicides in a storage wax were sprayed on the fruit with an air-less power sprayer. They were inoculated with *P. digitatum* imazalil-resistant isolate D201 or imazalil-sensitive isolate M6R. The fruit were stored for 1 week at 20 °C, and then the level of sporulation was recorded. A value of 5 indicates the lesions were 100% covered with spores, while a value of 0 indicates no spores were produced from decay lesions.
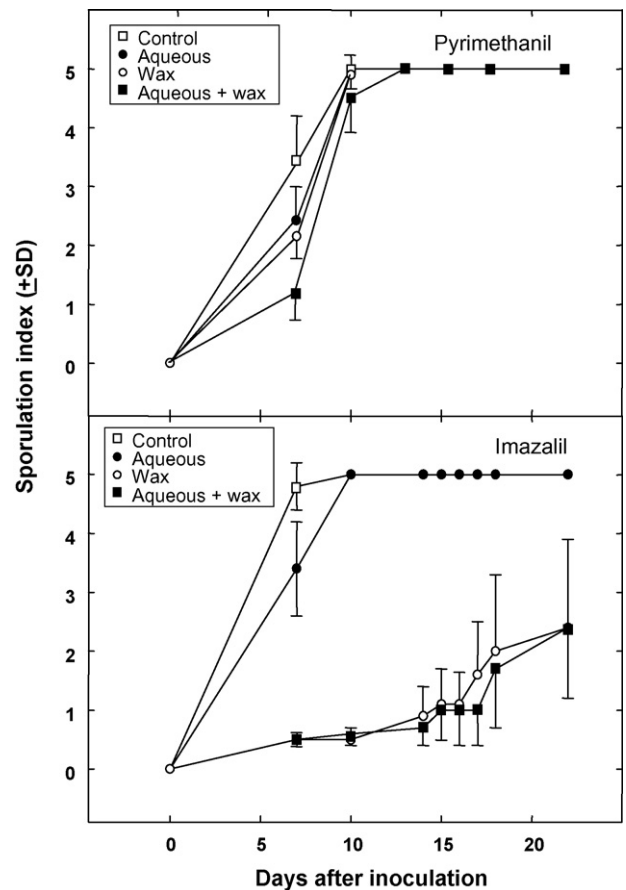

Fig. 11. Control of sporulation from lesions caused by *Penicillium digitatum* on lemons by water or wax formulations containing imazalil (IMZ) or pyrimethanil (PYR). *P. digitatum* isolate PD0490 used to inoculate the fruit was sensitive to both fungicides. The fungicides were applied: (1) by drenching the fruit for 15 s with aqueous suspensions at 46 °C containing 500 mg/L PYR or 350 mg/L IMZ; (2) by spraying the fruit with a finishing wax containing 2000 mg/L PYR or IMZ; and (3) by drenching the fruit for 15 s with the heated aqueous suspensions followed by finishing wax application. Residues of PYR after the aqueous, wax, and aqueous plus wax treatment were 0.4, 1.2, and 1.7 μg/g fresh weight, respectively. Residues of IMZ after the aqueous, wax, and aqueous plus wax treatment were 0.3, 1.5, and 1.5 μg/g fresh weight, respectively. Observations of sporulation were made repeatedly during storage at 10 °C. A value of 5 indicates the lesions were 100% covered with spores, while a value of 0 indicates no spores were produced from decay lesion.

4. Discussion

Pyrimethanil inhibited the germination of *P. digitatum* spores in vitro at 0.2–0.4 mg/L and this concentration was not influenced by pH. This differs from imazalil, another citrus postharvest fungicide in common use, where pH greatly influenced its toxicity (Holmes and Eckert, 1999; Smilanick et al., 2005). Toxicity that does not change with pH is an advantage because the pH of water or waxes in which pyrimethanil is used in packinghouses would not require adjustment.

Pyrimethanil was more effective in aqueous suspensions than when it was suspended in fruit waxes. In general, waxes reduce the effectiveness of citrus fungicides (Eckert and Eaks,

was combined with imazalil, and results with wax formulations were only slightly superior. In the third experiment to evaluate control of sporulation, where commercial packingline equipment was used, residues of imazalil after the aqueous, wax, and aqueous plus wax treatment were 0.3, 1.5, and 1.5 μg/g fresh weight, respectively, and those of pyrimethanil were 0.4, 1.2, and 1.7 μg/g fresh weight, respectively. At similar levels of residue, inhibition of sporulation by pyrimethanil was inferior to that of imazalil. Inhibition of sporulation by imazalil was persistent, while that of pyrimethanil was not. Aqueous formulations of both fungicides left much lower residues and were inferior to wax formulations for the control of sporulation (Fig. 11).

1989; Eckert et al., 1994; Smilanick et al., 1997). To compensate for the reduced effectiveness when combined with waxes, fungicides are applied in aqueous suspensions before waxing or used at much higher rates in waxes (Eckert and Eaks, 1989).

The EC_{50} values we observed for *P. digitatum* were similar to those reported by Sholberg et al. (2005) for *P. expansum*. EC_{50} concentrations of pyrimethanil that inhibited growth of *B. cinerea* and *P. expansum* were 0.039 and 0.331 mg/L, respectively, in potato dextrose agar (Sholberg et al., 2005). Pyrimethanil has been shown to effectively control decay on other fruit when applied either before or after harvest. Its application before harvest to apples controlled postharvest decay caused by *B. cinerea* or *P. expansum* (Bedford et al., 2002; Sholberg et al., 2005). Postharvest pyrimethanil applications controlled both pathogens on apples with rates as low as 250 mg/L, which was similar to the rates we found to control green mold on citrus fruit. This low rate controlled a thiabendazole-resistant isolate of *P. expansum* indicating that thiabendazole resistance did not confer cross-resistance to pyrimethanil (Sholberg et al., 2005). Similarly in our work, an isolate of *P. digitatum* resistant to three fungicides, each of different mode of action classes (sodium *o*-phenylphenate, imazalil, and thiabendazole), was effectively controlled by pyrimethanil.

Sanitation of fungicide solutions used in packinghouse tanks or drenches, where the solution is recycled or used in tanks for long periods, is needed to prevent the accumulation of inoculum and nuisance microbes. Chlorine is commonly used to sanitize thiabendazole solutions in packinghouses, while imazalil reacts with chlorine so heat pasteurization or filtration is used for its sanitation. Because chlorine did not persist in pyrimethanil solutions, some other method of sanitation for this fungicide is needed, such as heat pasteurization, filtration, or other chemical sanitizers. Heat sanitation of pyrimethanil is feasible as it is now used to sanitize imazalil solutions. We used pyrimethanil repeatedly at 55 °C in the present work and it was stable in solutions heated up to 60 °C for long periods (data not shown). *P. digitatum* spores did not survive for 30 s in water at 60 °C (Smilanick et al., 2003).

The addition of sodium bicarbonate further improved pyrimethanil performance and the combination was significantly synergistic. This measure might be particularly useful if less sensitive isolates of *P. digitatum* occur in packinghouses, because as we previously reported, improved control of imazalil and thiabendazole resistant isolates occurred when these fungicides were combined with sodium bicarbonate (Smilanick et al., 2005, 2006). We found heating the pyrimethanil solution greatly increased fruit residues, however, the effectiveness of the fungicide was not proportionately increased. The benefits of heating the solution are: (1) a modest improvement in performance; and (2) continuous sanitation of the solution of inoculum of *P. digitatum* and other pathogens, if the temperature used exceeds 40 °C (Smilanick et al., 2003). The disadvantages of heating the solution are: (1) the accelerated depletion of pyrimethanil

from the solution caused by its increased incorporation into the fruit; (2) the energy needed to heat the solution; (3) the risk that heat may increase residues above regulatory tolerances; and (4) the risk of heat injury to the fruit. Residues on lemons after some of the heated pyrimethanil treatments in our experiments approached the US pyrimethanil tolerance for citrus fruit of 10 µg/g (Anon, 2004). Residues of two other citrus postharvest fungicides, imazalil and thiabendazole, also increased when used at higher temperatures, however, heat profoundly improves their performance even when their residues are not increased (Cabras et al., 1999; Smilanick et al., 1997, 2006).

Pyrimethanil residues in lemons or oranges following immersion in solutions containing 300–500 mg/L of pyrimethanil were typically 1–2 µg/g and higher residues were unnecessary. A residue of at least 1 µg/g is the minimum recommended to control postharvest decay on pomes (Bylemans and Goodwine, 2005). We also found pyrimethanil had effective curative activity, but poor protective activity against citrus green mold, even when used at 1000 mg/L where the residues would have been comparatively high. We did not examine residue persistence in our work. The long-term persistence of postharvest residues of pyrimethanil has not been reported. Angioni et al. (2004) determined the persistence of pyrimethanil in strawberry fruit after field treatment. They found that, from an initial content of 2.98 µg/g, the residues declined with a half-life of 4.8 days, and that washing the fruit with tap water did not affect pyrimethanil residues. The persistence of pyrimethanil residues is less important for the control of green mold than those of imazalil or thiabendazole. Residues of imazalil or thiabendazole control sporulation and protect the fruit from subsequent infections that occur after treatment, while those of pyrimethanil, like those of sodium *o*-phenylphenate, do not and were primarily curative in nature.

Pyrimethanil is a useful fungicide for the practical management of citrus postharvest green mold. It is of a different mode of action class than the fungicides now in use for this purpose so it is of particular value to control isolates of *P. digitatum* resistant to imazalil, thiabendazole, and sodium *o*-phenylphenate that are commonly used in packinghouses. It effectively controlled an isolate of *P. digitatum* resistant to these fungicides. It was more effective than imazalil and thiabendazole, even when these materials were used with sodium bicarbonate and heat to maximize their effectiveness against resistant isolates. Recently, we measured the pyrimethanil sensitivity of a collection of more than 100 isolates of *P. digitatum* collected from 12 packinghouses in California and all were sensitive to pyrimethanil regardless of their sensitivity to other fungicides. However, Kanetis (2005) selected *P. digitatum* isolates after repeated exposures to pyrimethanil that were less sensitive to the fungicide and poorly controlled by recommended rates of the fungicide. Thus, the introduction of pyrimethanil into packinghouses should be done with strategies to minimize the development of resistant isolates, such as its use in mixtures or in alterna-

tions with other fungicides, good packinghouse design and sanitation practices (Goodwine, 2005), to prolong the effective life of the fungicide.

There are several reasons to use pyrimethanil and imazalil together, and a commercial formulation of the two has been prepared. Sporulation control from decay lesions by pyrimethanil requires relatively high residue levels that would be difficult to obtain with typical industry equipment and practices today, while excellent sporulation control with relatively low residue levels was obtained with imazalil when it was used in wax. Although pyrimethanil has not been widely used in citrus packinghouses at this time and little is known about the characteristics of *P. digitatum* isolates that may become resistant to this fungicide, imazalil resistance in this pathogen has been well characterized. The proportion of imazalil resistant spores produced from successive cycles of decayed fruit produced by infections caused from mixtures of sensitive and resistant spores declines rapidly after several cycles (Holmes and Eckert, 1995). Therefore, it is likely that isolates resistant to imazalil and pyrimethanil would also have this characteristic and might be easier to manage than those resistant to pyrimethanil alone.

Acknowledgements

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