Sensitivity of *Penicillium digitatum* and *P. italicum* to Postharvest Citrus Fungicides in California

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ABSTRACT

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Penicillium digitatum isolates (326), collected in California citrus groves and packinghouses, were assayed qualitatively for their sensitivity to imazalil, thiabendazole, and *o*-phenylphenol. Eighteen typical triple-resistant isolates, acquired in each of 3 years (1988, 1990, and 1994), were assayed quantitatively for their sensitivity to each of the three fungicides. No significant differences were found in the mean sensitivity of the isolates collected in different years. However, the proportion of isolates that were resistant to all three fungicides increased from 43% in 1988 to 77% in 1990 and 74% in 1994. Imazalil-resistant biotypes of *P. digitatum* were isolated

frequently in California packinghouses, while resistant *P. italicum* was rare. No fungicide-resistant biotypes of either species were collected from citrus groves. Wild-type *P. italicum* was slightly less sensitive than wild-type *P. digitatum* to all three fungicides. The concentration of imazalil producing 50% growth inhibition (EC_{50}) was three times greater when the age of the *P. digitatum* assay inoculum was increased from 12 to 24 h. Activity of imazalil increased with pH of the assay medium in the range pH 5.1 to 5.9, reflecting the greater concentration of dissociated imazalil at the higher pH value.

Additional keywords: demethylation inhibitor, fungicide resistance, sterol inhibitor.

Green mold (caused by *Penicillium digitatum* (Pers.:Fr.) Sacc.) and blue mold (*P. italicum* Wehmer) are the most important postharvest diseases of citrus fruits produced in California. These wound-obligate pathogens have a relatively short disease cycle (3 to 5 days at 25°C) and, on a single fruit, can produce 1 to 2 billion conidia that are efficiently dispersed via air currents (19).

In most California packinghouses, citrus fruits are treated with sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole to control *Penicillium* decay. These fungicides are used in a manner that is highly conducive to the selection and proliferation of resistant biotypes of *P. digitatum* and *P. italicum*. The fruit surface is completely covered by the fungicides, and the residue is persistent for the life of the fruit. Many packinghouses process fruit the year around, resulting in continuous selection pressure on the pathogen population. The common practice of repacking after storage to remove diseased fruit results in efficient aerial dispersal of conidia of fungicide-resistant biotypes to recently harvested fruit being processed in the same packinghouse.

o-Phenylphenol and thiabendazole have been used routinely on citrus fruits over the past 3 decades, resulting in a serious problem of resistance to these two fungicides by the late 1970s (16,17). In 1976, Harding (18) recognized that imazalil, successfully used in Mediterranean citrus production areas for several years, could be an effective treatment for control of thiabendazole-resistant isolates of *P. digitatum* and *P. italicum* in California packinghouses. Imazalil was enthusiastically adopted by the California citrus industry in 1981 as an additive to fruit coating formulations to re-

place thiabendazole, which was largely ineffective due to the large population of thiabendazole-resistant *Penicillium* spp. in California packinghouses.

Despite earlier speculation that fungicide resistance would not become a problem with sterol demethylation inhibitors (12), imazalil-resistant biotypes of *P. digitatum* were reported in 1987 (10), just 5 years after the introduction of imazalil as a commercial treatment in California packinghouses. The wide distribution of these isolates in California was confirmed (5), and their potential impact on decay control was demonstrated in commercial packinghouse trials (11).

Although the existence of biotypes of *P. digitatum* and *P. italicum* resistant to *o*-phenylphenol, thiabendazole, or imazalil has been documented in California (5,10,11,17), other citrus production areas (9,24,27,35), and terminal markets (4), the degree and multiplicity of resistance has not. This investigation was undertaken to characterize, both qualitatively and quantitatively, resistance of *P. digitatum* and *P. italicum* in California to imazalil, thiabendazole, and *o*-phenylphenol and to determine if resistance patterns have changed between 1988 and 1994, a 6-year period following the first detection of imazalil resistance in *P. digitatum* (10).

MATERIALS AND METHODS

Origin, qualitative sensitivity, and storage of *Penicillium* isolates. Isolates of *P. digitatum* (326) and *P. italicum* (192) were collected from the atmosphere and from diseased fruit in 30 citrus groves and 26 packinghouses in California between 1986 and 1994. These isolates were evaluated for fungicide sensitivity in comparison with reference isolates from our culture collection.

Isolates were collected from the atmosphere by exposing petri dishes containing Harding's medium (H-25; potato-dextrose agar [PDA, Difco Laboratories, Detroit] plus 2 g of neopeptone per liter and 2 g of yeast extract per liter) (17) amended with imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole; techni-

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cal, 97.5% a.i.; Janssen Pharmaceutica, Beerse, Belgium) (0.1 µg/ml), thiabendazole (2-[4-thiazolyl] benzimidazole; Freshgard 598, 98.5% a.i.; FMC BioProducts, Riverside, CA) (15 µg/ml), or *o*-phenylphenol (Dow Chemical, Midland, MI) (10 µg/ml). These fungicide concentrations prevented colony development of wild-type *P. digitatum* and *P. italicum*, but had little or no effect on the growth of fungicide-resistant biotypes.

Conidia were collected from diseased citrus fruits with a sterile, wooden applicator stick that was scored 2 to 3 cm from the end. The scored end of the stick was used to contact sporulating areas of fruit and then broken off inside a sterile microcentrifuge tube (1.5-ml capacity). A 0.01% (wt/vol) aqueous Triton X-100 (Sigma-Aldrich, St. Louis) solution (1 ml) was added to the microcentrifuge tube and vortexed 1 to 2 s to suspend the conidia. This suspension of conidia was used to inoculate fruit.

Each isolate was inoculated into imazalil-treated and nontreated lemons to confirm its identity and pathogenicity and to determine its qualitative sensitivity to imazalil. Lemons (*Citrus limon* (L.) N.L. Burman f. 'Eureka') were surface-sterilized in 70% (vol/vol) aqueous ethanol (1 min) and dipped for 1 min in imazalil solution (1 mg/ml) or not treated. Each isolate was injected by hypodermic needle (200 µl of spore suspension containing >10⁷ conidia per ml) into the albedo (i.e., spongy, white endocarp) of three nontreated and three imazalil-treated fruit. The fruit were stored at 15°C for 2 to 3 weeks. Imazalil-sensitive isolates decayed imazalil-treated lemons, but did not sporulate on the fruit surface. Imazalil-resistant isolates decayed and sporulated freely on treated fruit.

Of the 326 isolates tested, 186 of those found to be resistant to imazalil were assayed for their resistance to thiabendazole and *o*-phenylphenol. This was determined by streaking spore suspensions (>10⁷ conidia per ml) onto H-25 medium amended with imazalil, thiabendazole, or *o*-phenylphenol as described. Isolates that grew on a fungicide-amended medium were maintained on silica gel at 3°C (26). Slant cultures of the above isolates were later obtained by sprinkling a few silica gel crystals onto H-25 medium in slant tubes and incubating at 25°C for 5 to 7 days.

Determination of fungicide sensitivity (concentration producing 50% growth inhibition [EC₅₀ values]). Conidia on 7- to 10-day-old, H-25 slant cultures were suspended in 4 to 5 ml of sterile distilled water containing 0.01% Triton X-100, vortexed 5 to 10 s, and filtered through double-layered cheesecloth to remove hyphal fragments. The suspension was adjusted to 10⁶ conidia per ml, and 1 ml was added to 15 ml of molten "disk agar" (H-25 agar supplemented with agar (6 g/liter) to increase firmness) at 50°C, dispensed immediately into petri dishes, and incubated for 24 h at 25°C. Disks were cut from these cultures with a cork borer (4-mm diameter), and one disk was placed in the center of each dish containing fungicide-amended or nonamended H-25 agar (pH = 5.9 ± 0.2). Colony diameters were measured at 7 to 10 days at 25°C. Percent inhibition at each fungicide concentration was calculated from the mean colony diameter on medium without fungicide and from the colony diameter on each fungicide-amended plate

$$\% \text{ inhibition} = \frac{\bar{X} - X_i}{\bar{X}} \times 100$$

in which $\bar{X}$ is the mean colony diameter on nonamended medium (minus the 4-mm inoculum disk) and X_i is the colony diameter on a single fungicide-amended plate (minus the 4-mm inoculum disk). A linear regression of percent inhibition versus fungicide concentration was calculated, and the fungicide concentration estimated to produce 50% inhibition (EC₅₀) was determined by interpolation from the regression line.

Factors affecting EC₅₀ value. Inoculum age. The effect of inoculum age on the EC₅₀ value was determined for triple-resistant isolates 151 and 1116 in the manner described above, except that the inoculum disks were transferred to H-25 medium amended with several concentrations of imazalil, thiabendazole, or *o*-phenylphenol (three dishes per concentration) at 0, 4, 8, 12, 16, 20, 24, 32, 40,

and 48 h after the disk agar was seeded with conidia. The experiment was repeated using two other triple-resistant isolates (1693 and 1750) and one wild-type isolate (M6R).

pH. The effect of medium pH on imazalil activity (EC₅₀) was investigated with imazalil-resistant isolate 151 on H-25 medium nonbuffered (pH 5.9) and buffered at pHs 5.7, 5.5, and 5.1 with 2(*N*-morpholino)ethanesulfonic acid (MES) (0.15 M)/NaOH (0.1 M). The buffers contained various proportions of MES/NaOH (25/0, 21.1/3.9, and 17.7/7.3 ml) to buffer the medium at pHs 5.1, 5.5, and 5.7, respectively. Initial and final pH of the media was measured by applying a surface electrode to the surface of cooled media in petri dishes. The buffer solution (25 ml) was mixed with 75 ml of concentrated (1.33×) H-25 medium (total volume, 100 ml). The imazalil concentrations in H-25 medium at each pH were 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75 µg/ml. Five replicate dishes were used at each concentration of imazalil at each pH. The experiment was repeated.

Sensitivity of *Penicillium* isolates to postharvest fungicides. As described above, EC₅₀ values were determined for imazalil-sensitive *P. digitatum* (24 isolates) and *P. italicum* (16 isolates) for imazalil, thiabendazole, and *o*-phenylphenol. Fungicide concentrations in amended H-25 medium were 0, 0.015, 0.020, 0.025, 0.030, 0.035, 0.040, 0.045, and 0.050 µg of imazalil per ml; 0, 0.1, 0.5, and 1.0 µg of thiabendazole per ml; and 0, 1, 5, 10, and 15 µg of *o*-phenylphenol per ml. All isolates were collected from citrus groves.

EC₅₀ values for imazalil-resistant *P. digitatum* (54 isolates) and *P. italicum* (4 isolates), collected from citrus packinghouses, were determined in the same manner as imazalil-sensitive isolates, except that the fungicide concentrations in the medium were 0, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 µg of imazalil per ml; 0, 10, 20, 30, and 40 µg of thiabendazole per ml; and 0, 5, 10, 20, and 30 µg of *o*-phenylphenol per ml. The imazalil-resistant isolates of *P. digitatum* were collected in 1988, 1990, and 1996 (18 each year) and were resistant also to thiabendazole and *o*-phenylphenol. The mean EC₅₀ values for isolates collected each year were compared by analysis of variance (ANOVA) to detect significant changes in sensitivity of *P. digitatum* to the three fungicides over time.

Imazalil concentrations used in assays with imazalil-sensitive and -resistant isolates were not replicated because *R*² values for regression lines averaged >0.95 and because additional concentrations of the fungicides yielded more valuable data than replicates of concentrations. However, other measures were taken to ensure the accuracy of EC₅₀ values: each test, which included 30 isolates, was repeated and included a reference isolate. The fungicide sensitivity of the reference isolates was determined previously in five tests in which each fungicide concentration was replicated three times.

Influence of triple resistance on growth. Thirty-five isolates each of triple-resistant and imazalil-resistant (only) *P. digitatum* were used to determine if the level of resistance to imazalil differed in the two populations. A loopful of spore suspension (approximately 10⁸ spores per ml) of each isolate was streaked onto the following H-25 media: nonamended and amended with 0.4 µg of imazalil per ml. Fungal growth (presence or absence) was evaluated at 7 days at 25°C.

RESULTS

***Penicillium* isolates.** Conidia of 300 isolates of imazalil-resistant *P. digitatum* were collected in 26 California citrus packinghouses in 1986 to 1994. These isolates produced typical green mold on imazalil-treated lemons and sporulated heavily on H-25 medium amended with imazalil (0.1 µg/ml). In contrast, all 26 isolates of *P. digitatum* collected in citrus groves decayed, but did not sporulate on, imazalil-treated lemons and did not grow on H-25 medium amended with 0.1 µg of imazalil per ml.

Imazalil-resistant *P. italicum* was relatively rare; out of 59 total isolates collected in 1994 (41 from packinghouses and 18 from groves), resistance was found at two packinghouses. A total of

four isolates was collected. Previous work in this laboratory looked at an additional 133 isolates of *P. italicum* (58 from packinghouses and 75 from groves) collected between 1988 and 1992, and no imazalil-resistant isolates were detected (J. W. Eckert, unpublished data).

The proportion of collected isolates that were resistant to imazalil, thiabendazole, and *o*-phenylphenol increased from 43.1% (22 out of 51) in 1988 to 77.2% (61 out of 79) in 1990 and 74.2% (46 out of 62) in 1994. All 114 thiabendazole-resistant isolates of *P. digitatum* collected from 1988 to 1994 were resistant to *o*-phenylphenol. Resistant biotypes from packinghouses fell into three groups: resistant to (i) imazalil only; (ii) thiabendazole and *o*-phenylphenol; and (iii) imazalil, thiabendazole, and *o*-phenylphenol.

Fig. 1. Effect of inoculum age on the fungicide concentration estimated to produce 50% inhibition (EC_{50}) for triple-resistant *Penicillium digitatum* isolate 1116 to imazalil, thiabendazole, and *o*-phenylphenol.

Factors affecting EC_{50} value. Inoculum age. An increase in inoculum age from 12 to 24 h increased the imazalil EC_{50} for *P. digitatum* isolates 151 and 1116 over twofold (from 0.40 $\mu\text{g/ml}$ at 12 h to 0.91 $\mu\text{g/ml}$ at 24 h) (Fig. 1). Two other imazalil-resistant isolates (1693 and 1750) and an imazalil-sensitive isolate (M6R) showed the same inoculum age- EC_{50} relationship. The EC_{50} for thiabendazole for isolate 1116 increased slightly with inoculum age (0 to 48 h), while the EC_{50} for *o*-phenylphenol was not significantly affected by inoculum age between 0 and 48 h.

pH. The EC_{50} for imazalil-resistant *P. digitatum* (isolate 151) was inversely related to the pH of the medium (Fig. 2A). The EC_{50} at pH 5.7 (0.88 $\mu\text{g/ml}$) was 1.7 and 5.3 $\times$ lower than the values determined at pH 5.5 (EC_{50} = 1.53 $\mu\text{g/ml}$) and pH 5.1 (EC_{50} = 4.66 $\mu\text{g/ml}$), respectively. In the medium buffered with MES/NaOH, growth inhibition appeared to be a function of the concentration of dissociated (neutral) imazalil, because the dosage response curves for pHs 5.1, 5.5, and 5.7 are superimposed when growth inhibition is plotted against concentration of dissociated imazalil (Fig. 2B). A comparison of dosage-response curves for dissociated imazalil at pH 5.9 (nonbuffered) and pH 5.7 (buffered) suggests that MES increased the activity of imazalil (Fig. 2B). Final pH of the media was ± 0.25 units from the initial pH.

Fungicide sensitivity. All *P. digitatum* isolates collected from citrus groves were sensitive to imazalil, thiabendazole, and *o*-phenylphenol with mean EC_{50} values of 0.026, 0.1, and 6.3 $\mu\text{g/ml}$, respectively (Table 1). Mean percent inhibition of mycelial growth by thiabendazole at the lowest concentration tested (0.1 $\mu\text{g/ml}$) was $57.8 \pm 9.2\%$ for 24 isolates.

Imazalil-resistant *P. digitatum* was approximately 35 $\times$ more resistant to imazalil than the wild type. The level of imazalil resistance in isolates of *P. digitatum* collected from packinghouses remained relatively constant (EC_{50} range = 0.87 to 0.92 $\mu\text{g/ml}$) from 1988 to 1994 (Table 2). The mean EC_{50} values for 1988, 1990, and 1994 were not significantly different (ANOVA; $P = 0.05$).

Isolates of *P. italicum* collected from citrus groves were sensitive to imazalil. The mean EC_{50} value for 17 isolates was 0.031 ± 0.009 μg of imazalil per ml. Wild-type *P. italicum* was slightly less sensitive to imazalil, thiabendazole, and *o*-phenylphenol than was

Fig. 2. A, Effect of medium pH on imazalil activity (inhibition of mycelial growth) against imazalil-resistant *Penicillium digitatum* isolate 151. B, Effect of the concentration of dissociated imazalil (calculated) on growth inhibition at the same pH values. Each data point represents the mean of five replicates. H-25 medium was buffered with 2-(*N*-morpholino)ethanesulfonic acid/NaOH to give the desired pH. The colony diameter was measured at 6 days.

$$\% \text{ dissociated imazalil} = \left[\frac{100}{1 + 10^{(\text{pH} - \text{pK}_a)}} \right] - 100 \quad (1). \text{ Imazalil } \text{pK}_a = 6.53 \text{ (29).}$$

wild-type *P. digitatum*. Four imazalil-resistant isolates of *P. italicum* were isolated from two packinghouses in 1994. The mean EC₅₀ value for these isolates was 0.52 ± 0.08 µg of imazalil per ml, approximately half the mean EC₅₀ value for imazalil-resistant biotypes of *P. digitatum* (Table 2).

Cost of triple resistance. Thirty-five isolates that were resistant only to imazalil produced colonies from conidia streaked on H-25 medium containing 0.4 µg of imazalil per ml, whereas colony development of 35 triple-resistant isolates was inhibited completely on the same medium.

DISCUSSION

Intensive postharvest use of the chemically unrelated fungicides imazalil, thiabendazole, and *o*-phenylphenol on citrus fruits in California has resulted in the proliferation of triple-resistant biotypes of *P. digitatum*. However, this did not lead to an increase in the level of resistance (EC₅₀) to any of the fungicides in isolates collected between 1988 and 1994. Furthermore, none of the isolates collected during that period possessed a significantly higher level of resistance than our original imazalil-resistant isolates collected in 1986.

Concern over imazalil resistance prompted packinghouse service companies to combine imazalil and thiabendazole in their fruit coating formulations. Theoretical studies of resistance development have revealed that the combination of two selective fungicides to combat resistance is a reasonable strategy only when used against wild populations of the pathogen with an extremely low frequency of resistance to both fungicides (28). Even then, sequential use of two unrelated fungicides may be a more effective strategy. When the combination strategy was implemented in California, the population of *Penicillium* spp. in packinghouses was well endowed with independent resistance genes for imazalil, thiabendazole, and *o*-phenylphenol. Treatment of fruit with all of these fungicides provided a substrate that selected for mutant biotypes possessing genes for resistance to each of the fungicides. Furthermore, laboratory experiments suggest that multiple-resistant biotypes could be sustained in the pathogen population, at least in the short term, by application of only one of the fungicides (12). In our survey, the proportion of triple-resistant isolates in the *P. digitatum* population in packinghouses increased from 43% in 1988 to over 70% by 1994.

Resistance to imazalil and other demethylation inhibitors (DMI) is controlled by a polygenic system in certain fungi (21,33), and the development of resistance in *P. expansum* (34) and *P. italicum* (7,8) suggests that several minor genes control imazalil resistance in these species as well. Surveys of wild populations of *Penicillium* spp. (2,7,25) revealed biotypes with varying degrees of sensitivity to imazalil, but none were resistant to dosages recommended for disease control. Likewise, we have never isolated a *P. digitatum* isolate from a citrus grove that was capable of growing

on PDA with 0.1 ppm of imazalil or of sporulating on lemons dipped in a 1,000-ppm imazalil solution.

de Waard et al. (7,8) demonstrated a stepwise progression in imazalil resistance in *P. italicum* by repeated mass selection on increasing concentrations of imazalil in the culture medium. A biotype that resulted from five selection steps was not well controlled on oranges dipped in a 500-ppm imazalil solution. de Waard and van Nistelrooy (8) suggested that this biotype may have accumulated at least five minor genes for imazalil resistance and these genes interacted in an additive fashion to confer a significant level of resistance to this fungicide. One additional selection step resulted in a biotype with a higher level of resistance, but it was not stable in the absence of imazalil.

Biotypes of *P. digitatum* and *P. italicum* with a moderate level of imazalil resistance are virulent and stable in the absence of imazalil (8,19). They are more fit than wild isolates on imazalil-treated fruit, but most of these resistant biotypes are less competitive than wild biotypes on nontreated fruit, and their frequency decreases in the spore population over several disease cycles (7,8,19). However, fitness of resistant biotypes could improve with time if selection pressure continues. Biotypes of *P. italicum* with high levels of imazalil resistance, produced with mutagenizing agents, tend to have reduced virulence, saprophytic fitness, and sporulation capacity (7,8,33). Such observations nurtured the speculation that resistance to imazalil and other DMIs was not likely to become a practical problem in disease control (5,32).

Our observation that the degree of imazalil resistance in isolates of *P. digitatum* did not increase significantly from 1986 to 1994 may be explained by the relatively constant selection pressure produced by the standard treatment of 2,000 ppm of imazalil incorporated in a fruit coating formulation. The resistant biotypes that evolved under this degree of selection pressure are as virulent as wild types and more fit on imazalil-treated citrus fruits (19). Therefore, they have become the dominant biotypes of *P. digitatum* in California packinghouses that routinely store imazalil-treated fruit. While natural mutants with greater resistance to imazalil are possible, they probably would be burdened with diminished virulence, sporulation, and competitive fitness (7,8,13,14,23,30,34). They would have no evolutionary advantage over prevailing resistant biotypes that are not controlled by the standard imazalil spray treatment. However, increasing the effectiveness of imazalil by using a different method of application (e.g., heated aqueous solution instead of wax) may alter this situation (31).

The increase in the proportion of triple-resistant biotypes in the *P. digitatum* population in California packinghouses from 1988 to 1994 may be attributed to the selection of independent mutant genes in individual biotypes subjected to continuous selection pressure of the three fungicides applied to the same fruit.

Imazalil, like other DMIs, is considered a low- to medium-risk fungicide with respect to the probability that a practical resistance problem will develop (14,23). Similar imazalil-resistant isolates of *P. digitatum* and *P. italicum* have been isolated in Rotterdam from citrus fruits produced in Spain and Israel (3,4). Imazalil-resistant

TABLE 1. Sensitivity of wild-type citrus grove isolates of *Penicillium digitatum* and *P. italicum* to imazalil, thiabendazole, and *o*-phenylphenol

Fungicide, species	Mean EC ₅₀ ^w	Mean % inhibition	n ^x
Imazalil			
<i>P. digitatum</i>	0.026 ± 0.003 ^y	...	24
<i>P. italicum</i>	0.031 ± 0.009	...	17
<i>o</i> -Phenylphenol			
<i>P. digitatum</i>	6.3 ± 1.0	...	28
<i>P. italicum</i>	6.8 ± 1.2	...	16
Thiabendazole ^z			
<i>P. digitatum</i>	...	57.8 ± 9.2	24
<i>P. italicum</i>	...	35.1 ± 7.8	16

^w Fungicide concentration estimated to produce 50% inhibition.

^x Number of isolates tested.

^y Values are means of multiple isolates from a single experiment followed by the standard deviation of the mean.

^z 0.1 µg/ml.

TABLE 2. Sensitivity of 54 triple-resistant isolates (18 each in 1988, 1990, and 1994) of *Penicillium digitatum* to imazalil, thiabendazole, and *o*-phenylphenol^x

Year	Mean EC ₅₀ (µg/ml) ^y		
	Imazalil	Thiabendazole	<i>o</i> -Phenylphenol
1988	0.88 ± 0.17 a ^z	49.83 ± 6.20 a	37.74 ± 6.64 a
1990	0.92 ± 0.09 a	47.84 ± 8.94 a	36.82 ± 6.83 a
1994	0.87 ± 0.18 a	47.12 ± 9.35 a	34.63 ± 6.59 a

^x All isolates were collected in California citrus packinghouses.

^y Fungicide concentration estimated to produce 50% inhibition. Values are means of multiple isolates from a single experiment followed by the standard deviation of the mean.

^z Values within columns followed by the same letter are not significantly different (*P* = 0.05).

isolates have not been reported in Florida (2). The 5-year lag period between the introduction of imazalil into commercial California packinghouses and the isolation of resistant isolates of *P. digitatum* may support the hypothesis that imazalil resistance in *Penicillium* spp. is controlled by a polygenic system and several minor genes for resistance must be accumulated for a practical level of resistance to this fungicide to develop (7,8,14,30). However, field isolates with intermediate levels of resistance have not been found. Thiabendazole resistance, due to a single major gene mutation (6,34), appeared in *P. italicum* isolates in California lemon packinghouses after only 15 months of continuous use of this fungicide (17).

P. digitatum isolates that were resistant only to imazalil had a higher level of imazalil resistance than isolates resistant to imazalil, *o*-phenylphenol, and thiabendazole. The two groups of imazalil-resistant isolates were discriminated by streaking spore suspensions of both groups of isolates onto H-25 medium amended with 0.4 µg of imazalil per ml and 1.0 µg of thiabendazole per ml. The triple-resistant isolates did not form colonies on the imazalil-amended medium, whereas isolates resistant to imazalil only did form colonies. The increased imazalil sensitivity of the triple-resistant isolates appears to be expressed in the early stages of conidial development, because there was no apparent difference in sensitivity expressed in our EC₅₀ and EC₉₀ values. Guan et al. (15) reported that conidial germination in *P. italicum* was more sensitive to imazalil than was mycelial growth of hyphae approximately 18 h old. The hyphal inoculum for our EC₅₀ studies was approximately 24 h old. This difference may account for the difference between the two assays.

Variability in the EC₅₀ values reported for imazalil by various researchers can be explained by differences in experimental procedures and the calculation of the EC₅₀ values. We observed that pH of the culture medium and inoculum age have a significant effect upon the EC₅₀ value. The pK_a of imazalil, 6.53 (29), is sufficiently close to the pH of PDA (5.6 ± 0.2) and H-25 medium (5.9 ± 0.2) that a slight difference in the pH of the culture medium in different assays could have a significant effect on the concentration of the neutral (dissociated) form of imazalil, which is largely responsible for the antifungal activity of this compound (15,29). At pH 5.5, 9.1% of the imazalil in the culture medium is in the neutral (active) form; at pH 6.0, 24% of the imazalil is in the neutral form (1). The striking effect of pH upon the EC₅₀ value is shown in Figure 2. To minimize the effect of pH on EC₅₀ values, all petri dishes in a single 30-isolate test contained the same batch of H-25 medium (pH 5.9 ± 0.2) and included a well-characterized isolate of a *Penicillium* sp. as a reference.

The striking effect of inoculum age upon the EC₅₀ value for imazalil, but not for thiabendazole or *o*-phenylphenol, may be due to cell permeability to the fungicide or to action sites unique to different stages of fungal growth. Guan et al. (15) noted that conidial germination was more sensitive to imazalil than was the growth of 18-h-old hyphae. The mass of fungus tissue interacting with a relatively low concentration of imazalil in the culture medium may also be a factor, since the estimate of minimum inhibitory concentrations (MICs) can be influenced by the mass of fungal inoculum (J. W. Eckert, unpublished data).

In our experiments, the dose response of *P. digitatum* and *P. italicum* to imazalil at the concentrations tested was linear, with R² values generally greater than 0.95. The calculation of EC₅₀ values using the original experimental units was simpler and provided more accurate estimates of EC₅₀ values than the log-dose probit transformation.

de Waard et al. (8) reported the imazalil sensitivity of a single wild-type *P. italicum* isolate (EC₅₀ = 0.07 µg/ml) and of several laboratory-induced imazalil-resistant mutants. The EC₅₀ for their wild-type *P. italicum* was more than twice the mean of 17 wild-type isolates that we tested (mean EC₅₀ = 0.031 µg/ml). This difference in imazalil sensitivity is easily within the error range of the assay procedure.

The scarcity of imazalil-resistant isolates of *P. italicum* (4,8,19,22) in the packinghouse is puzzling. Abundance of the species in citrus groves ensures that it is continually introduced to the packinghouse environment, where there is intense selection pressure for fungicide resistance. The existence of a few stable, virulent, imazalil-resistant isolates of *P. italicum* that have been characterized by us and several other investigators (3,4,7,8) suggests that significant populations of the species should develop under the selection pressure of this fungicide. However, this has not occurred under field conditions, which suggests a fundamental difference in the biology of *P. italicum* and *P. digitatum*. This difference could result from factors such as smaller populations of *P. italicum* in packinghouses available for selection, greater imazalil sensitivity of resistant mutants, and the lower parasitic fitness and reproductive capacity of this species compared with *P. digitatum* (20). A better understanding of the biology and ecology of *P. digitatum* and *P. italicum* may offer important insight into the management of fungicide resistance in citrus green mold and other diseases.

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