Determination of Natural Resistance Frequencies in *Penicillium digitatum* Using a New Air-Sampling Method and Characterization of Fludioxonil- and Pyrimethanil-Resistant Isolates

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ABSTRACT

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Fungicide resistance was identified in natural populations of *Penicillium digitatum,* the causal agent of green mold of citrus, to two of three new postharvest fungicides before their commercial use. Using a new air-sampling method where large populations of the pathogen in citrus packinghouses were exposed to agar plates with a continuous, wide-range fungicide concentration gradient, isolates with reduced sensitivity to fludioxonil or pyrimethanil were obtained. Resistance frequencies to fludioxonil and pyrimethanil were calculated as 9.5×10^{-7} to 1.5×10^{-5} and 7.3×10^{-6} to 6.2×10^{-5}, respectively. No isolates resistant to azoxystrobin were detected. Isolates with reduced sensitivity to fludioxonil or pyrimethanil were also obtained in laboratory selection studies, where high concentrations of conidial mixtures of isolates sensitive to the three fungicides were plated onto agar amended with each fungicide at 10 µg/ml. Isolates obtained from fludioxonil selection plates in laboratory and packinghouse experiments were placed into two categories based on mycelial growth: moderately resistant isolates had 50% effective concentration (EC_{50}) values of 0.1 to 0.82 µg/ml and highly resistant isolates had EC_{50} values > 1.5 µg/ml. Isolates resistant to pyrimethanil all had EC_{50} values >8 µg/ml. Representative isolates of the two categories with reduced sensitivity to fludioxonil varied widely in their virulence and sporulation capacity as measured by the incidence of decay and degree of sporulation on inoculated fruit, respectively, whereas pyrimethanil-resistant isolates were mostly similar to the wild-type isolate. Fungicide sensitivity characteristics for isolates from fludioxonil and pyrimethanil selection plates remained stable after passages on nonamended agar, and disease could not be controlled after treatment with the respective fungicides. Types of fungicide resistance were visualized on thiabendazole- (TBZ) and imazalil-amended selection plates that were exposed in packinghouses where resistance to these fungicides was known to occur. The qualitative, single-site resistance to the benzimidazole TBZ was visualized by two distinct subpopulations in regard to fungicide sensitivity, whereas the quantitative, multi-site resistance to the demethylation inhibitor imazalil was apparent as a continuous density gradient of colonies along the fungicide concentration gradient. Types of resistance could not be assigned to fludioxonil or pyrimethanil because a limited number of resistant colonies was obtained on each plate. Thus, with this new method, we were able to estimate fungicide resistance frequencies as well as characterize and visualize types of resistance within populations of a fungal species. This information will be used to design resistance management strategies for previous and newly registered postharvest fungicides of citrus.

The development of fungicide resistance has greatly impacted the management of many pre- and postharvest fungal diseases. Among postharvest pathogens, species of *Penicillium* have a particularly high risk of becoming resistant to fungicides, mainly due to the pathogens' enormous asexual reproduction potential (16). Thus, a higher number of individuals less sensitive to a fungicide within these pathogen populations may occur than for many other fungi, and these individuals may be favored if the selection pressure continues. In postharvest management of citrus decays, this risk of fungicide resistance development in *Penicillium* populations is exacerbated by the year-round availability of susceptible host tissues because, in packinghouse operations, fruit are received, processed, and stored almost continuously. Furthermore, in California lemon (*Citrus limon* (L.) N. L. Burm.) production, fruit are often stored for extended times based on market needs and economic incentives. Decay commonly develops in storage and, during fruit processing before marketing, fungal inoculum easily spreads in the packinghouse to healthy fruit and to fruit coming from the field if strict sanitation practices are not followed.

Thiabendazole (TBZ) and imazalil, two postharvest fungicides that have been used by the citrus industry for >25 years, have been the primary postharvest treatments for managing the major postharvest decays of citrus, green mold caused by *Penicillium digitatum* (Pers.) Sacc. and blue mold caused by *P. italicum* Wehmer. Resistance to these two fungicides is widespread in pathogen populations, and treatment efficacy of these originally highly effective compounds is often compromised (5,10,17). Resistance to the benzimidazole TBZ is characterized as qualitative because a sudden shift from sensitivity to high-level resistance occurs due to mutation of a single or small number of major genes (16). Resistance to the demethylation inhibitor imazalil is characterized as quantitative or multi-step due to the mutation of several genes, each contributing to the development of fungicide insensitivity and leading to a gradual decrease in fungicide efficacy (16). Three new postharvest fungicides have been recently registered in the United States on citrus: the quinone outside inhibitor (QoI) azoxystrobin, the phenylpyrrole fludioxonil, and the anilinopyrimidine pyrimethanil (12). No resistance to these

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new fungicides in *Penicillium* spp. of citrus has been reported to date in packinghouses and the potential to develop resistance is not known.

To prevent or delay the development of resistance, treatment strategies that include sanitation, the use of fungicide mixtures, and optimal application methods have been developed for an integrated decay management system (14). In addition, routine monitoring for fungicide resistance is a common practice in many packinghouses, with the goal of detecting any shifts in fungicide sensitivity in the pathogen population at an early stage so that treatment strategies can be adjusted and the build-up of resistance may be avoided.

The standard monitoring method involves the exposure of agar plates amended with a discriminatory concentration of the fungicide of interest in the packinghouse (1). These plates are incubated and development of *Penicillium* spp. colonies is compared with nonamended plates that were exposed at the same time. This method has served the industry well in determining current resistance frequencies for imazalil and TBZ where resistance has been well characterized. For the newly registered fungicides, however, where baseline sensitivity ranges are known but resistance has not been described under field conditions, single discriminatory concentrations needed for effective monitoring programs have not been determined. This deficiency will render the use of the standard air-sampling technique problematic and, especially if quantitative resistance with a range of insensitivities is involved, an accurate assessment of fungicide sensitivity in a pathogen population will not be obtained. Furthermore, rare phenotypes are likely to remain undetected using this method because only a limited number of isolates is being evaluated.

Because the early detection of resistance is important for prolonging the effectiveness of the newly registered citrus postharvest fungicides (i.e., azoxystrobin, fludioxonil, and pyrimethanil) (22), one objective of our study was to develop a new air-sampling technique for citrus packinghouses that does not require prior knowledge about potential degrees of resistance. Other objectives were to obtain an estimate of the natural resistance frequencies in populations of *P. digitatum* for these new fungicides as an indicator for the risk for resistance development under field conditions and to characterize resistant isolates that were recovered in these studies. Furthermore we used the new air-sampling method to visualize frequencies and degrees as well as types of resistance for imazalil and TBZ in air samplings of packinghouses where resistance in *P. digitatum* to these two fungicides was known to occur.

MATERIALS AND METHODS

Fungal isolates and fungicides. Isolates of *P. digitatum* were maintained as mycelial plugs in sterile water at 4°C for up to 1 year. The single-spore isolate Pd that is sensitive to all fungicides included in the current study was used as a reference culture. Isolates used in the laboratory selection studies (see below) were part of baseline populations that were previously characterized for their fungicide sensitivity (13). Additional isolates were collected from decaying fruit in citrus packinghouses and were single spored before use. For conidial production, mycelial plugs were transferred to potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) and petri dishes were incubated for 5 to 10 days at 25°C. Spore inoculum was prepared in 0.01% Tween 20 (Sigma-Aldrich, St. Louis, MO) and adjusted to 1×10^6 conidia/ml with sterile water. Single-spore colonies were produced from spore suspensions (≈2,000 conidia/ml) that were vortexed for 1 min to separate conidial chains before plating onto 10-cm PDA dishes using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA) set for the exponential application mode. Colonies developing in the most diluted area of the agar plate were transferred and were considered single-spore isolates.

Fungicides used were formulated products of azoxystrobin (Abound 2.08F; Syngenta Crop Protection, Greensboro, NC), fludioxonil (Scholar 50WP; Syngenta Crop Protection), imazalil (Freshgard 700; Janssen Pharmaceutica, Titusville, NJ), pyrimethanil (Penbotec 400SC; Janssen Pharmaceutica and Scala 600SC; Bayer CropScience, Kansas City, MO), and thiabendazole (TBZ; Mertect 340-F; Syngenta Crop Protection). Aqueous solutions of the fungicides were used in all assays. All fungicide concentrations were based on the active ingredient.

Estimation of resistance frequencies in populations of *P. digitatum* for fludioxonil and pyrimethanil in laboratory selection studies. For selection of resistant isolates, conidial suspensions of mixtures of single-spore isolates of *P. digitatum* were plated onto petri dishes (150 mm in diameter) containing PDA amended with either fludioxonil or pyrimethanil at 10 µg/ml. For fludioxonil, mixtures of 24 baseline isolates from a previous study (13) (2.5×10^9 conidia/ml) or 42 newly obtained isolates from decayed lemon fruit in a packinghouse (3×10^9 conidia/ml) were used. For pyrimethanil, mixtures of 24 baseline isolates or an additional 21 isolates from lemon fruit in a citrus packinghouse (both at 1×10^6 conidia/ml) were used. In addition to the baseline isolates, all newly obtained isolates were determined to be azoxystrobin-, fludioxonil-, and pyrimethanil-sensitive (described below). Each of these conidial suspensions contained an equal number of conidia from each mixture isolate. Suspensions were plated out with a spiral plater using the uniform mode of application, where 63.5 µl of suspension is uniformly spread onto the agar surface. This resulted in a deposition of 1.6×10^8 or 1.9×10^8 conidia/plate for fludioxonil and 6.3×10^4 conidia/plate for pyrimethanil. For each spore suspension, 5 to 10 selection plates (replications) were used. After 3 days of incubation at 25°C, the number of green sporulating colonies of *P. digitatum* was recorded for each plate. The resistance frequency (Rf) was calculated as the proportion of the number of these putative resistant colonies of the total number of conidia plated. A subset of 13 colonies for each fungicide was transferred to nonamended PDA plates using a sterile toothpick and single-spore isolates were obtained that were characterized for their sporulation capacity using a scale of 0 to 4 (where 0 = no or negligible sporulation and 1 = ≤25, 2 = 26 to 50, 3 = 51 to 75, and 4 = ≥76% of the colony surface with green sporulation) and evaluated in fungicide sensitivity and lemon fruit inoculation tests.

Estimation of resistance frequencies in populations of *P. digitatum* for fludioxonil, pyrimethanil, and azoxystrobin in air samplings of citrus packinghouses. Resistant isolates in citrus packinghouses were selected in samplings for airborne spores. These studies were conducted in three packinghouses in the late spring season when decay levels of stored fruit are usually the highest of the year. Fungicide-amended agar plates (15 cm in diameter) that were used for sampling were prepared by spiral gradient dilution (SGD) 4 to 24 h before sampling using the exponential deposition mode of the spiral plater (6). For fludioxonil and pyrimethanil, PDA was used, whereas, for azoxystrobin, PDA was amended with salicylhydroxamic acid (SHAM; Sigma-Aldrich) at 100 µg/ml prepared in 50% ethanol and added to the cooled media. After spiral plating of the fungicides, these plates contained a continuous radial fungicide concentration gradient, with the highest concentration in the center of the plate and the lowest concentration at the edge of the plate. The 95% effective concentration (EC_{95}) sensitivity range for each fungicide as established for mycelial growth of baseline populations (i.e., 0.13 µg/ml for fludioxonil, 0.21 µg/ml for azoxystrobin, and 0.30 µg/ml for pyrimethanil) was located ≈1.5 to 2.5 cm from the edge of the petri dish (13). The required stock solutions to obtain this were calculated using the SGE software (version 1.1; Spiral Biotech, Inc.) and were determined to be 500 µg/ml for fludioxonil and 1,000 µg/ml for azoxystrobin and pyrimethanil. These stock solutions resulted in radial concentration gradients of 0.026 to 4.0 µg/ml

for fludioxonil, 0.037 to 7.7 μg/ml for azoxystrobin, and 0.063 to 7.8 μg/ml for pyrimethanil.

For air sampling, plates were exposed with their lids removed in areas of the packinghouse with a high spore load (i.e., areas where lemon fruit are taken out of storage to be processed for shipping) or in storage rooms. Exposure times were 0.5 to 5 min, depending on the amount of Penicillium decay observed on the fruit. To minimize the amount of contamination with *Rhizopus* and *Mucor* spp., plates were placed at least 1 m above the floor level. For each fungicide, five selection plates were used in each of four sampling studies (two samplings in each of two packinghouses conducted on different dates). Plates were taken back to the laboratory and incubated as described for the laboratory selection studies. Similarly, the number of colonies growing at fungicide concentrations $>EC_{95}$ were enumerated and a subsample of colonies (5 to 20 sporulating colonies for each experiment, resulting in a total of 40 colonies) was cultured, single spored, and further characterized similar to isolates derived from the laboratory selection studies.

To estimate the average number of spores deposited onto the plates at each sampling, 15-cm petri dishes containing 50 ml of 0.01% (vol/vol) Tween 20 were exposed simultaneously with the fungicide selection plates. Conidial counts were obtained using a hemacytometer and the average total number of conidia in the 50 ml of solution was calculated from three replicated plates and used for calculating Rf values as described above for the laboratory selection studies.

Determination of in vitro fungicide sensitivities of putative resistant isolates of *P. digitatum*. The 50% effective concentration (EC_{50}) values for inhibition of mycelial growth were determined using the SGD method as described previously (6,13). Stock concentrations plated onto PDA plates for azoxystrobin (PDA-SHAM was used for this fungicide), fludioxonil, imazalil, pyrimethanil, and TBZ were 100, 50 and 200, 30 and 200, 100 and 1,000, and 200 and 1,000 μg/ml, respectively. After 3 to 4 h of incubation to allow a continuous fungicide gradient to form, 10-μl droplets of conidial suspensions (1×10^6 conidia/ml) of *P. digitatum* were streaked out radially across the gradient. After 3 days of incubation at 25°C, the radial distance where mycelial growth was inhibited by 50% compared with the control was entered into the SGE software and translated into a local fungicide concentration (6). Isolates were rated for their sensitivity to each fungicide in comparison with baseline sensitivity values (13) and were categorized as sensitive (S; EC_{50} value within baseline), sensitive but outside baseline (S-OBL; EC_{50} value <10 times above baseline), or resistant (R; EC_{50} value at least 10 times the baseline value). For fludioxonil, resistant isolates were differentiated as moderately resistant (MR; EC_{50} value of 0.1 to 0.82 μg/ml) or highly resistant (HR; EC_{50} value >1.5 μg/ml).

Evaluation of pathogenicity, virulence, sporulation capacity, and response of resistant isolates to fungicide treatments in lemon fruit inoculation studies. Lemon fruit cv. Eureka grown using commercial practices and without the use of any preharvest fungicide treatments were hand washed in water with a dishwashing detergent, surface disinfested by dipping into sodium hypochlorite (100 μg/ml), rinsed with tap water, and placed into plastic fruit trays in cardboard fruit boxes. For evaluation of pathogenicity (ability to cause disease), virulence (incidence of decay), sporulation capacity (amount of asexual reproduction), and response to fungicide treatments, fruit were wounded using a 1-by-2-mm nail-like, stainless-steel probe without injuring the juice sacks below the albedo and then inoculated with 20-μl drops of inoculum (1×10^6 conidia/ml) of *P. digitatum*. For evaluation of the sporulation capacity on decaying fruit, lemon fruit were inoculated at the center of the fruit using a syringe. Representative isolates from packinghouse selections less sensitive to fludioxonil (12MR, 13HR, FL7, and FL12) or pyrimethanil (1, 10a, 5AP, and BF) were compared with the sensitive isolate Pd. Fruit boxes were covered with plastic bags and incubated at 20°C and >90% relative humidity for 14 to 16 h. For treatment, fruit were sprayed with aqueous solutions of azoxystrobin, fludioxonil, imazalil, pyrimethanil, or TBZ, (all at 1,000 μg/ml) on the inoculated side using a hand-operated atomizer (Model 15-RD; DeVilbiss Health Care, Somerset, PA). Fruit for sporulation evaluation were only treated with fludioxonil or pyrimethanil. Fruit of the control treatment were sprayed with water. Fruit boxes covered with plastic bags were incubated for 6 to 7 days at 20°C and >90% relative humidity. Fruit were then inspected carefully for green mold development that was either easily visible as mycelium- or conidia-covered decay or was present as soft, often watery lesions around the inoculation site. Virulence was determined based on the disease incidence that was calculated using the number of decayed fruit divided by the total number of fruit inoculated. Sporulation capacity was determined based on fungal sporulation on the fungicide-treated side of the fruit and was rated using a scale of 0 to 4, where 0 = no or negligible sporulation and 1 = ≤25, 2 = 26 to 50, 3 = 51 to 75, and 4 = ≥76% of the fruit surface with green sporulation. There were three to four replications of 10 to 15 fruit for each treatment and experiments were performed two or three times.

Visualization of qualitative and quantitative types of fungicide resistance on fungicide selection plates after air sampling of *P. digitatum* populations in citrus packinghouses. SGD selection plates were prepared as described above using a spiral plater but plates were amended with imazalil or TBZ. Stock concentrations applied were 200 μg/ml for imazalil and 600 μg/ml for TBZ. This again localized the 95% inhibitory concentrations of the two fungicides (i.e., 0.047 and 0.29 μg/ml for imazalil and TBZ, respectively) within 10 to 15 mm of the outer periphery of the agar plates. Three replicate plates in each of two experiments were exposed to airborne populations of *P. digitatum* as described above in a citrus packinghouse where resistance was known to occur. After incubation, fungal growth on the plates was characterized based on the distribution and density of colonies. Cultures were established from colonies derived within the inner zone, where fungicide concentrations are greater than the EC_{95} values (i.e., 20 to 49 mm from the center of the plate) and the outer zone, where fungicide concentrations are lower than the EC_{95} values (i.e., 15 to 25 mm from the periphery) of the plate and, in total, 9 to 21 colonies from each zone were evaluated for their fungicide sensitivity. Stock concentrations applied to SGD plates for colonies from imazalil selection plates were imazalil at 30 and 200 μg/ml and concentrations for colonies from TBZ selection plates were TBZ at 200 and 1,000 μg/ml.

Statistical analysis of data. Data for decay incidence were arcsine transformed. Bartlett's test for homogeneity of variances was performed for repeated experiments. Data sets with homogeneous variances ($P < 0.05$) were combined and then analyzed using a one- or two-way arrangement of data depending on the experiment. For error control, all treatments were in a randomized complete-block design. Values were analyzed using general linear model or analysis of variance and least significant difference (LSD) mean separation procedures of SAS (version 9.1; SAS Institute, Cary, NC).

RESULTS

Estimation of resistance frequencies in populations of *P. digitatum* for fludioxonil and pyrimethanil in laboratory selection studies. In each of the two experiments conducted, sporulating colonies of *P. digitatum* developed on selection plates amended with fludioxonil or pyrimethanil at 10 μg/ml. For these putative resistant isolates, Rf values were calculated based on the total number of conidia applied to a single plate. For fludioxonil, similar ($P = 0.069$) average Rf values were obtained for the two

experiments that ranged from 9.7×10^{-8} to 1.3×10^{-7} (Table 1). For pyrimethanil, values from different experiments were significantly ($P = 0.017$) different and ranged from 6.9×10^{-5} to 1.1×10^{-4}.

When colonies were transferred onto nonamended agar media, cultural morphology, including the amount of sporulation of colonies obtained from pyrimethanil-amended plates, was similar to the wild-type isolate Pd (rating of 4 for each). Colonies originating from the fludioxonil selection plates, however, showed reduced sporulation (rating of 2.5) compared with isolate Pd (rating of 4).

Estimation of resistance frequencies in populations of *P. digitatum* for fludioxonil, pyrimethanil, and azoxystrobin in air samplings of citrus packinghouses. Representative air-sampling plates for fludioxonil, pyrimethanil, and azoxystrobin after 3 days of incubation are shown in Figure 1A, B, and C, respectively. For fludioxonil and pyrimethanil, a high density of *P. digitatum* colonies was found as a concentric ring in the outer periphery of the plate where local fungicide concentrations were less than the EC_{95} values. After 5 days of incubation, most of these colonies were sporulating with the typical green color of *P. digitatum*. A low number of colonies (generally <20) was present in the higher fungicide concentration range toward the center of the plate. Most of these colonies remained <1 mm in diameter and never produced spores but a few started expanding and sporulating. These latter colonies were considered putative resistant isolates and were cultured on PDA not amended with fungicides, single spored, and characterized in subsequent studies. Based on the number of putative resistant colonies and the total number of spores deposited on each plate that was derived from spore counts in dishes with an aqueous Tween 20 solution (estimated between 1.0×10^5 and 4.1×10^6 conidia/plate), Rf values were calculated. For fludioxonil, no significant ($P = 0.267$) difference among average Rf values for the four air samplings was found although values ranged from 9.5×10^{-7} to 1.5×10^{-6} (Table 1). For pyrimethanil, three of the samplings resulted in similar Rf values (7.3×10^{-6} to 4.7×10^{-6}), whereas a forth one had a significantly ($P < 0.0001$) higher frequency of 6.2×10^{-5} (Table 1).

TABLE 1. Estimates of resistance frequencies for fludioxonil and pyrimethanil in populations of *Penicillium digitatum* based on laboratory studies or packinghouse air samplings

Fungicide, population[y]	Resistance frequency[z]
Fludioxonil	
Lab-1	1.3×10^{-7} a
Lab-2	9.7×10^{-8} a
Air-sampling 1	7.4×10^{-7} a
Air-sampling 2	3.9×10^{-7} a
Air-sampling 3	9.5×10^{-7} a
Air-sampling 4	1.5×10^{-6} a
Pyrimethanil	
Lab-1	1.1×10^{-4} a
Lab-2	6.9×10^{-5} b
Air-sampling 1	7.3×10^{-6} a
Air-sampling 2	5.6×10^{-6} a
Air-sampling 3	6.2×10^{-5} b
Air-sampling 4	4.7×10^{-6} a

[y] In laboratory studies, conidial mixtures of 24 (experiment Lab-1) or 42 (experiment Lab-2) isolates (1.5 to 1.9×10^8 conidia/selection plate) for fludioxonil and 24 (experiment Lab-1) and 21 (experiment Lab-2) isolates (6.3×10^4 conidia/plate) for pyrimethanil were plated onto fungicide-amended media. In packinghouse studies, 5.3×10^5 to 4.1×10^6 conidia/plate were air sampled for fludioxonil and 1.0×10^5 to 1.6×10^6 conidia/plate were air sampled for pyrimethanil in four experiments.

[z] Resistance frequency (Rf) is the proportion of resistant isolates found per total number of conidia sampled. Each population (laboratory or packinghouse air sampling) was statistically analyzed separately. Rf values within each population followed by the same letter are not significantly different ($P > 0.05$) based on an analysis of variance and least significant difference mean separation procedures.

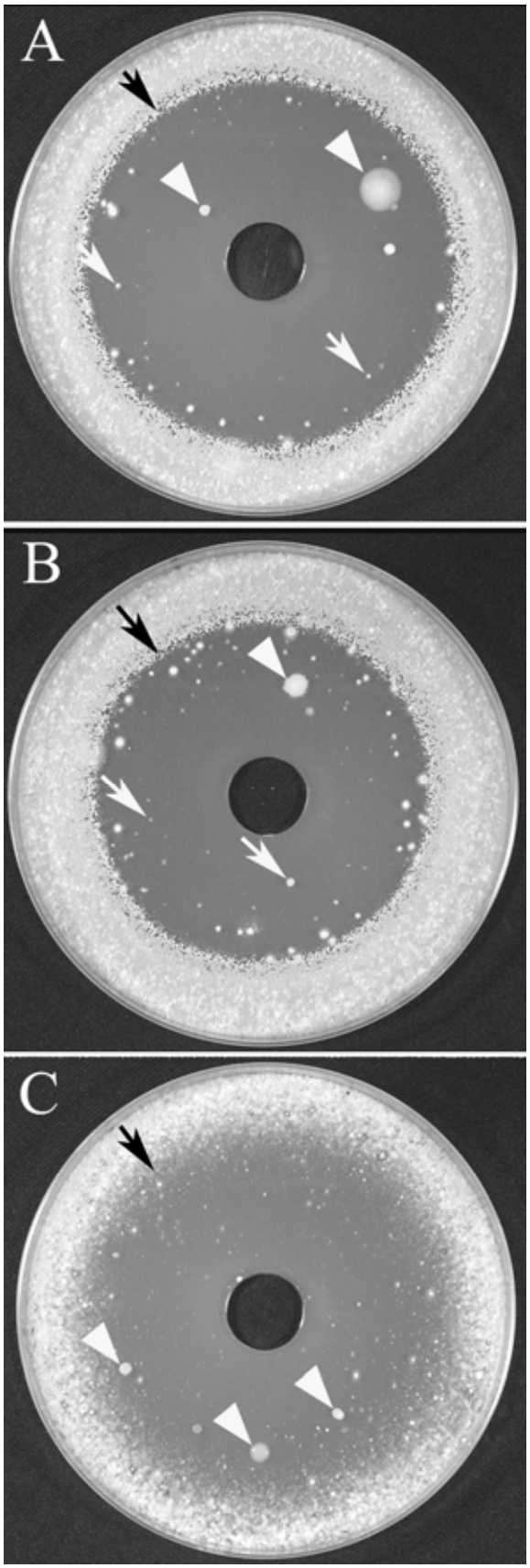

Fig. 1. Air-sampling spiral gradient dilution plates amended with **A,** fludioxonil; **B,** pyrimethanil; or **C,** azoxystrobin. Black arrows indicate the location of the 95% effective concentration of each fungicide. White arrows indicate some of the putative resistant isolates of *Penicillium digitatum*. Arrowheads indicate some of the contaminating fungal or bacterial colonies that develop during air-sampling of commercial packinghouses.

Selection plates amended with azoxystrobin also showed a high colony density in a concentric outer ring defined by fungicide concentrations of $\leq EC_{95}$ (Fig. 1C). Colonies in this area enlarged and sporulated after 5 days of incubation. A density gradient of smaller nonsporulating colonies developed in areas of the selection plates with higher concentrations of azoxystrobin, with fewer colonies present at increasing concentrations. When these colonies were transferred to nonamended PDA, they slowly enlarged but then stopped growing and never produced conidia. These colonies were not further characterized.

The vast majority of fungi developing on the selection plates was identified as *P. digitatum*. In most samplings, colonies of *P. italicum* as well as *Alternaria, Cladosporium,* and *Geotrichum* spp. developed but did not interfere with our selection studies. Contamination with *Rhizopus* and *Mucor* spp. was minimized by placing selection plates at least 1 m above the ground level.

Fungicide sensitivities of putative resistant isolates of *P. digitatum*. Putative fludioxonil- or pyrimethanil-resistant isolates were evaluated for their sensitivities to azoxystrobin, fludioxonil, pyrimethanil, imazalil, and TBZ. Results for representative isolates are shown in Table 2. All isolates evaluated were found to be sensitive to azoxystrobin, although EC_{50} values of some isolates were above the previously established baseline range (i.e., 0.007 to 0.028 µg/ml) (13).

Isolates obtained from fludioxonil selection plates in laboratory and packinghouse experiments were designated as moderately or highly resistant (Table 2). EC_{50} values of moderately resistant isolates from the laboratory selections were all close to 1 µg/ml, whereas those of packinghouse selections were 0.1 to 1 µg/ml. All isolates from the fludioxonil selection plates were categorized as sensitive to pyrimethanil although one isolate had an EC_{50} value of 0.909 µg/ml and, thus, was above the baseline range of 0.208 to 0.413 µg/ml. Isolates obtained in laboratory selections were sensitive to TBZ, whereas some isolates were resistant to imazalil. All isolates from the packinghouse selections were resistant to the latter two fungicides.

Isolates obtained from pyrimethanil selection plates in laboratory and packinghouse experiments were all resistant to pyrimethanil, with EC_{50} values >8 µg/ml, the highest concentration evaluated for pyrimethanil in the sensitivity studies. Highly resistant isolates had EC_{50} values >1.58 µg/ml. All isolates were sensitive to fludioxonil. Of the three isolates from laboratory studies that were evaluated for their imazalil and TBZ sensitivity, two were sensitive and one was resistant to both fungicides. All isolates from the packinghouse selections were resistant to imazalil and TBZ.

Fungicide sensitivities for fludioxonil and pyrimethanil of representative isolates were re-evaluated over the course of the studies. Resistance was found to remain stable using the same spiral gradient plate procedures and fruit inoculation assays (described below) after five to seven transfers on nonamended media and after storage at 4°C for 1 year (the longest storage time evaluated). These results are not presented.

Pathogenicity, virulence, sporulation capacity, and response of resistant isolates to fungicide treatments in lemon fruit inoculation studies. Pathogenicity was evaluated as the ability of isolates to cause decay of lemon fruit. All pyrimethanil- and fludioxonil-resistant isolates collected from selection plates were pathogenic. Fludioxonil-resistant isolates were either similarly (isolate 13HR) or significantly less (isolates FL7HR, FL12HR, and 12MR) virulent than the wild-type isolate Pd (Table 3, data for "untreated"). Isolate 12MR caused green mold decay on 65% of the fruit but only 23.3% of the fruit developed decay after inoculation with isolates FL7HR or FL12HR compared with 100% for isolate Pd. Three of the pyrimethanil-resistant isolates

TABLE 3. Efficacy of postharvest treatments against green mold decay of lemon fruit caused by isolates of *Penicillium digitatum* exhibiting different levels of sensitivity to fludioxonil

	Incidence (%)[z]				
	Wild type	Resistant isolates			
Treatment[y]	Pd	FL7HR	FL12HR	12MR	13HR
Untreated	100.0 A a	23.3 C b	23.3 C b	65.0 B a	90.0 A a
Azoxystrobin	20.0 A b	3.3 B c	0.0 B c	6.7 B bc	6.7 B c
Fludioxonil	14.2 C b	41.7 B a	33.3 B a	20.0 C b	68.3 A b
Imazalil	0.0 B c	0.0 B d	0.0 B c	8.3 A bc	3.3 AB c
Pyrimethanil	0.0 A c	0.0 A d	0.0 A c	0.0 A c	0.0 A c
Thiabendazole	0.0 C c	0.0 C d	5.0 C c	16.7 B b	70.0 A b

[y] All fungicides were applied as aqueous solutions at a concentration of 1,000 µg/ml using an air-nozzle sprayer 12 h after inoculation.

[z] Decay incidence is the percentage of inoculated fruit with decay. Incidence values followed by the same letter within a row (capitalized letters) or column (small letters) are not significantly different ($P > 0.05$) following an analysis of variance and least significant difference mean separation procedures. Fludioxonil-resistant isolates were selected either in laboratory assays (FL7HR and FL12HR) or in air samplings in citrus packinghouses (12MR and 13HR) (see text for details). Isolate Pd is sensitive to all currently registered and new fungicides.

TABLE 2. Sensitivities (50% effective concentration [EC_{50}] values) of fludioxonil- or pyrimethanil-resistant isolates of *Penicillium digitatum* selected from laboratory or packinghouse populations against citrus postharvest fungicides[w]

		Azoxystrobin[y]		Fludioxonil[y]		Pyrimethanil[y]		Imazalil[z]		TBZ[z]	
Fungicide, loc.[x]	Isolate	EC_{50} (µg/ml)	Sensitivity	EC_{50} (µg/ml)	Sensitivity	EC_{50} (µg/ml)	Sensitivity	EC_{50} (µg/ml)	Sensitivity	EC_{50} (µg/ml)	Sensitivity
Fludioxonil											
Laboratory	FL2MR	0.028	S	0.817	MR	0.289	S	0.105	S	0.046	S
	FL7HR	0.038	S-OBL	≥1.58	HR	0.310	S	0.022	S	0.055	S
	FL12HR	0.027	S	≥1.58	HR	0.190	S	≥0.24	R	0.810	S
Packinghouse	7MR	0.064	S-OBL	0.124	MR	0.162	S	≥0.24	R	≥1.57	R
	12MR	0.014	S	0.191	MR	0.109	S	≥0.24	R	≥1.57	R
	13HR	0.055	S-OBL	≥1.58	HR	0.919	S-OBL	≥0.24	R	≥1.57	R
	15HR	0.077	S-OBL	≥1.58	HR	0.160	S	≥0.24	R	≥1.57	R
Pyrimethanil											
Laboratory	10a	0.020	S	0.009	S	≥8	R	0.020	S	0.066	S
	10b	0.015	S	0.009	S	≥8	R	0.020	S	0.060	S
	5AP	0.022	S	0.023	S	≥8	R	≥0.24	R	≥1.57	R
Packinghouse	1AP	0.078	S-OBL	0.026	S	≥8	R	≥0.24	R	≥1.57	R
	9AP	0.061	S-OBL	0.022	S	≥8	R	≥0.24	R	≥1.57	R
	BF	0.029	S	0.026	S	≥8	R	≥0.24	R	≥1.57	R

[w] Sensitivity is defined as S = sensitive (within baseline), S-OBL = sensitive but outside baseline, R = resistant, MR = moderately resistant, and HR = highly resistant (see text for details).

[x] Fungicide and location.

[y] Baseline sensitivity ranges for azoxystrobin, fludioxonil, and pyrimethanil are 0.007 to 0.028, 0.009 to 0.072, and 0.208 to 0.413 µg/ml, respectively (13).

[z] Discriminatory concentrations used to characterize an isolate as resistant to imazalil or thiabendazole (TBZ) were 0.15 or 1 µg/ml, respectively.

showed virulence similar to the wild-type isolate Pd whereas one isolate (i.e., 10a) was slightly but significantly less virulent, causing 79.1% of the fruit to decay compared with 94.3% of the wild-type isolate (Table 4, data for "untreated").

Inoculated fruit were treated with each of the fungicides and their efficacy in reducing decay caused by the wild-type and resistant isolates was evaluated. The effect of the applied fungicides on disease incidence was highly significant ($P < 0.0001$) and there was a highly significant ($P < 0.0001$) interaction between isolates and treatments. Thus, data are presented separately for each treatment and isolate in Tables 3 and 4.

Green mold development on fruit inoculated with the fungicide-sensitive wild-type isolate Pd was significantly reduced by treatments with all five fungicides at a concentration of 1,000 µg/ml. Decay incidences were 0 to 20% compared with the untreated control, where 94.8 to 100% of the fruit developed green mold (Tables 3 and 4). After inoculation with either one of four fludioxonil-resistant isolates and treatment with fludioxonil, 20.0 to 68.3% of the fruit developed decay. The lowest incidence was observed for the intermediately resistant isolate 12MR (Table 3). Interestingly, incidence of decay caused by the two laboratory-selected highly resistant isolates FL7HR and FL12HR was significantly increased after treatment with fludioxonil compared with the untreated control for each isolate. Decay caused by fludioxonil-resistant isolates, however, was effectively controlled by treatments with azoxystrobin or pyrimethanil, resulting in a decay incidence of 0 to 6.6%. Although one of the laboratory-selected isolates (FL12HR) and the two packinghouse-selected isolates were characterized as resistant to imazalil (Table 2), this fungicide reduced decay levels to ≤8.3%. TBZ treatments prevented decay development after inoculation with the TBZ-sensitive isolate FL7HR whereas, for the three TBZ-resistant isolates, decay incidence was reduced by 22.2 to 78.5% compared with the untreated control for each isolate.

In contrast to fruit inoculated with the sensitive wild-type isolate Pd, treatments with pyrimethanil were not very effective in reducing green mold when inoculated with any of the four pyrimethanil-resistant isolates evaluated (Table 4). For isolate BF, decay incidence was reduced to 72% compared with the control with 100%. For isolate 10a, all fruit developed green mold after pyrimethanil treatment. Treatments with azoxystrobin or fludioxonil were effective for all isolates of *P. digitatum* evaluated but there was a significant difference in efficacy, although in vitro sensitivities to these fungicides were very similar among isolates (Table 2). Treatments with imazalil reduced decay to ≤15.0% for all isolates, whereas TBZ was highly effective in reducing decay caused by one of the pyrimethanil-resistant isolates but not for three additional ones that were found to be TBZ resistant (Table 2).

In evaluation of sporulation capacity, fludioxonil-resistant isolates sporulated significantly less on decaying fruit, with ratings of 1.0 to 2.63 compared with the wild-type isolate, with a rating of 4.0 (Table 5). After fludioxonil treatment, sporulation on lemon fruit inoculated with the wild-type isolate Pd was significantly reduced to a rating of 0.25. For three fludioxonil-resistant isolates, the degree of sporulation increased after fludioxonil treatment whereas, for the fourth isolate, there was no difference between treated and untreated fruit. All pyrimethanil-resistant isolates had high sporulation capacities, with ratings of 4.0 (Table 6). After treatment with pyrimethanil, sporulation was significantly reduced in inoculations with the sensitive and resistant isolates (ratings of 1.65 to 2).

Visualization of qualitative and quantitative types of fungicide resistance on fungicide selection plates after air sampling of *P. digitatum* populations in citrus packinghouses. Spiral gradient air-sampling plates amended with imazalil or TBZ were exposed in a citrus packinghouse where resistance to these fungicides was known to occur. Selection plates for both fungicides at local concentrations ≤EC_{95} again showed an outer concentric ring with a high colony density (>100/cm^2) (Fig. 2A and B). For both fungicides, a large number of colonies was present at higher fungicide concentrations toward the center of the plate, and these colonies sporulated after 5 days of incubation. The spatial distribution of these latter colonies was markedly different for the two fungicides. For TBZ, there was a distinct border between the high-density outer ring and the lower-density inner zone, and

TABLE 4. Efficacy of postharvest treatments against green mold decay of lemon fruit caused by isolates of *Penicillium digitatum* resistant to pyrimethanil

	Incidence (%)[z]				
	Wild type	Resistant isolates			
Treatment[y]	Pd	5AP	10a	1AP	BF
Untreated	94.3 A a	95.8 A a	79.2 B a	100.0 A a	100.0 A a
Azoxystrobin	3.8 BC b	1.3 C d	1.3 C b	10.0 B e	23.2 A c
Fludioxonil	5.0 BC b	12.5 B c	2.5 C b	23.3 A d	11.6 B c
Imazalil	0.0 C c	7.5 B cd	2.5 BC b	8.3 B e	15.0 A c
Pyrimethanil	0.0 D c	86.3 B b	100.0 A a	80.0 BC c	75.0 C b
Thiabendazole	0.0 D c	100.0 A a	0.0 D b	88.3 C b	95.0 B a

[y] All fungicides were applied as aqueous solutions at a concentration of 1,000 µg/ml using an air-nozzle sprayer 12 h after inoculation.

[z] Decay incidence is the percentage of inoculated fruit with decay. Incidence values followed by the same letter within a row (capitalized letters) or column (small letters) are not significantly different ($P > 0.05$) following an analysis of variance and least significant difference mean separation procedures. Pyrimethanil-resistant isolates were selected either in laboratory assays (5AP and 10a) or in air samplings in citrus packinghouses (1AP and BF) (see text for details). Isolate Pd is sensitive to all currently registered and new fungicides.

TABLE 5. Sporulation of *Penicillium digitatum* isolates with different sensitivities to fludioxonil on lemon fruit treated with fludioxonil

	Rating[z]				
	Wild type	Resistant isolates			
Treatment[y]	Pd	FL7HR	FL12HR	12MR	13HR
Untreated	4.00 A a	1.27 C b	1.27 C b	2.63 B a	1.00 C b
Fludioxonil	0.25 A b	2.60 A a	2.62 A a	3.13 A a	2.50 A a

[y] Fludioxonil was applied as an aqueous solution at 1,000 µg/ml using an air-nozzle sprayer 12 h after inoculation.

[z] Sporulation rating is based on a scale from 0 = no sporulation to 4 = fruit completely green. Incidence values followed by the same letter within a row (capitalized letters) or column (small letters) are not significantly different ($P > 0.05$) following an analysis of variance and least significant difference mean separation procedures. Fludioxonil-resistant isolates were selected either in laboratory assays (FL7HR and FL12HR) or in air samplings in citrus packinghouses (12MR and 13HR) (see text for details). Isolate Pd is sensitive to all currently registered and new fungicides.

TABLE 6. Sporulation of *Penicillium digitatum* isolates resistant to pyrimethanil on lemon fruit treated with pyrimethanil

	Rating[z]				
	Wild type	Resistant isolates			
Treatment[y]	Pd	5AP	10A	1AP	BF
Untreated	4.00 A a	4.00 A a	4.00 A a	4.00 A a	4.00 A a
Pyrimethanil	2.10 A b	1.90 AB b	1.79 BC b	1.65 C b	1.68 C b

[y] Pyrimethanil was applied as an aqueous solution at 1,000 µg/ml using an air-nozzle sprayer 12 h after inoculation.

[z] Sporulation rating is based on a scale from 0 = no sporulation to 4 = fruit completely green. Incidence values followed by the same letter within a row (capitalized letters) or column (small letters) are not significantly different ($P > 0.05$) following an analysis of variance and least significant difference mean separation procedures. Pyrimethanil-resistant isolates were selected either in laboratory assays (5AP and 10A) or in air samplings in citrus packinghouses (1AP and BF) (see text for details). Isolate Pd is sensitive to all currently registered and new fungicides.

colony density was quite uniform over the entire inner zone, indicating the presence of two populations of the fungus (Fig. 2A). For imazalil, there was a gradual decrease in colony density from the EC_{95} ring toward the center of the plate, indicating that isolates had a range of different sensitivities and that high concentrations of the fungicide were still completely inhibitory to fungal growth (Fig. 2B).

Colonies that were cultured from two areas (low and high fungicide concentrations) of imazalil and TBZ selection plates were evaluated for their fungicide sensitivity. For both fungicides, colonies from the outer zone were either sensitive or resistant to the respective fungicides whereas, for the inner zone, all isolates were identified as resistant (Table 7). EC_{50} values of resistant isolates for TBZ were all >7.825 μg/ml and, for imazalil, they were 0.25 to 1.136 μg/ml.

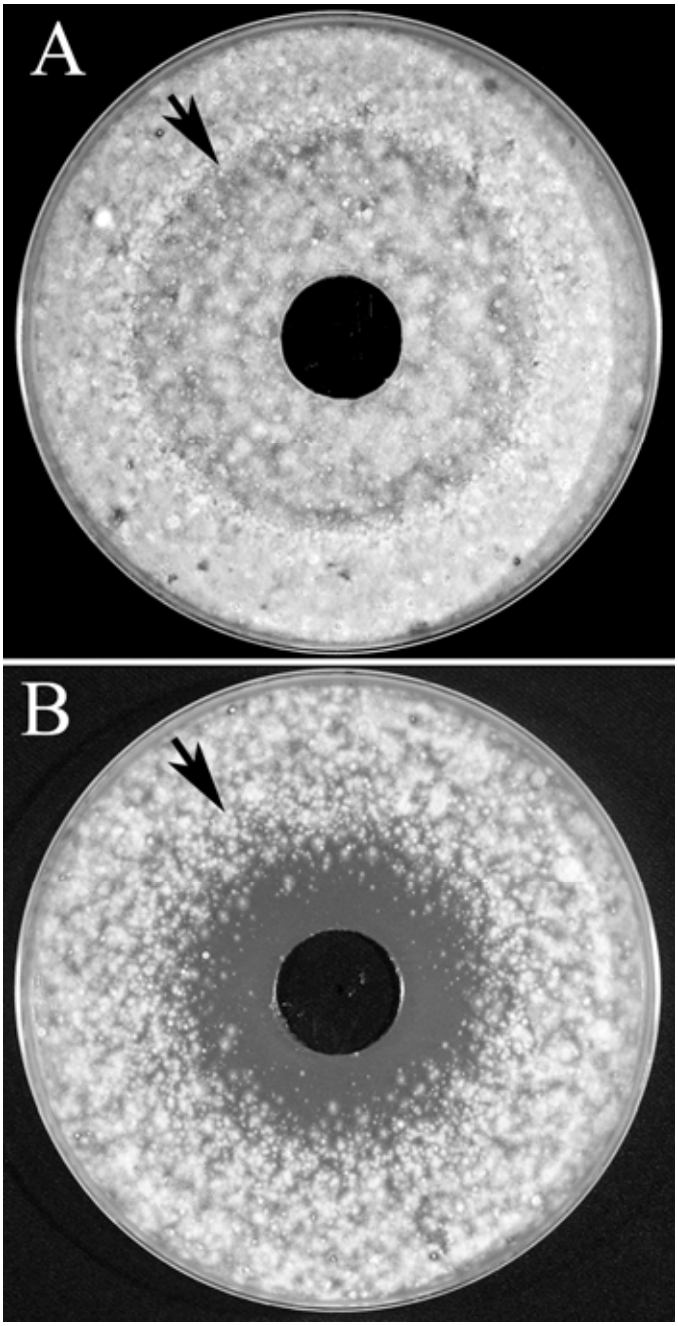

Fig. 2. Air-sampling spiral gradient dilution plates amended with **A,** thiabendazole (TBZ) or **B,** imazalil. Qualitative and quantitative resistance is visualized for TBZ and imazalil, respectively. Black arrows indicate the location of the 95% effective concentration of each fungicide.

DISCUSSION

The new spore air-sampling method described in this study allowed both visual assessment and quantitative analysis of a pathogen population regarding its fungicide sensitivity. Citrus packinghouses provide an ideal environment to use this method because spore densities of *Penicillium* spp. can be very high and, more importantly, there is a need for the continuous monitoring of fungicide sensitivities to aid in reassessing decay management strategies. Still, the method might be useful for other host–pathogen systems such as *Penicillium* spp. of pome fruit, where a similar high risk for resistance development exists, and in other systems, where high numbers of pathogen propagules are present. Because selection plates contain a wide gradient of fungicide concentrations (i.e., a 2.5-log concentration dilution from the center to the edge of a 15-cm agar plate), the assay can be used for fungicides where resistance has not been characterized and where discriminatory concentrations needed for traditional single-concentration selection plates have not been determined. EC_{95} ranges for sensitive baseline populations for each fungicide evaluated were derived from previous baseline sensitivity studies (13) and were positioned toward the outer edge of the plate. In our evaluations of large pathogen populations, this allowed most of the agar surface to be available for the selection of resistant isolates while, at the same time, visualizing the aerial spore density within the fungicide concentration range of $< EC_{95}$. Real-time polymerase chain reaction technology has been suggested to be used for the screening of large mixed populations by others (4). This strategy, however, can only be used when all loci conferring resistance have been thoroughly characterized on a molecular basis. Still, isolates with noncharacterized resistance mechanisms will remain undetected unless fungicide-amended media assays are used.

Isolates of *P. digitatum* less sensitive to fludioxonil or pyrimethanil were readily recovered in packinghouse samplings although, at the time when these experiments were conducted, neither fungicide was in commercial use. Thus, these isolates were selected from natural populations in contrast to other studies where resistance in *Penicillium* spp. was generated by mutagenesis in the laboratory (19,25). None of the selected isolates was resistant to both fludioxonil and pyrimethanil.

In our studies, we determined resistance frequencies by quantifying the number of *P. digitatum* colonies developing on fungicide-amended plates relative to the total number of spores deposited on control plates. Thus, this is the first time that natural resistance frequencies in a plant pathogen population were measured directly rather than theoretically estimated. Others have determined that thousands of isolates would need to be assessed to detect a resistant variant that occurs at a frequency of 1 in 1,000 within populations of *Erysiphe graminis* (4,9,24). Using our method, we sampled hundreds of thousands of isolates in each replication and experiment (Table 1); thus, the large sample population provided the most accurate estimate of frequencies of natural variants within a fungal population. Samplings at the packingline where lemon fruit were taken out of storage and processed for shipping yielded much higher colony densities on the selection plates than samplings in storage rooms. Thus, most samplings were conducted at the packingline. Furthermore, resistance frequencies within a single fungal species were shown to be different for the two fungicides with different modes of action in both laboratory and packinghouse populations. Therefore, we have described an intrinsic characteristic of resistance potential in this pathogen that varies with the fungicide being tested. Resistance frequencies in most samplings were higher for pyrimethanil than for fludioxonil, indicating that pyrimethanil-insensitive isolates occur at higher frequencies within the wild-type population of the fungus. Resistance frequencies for both fungicides and the potential risk of these isolates to become established, however,

must be considered high when taking into account the high reproduction potential of species of *Penicillium*.

Isolates with reduced sensitivity that occur at frequencies of 6.2×10^{-5} or lower, as determined for *P. digitatum* in our study, are unlikely to be detected in baseline sensitivity studies where only a limited number of isolates is included. Thus, by sampling large populations, our method allows the early detection of rare variants. Additionally, based on their natural occurrence, these less-sensitive isolates should be considered part of the baseline population. Therefore, resistance monitoring more accurately could be based on changes in resistance frequencies compared with a baseline population rather than on sensitivities of a limited number of isolates of baseline and test populations. This approach is feasible for *Penicillium* spp. populations in citrus packinghouses using the method described in this study, and shifts in sensitivities in large populations can be detected at an early stage.

For our selection plates, we used PDA. A minimum growth medium for evaluation of anilinopyrimidine (AP) fungicides such as pyrimethanil was evaluated in our previous studies with *P. digitatum* (13). We found that mean EC_{50} values using the AP medium were slightly, although significantly, lower compared with PDA but were consistently proportional. Because the AP medium is laborious to prepare and requires much longer incubation times before fungal growth can be measured (i.e., 5 to 6 days compared with 3 days on PDA), we chose to do our studies using PDA medium.

In contrast to fludioxonil and pyrimethanil, only atypical, slow-growing, nonsporulating colonies of *P. digitatum* were observed on azoxystrobin-amended selection plates that were exposed in citrus packinghouses. Thus, no resistance to azoxystrobin was detected in natural populations of this fungus, although resistant isolates were obtained by UV mutagenesis in the laboratory by others (25). This indicates that laboratory mutation studies may not give a realistic estimate of resistance risk. Resistance to QoI fungicides in the field has been described for a diverse range of fungal species, including powdery and downy mildew pathogens (11), as well as species of *Mycosphaerella* (7), *Alternaria* (20), and *Venturia* (18). In contrast, resistance to QoIs has not been reported to date for rust species and for *Alternaria solani*, and this has been correlated with the presence of an intron in the cytochrome b sequence directly after the codon for glycin at position 143, where mutations most commonly confer resistance (8). Such an intron, however, was not found in a comparison of laboratory-generated resistant and wild-type sensitive isolates of *P. digitatum* (25); thus, this molecular characteristic cannot explain the absence of resistant isolates in our selections. Although no naturally resistant isolates were found in our evaluations of very large populations of *P. digitatum*, the risk of development of resistance to azoxystrobin in this pathogen cannot be ignored. Perhaps, resistance frequencies for this fungicide are lower than could be detected in our studies.

Similar to the packinghouse samplings, isolates of *P. digitatum* with reduced sensitivity to fludioxonil or pyrimethanil but not azoxystrobin also developed on our laboratory selection plates that were inoculated with diverse conidial mixtures of isolates sensitive to these three fungicides. Individual isolates of the mixtures were also mostly sensitive to imazalil and TBZ; thus, in contrast to the packinghouse selections, isolates less sensitive to fludioxonil or pyrimethanil were mostly sensitive to imazalil and TBZ. The selection of resistant isolates from mass platings of sensitive, single-spore isolates cannot be easily explained. Neither of the fungicides (i.e., pyrimethanil or fludioxonil) is known to be a mutagen; thus, other mechanisms must be responsible. Parasexuality has been demonstrated for *P. chrysogenum, P. expansum,* and *P. italicum* based on the presence of heterokaryons and the subsequent formation of segregants from diploids (3,21). For *P. digitatum*, heterokaryosis could not be demonstrated positively (23) but rearrangement of genetic material in similar processes cannot be ruled out.

Representative isolates with reduced sensitivity to pyrimethanil were mostly similar to the sensitive wild-type isolate in their pathogenicity, virulence, and sporulation capacity. In contrast, anilinopyrimidine-resistant isolates of *Botrytis cinerea* were highly variable in their levels of sporulation but, on average, produced more spores on diseased host tissue than sensitive isolates (2). For fludioxonil, all isolates of *P. digitatum* in our studies were pathogenic but varied widely in their virulence and sporulation capacity. In addition, as described previously (15), isolates less sensitive to fludioxonil were variable in their in vitro sensitivity to the fungicide and were categorized as either moderately or highly resistant. Moderately resistant isolates from the laboratory selections all had relatively high EC_{50} values (close to 1 µg/ml) whereas those from the packinghouse displayed a wider distribution of sensitivities. This can be explained by the use of single-discriminatory concentration (i.e., 10 µg/ml) selection plates in the laboratory in contrast to selection plates in the packinghouse studies that contained a wide fungicide concentration range. Because insensitivity to pyrimethanil occurred at a higher frequency and insensitive isolates were all highly resistant and sporulated at the highest rating, the risk of field resistance to develop to this fungicide is likely to be higher than for fludioxonil. Still, isolates from both fludioxonil and pyrimethanil selection plates were stable in their fungicide sensitivity characteristics after several passages on nonamended agar, and disease could not be sufficiently controlled after treatment with the respective fungicides at a rate common to the commercial label of each fungicide. Thus, under selection pressure, these isolates could become predominant in a population. Studies on the com-

TABLE 7. Fungicide sensitivity of *Penicillium digitatum* isolates collected from selected locations of thiabendazole- (TBZ) or imazalil-amended spiral gradient dilution (SGD) plates used in air samplings in citrus packinghouses

Type of resistance[w]	Fungicide	Selection area on SGD plate[x]	Sensitivities[y]	*n*	EC_{50} (µg/ml)[z]	
					Mean	Range
Qualitative	TBZ	Outer zone	R	9	>7.825	>7.825
	…	…	S	11	0.205	0.059–0.852
	…	Inner zone	R	20	>7.825	>7.825
	…	…	S	0	…	…
Quantitative	Imazalil	Outer zone	R	3	0.343	0.254–0.400
	…	…	S	18	0.042	0.023–0.060
	…	Inner zone	R	9	0.783	0.329–1.136
	…	…	S	0	…	…

[w] Qualitative and quantitative types of fungicide resistance (16) are represented by TBZ and imazalil, respectively.

[x] Isolates of *P. digitatum* were arbitrarily selected from the outer or inner zones (see Materials and Methods) separated by the 95% effective concentration of a 15-cm SGD plate.

[y] R = resistant and S = sensitive.

[z] Discriminatory concentrations (EC_{50} = 50% effective concentration) used to characterize isolates using the SGD method as resistant to TBZ or imazalil were 1 or 0.15 µg/ml, respectively.

petitiveness of less-sensitive isolates for each fungicide in the presence of wild-type sensitive isolates will provide insight into their persistence in the absence of selection pressure.

Selection plates amended with TBZ or imazalil that were exposed in packinghouses where resistance to these fungicides was known to occur were used to visualize resistance quantitatively and qualitatively. Resistance was quantified based on the number of fungal colonies growing at fungicide concentrations $>EC_{95}$ compared with lower concentrations. Resistance was qualified based on the distribution of colonies on the agar surface that differed between the two fungicide classes. The two growth patterns could be correlated with the two known types of resistance (16). The qualitative type of resistance for the benzimidazole TBZ that is based on a single genetic site was represented by two distinct subpopulations in regard to fungicide sensitivity, whereas the step-wise changes characteristic for quantitative resistance that is typical for the demethylation inhibitor group of fungicides (e.g., imazalil) were apparent as a continuous density gradient of colonies along the fungicide concentration gradient. Thus, the two types of resistance could be verified by mass samplings of fungal propagules on selection plates. Types of resistance could not be assigned to fludioxonil or pyrimethanil because a limited number of resistant colonies were obtained on each plate. The air-sampling method described is to serve primarily for monitoring purposes but eventually may be used, in part, to characterize presumptive resistance types if resistance phenotypes become prevalent.

The method described in this study is amenable for use in routine monitoring for resistance in airborne organisms where large populations can be sampled. During our relatively short exposure times, contamination of selection plates was negligible. For situations or host–pathogen systems that require plates to be exposed for longer times, the use of a selective medium may be necessary.

The results presented provide information for characterizing the resistance risks for the newly introduced citrus postharvest fungicides azoxystrobin, fludioxonil, and pyrimethanil for managing green mold. Because our method subjected large natural pathogen populations to selection, it is more likely to reflect the potential of the pathogen compared with the use of mutagens in the laboratory (24). Furthermore, properties of resistant isolates selected from natural populations will be more realistic because laboratory mutants may be affected at loci not involved in resistance. The ultimate establishment of resistant isolates in a population will depend on the interaction between stabilizing, directional, and disruptive selection forces as well as fungicide application strategies (24) and integrated disease management approaches, including sanitation practices.

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