Reduction of Imazalil Effectiveness Against Citrus Green Mold in California Packinghouses by Resistant Biotypes of *Penicillium digitatum*

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ABSTRACT

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Oranges and lemons inoculated with imazalil-sensitive and imazalil-resistant isolates of *Penicillium digitatum* were dipped in imazalil suspensions in the laboratory or sprayed with water or wax formulations in commercial packinghouses. Dip treatments (1–2 g a.i./L) significantly reduced infection of lemons by resistant biotypes but did not control their sporulation on diseased fruit. Control of infection by resistant biotypes was inferior to that by sensitive biotypes. Commercial packinghouse treatments at 1–4 g a.i./L (maximum registered dosage) gave poor control of infection by resistant isolates and had no effect on their sporulation on fruit. When biotypes with resistance to both imazalil and thiabendazole were inoculated into lemons, infection was poorly controlled by dip treatment with either or both fungicides.

Imazalil has been used successfully to control postharvest diseases of citrus fruits worldwide since the mid-1970s and was introduced for this use in the United States in 1981. The principal target organisms of imazalil are *Penicillium digitatum* (Pers.:Fr.) Sacc. and *P. italicum* Wehmer (incitants of green and blue mold, respectively), especially biotypes of these pathogens that are resistant to the benzimidazole fungicides (benzimidazole-R). Benzimidazole-R biotypes were responsible for large postharvest decay losses in the 1970s (2,14,16,19).

Despite several reports that imazalil applied in an aqueous spray is more effective, California packinghouses usually apply imazalil in a water-based wax formulation (3,5,6). Imazalil can eradicate 24-hr infections of wild-type sensitive isolates of *P. digitatum* and *P. italicum* or suppress sporulation of these fungi on decaying fruits (5). The U.S. registration permits a maximum of 4,000 ppm (wt/vol) of imazalil in a wax formulation to suppress sporulation of *Penicillium* spp. on fruit, but because of cost, the fungicide is not commonly used above 2,000 ppm. The residue tolerance for imazalil is 10 ppm (mg/kg whole fruit) in the United States and 5 ppm in most other countries.

Imazalil, like other azole fungicides (triazole and imidazole), has been considered a low- to medium-risk fungicide with respect to the development of pathogen resistance (20,23). Nevertheless, imazalil-resistant (imazalil-R) isolates of *P. digitatum* were reported in California packinghouses in 1987 (13), following 5 yr of intensive imazalil use. The existence of these resistant isolates in California has been confirmed (9) and similar resistant biotypes have been isolated in Rotterdam from citrus fruits produced in Spain and Israel (7,8).

Imazalil-R isolates of *P. digitatum* have a rather low order of resistance compared with benzimidazole-R isolates (7,8,18). Experience with similar azole-resistant pathogens has indicated that they do not always cause a loss in disease control by these fungicides (20). Therefore, reports of pathogen resistance to these fungicides should include, when possible, a statement of their potential impact on disease control (10). This investigation was undertaken to evaluate the potential of imazalil to control infection by resistant biotypes of *P. digitatum* and the performance of commercial imazalil treatments against green mold initiated by imazalil-R biotypes.

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MATERIALS AND METHODS

Isolates. Imazalil-sensitive (imazalil-S) and imazalil-R biotypes of *P. digitatum* were isolated in California citrus packinghouses and from fallen fruit in groves. The isolates were single-spored and stored on silica gel (15). Conidial inoculum was produced on potato-dextrose agar (PDA) slants. Imazalil-S biotypes M6R and CCH did not form colonies on PDA containing 0.1 mg/L of imazalil. Imazalil-R biotypes S2, P3, F1, J179, and J229 formed colonies on PDA containing 1.0 mg/L of imazalil. In addition, imazalil-S isolates did not sporulate on lemons dipped in an aqueous suspension of imazalil (2 g/L); imazalil-R isolates sporulated on these treated lemons to the same degree as on untreated fruit.

Laboratory dip tests. *Infection study.* Although imazalil dip treatments are not commercially feasible in California citrus packinghouses, we used highly efficient dip treatments to evaluate the potential of imazalil to control resistant isolates of *P. digitatum*. Lemons (*Citrus limon* (L.) N.L. Burm. 'Eureka') were obtained from commercial packinghouses, washed in detergent solution, and surface-sterilized by dipping in 70% ethyl alcohol for 1 min. To determine the effect of imazalil upon the incidence of infection, fruit were puncture-inoculated with a 1 × 3 mm steel tool dipped in a suspension of *P. digitatum* spores (1×10^6/ml). An equal number of fruit were inoculated with each isolate. Imazalil-S isolates (M6R and CCH), imazalil-R isolates (F1 and P3), and isolates resistant to both imazalil and thiabendazole (J179 and J229) were used in different tests. The inoculated fruit were incubated at 20 C for 18–20 hr, and fruit with each *Penicillium* isolate were randomized into sublots of 30–35 fruit each. Three sublots were dipped for 15–30 sec in a suspension of imazalil (0.5–2 g a.i./L) or a mixture of imazalil and thiabendazole (1 g a.i./L each). Imazalil (Deccozil EC-289, 22.2% a.i) was obtained from Decco Atochem

Inc., Monrovia, California, and thiabendazole (technical, 98.5%) was obtained from Merck & Co., Rahway, New Jersey. The fruit were stored at 20 C for 2 wk or 13 C for 2–3 wk, depending upon the fruit condition, before evaluation of disease incidence.

Sporulation study. To evaluate the effect of imazalil upon *P. digitatum* sporulation on diseased fruit, 12–18 noninoculated fruit in each test were dipped in an imazalil suspension or a mixture of imazalil and thiabendazole and laid out in a single layer on a rack in a steel tray (Fig. 1). Fruit treated with imazalil in a commercial packinghouse were often used for comparison. When dry, each fruit was injected on its equator with 0.2 ml of a conidial suspension of one isolate delivered into the albedo with a hypodermic syringe. These fruit were stored at 13 C for 2–3 wk, and sporulation was evaluated on a scale of 0 = nil to 5 = normal heavy sporulation on untreated fruit (Fig. 1). The procedures followed in these tests have been described in detail (15).

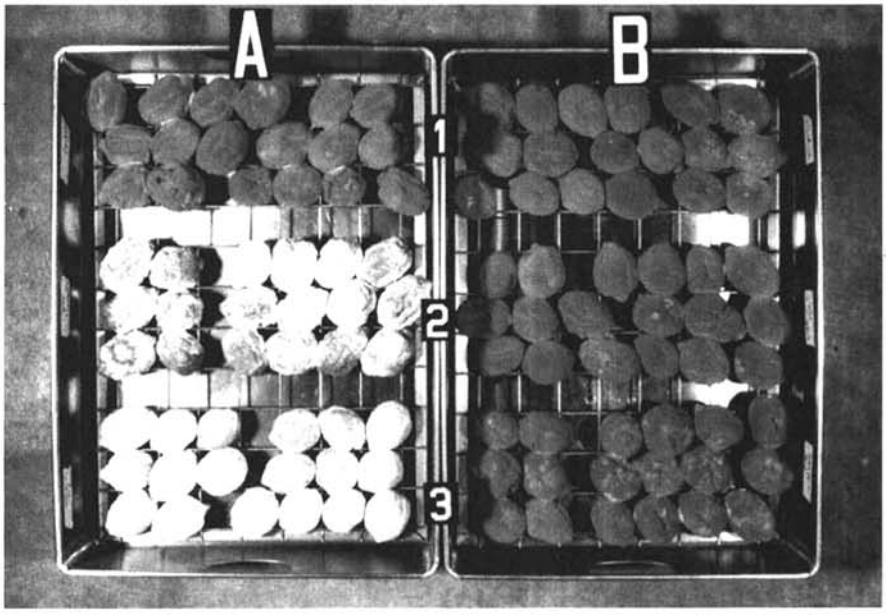

Fig. 1. Effect of imazalil treatments on sporulation of **(A)** sensitive and **(B)** resistant isolates of *Penicillium digitatum* on lemons. The fruit were: 1) dipped in water-wax only, 2) run through a commercial brush waxer applying 2 g/L of imazalil in the water-wax formulation, or 3) dipped for 30 sec in 1 g of imazalil per liter of water. After drying, the fruit were injected with 0.2 ml of 10^6 spores per liter of (A) imazalil-sensitive isolate M6R or (B) imazalil-resistant isolate F1 and stored 3 wk at 13 C.

Table 1. Effect of commercial imazalil (IMZ) treatments on decay of citrus fruits inoculated with sensitive (S) and resistant (R) isolates of *Penicillium digitatum*

Packinghouse test	Fruit	Inoculum: Isolate	Inoculum: IMZ sensitivity	Fruit treatment (g/L IMZ)	IMZ residue (mg/kg fruit)	Percent decay[v]	*Penicillium* sporulation[v,w]
1	Orange[x]	M6R	S	0.0	0.05	85.0 b	4.7 a
		S2	R	0.0	0.05	98.3 a	4.8 a
		M6R	S	1.0	0.33	38.3 d	3.2 b
		S2	R	1.0	0.33	85.0 b	4.8 a
		M6R	S	2.0	1.15	55.3 c	1.8 c
		S2	R	2.0	1.15	83.3 b	4.7 a
2	Lemon[y]	M6R	S	0.0	0.09	64.4 b	4.5 a
		F1	R	0.0	0.09	98.7 a	4.6 a
		M6R	S	2.0	1.03	27.6 c	0.8 b
		F1	R	2.0	1.03	98.7 a	4.4 a
3	Lemon[z]	M6R	S	0.0	...	100.0 a	5.0 a
		P3	R	0.0	...	100.0 a	5.0 a
		M6R	S	2.0	1.63	58.2 c	0.3 c
		P3	R	2.0	1.63	80.1 b	3.5 b
		M6R	S	4.0	4.05	58.3 c	0.3 c
		P3	R	4.0	4.05	81.9 b	3.8 b

[v] Within each packinghouse, values in this column followed by the same letter are not significantly different at $P \geq 0.01$ according to the Waller-Duncan k-ratio test with $k = 100$.

[w] Sporulation was evaluated on 12 fruit on a scale of 0 = nil to 5 = normal heavy sporulation on nontreated fruit.

[x] Three treatments were applied to fruit on a commercial packingline; 1g/L of imazalil was applied in a water spray and 2 g/L was applied in a resin solution water-wax formulation. Percent decay is the mean of seven replications of 38 fruit each and was evaluated after 3 wk at 13 C.

[y] Imazalil was applied in a water-emulsion wax. Percent decay is the mean of three replications of 90 fruit each and was evaluated after 3 wk at 13 C.

[z] Imazalil treatments were applied in a resin solution water-wax formulation on a commercial packingline; a wax-only control treatment was applied as a fruit dip. Percent decay is the mean of three replications of 80 fruit each and was evaluated after 2 wk at 13 C.

Commercial packinghouse tests. After 18 hr of incubation, lemons or oranges (*C. sinensis* (L.) Osbeck 'Valencia'), puncture-inoculated with an imazalil-S or imazalil-R isolate or not inoculated, were taken to a local packinghouse and placed on the line with a commercial fruit lot. Imazalil was incorporated into a resin solution water-wax (packout wax) in tests 1 and 3 and to a water emulsion wax (storage wax) in test 2 (Table 1). The wax formulations are proprietary, but their general composition has been described (17). The application of the imazalil treatment was supervised by a representative of the fungicide/wax service company. Isolates used in each test and the imazalil rates are given in Table 1. The treated fruit were returned to the laboratory and evaluated for decay and pathogen sporulation by methods described above. Decay incidence in tests 1, 2, and 3 was evaluated on seven replications of 38 fruit each, three replications of 90 fruit each, and three replications of 80 fruit each, respectively. Sporulation was evaluated on 12 fruit in each treatment in all three tests.

Residue analysis. Twelve noninoculated fruit from each treatment were pooled and analyzed for imazalil by a cooperating service company. Fruit in the laboratory dip test were analyzed by Sunkist Inc. using high-pressure liquid chromatography and a UV detector (21). Fruit in the commercial packinghouse test were analyzed by Brogdex Co., FMC Corp., or Decco Atochem Inc. by gas chromatography using a capillary column and an electron capture detector (22).

RESULTS

Effect of imazalil dip concentration on control of imazalil-S and imazalil-R biotypes of *P. digitatum*. The infection of lemons by imazalil-S isolates M6R and CCH was controlled well by dipping fruit for 30 sec in 0.25 g/L of the fungicide, although 0.5 g/L was required for control of pathogen sporulation on decaying fruit (Fig. 2). In contrast, imazalil-R isolates F1 and P3 were not controlled by 1 g/L; 2 g/L gave 88 and 82% reduction, respectively, in decay by these isolates. None of the imazalil dip

treatments reduced sporulation of imazalil-R isolates F1 or P3 (Fig. 1). Imazalil residues on lemons treated with 0.25, 0.5, 1.0, and 2.0 g/L of imazalil were 1.4, 2.3, 3.0, and 3.4 mg/kg of fruit, respectively.

Effect of commercial packinghouse treatments on green mold caused by imazalil-S and imazalil-R biotypes of *P. digitatum*. In the first packinghouse experiment with Valencia oranges, a water spray (1 g/L of imazalil) reduced decay by the imazalil-S isolate M6R by 55%, whereas decay by the imazalil-R isolate S2 was reduced by only 13% compared with the untreated control (Table 1). Treatment of oranges with 2 g/L in the wax formulation gave good control of sporulation of imazalil-S isolate M6R on diseased fruit but had no effect on sporulation of imazalil-R isolate S2.

In the second packinghouse test, the storage wax with imazalil (2 g/L) reduced decay in lemons inoculated with M6R from 64% in the controls to 28% in fruit treated with the imazalil wax (Table 1). The imazalil in wax had no effect upon decay or fungus sporulation on lemons inoculated with imazalil-R isolate F1.

The third packinghouse experiment evaluated imazalil at 2 and 4 g/L (the highest dosage registered in the United States) in a lemon pack wax formulation. The wax formulation with 2 g/L of imazalil reduced decay in fruit inoculated with M6R by 42%, with good sporulation control, whereas this treatment reduced decay incited by imazalil-R isolate P3 by only 20% and gave poor control of sporulation of this isolate on treated fruit (Table 1). Imazalil at 4 g/L in the wax formulation did not significantly reduce decay or fungus sporulation compared with the 2 g/L wax formulation.

Effect of thiabendazole, imazalil, and their mixture on green mold of lemons inoculated with biotypes resistant to either or both fungicides. Biotype M6R, sensitive to both imazalil and thiabendazole, was controlled well by thiabendazole, imazalil, and a mixture of both fungicides (Table 2). Imazalil-R isolates F1 and P3 were controlled by thiabendazole and a mixture of thiabendazole and imazalil. Isolates J179 and J229, resistant to both imazalil and thiabendazole, were not controlled by either fungicide or by their mixture.

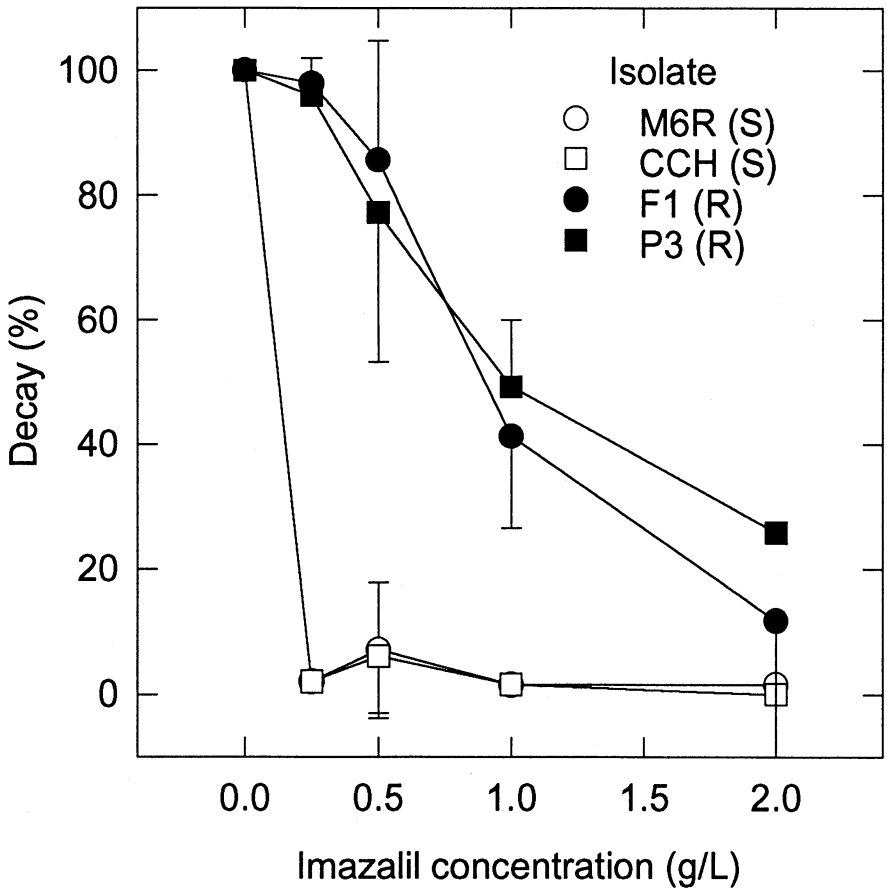

Fig. 2. Effect of imazalil concentration in laboratory dip treatments on decay of lemons inoculated with an imazalil-sensitive isolate (M6R or CCH) or an imazalil-resistant isolate (F1 or P3). The fruit were dipped for 30 sec and stored 3 wk at 13 C. Each data point and bar represent the mean and standard error of three replications of 35 fruit each.

DISCUSSION

In vitro resistance of *Penicillium* spp. to the postharvest azole fungicides imazalil and prochloraz has been reported by several investigators (1,7–9, 24–26,28), with little or no attempt to establish the impact of their observations upon the effectiveness of a commercial fungicide treatment. De Waard et al (11,12) produced imazalil-R biotypes of *P. italicum* in the laboratory and showed that these isolates were more difficult to control than wild-type isolates on inoculated oranges dipped in imazalil solutions. These results are difficult to inter-

Table 2. Effect of thiabendazole (TBZ) and imazalil (IMZ) on decay and pathogen sporulation on lemons inoculated with isolates of *Penicillium digitatum* susceptible (S) or resistant (R) to TBZ or IMZ or both

Inoculum			Fungicide treatment							
			None		TBZ		IMZ		TBZ + IMZ	
Isolate	TBZ sensitivity	IMZ sensitivity	Percent decay[y]	Sporulation[z]	Percent decay[y]	Sporulation[z]	Percent decay[y]	Sporulation[z]	Percent decay[y]	Sporulation[z]
M6R	S	S	98.7 a	5.0 a	0.0 e	4.0 c	2.0 e	0.8 g	1.0 e	0.0 h
F1	S	R	95.3 a	5.0 a	2.3 e	4.0 c	29.7 d	3.5 d	1.0 e	2.3 e
P3	S	R	99.0 a	5.0 a	2.0 e	4.3 b	49.3 bc	5.0 a	0.0 e	1.8 f
J179	R	R	99.0 a	5.0 a	95.7 a	5.0 a	35.0 d	5.0 a	30.0 d	5.0 a
J229	R	R	100.0 a	5.0 a	100.0 a	5.0 a	44.3 c	5.0 a	53.7 b	5.0 a

[y]Lemons were puncture-inoculated with a suspension of *P. digitatum* spores and incubated 18 hr at 20 C before treatment. Fruit were dipped for 15 sec in solutions containing 1 g/L of the specified fungicide(s). Decay was evaluated after 2 wk at 20 C. Each value is the mean of three replications of 30 fruit each. Values followed by the same letter are not significantly different at $P \geq 0.01$ according to the Waller-Duncan k-ratio test with $k = 100$.

[z]Twelve lemons were dipped for 1 min in TBZ (3.5 g/L), IMZ (2.0 g/L), a mixture of both fungicides, or water only. Treated fruit were placed in a metal tray and injected with 0.2 ml of a spore suspension (10^6 spores per milliliter) of the specified *P. digitatum* isolate. Fruit were stored at 13 C for 3 wk. Fungus sporulation was evaluated on a scale of 0 = nil to 5 = normal heavy sporulation on nontreated fruit. Values followed by the same letter are not significantly different at $P \geq 0.01$ according to the Waller-Duncan k-ratio test with $k = 100$.

pret on a practical level because imazalil-R *P. italicum* has rarely been found on commercial lots of citrus fruits (7,8,13, 18) and imazalil is applied to citrus fruits as a spray over brushes, never in a dip solution (3,5,16).

Imazalil-R *P. digitatum* biotypes have been isolated from diseased citrus fruits from production areas where imazalil has been intensely applied postharvest and the treated fruit stored for several weeks in the packinghouse before shipment (8,9,13,18). These conditions favor the selection, proliferation, and dissemination of fungicide-R biotypes (14,16). In Florida, where fruit are shipped within a few days after treatment, imazalil-R biotypes of *P. digitatum* were not detected (4).

We found that the highest imazalil dosage (4 g/L) registered in the United States will not control sporulation of imazalil-R biotypes, despite an earlier report to the contrary (9). Wound infection of citrus fruit by imazalil-R biotypes was significantly reduced by dipping inoculated lemons in 1–2 g/L of imazalil for 15–30 sec in the laboratory, but the effectiveness of the treatment was much greater on fruit inoculated with imazalil-S isolates. Earlier tests showed that imazalil applied in an aqueous spray resulted in higher residues than a wax formulation and better control of *Penicillium* decay and sporulation on diseased oranges (3,6). In our investigation, lemons dipped in aqueous suspensions of imazalil had two to three times higher residues than fruit treated with water or wax formulations on brushes in a commercial packinghouse. Commercial applications of imazalil provided little, if any, control of decay or sporulation of imazalil-R biotypes of *P. digitatum*. These treatments provided substantial control of green mold in fruit inoculated with imazalil-S *P. digitatum*.

The frequency of imazalil-R biotypes of *P. digitatum* and *P. italicum* declines in mixed populations with imazalil-S biotypes during several disease cycles on untreated fruit (11,12,14,26,27). This observation has led to the opinion that the lowered fitness of imazalil-R biotypes will prevent them from becoming a major problem in disease control (9,27). This conclusion ignores the fact that imazalil-R biotypes are the most fit on imazalil-treated fruit and they increase in frequency during several disease cycles on imazalil-treated fruit (12,26).

Because the frequency of imazalil-R biotypes in groves is extremely rare, presumably due to their reduced fitness on untreated fruit, strategies for resistance management are focused on the packinghouse. Strategies begin with packinghouse sanitation before the harvest season begins and continue with alternation of fungicides with different biochemical targets, complete isolation of fruit repacking operations, and monitoring of resistant biotypes in the packinghouse environment. Mixing of fungicides after imazalil-resistance is detected is a totally inappropriate strategy because it can lead to double resistance, a common phenomenon in California packinghouses today (18).

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