Relative Fitness of Imazalil-Resistant and -Sensitive Biotypes of *Penicillium digitatum*

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ABSTRACT

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

Imazalil-resistant (R) and wild (S) biotypes of *Penicillium digitatum* collected in California citrus packinghouses and groves were evaluated alone and in R/S combinations in lemons and on culture medium for phenotype stability and relative fitness. R and S phenotypes were stable in the presence or absence of imazalil over several disease cycles in fruit and several spore generations on culture medium. The proportion of R spores in R/S mixtures (1:1) declined more rapidly on fruit than on culture medium. Competitiveness was studied with 11 random combinations of 11 S and 11 R isolates over two disease cycles in nontreated lemons. A significant reduction in percent R spores was observed in eight of 11 mixtures. However, percent R spores increased in three of the mixtures, suggesting that some R biotypes are more competitive than some S biotypes, at least during two disease cycles. In seven of nine possible combinations of three R and three S biotypes, R biotype spore populations declined to almost zero by the end of the second disease cycle in lemons. With the same R/S combinations on culture medium, R biotypes accounted for greater than 15% of the spore populations at the end of the third spore generation. There was no correlation between isolate competitiveness in R/S mixtures and spore production, radial growth in vitro, or latent period in fruit when isolates were evaluated alone.

Additional keywords: *Citrus limon*, fungicide resistance, green mold, sterol biosynthesis inhibitor

Green mold caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. is the major cause of postharvest decay of citrus fruits in all arid production areas of the world (8). Three fungicides, sodium *o*-phenylphenate, thiabendazole, and imazalil, are applied to citrus fruits after harvest to control green mold. Several years after the introduction of each of these fungicides, resistant (R) biotypes of *P. digitatum* were found in packinghouses and in terminal markets (3,4,7,10,15,16,21). Presumably, these isolates became abundant due to the intense selection pressure of fungicide residues on treated fruit.

Several reports have indicated that some isolates of *P. digitatum* and *P. italicum* resistant to thiabendazole or imazalil were less fit in nontreated fruit or fungicide-free media than sensitive (S) biotypes. Several investigators have reported reduced fitness of R biotypes of *Penicillium* spp. but their studies have differed in methodology, source of isolates, fungicide(s) tested, and *Penicillium* spp. used (5,6,10, 19,29,31). Reduced fitness of R biotypes is an extremely important phenomenon in the development of strategies for control of fungicide-resistant biotypes of *P. digitatum* because it implies that the use of nonselective fungicides or fungicide-free periods will bring about a reduction in the frequency of R biotypes. This would permit effective use of the selective fungicide at a later time. Currently, imazalil is the cornerstone of the practical program for control of this disease in California because it is the only available postharvest fungicide with both curative and antisporulation action against *P. digitatum*. Imazalil is especially effective on biotypes of the pathogen that are resistant to thiabendazole (1,10). Isolates of *P. digitatum* resistant to imazalil were first reported in 1987 (7) and have increased at an alarming rate in California packinghouses over the past 7 years (16). The deleterious impact of these R biotypes on control of green mold has been demonstrated in commercial packinghouses (9).

This investigation was undertaken to determine if R biotypes are less competitive in a mixed population with S biotypes in nontreated citrus fruit. We also evaluated possible mechanisms to account for changes in the frequency of R/S biotypes in diseased fruit.

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This publication reports research involving pesticides. It does not contain recommendations for their use.

Accepted for publication 13 July 1995.

MATERIALS AND METHODS

Isolates. Imazalil-S and -R isolates of *P. digitatum* were collected in citrus groves and packinghouses (Tables 1 and 2). Sensitivity to imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole; 97.5% a.i.) (Janssen Pharmaceutica, Beerse, Belgium), thiabendazole (2-[4-thiazolyl]-1*H*-benzimidazole; Freshgard 598, 98.5% a.i.) (FMC, Riverside, Calif.), and *o*-phenylphenol (99.7% a.i.) (Dow Chemical, Midland, Mich.) was determined by streaking a spore suspension of each isolate onto H-25 medium (H-25: 39 g of potato-dextrose agar [PDA, Difco, Detroit, Mich.], 2 g of neopeptone, and 2 g of yeast extract per liter) (15) containing imazalil (0.1 µg/ml), thiabendazole (10 µg/ml), or *o*-phenylphenol (15 µg/ml). Growth on fungicide-amended medium equivalent to that observed on nonamended medium indicated that the isolate was resistant to that fungicide. Isolates were stored on silica gel at 3°C (25). To obtain spores, a few silica gel granules were dropped onto PDA slants and the cultures were incubated at 25°C for 7 to 10 days. The slant cultures were flooded with 4 to 5 ml of sterile, deionized water containing 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), vortexed 5 to 10 s, and the spore suspension was filtered through double-layered cheesecloth to remove hyphal fragments.

Frequency of imazalil-R biotypes in spore population. The following procedure was used to determine the frequency of R biotypes in a spore population. Spore suspensions of R and S isolates were adjusted to 10^6 spores/ml on the basis of optical density (23). These spore suspensions, alone or mixed with an equal volume of spore suspension of another isolate, were inoculated into lemons (*Citrus limon* (L.) N. L. Burm. 'Eureka') or streaked onto H-25 medium with and without imazalil (0.1 µg/ml). To verify the R/S composition of spore inoculum, each mixture of two isolates was diluted to 10^3 spores/ml and 200 µl was streaked with a glass rod onto three plates of H-25 medium, containing 3 µg of dicloran per ml (2,6-dichloro-4-nitroanaline; Botran technical, 90% a.i.) (Nor-Am Chemical Co., Wilmington, Del.) and 0.1 µg of imazalil per ml, or dicloran alone. Dicloran restricted radial colony growth and facilitated counting of individual colonies. Culture plates were incubated at 25°C for 2 days and the developing colonies were

counted. A single visible colony after 2 days incubation on imazalil-amended medium was assumed to have developed from one R spore. The percentage of spore population resistant to imazalil was calculated from the mean number of *P. digitatum* colonies on medium with and without imazalil.

Phenotype stability of imazalil-R and -S biotypes. Prior to more definitive competition studies in fruit and on culture medium, preliminary experiments were undertaken to determine the stability of R and S phenotypes. One S and three R biotypes (S4 and R1, R3, R4; Table 2) were inoculated into nontreated lemons and H-25 medium in petri plates as follows. The exocarp of surface-sterilized lemons (1 min dip in 70% ethanol) was punctured at a 45 degree angle, 3 to 4 mm deep, using a sewing needle (Singer, style 2020, size 16, 1 mm diameter) that was dipped into a 10^6 spores/ml suspension. One lemon was inoculated with each isolate and each fruit was placed on a Syracuse watch glass (60 mm outside diameter, Thomas Scientific Co., Swedesboro, N.J.) in a 1-quart mason jar so that fruit did not contact the wall of the jar. The mason jar and fruit were capped with sterile paper, secured with a rubber band, and held at 25°C for 7 days. At 7 days the entire fruit surface was covered with the sporulating fungus; 250 ml of sterile 0.05% Triton X-100 was added to each mason jar and the contents were agitated by hand until spores were wetted and removed uniformly from the fruit surface. The spore suspension from each fruit was immediately adjusted to 10^6 spores/ml and inoculated into another group of lemons for the next disease cycle. The same suspension was diluted to 10^3 spores/ml and assayed for percent resistance as described above.

Phenotype stability on culture medium was evaluated by assaying imazalil-R isolates in three consecutive spore generations. Petri plates with H-25 medium amended with 0.1 µg of imazalil per ml were streaked with 200 µl of a suspension of 10^6 spores/ml and incubated as described above. The cultures were flooded with approximately 10 ml of 0.05% sterile Triton X-100 and spores were scraped from the mycelial mat using a wire loop. The recovered spore suspension was used to streak the next set of petri plates and growth of the isolates was recorded.

The stability of three R/S mixtures (R1/S4, R3/S4, and R4/S4) was followed over two disease cycles in fruit and three spore generations on culture medium. The first disease cycle/spore generation was completed on substrate containing imazalil (fruit dipped 1 min in 1,000 µg of imazalil per ml, or 0.1 µg of imazalil per ml in H-25 medium) followed by a second disease cycle in nontreated lemons or two additional spore generations on medium without imazalil. Spores were harvested and assayed for imazalil resistance in the manner described above.

Competition between S and R biotypes in nontreated fruit. Since the isolates used had been stored on silica gel for varying periods of time (Table 1), each was grown out as described above and the resulting spore suspensions were puncture-inoculated into lemons on the same day and reisolated from sporulating lesions after 5 days at 25°C. The re-vitalized cultures were stored on silica gel and used in competition experiments carried out over a period of 3 months. Spore suspensions (10^6 spores/ml) of 11 R and 11 S isolates (Table 1) were combined (at 1:1 ratio) in 11 isolate mixtures. Each spore suspension mixture was assayed for resistance and also inoculated into three lemons. The fruit were incubated at 25°C for 7 days, and the spore suspension derived from each lemon was assayed for imazalil resistance. Inoculum for the next disease cycle was prepared by mixing equal portions of the adjusted spore suspensions (10^6 spores/ml) from three replicate lemons. The frequency of resistance in the spore popula-

Table 1. Composition of *Penicillium digitatum* isolate mixtures used in two-generation competition experiments in lemons

	Imazalil-sensitive isolates[a]		Imazalil-resistant isolates[b]		
Mixture No.	Isolate	Date isolated	Isolate	Type of resistance	Date isolated
1	1	April 1965	666	Triple[c]	October 1988
2	76	December 1981	152	Single[d]	December 1986
3	481	March 1988	571	Triple	August 1988
4	279	July 1987	1809	Single	March 1994
5	1049	March 1989	1781	Single	March 1994
6	1832	April 1994	680	Triple	October 1988
7	1670	March 1994	1751	Single	March 1994
8	1755	March 1994	1715	Triple	March 1994
9	1791	March 1994	1685	Triple	April 1994
10	1048	March 1989	973	Single	February 1989
11	1023	March 1989	674	Single	October 1991

[a] All sensitive isolates were collected from air or from fruit in citrus groves.
[b] All resistant isolates were collected from air or from fruit in citrus packinghouses.
[c] Resistance to imazalil, thiabendazole, and *o*-phenylphenol.
[d] Resistance to imazalil only.

Table 2. Origin of *Penicillium digitatum* isolates used in experiments on four-generation competition and phenotype stability

Isolate	Imazalil sensitivity	Source	Location	Date isolated
S1 (55)[a]	Sensitive	Air	Packinghouse, Yuma, Ariz.	March 1981
S2 (355)	Sensitive	Orange	Grove, Ivanhoe, Calif.	October 1987
S3 (1755)	Sensitive	Orange	Grove, Santa Paula, Calif.	March 1994
S4 (1)	Sensitive	Lemon	California	April 1965
R1 (151)	Resistant	Lemon	Packinghouse, Santa Paula, Calif.	December 1986
R2 (1698)	Resistant	Air	Packinghouse, Santa Paula, Calif.	March 1994
R3 (1716)	Triple resistant[b]	Air	Packinghouse, Saticoy, Calif.	March 1994
R4 (1718)	Resistant	Air	Packinghouse, Saticoy, Calif.	March 1994

[a] Numbers in parentheses correspond to laboratory culture collection accession numbers.
[b] Resistant to imazalil, thiabendazole, and *o*-phenylphenol.

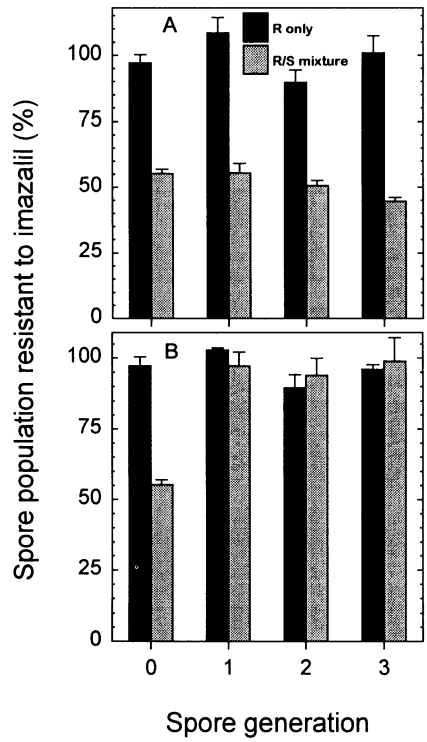

Fig. 1. Phenotype stability of imazalil-resistant (R) *Penicillium digitatum* (solid bars) and changes in R/sensitive (S) ratios (gray bars) in mixtures of two isolates seeded on petri plates with **(A)** H-25 medium and **(B)** H-25 medium amended with imazalil (0.1 µg/ml) in the first spore generation. Spore generations 2 and 3 were produced in the absence of imazalil. Each bar represents the mean and standard error of three different isolates (R1, R2, R4) or R/S isolate mixtures (R1/S4, R3/S4, R4/S4; Table 2).

tion was assayed after the second disease cycle. A two-sample *t* test was performed on the means of three replicates of original inoculum (generation zero) and harvested spores from generation two. This test was repeated.

A similar experiment was conducted with three R and three S isolates (Table 2). Each R isolate was mixed with an S isolate in nine possible combinations to confirm the correlation between imazalil-R biotypes and fitness. The frequency of resistance in the spore population was followed over four disease cycles.

Competition between S and R biotypes on solid culture medium. The spore suspensions used in the second competition test in lemons (i.e., nine combinations of R and S isolates) (Table 2) were also used for the competition study in vitro. Aliquots of 600 µl of each R/S spore suspension mixture (10^6 spores/ml) were added to three 125-ml DeLong culture flasks containing 20 ml of H-25 medium. The cultures were incubated at 25°C for 7 days. Spores were harvested by adding 20 ml of sterile 0.05% Triton X-100 solution and shaking on a rotary shaker for 10 min at 350 oscillations/min. The spore suspensions were filtered through double-layered cheesecloth and adjusted to 10^6 spores/ml by optical density (23). Percent imazalil-R in these spore populations was determined as described above.

Spore production on solid culture medium and on diseased fruit. The spore suspensions containing single isolates inoculated into fruit for the spore production tests were also used to study spore production on solid culture medium. Aliquots of 600 µl of spore suspension (10^6 spores/ml) were added to three 125-ml DeLong culture flasks containing 20 ml of H-25 medium. The flasks were incubated at 25°C for 7 days and spores were harvested and counted as described above.

Three lemons, size 95 (i.e., 95 fruit/17.2 kg carton; approximately 146 cm^2 surface area per fruit) (30), were inoculated as previously described with one of the following isolates: S1, S2, S3, R1, R2, R3 (Table 2). The lemons were incubated at 25°C and each fruit was examined macroscopically every 12 h to determine the latent period (time from inoculation to first signs of sporulation) for each isolate. After 7 days, the spores were harvested from each lemon as described for the competition in fruit experiment and the number of spores determined by hemacytometer counts.

Growth rate on culture medium. Spore suspensions (10^6 spores/ml) of each of six isolates (S1, S2, S3, R1, R2, R3) were stab-inoculated in the center of petri plates (five replicates per isolate) containing 15 ml of H-25 medium. The cultures were incubated at 25°C for 7 days and the colony diameters were measured. This test was repeated three times. Differences between the mean colony diameters of the six isolates were evaluated by analysis of variance (ANOVA) and the Waller-Duncan k-ratio *t* test.

RESULTS

Phenotype stability of imazalil-R and -S biotypes. Phenotypes of both R and S biotypes were stable with respect to their responses to imazalil over three spore generations on H-25 medium (Fig. 1A) and over two disease cycles in nontreated lemons (Fig. 2A) fruit. The S biotypes in each spore population declined rapidly when R/S mixtures were exposed to imazalil during the first spore generation on H-25 medium (Fig. 1B) and on lemons (Fig. 2B). Spore populations produced in subsequent generations in the absence of imazalil remained virtually 100% resistant.

Competition between R and S biotypes in nontreated fruit. In nontreated lemons inoculated with a mixture of R and S biotypes, the frequency of R biotypes in the spore population decreased significantly (*t* test, $P = 0.05$) over two disease cycles in 8 of 11 mixtures, and increased in 3 of 11 mixtures (Fig. 3). This experiment was repeated with similar results.

In a confirmatory experiment with nine R/S combinations followed over four disease cycles, seven of nine combinations decreased in percent resistance to almost zero in the second spore generation (Fig. 4). Resistance declined more slowly in spore populations that contained isolate R2, in which resistant spores constituted between 3 and 23% of the spore population after the fourth disease cycle.

Competition between R and S biotypes on solid culture medium. The proportion of imazalil resistance in nine R/S

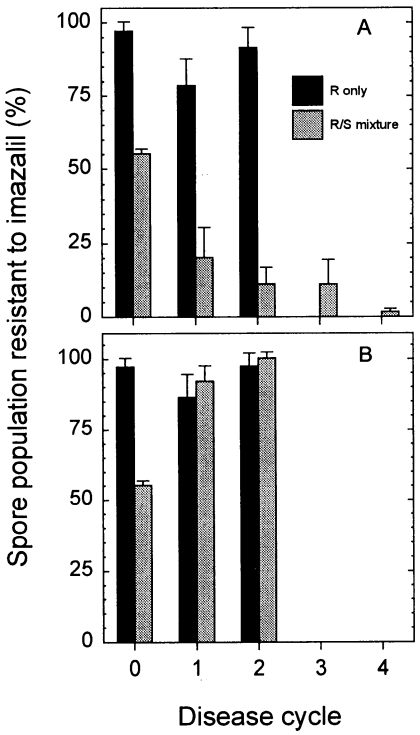

Fig. 2. Phenotype stability of imazalil-resistant (R) *Penicillium digitatum* alone (solid bars) and changes in R/sensitive (S) ratios (gray bars) in mixtures of two isolates inoculated into lemons **(A)** nontreated and **(B)** treated with imazalil (1g/liter) in the first disease cycle. Fruit for the second disease cycle were nontreated. Third and fourth disease cycles were not assayed for **(A)** R only and **(B)** R only and R/S mixture. Each bar represents the mean and standard error of three single (R1, R2, R4) or R/S isolate mixtures (R1/S4, R3/S4, R4/S4; Table 2).

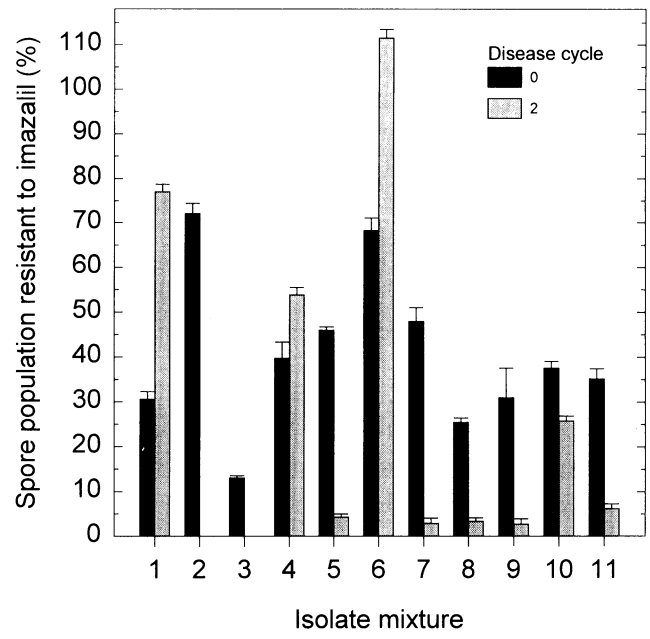

Fig. 3. Competition for sporulation between random pairs of 11 imazalil-sensitive and 11 imazalil-resistant isolates of *Penicillium digitatum* (Table 1) over two disease cycles in nontreated lemons. Each bar represents the mean and standard error obtained from three replicate lemons. Values for second disease cycle of isolate mixtures 2 and 3 are zero.

mixtures decreased gradually over four generations on H-25 medium without imazalil (Fig. 5). In general, frequency of isolate R2 declined more rapidly in the R/S mixtures than that of isolates R1 and R3. Isolate mixtures that were most stable in fruit (i.e., R2/S2 and R2/S3), did not show this characteristic on culture medium. Mixtures R2/S3 and R3/S2 could not be taken through the fourth generation due to contamination.

Spore production on culture medium and on diseased fruit. The number of spores harvested from cultures of R or S isolates on H-25 medium after 7 days at 25°C was between 3.9×10^7 and 1.9×10^8 (Fig. 6A). The number of spores harvested from a single lemon after inoculation with an S or R isolate and incubation under the same conditions was between 1.2×10^{10} and 2.1×10^{10}. Spore production of individual R and S isolates on culture medium or on fruit did not correlate with competitiveness in fruit. The latent period for all tested isolates ranged from 72 to 84 h with no apparent difference between R and S isolates.

Growth rate on culture medium. Mean colony diameters of isolates S2, R1, and R2 after 7 days at 25°C were significantly ($P < 0.05$) different from those of other tested isolates (Fig. 6B). Repeated experiments gave similar results. There was no evident correlation between the radial growth rate of an isolate and its competitiveness on fruit or on culture medium. Similarly, there was no obvious relationship between radial growth rate and spore production (Fig. 6A, B).

DISCUSSION

Major factors underlying the development of a fungicide resistance problem in *P. digitatum* include magnitude and duration of selection pressure, dispersal of resistant inoculum, and relative fitness of R and S biotypes (10,12,13,27). Imazalil is currently the most popular postharvest fungicide for control of decay of citrus fruits because it is highly effective against S biotypes of *P. digitatum* and *P. italicum* (1,2,8). This fungicide is applied one or more times at relatively high dosage rates (2 to 4 g/liter) in a wax formulation that covers the surface of the fruit. Substantial antifungal residues of imazalil persist for the postharvest life of the fruit (2). These conditions produce intense pressure for selection of imazalil-R biotypes in the *P. digitatum* population. In California, citrus fruits are often stored for several weeks, making it necessary to cull decayed fruit before marketing. This culling provides an efficient means for air-borne dispersal of spores of fungicide-R biotypes (8).

Relative fitness has been defined as "the ability of a given genotype to survive and reproduce in a given environment in comparison to the rest of the population in the same environment (13)." The observation that imazalil-R biotypes have reached an alarming frequency in California citrus packinghouses with a history of heavy imazalil use (16) indicates that R biotypes possess an advantage in relative fitness over the wild-type (S biotype) in imazalil-treated fruit. Conversely, the absence of R biotypes in groves, where imazalil is never applied, despite regular introduction of resistant spores on picking boxes, suggests that R biotypes may be less fit than S biotypes of *P. digitatum* in nontreated fruit. A significant fitness cost associated with imazalil resistance could justify a resistance management program based on rotation of imazalil with an unrelated fungicide (13,22).

We evaluated both parasitic fitness (latent period) and reproductive fitness (sporulation) of imazalil-R and -S biotypes alone and in pairs on lemons and culture medium. The relative fitness of R biotypes in R/S mixtures is important because the probability of successful infection increases with the number of *Penicillium* spores that occupy a wound site (17,33). Under natural packinghouse conditions, it is reasonable to expect a mixture of R and S biotypes at each wound site. The relative fitness of different biotypes in a mixed infection should be reflected in the frequency of their spores in each disease cycle (12).

We found that R biotypes of *P. digita-*

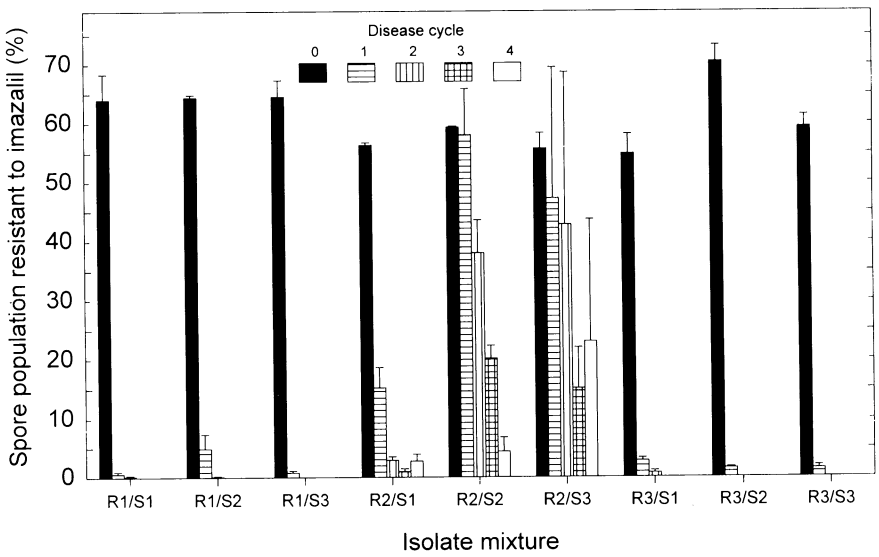

Fig. 4. Competition for sporulation between pairs of imazalil-sensitive (S) and -resistant (R) biotypes (nine possible combinations of three S and three R isolates) of *Penicillium digitatum* (Table 2) over four disease cycles in nontreated lemons. In S/R mixtures containing R1 or R3, percent R in spore population was zero in generation 3; these mixtures were not carried into the fourth generation. Each bar represents the mean and standard error of spore populations obtained from three replicate lemons.

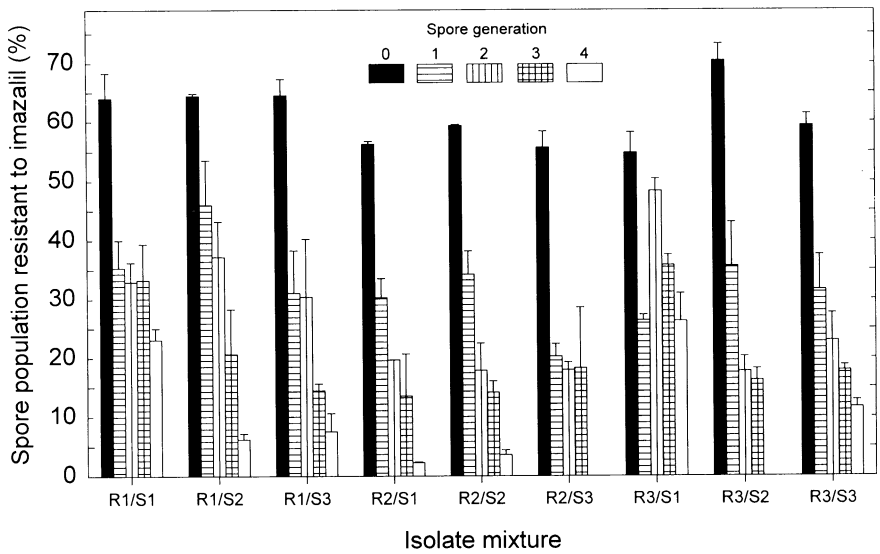

Fig. 5. Competition for sporulation between pairs of imazalil-sensitive (S) and -resistant (R) biotypes (nine possible combinations of three S and three R isolates) of *Penicillium digitatum* (Table 2) over four generations on H-25 culture medium. Data for mixtures R2/S3 and R3/S2 at generation four are missing. Each bar represents the mean and standard error of three replicate cultures.

tum were more fit than S biotypes on imazalil-treated fruit, as reported earlier for *P. italicum* (6). On nontreated fruit and culture medium without imazalil, R biotypes were generally less competitive than S biotypes, a phenomenon that is in agreement with other studies on *P. digitatum* and *P. italicum* (5,6,29,31). The reduced competitiveness of R biotypes was expressed to a greater degree on nontreated lemons inoculated with R/S mixtures than on nonamended culture medium.

The imazalil-R biotypes varied in relative fitness; some persisted or increased through several spore generations in R/S mixtures on fruit while others did not. The generally lower competitiveness of an R biotype in mixture with an S biotype cannot be explained by phenotype instability of the R biotypes as suggested by other investigators (4,19,31). We found that R biotypes were stable in pure culture on H-25 medium through six transfers, and in fruit over two disease cycles while the S component in R/S mixtures was virtually eliminated by passage through an imazalil-treated fruit or imazalil-amended culture medium.

We confirmed that 0.1 µg of imazalil per ml in the culture medium was the optimum selective concentration for enumerating spores of R biotypes (6). Culture medium amended with 0.25 µg of imazalil per ml, as specified in other investigations (29,31), suppressed colony formation by some R isolates, resulting in an underestimation of their frequency in the spore population.

Resistance to imazalil and other demethylation inhibitors is controlled by a polygenic system in some fungi (20,32). There is evidence that the fitness cost associated with fungicide resistance increases with the number of genes that must mutate to produce a practical level of resistance to demethylation inhibitors (12,13, 27) and dicarboximides (12,18). However, a recent investigation did not find a correlation between fitness of *Pyrenophora teres* isolates and resistance to the demethylation inhibitors triadimenol and propiconazole (24). Other investigators have observed no fitness loss in pathogens resistant to sterol biosynthesis inhibitors and other site-specific fungicides (12,14,26, 28). We were not able to correlate the general low competitive fitness (in R/S pairs) of imazalil-R biotypes of *P. digitatum* with reduced parasitic fitness (latent period) or reproduction rate (sporulation) of imazalil-R biotypes in nontreated lemons or culture medium. The general loss in fitness of imazalil-R isolates apparently is expressed only in competition with S biotypes.

The reduced competitive behavior of imazalil-R biotypes, in mixed infections with S biotypes, should encourage an evaluation of resistance-management programs involving the rotation of imazalil with a nonselective fungicide. This strategy should be especially effective following packinghouse sanitation to reduce the overall *Penicillium* spore population since only imazalil-S spores are brought in on fruit from the grove. However, the continued intensive use of imazalil could adversely affect this strategy. The reduced competitive behavior of imazalil-R biotypes of *P. digitatum*, expressed only in nontreated fruit, could be jeopardized by the acquisition of fitness-modifying genes under selection pressure. The development of increased fitness among pesticide-resistant fungi, insects, and weeds has been discussed (11). The build-up of biotypes with multiple resistance to imazalil, thiabendazole, and *o*-phenylphenol in recent years increases our concern that treatment of fruit with either of the latter fungicides could sustain the imazalil-R phenotype in the *Penicillium* spore population, at least temporarily. Multiple-resistant biotypes appeared to be responsible for maintenance of benzimidazole resistance in the *P. digitatum* population long after the thiabendazole treatment was discontinued (10).

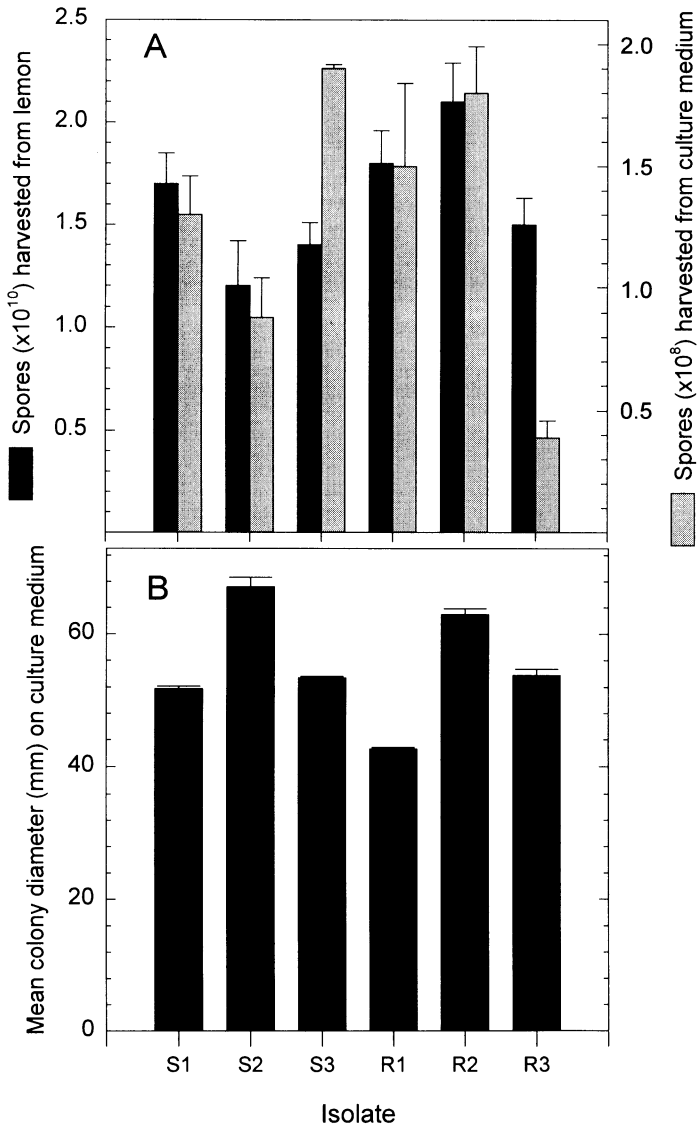

Fig. 6. (A) Mean number of spores of imazalil-resistant (R) and -sensitive (S) biotypes of *Penicillium digitatum* harvested from a single lemon or from a single culture on H-25 medium incubated at 25°C for 7 days. Each bar represents the mean and standard error of three replicate lemons or three culture plates. **(B)** Mean colony diameter of *P. digitatum* isolates after 7 days at 25°C on H-25 medium. Each bar represents the mean and standard error of five replicate cultures.

ACKNOWLEDGMENTS

This research was supported by grants from the California Citrus Research Board. The assistance of M. Ratnayake and J. Sievert is gratefully acknowledged.

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