Baseline Sensitivities for New Postharvest Fungicides Against *Penicillium* spp. on Citrus and Multiple Resistance Evaluations in *P. digitatum*

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ABSTRACT

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For the first time in over 25 years, three new fungicides (azoxystrobin, fludioxonil, and pyrimethanil), all belonging to different chemical classes, are being registered for postharvest use against *Penicillium* decays of citrus fruit in the United States. Baseline sensitivities of *Penicillium digitatum* and *P. italicum* were developed using isolates collected before the commercial use of these new fungicides. In a comparison of methods, EC₅₀ values obtained using the spiral gradient dilution method were very similar to those obtained using traditional agar dilutions of fungicides. For azoxystrobin, the addition of salicylhydroxamic acid (SHAM) did not significantly affect EC₅₀ values for mycelial growth of both species. In additional studies on conidial germination of *P. digitatum*, SHAM significantly reduced EC₅₀ values for azoxystrobin. For pyrimethanil, the mean EC₅₀ value for mycelial growth obtained using a minimum growth medium for anilinopyrimidine fungicides was significantly lower but comparable to values obtained when using potato dextrose agar. For mycelial growth of *P. digitatum*, mean EC₅₀ values were 0.014, 0.025, and 0.313 µg/ml, whereas for conidial germination, they were 0.074, 0.163, and 1.195 µg/ml for azoxystrobin, fludioxonil, and pyrimethanil, respectively. For *P. italicum*, mean EC₅₀ values for mycelial growth for fludioxonil and pyrimethanil were 0.005 and 0.040 µg/ml, respectively. For azoxystrobin, the mean EC₅₀ value for mycelial growth for 33 isolates was 0.029 µg/ml. Four isolates had EC₅₀ values ≥0.772 µg/ml and were considered part of a resistant subpopulation. Multiple resistance between the older and new postharvest fungicide classes on citrus was not detected in *P. digitatum*, and all isolates that were sensitive or resistant to imazalil or thiabendazole were sensitive to the new compounds. This information is important for monitoring populations of *P. digitatum*, where resistance against the older fungicides has commonly developed.

Additional keywords: fungicide resistance

Green mold, caused by the fungus *Penicillium digitatum* (Pers.:Fr.) Sacc., is a major postharvest decay of citrus fruit in all citrus growing areas of the world, especially in locations with arid and subtropical climates (6,8). Blue mold, caused by *P. italicum* Wehmer, is generally less prevalent, but may become a major problem under conditions that suppress development of green mold such as storage temperatures below 10°C (4). Both species of *Penicillium* mainly infect citrus fruit (6). The reproductive capacity of these fungi is extremely high, and the surface of literally every citrus fruit is contaminated with their airborne spores at harvest time (6). Both fungi are wound-invading pathogens. Conidia of *P. digitatum* have been reported to

be unable to germinate on and penetrate healthy fruit and can only infect wound sites in the fruit's flavedo (8). Injuries, however, commonly occur during harvesting, transportation, and handling. Applications of effective fungicides soon after harvest can eradicate or inhibit early infections.

In California, both diseases, but especially green mold, may cause significant economic losses to citrus growers and packers. The control of postharvest decays of citrus is based on an integrated program, with the use of sanitation practices and fungicides as core components. Registered postharvest fungicides against *Penicillium* species and other postharvest pathogens in California include sodium *o*-phenylphenate (SOPP), thiabendazole (TBZ), and imazalil. The former two fungicides have been used routinely by the citrus industry for nearly four decades, while the latter one was first introduced in California in 1981. The extensive use of these fungicides over many years has resulted in the selection and proliferation of fungicide-resistant individuals of *P. digitatum* and *P.*

italicum. Furthermore, pathogen populations that are resistant to more than one fungicide class (i.e., multiple-resistance) have developed (5,7,8,14).

Until the current registration of new fungicides, there were no effective alternatives to the old compounds, and new fungicides needed to be developed to maintain and improve postharvest disease management. As compared to the introduction of the older fungicides, which took place over decades, the three new compounds—azoxystrobin, fludioxonil, and pyrimethanil—will be registered over a 3-year period for postharvest use on citrus. These fungicides belong to different chemical classes and have never been used against *Penicillium* spp. on citrus. This pluralism of postharvest fungicides allows implementation of resistance management practices from the onset of commercial use. Thus, management options for green mold and blue mold will include mixtures and rotations of all registered fungicides, minimizing the risk of resistance development to the new fungicides.

The three new fungicides have different modes of action and different effects on growth of the pathogen. Azoxystrobin is a representative of the broad-spectrum QoI class of fungicides. It is a synthetic compound based on the antifungal molecule strobilurin A (33). Like all strobilurins, azoxystrobin interferes with electron transport during mitochondrial respiration by binding to the ubiquinol oxidation center (Qp) of the mitochondrial cytochrome *bc*₁ complex (2,33). Fludioxonil belongs to the phenylpyrroles, a class of nonsystemic compounds derived from the antifungal antibiotic pyrrolnitrin that is produced by bacterial species in the genus *Pseudomonas* (25). This synthetic fungicide is used to control turfgrass diseases (32) and postharvest decays of stone fruit (10) as well as several seedborne pathogens (12). It inhibits spore germination, germ tube elongation, and mycelial growth (25). It also induces germ tube distortions and cell bursting (23,25). Although the mode of action of fludioxonil is not completely understood, it has been reported to interfere with the osmoregulatory signal transduction pathway by inhibition of a protein kinase in laboratory-selected mutants of *Neurospora crassa* and other fungi (23). Pyrimethanil is a member of the anilino-pyrimidines, another class of fungicides. It

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is effective against diseases caused by *Botrytis cinerea* on a variety of crops and scab of pome fruits caused by *Venturia inaequalis* (18,25). Anilino-pyrimidine fungicides are thought to interfere with the biosynthesis of methionine and other amino acids and inhibit secretion of hydrolytic enzymes such as pectinases, cellulases, and proteases, but their primary site of action is still unknown (13). Anilino-pyrimidines have little effect on spore germination, but strongly inhibit germ tube elongation and mycelial growth (25).

The process of replacing less effective fungicides with new, improved products is difficult due to the high cost of research, development, and registration. Additionally, in order to maintain and extend the effectiveness of new compounds, fungicide resistance must be managed after the fungicide is introduced. An essential component of such management is the periodic monitoring of the target fungal populations for their sensitivity, as is commonly being done in California citrus packinghouses for the older fungicides. Monitoring for the new compounds should be done upon their market introduction in order to detect shifts to lower sensitivities at early stages of selection (3,16). Monitoring should be based upon comparisons of fungal sensitivities with baseline sensitivity data that are determined prior to the commercial introduction of the compounds. For this, reliable and rapid methods have to be employed. The currently most common technique used to determine in vitro toxicity of fungicides is based on measuring mycelial growth or spore germination on culture media amended with serial dilutions of the tested fungicides. Because this technique is laborious and resource intensive, the spiral gradient dilution (SGD) method was developed (11,15).

Thus, the purpose of this study was to develop baseline sensitivity data for *P. digitatum* and *P. italicum* for azoxystrobin, fludioxonil, and pyrimethanil. The specific objectives were to: (i) evaluate the SGD method for the determination of EC₅₀ values of *P. digitatum* isolates by comparison to EC₅₀ values obtained using the serial dilution method; (ii) establish baseline sensitivity ranges of EC₅₀ values for *P. digitatum* and *P. italicum*; (iii) compare the effect of azoxystrobin-specific media to a standard medium in the determination of EC₅₀ values for both species and of pyrimethanil-specific media to a standard medium for *P. digitatum*; and (iv) evaluate the in vitro potential of multiple-resistance in *P. digitatum* between two of the currently used postharvest fungicides, imazalil and thiabendazole, and the new compounds.

MATERIALS AND METHODS

Fungicides. Formulated products of azoxystrobin (Abound 2.08F; Syngenta Crop Protection, Greensboro, NC), fludio-

xonil (Scholar 50WP; Syngenta Crop Protection), imazalil (Freshgard 700; FMC Corporation, Philadelphia, PA), and pyrimethanil (Penbotec 400SC; Janssen Pharmaceutica, Titusville, NJ) were used for this study. Thiabendazole (Decco Salt No. 19; Cerexagri-Decco, King of Prussia, PA) was used as technical grade. Aqueous solutions of the fungicides were used in all assays. Stock solutions for thiabendazole were prepared in 95% ethanol.

Isolates of *P. digitatum* and *P. italicum* and culture methods. Isolates of *P. digitatum* originated from citrus groves and packinghouses throughout the citrus growing areas of California and were collected between 1986 and 1994 (collection of G. J. Holmes) and between 2000 and 2005 (Table 1). To obtain the majority of the total of 67 isolates, we collaborated with three service companies and a citrus growers' cooperative. Multispore isolates were collected on cotton swabs from infected citrus fruit. Isolates of *P. italicum* were obtained from citrus fruit or air samplings in California packinghouses or from fruit in orchards between 2000 and 2005 (Table 2). Isolates were cleaned from contaminants and then single-spored by exponential dilution of a spore suspension using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA). Single-spore isolates were plated out on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) and incubated for 3 to 4 days until the fungus sporulated. A conidial suspension (50 µl of 1 × 10⁴ conidia/ml) was then evenly spread onto 60-mm PDA plates. Plates were incubated for 24 h, mycelial plugs were transferred into 2-ml microcentrifuge tubes containing 1 ml of sterile water, and tubes were stored at 4°C for up to 12 months. These cultures served as working stocks during this study.

Determination of EC₅₀ values for mycelial growth using the agar dilution and SGD methods. Using the agar dilution method, EC₅₀ values for azoxystrobin, fludioxonil, pyrimethanil, and imazalil were determined for seven randomly chosen isolates of *P. digitatum*. Aqueous solutions of fungicides were added to molten PDA after cooling to 45 to 50°C. Fungicide concentrations used were a series of twofold dilutions from 0.125 to 0.0039 µg/ml for azoxystrobin and 0.1 to 0.0031 µg/ml for fludioxonil. For pyrimethanil, concentrations of 0.5, 0.30, 0.25, 0.20, 0.15, 0.10, and 0.05 µg/ml were used. For imazalil-sensitive and -resistant isolates, imazalil concentrations used were twofold dilutions from 0.1 to 0.0031 µg/ml and 2 to 0.125 µg/ml, respectively. Mycelial plugs prepared as described above were placed in the center of 10-cm agar plates. Three replicate plates were used for each fungicide concentration and the nonamended control. Plates were incubated for 5 days at 25°C. Radial growth was determined as an average of four evenly spaced locations of

each fungal colony, and this value was averaged for the three replications of each fungicide concentration evaluated. Percent inhibition for the fungicide-amended plates was calculated based on growth on nonamended control plates (14). EC₅₀ values were determined as described previously (11). This experiment was done three times.

The SGD method was used for all isolates of both species of *Penicillium* and was conducted as described in detail by Förster et al. (11). Mycelial inoculum for the SGD plates was grown on hydrophilic cellophane strips (du Pont de Nemours, Wilmington, DE). Eight to 10 cellophane strips were placed on the surface of PDA in 10-cm petri dishes, 10 µl of a conidial suspension (10⁶ conidia/ml) was applied evenly onto each cellophane strip, and strips were incubated at 25°C for 24 h. Agar plates 15 cm in diameter were prepared at least 24 h before fungicide solutions were applied with a spiral plater using the exponential deposition mode. Concentrations for azoxystrobin, fludioxonil, and pyrimethanil were 100, 50, and 100 µg/ml, respectively, for both *P. digitatum* and *P. italicum*. For TBZ and imazalil, concentrations were 200/1,000 µg/ml and 30/200 µg/ml for sensitive/resistant isolates of *P. digitatum*, whereas concentrations were 500 and 30 µg/ml for isolates of *P. italicum*, respectively. Plates were incubated for 2 to 4 h to allow fungicides to diffuse into the medium and form a gradient of concentrations along the radius of the plate. Mycelium-covered strips were placed radially along the gradient at predetermined plate positions. For each fungal isolate, a duplicate sample was placed at opposite locations on the plate, and three replicate plates were prepared for each fungicide. The 2-day incubation option was used in the SGE software (Spiral Biotech) for the calculation of the local concentrations where 50% growth inhibition was observed (11). Molecular weights used for the calculations were 403 for azoxystrobin, 248 for fludioxonil, 297 for imazalil, 199 for pyrimethanil, and 201 for TBZ. Additionally, to evaluate the presence of multiple-resistance in *P. digitatum* between the new fungicides and the older ones (i.e., imazalil and TBZ), a subset of 38 single-spore isolates with a range of sensitivities to imazalil (EC₅₀ values from 0.027 to 1.28 µg/ml) and TBZ (EC₅₀ values from 0.057 to ≥7.825 µg/ml) were selected (Table 1) for statistical comparisons. All experiments were done three times.

Determination of EC₅₀ values for conidial germination of *P. digitatum* using the SGD method. A subset of 21 *P. digitatum* isolates from the baseline population representing different hosts and geographic origins was used (Table 1). Spiral gradient plates were prepared as described above. Concentrations applied to the plates were 100 µg/ml for azoxystrobin, 50 µg/ml for

Table 1. Isolates of *Penicillium digitatum* used for development of baseline sensitivities to azoxystrobin, fludioxonil, and pyrimethanil

No.	Code no.	Fruit type	Location	County of origin	Additional experiments ^b	Sensitivity ^a	
						Imazalil	TBZ
1	1791 ^c	Grapefruit	NA ^d	NA	AP, C, M, MR, SHAM	R	S
2	J-286 ^c	Grapefruit	Orchard	Riverside	...	S	S
3	439	Grapefruit	Orchard	Riverside	...	S	S
4	12I	Lemon	Packinghouse	Fresno	AP, C, MR	R	S
5	12H	Lemon	Packinghouse	Fresno	...	R	S
6	12G	Lemon	Packinghouse	Fresno	AP, C	R	S
7	12F	Lemon	Packinghouse	Fresno	AP	R	S
8	12E	Lemon	Packinghouse	Fresno	AP	R	S
9	L1	Lemon	Packinghouse	Kern	AP, C, M, MR, SHAM	R	S
10	6D4	Lemon	Packinghouse	Kern	MR	S	S
11	6D3	Lemon	Packinghouse	Kern	...	R	R
12	6D2	Lemon	Packinghouse	Kern	MR	R	R
13	6D1	Lemon	Packinghouse	Kern	...	R	R
14	6C4	Lemon	Packinghouse	Kern	MR	R	R
15	6C3	Lemon	Packinghouse	Kern	MR	R	R
16	6C2	Lemon	Packinghouse	Kern	MR	S	R
17	6C1	Lemon	Packinghouse	Kern	MR	R	R
18	6B3	Lemon	Packinghouse	Kern	MR	S	R
19	6B2	Lemon	Packinghouse	Kern	MR	S	S
20	6B1	Lemon	Packinghouse	Kern	MR	S	S
21	6A1	Lemon	Packinghouse	Kern	MR	S	S
22	5D1	Lemon	Packinghouse	Kern	MR	R	R
23	5C3	Lemon	Packinghouse	Kern	MR	R	R
24	5C2	Lemon	Packinghouse	Kern	MR	S	R
25	5B3	Lemon	Packinghouse	Kern	MR	R	R
26	5A1	Lemon	Orchard	Kern	MR	S	S
27	1E	Lemon	Packinghouse	Kern	MR	R	R
28	2152	Lemon	Packinghouse	Kern	AP, C, M, MR, SHAM	R	R
29	J-235 ^c	Lemon	Packinghouse	Los Angeles	...	R	S
30	J-227 ^c	Lemon	Packinghouse	Los Angeles	...	R	S
31	1726 ^c	Lemon	Orchard	Riverside	AP, C, M, MR, SHAM	S	S
32	Pd	Lemon	Orchard	Riverside	AP, C, M, MR, SHAM	S	S
33	H	Lemon	Orchard	Riverside	AP, C, M, MR, SHAM	S	S
34	J-348 ^c	Lemon	Orchard	San Bernardino	...	S	S
35	P4 ^c	Lemon	Packinghouse	Tulare	...	R	S
36	2A	Lemon	Packinghouse	Tulare	MR	R	S
37	1D	Lemon	Packinghouse	Tulare	C	R	S
38	12D	Lemon	Packinghouse	Tulare	AP, MR	R	S
39	12C	Lemon	Packinghouse	Tulare	AP, MR	R	S
40	12B	Lemon	Packinghouse	Tulare	AP, MR	R	S
41	12A	Lemon	Packinghouse	Tulare	AP, MR	R	S
42	J-976 ^c	Lemon	Packinghouse	Ventura	...	R	ND ^e
43	J-616	Lemon	Packinghouse	Ventura	...	R	ND
44	J-125 ^c	Lemon	Packinghouse	Ventura	...	R	S
45	4D	Lemon	Packinghouse	Ventura	...	R	S
46	4C	Lemon	Packinghouse	Ventura	...	R	S
47	4B	Lemon	Packinghouse	Ventura	...	R	S
48	4A	Lemon	Packinghouse	Ventura	MR	R	S
49	3B	Lemon	Packinghouse	Ventura	C, MR	R	S
50	3A	Lemon	Packinghouse	Ventura	C, MR	R	S
51	1792	Lemon	Packinghouse	Ventura	AP, C, M, MR, SHAM	R	S
52	J-893 ^c	Orange	Packinghouse	Fresno	...	R	ND
53	1C	Orange	Packinghouse	Fresno	C, MR	S	S
54	1B	Orange	Packinghouse	Fresno	C, MR	S	S
55	1A	Orange	Packinghouse	Fresno	C, MR	S	S
56	8F	Orange	Packinghouse	Tulare	AP, C	R	S
57	8E	Orange	Packinghouse	Tulare	AP, C, MR	R	S
58	8D	Orange	Packinghouse	Tulare	AP	R	S
59	8C	Orange	Packinghouse	Tulare	AP, C	S	S
60	8B	Orange	Packinghouse	Tulare	AP, C	R	R
61	8A	Orange	Packinghouse	Tulare	AP, C	R	S
62	2B	Orange	Packinghouse	Tulare	MR	S	S
63	J-713 ^c	Orange	Packinghouse	Ventura	...	R	ND
64	373	Orange	Packinghouse	Yuma	...	S	S
65	P2 ^c	Orange	NA	Fresno	...	S	S
66	1F ^c	NA	Orchard	NA	C, MR	S	S
67	P3F ^c	NA	NA	NA	...	S	S

^a Sensitivity to imazalil and thiabendazole (TBZ) was determined using the spiral gradient dilution (SGD) method: S = sensitive or R = resistant to the respective fungicide. For imazalil, resistance was considered at $EC_{50} \geq 0.15 \mu\text{g/ml}$; for TBZ at $EC_{50} \geq 1 \mu\text{g/ml}$.

^b Isolates used for additional experiments: AP = effect of anilinoypyrimidine (AP) medium on effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) for pyrimethanil; C = conidial germination studies; M = comparison between agar dilution and spiral gradient dilution (SGD) method; MR = multiple-resistance studies; and SHAM = effect of salicylhydroxamic acid (SHAM) on EC_{50} values of azoxystrobin.

^c Isolates from the collection of G. J. Holmes.

^d Information not available (NA).

^e Not determined (ND).

fludioxonil, and 200 µg/ml for pyrimethanil. For azoxystrobin, either PDA or PDA amended with 100 µg/ml salicylhydroxamic acid (SHAM; prepared in 50% ethanol and added to the cooled media) was used. A conidial suspension (10^6 conidia/ml) was prepared from 6- to 8-day-old cultures, and 10 µl was streaked radially along the concentration gradient (11). After incubation at 25°C for 16 to 18 h, conidial germination was evaluated under an inverted microscope (Axiovert S100; Carl Zeiss, Jena, Germany) using the 20× objective. A conidium was considered germinated if the germ tube was at least twice the diameter of the conidium. The location along the spore streak where germination was inhibited by 50% was determined by counting conidia within the field of view and was marked on the bottom side of the plate. The 1-day incubation option was used in the SGE software for this assay. Experiments were conducted three times.

Effect of SHAM and different culture media on EC_{50} values in mycelial growth assays of azoxystrobin and pyrimethanil, respectively. Sensitivities to azoxystrobin were determined for seven isolates of *P. digitatum* as indicated in Table 3

and for 33 isolates of *P. italicum* (Table 4) using the agar dilution method. For this, either PDA amended with azoxystrobin or PDA amended with azoxystrobin and 100 µg SHAM/ml was used as described above. EC_{50} values and percent inhibition of mycelial growth at 0.25 µg azoxystrobin/ml (done only for *P. digitatum*) in each of the two media as compared with the nonamended control medium were determined.

The anilinoypyrimidine (AP) working group of the Fungicide Resistance Action Committee (FRAC) has suggested the use of a synthetic medium for mycelial growth assays that contains 10 g glucose, 1.5 g K_2HPO_4 , 2 g KH_2PO_4 , 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, and 12.5 g of agar per liter of distilled water (13,19). Thus, EC_{50} values for mycelial growth for a subset of 21 baseline isolates of *P. digitatum* (Table 1) were also determined using this FRAC-AP medium in the SGD assay as described above. EC_{50} values and percent inhibition of mycelial growth at 0.35 µg pyrimethanil/ml in each of the two media as compared with the nonamended control medium were determined. The experiments for these media comparisons with azoxystrobin and pyrimethanil were done three times.

Statistical analysis of data. For repeated experiments, the homogeneity of variances was tested using Bartlett's test of homogeneity. Because variances were homogeneous for each experiment, data were combined and further statistically analyzed. For the comparison of the agar dilution and SGD methods, average EC_{50} values obtained by the SGD method were regressed against values obtained using the agar dilution method using SAS version 9.1 (SAS Institute, Cary, NC). The slope and intercept of the regression line were compared by means of *t* tests to a theoretical 1:1 relationship between the two methods with a slope of 1 and an intercept of zero (11). The accuracy of the SGD method was measured by estimating bias, which is defined as $100 \times (\beta - 1)$, where β is the slope of the linear regression. The precision of the method was estimated by calculating the coefficient of variation or CV ($100 \times s_p/\beta$) and a confidence interval or CI ($\beta \pm s_p t_{df, 0.05}$), where s_p is the standard error of the slope estimate, and $t_{df, 0.05}$ is the upper fifth percentile of a *t* distribution with *df* degrees of freedom (11).

Mean EC_{50} values of the baseline populations of *P. digitatum* and *P. italicum* for the three new fungicides were plotted in

Table 2. Isolates of *Penicillium italicum* used for development of baseline sensitivities to azoxystrobin, fludioxonil, and pyrimethanil

No.	Code no.	Source of isolation/ fruit type	Location	County of origin	Sensitivities ^a	
					Imazalil	TBZ
1	2AY	Air-sampling	Packinghouse	Riverside	S	S
2	2P2	Air-sampling	Packinghouse	Riverside	S	S
3	6CCH	Air-sampling	Packinghouse	Riverside	S	S
4	6CY	Air-sampling	Packinghouse	Riverside	S	S
5	PI1	Air-sampling	Packinghouse	Riverside	S	S
6	L1A	Lemon	Packinghouse	Riverside	S	S
7	L1B	Lemon	Packinghouse	Riverside	S	S
8	L2B	Lemon	Packinghouse	Riverside	S	S
9	L3A	Lemon	Packinghouse	Riverside	S	S
10	L5B	Lemon	Packinghouse	Riverside	S	S
35	24E	Lemon	Packinghouse	Riverside	S	S
37	9D	Lemon	Packinghouse	Riverside	S	S
22	KOR1688	Orange	Orchard	Fresno	S	S
24	KOR1734	Orange	Orchard	Fresno	S	S
27	KOR1876	Orange	Orchard	Fresno	S	S
29	KOR2010	Orange	Orchard	Fresno	S	S
34	305	Orange	Orchard	Fresno	S	S
36	1A	Orange	Packinghouse	Fresno	S	S
31	KOR2073	Orange	Orchard	Kern	S	S
11	O2A	Orange	Packinghouse	Riverside	S	S
12	O4A	Orange	Packinghouse	Riverside	S	S
13	O4B	Orange	Packinghouse	Riverside	S	S
14	O5	Orange	Packinghouse	Riverside	S	S
15	O7A	Orange	Packinghouse	Riverside	S	S
16	OP14	Orange	Packinghouse	Riverside	S	S
32	20L	Orange	Packinghouse	San Bernardino	S	S
17	KOR1483	Orange	Orchard	Tulare	S	S
18	KOR1505	Orange	Orchard	Tulare	S	S
19	KOR1572	Orange	Orchard	Tulare	S	S
20	KOR1642	Orange	Orchard	Tulare	S	S
21	KOR1671	Orange	Orchard	Tulare	S	S
23	KOR1727	Orange	Orchard	Tulare	S	S
25	KOR1775	Orange	Orchard	Tulare	S	S
26	KOR1795	Orange	Orchard	Tulare	S	S
28	KOR1935	Orange	Orchard	Tulare	S	S
30	KOR2055	Orange	Orchard	Tulare	S	S
33	742	Orange	Orchard	Tulare	S	S

^a Isolates were considered sensitive at $EC_{50} < 0.041$ µg imazalil/ml, or at $EC_{50} < 0.12$ µg thiabendazole (TBZ)/ml.

frequency histograms. For this, EC₅₀ values were first log₁₀-transformed and the bin (i.e., EC₅₀ category) width *h* for the transformed data was determined using the method of Scott (26) with the equation:

$$h_n = 3.49sn^{-1/3}$$

where *s* is an estimate of the standard deviation and *n* is the number of isolates used for each fungicide evaluated. Standard deviations were calculated from mean log₁₀-transformed EC₅₀ values for all isolates used in each fungicide evaluation. The number of bins was then determined over the range of EC₅₀ values for each fungicide.

For the comparisons of the toxicity of azoxystrobin in SHAM-amended and nonamended media and for the comparison of the toxicity of pyrimethanil on different culture media, mean EC₅₀ values were compared using analysis of variance (ANOVA) and least significant difference (LSD) separation procedures of SAS. Similarly, arcsine transformed percent inhibition values were compared for each fungicide at equivalent concentrations for the two media using ANOVA and LSD mean separation procedures. To determine if multiple-resistance between old (i.e., TBZ and imazalil) and each of the new fungicides was present, mean EC₅₀ values were plotted in pair-wise comparisons. EC₅₀ values of each of the new fungicides were then regressed against those obtained for imazalil or TBZ using ANOVA and regression procedures of SAS.

RESULTS

Comparison of EC₅₀ values for *P. digitatum* using the agar dilution and SGD methods. The regression of EC₅₀ values for mycelial growth determined by the agar dilution method on values obtained by the SGD method is shown in Figure 1. The model for the regression was highly significant (*P* < 0.01), and the coefficient of determination (*r*²) was 0.94. The slope of the regression line was not significantly different (*P* = 0.97) from the slope of a 1:1

relationship between the two methods. Furthermore, accuracy of the method was measured by the estimation of bias (*b*). This value was -1.87, and thus the SGD method underestimated a 1:1 relationship by 1.87%. The CV and CI, statistical estimates of the precision of a test, were 4.9% and 0.98 ± 0.08, respectively.

Baseline sensitivity for mycelial growth and conidial germination using the SGD assay. EC₅₀ values for mycelial growth were determined for both species of *Penicillium*, whereas values for conidial germination were only determined for *P. digitatum*. Mean EC₅₀ values for mycelial growth of the 67 isolates of *P. digitatum* for azoxystrobin, fludioxonil, and pyrimethanil using the SGD method were 0.014, 0.025, and 0.313 µg/ml, respectively. Ranges of EC₅₀ values were from 0.007 to 0.028 µg/ml for azoxystrobin, from 0.009 to 0.072 µg/ml for fludioxonil, and from 0.208 to 0.413 µg/ml for pyrimethanil. The frequency distribution of EC₅₀ categories for each fungicide is shown in the histograms of Figure 2A to C.

For the 37 isolates of *P. italicum*, mean EC₅₀ values for mycelial growth for fludioxonil and pyrimethanil using the SGD method were 0.005 and 0.040 µg/ml, respectively. Ranges of EC₅₀ values were from 0.004 to 0.010 µg/ml for fludioxonil and from 0.010 to 0.082 µg/ml for pyrimethanil. For azoxystrobin, the mean EC₅₀ value for mycelial growth for 33

isolates was 0.029 µg/ml with a range from 0.020 to 0.043 µg/ml. Four isolates had EC₅₀ values ≥ 0.772 µg/ml. The frequency distribution of EC₅₀ categories for each fungicide is shown in the histograms of Figure 3A to C. The histogram for azoxystrobin does not include the four insensitive isolates. The histogram developed for all isolates only had two bins (i.e., sensitive, *n* = 33; and insensitive isolates, *n* = 4). Scott's number for bin width using all isolates was 0.476, whereas for the sensitive isolates this value was 0.089. In comparing mean EC₅₀ values of the two pathogens, *P. italicum* was less sensitive to azoxystrobin, but more sensitive to fludioxonil and pyrimethanil than *P. digitatum*.

EC₅₀ values for conidial germination were determined for 21 single-spore isolates of *P. digitatum*. For azoxystrobin, no inhibition of conidial germination was observed on PDA without the addition of SHAM at any concentration of the fungicide evaluated. On SHAM-amended PDA, the range of EC₅₀ values was between 0.047 and 0.166 (mean = 0.074) µg azoxystrobin/ml. EC₅₀ values ranged between 0.106 and 0.204 (mean = 0.163) µg/ml for fludioxonil and between 0.863 and 1.608 (mean = 1.195) µg/ml for pyrimethanil. The frequency distribution of EC₅₀ categories for each fungicide is shown in the histograms of Figure 4A to C.

Effect of SHAM and different culture media on EC₅₀ values for mycelial

Table 4. Effect of salicylhydroxamic acid on the effective concentration of azoxystrobin to inhibit mycelial growth of *Penicillium italicum* by 50%

Method ^a	Fungicide	Treatment ^b	No. of isolates	Mean EC ₅₀ values (µg/ml) ^c	Statistics ^d	
					LSD	<i>P</i> > <i>F</i>
SGD	Azoxystrobin	+ SHAM	34	0.031	A	0.06
	Azoxystrobin	- SHAM	34	0.029	A	

^a Spiral gradient dilution (SGD) method was used.

^b Potato dextrose agar (PDA) was amended (+) or not amended (-) with salicylhydroxamic acid (SHAM) at 100 µg/ml.

^c Mean EC₅₀ values are averages from three experiments.

^d Mean values followed by the same letter are not significantly different according to analysis of variance and least significant difference (LSD) mean separation procedures (*P* > 0.05).

Table 3. Effect of salicylhydroxamic acid and different culture media on the in vitro toxicity of azoxystrobin and pyrimethanil, respectively, to mycelial growth of *Penicillium digitatum*

Method ^a	Fungicide	Treatment ^b	No. of isolates	Mean EC ₅₀ values (µg/ml) ^c	Mycelial growth assays			
					Statistics ^d		Inhibition (%) at equivalent concentrations ^e	Statistics ^d
					LSD	<i>P</i> > <i>F</i>		
Agar dilution	Azoxystrobin	+ SHAM	7	0.023	A	0.1315	99	<0.0001
	Azoxystrobin	- SHAM	7	0.028	A		83.5	
SGD	Pyrimethanil	PDA	21	0.31	A	<0.0001	99	0.0653
	Pyrimethanil	AP	21	0.21	B		95.6	

^a Agar dilution and spiral gradient dilution (SGD) methods were used.

^b For azoxystrobin, potato dextrose agar (PDA) was amended (+) or not amended (-) with salicylhydroxamic acid (SHAM) at 100 µg/ml. For pyrimethanil, an anilinopyrimidine (AP) minimal medium (13,19) was compared to PDA.

^c Mean EC₅₀ values are the averages from three experiments.

^d Mean values followed by the same letter are not significantly different according to an analysis of variance and least significant difference (LSD) mean separation procedures (*P* > 0.05).

^e Inhibition at equivalent concentrations for the two media in each comparison was determined using 0.25 µg azoxystrobin/ml or 0.35 µg pyrimethanil/ml and was based on growth on nonamended control media.

growth of azoxystrobin and pyrimethanil, respectively. The effect of SHAM on EC_{50} values of azoxystrobin was determined for both species of *Penicillium*, whereas the effect of culture media on EC_{50} values of pyrimethanil was only determined for *P. digitatum*. The mean EC_{50} value for azoxystrobin using PDA without SHAM was 0.028 $\mu\text{g/ml}$ for *P. digitatum*, whereas it was 0.023 $\mu\text{g/ml}$ when SHAM was added to the medium. In statistical comparisons of the values, the model was not significant ($P = 0.1315$) and the means did not separate using the LSD test (Table 3). On the PDA-SHAM medium at 0.25 μg azoxystrobin/ml, fungal growth was inhibited by 99% as compared to the control. At the same concentration of azoxystrobin on nonamended PDA, inhibition was significantly less (i.e., 85%) as compared to the medium amended with SHAM (Table 3), and higher concentrations of azoxystrobin did not further reduce growth. For *P. italicum*, also no significant differences in mean EC_{50} values were obtained using PDA without or with the addition of SHAM (Table 4).

For pyrimethanil, EC_{50} values of 21 isolates of *P. digitatum* were compared using PDA and AP media. Using the AP medium, values ranged from 0.14 to 0.25 $\mu\text{g/ml}$, whereas on PDA values ranged from 0.25 to 0.40 $\mu\text{g/ml}$. The ANOVA model in comparisons of the values was highly significant ($P < 0.0001$), and mean EC_{50} values were significantly different (Table 3). Additionally, mycelial growth was reduced on AP medium as compared with PDA. On PDA, an incubation time of 3 days was sufficient for mycelial growth to be accurately measured, whereas on AP medium 5 to 6 days were required. Using either medium at 0.35 μg pyrimethanil/ml (i.e., the concentration of pyrimethanil where growth on PDA was inhibited by 99%), fungal growth was inhibited similarly and mean values of percent inhibi-

tion were not significantly different (Table 3).

Evaluation of multiple-resistance in *P. digitatum*. In multiple-resistance studies, 38 isolates that were either sensitive or resistant to imazalil or TBZ were used. In pair-wise comparisons, mean EC_{50} values for imazalil for each isolate were regressed on values for each of the new fungicides. In Figure 5A to C, sensitive isolates, isolates resistant to only imazalil or TBZ, and isolates with multiple-resistance to imazalil and TBZ are shown as different symbols. There was no significant correlation between the sensitivities to imazalil and EC_{50} values of the new postharvest fungicides. More specifically, P values and coefficients of determination for each fungicide were: $P = 0.05$ and $r^2 = 0.12$ for azoxystrobin; $P = 0.08$ and $r^2 = 0.10$ for fludioxonil; and $P = 0.45$ and $r^2 = 0.02$ for pyrimethanil as shown in Figure 5A, B, and C, respectively. EC_{50} values for isolates resistant to TBZ could not be determined because values exceeded the solubility of the fungicide in the aqueous agar. Thus, regressions were not performed. Still, isolates resistant to TBZ were all sensitive and were found across the entire range of baseline sensitivities for each of the new fungicides (data not shown).

DISCUSSION

This is the first study to present baseline sensitivities of *P. digitatum* and *P. italicum* populations to the new citrus postharvest fungicides azoxystrobin, fludioxonil, and pyrimethanil. In vitro sensitivities for azoxystrobin have never been previously reported for any postharvest pathogen. For fludioxonil, Errampalli (9) determined mean EC_{50} values for mycelial growth and conidial germination of seven isolates of *P. expansum* to be 0.018 and 0.094 $\mu\text{g/ml}$, respectively. Förster et al. (10,11) found similar in vitro sensitivities for mycelial growth of the postharvest pathogens of

stone fruit *Monilinia fructicola*, *B. cinerea*, and *Rhizopus stolonifer* to fludioxonil. For pyrimethanil, Sholberg et al. (27) reported a mean EC_{50} value of 0.314 $\mu\text{g/ml}$ for mycelial growth of a baseline population of 17 isolates of *P. expansum* using a minimal growth medium. This mean EC_{50} value was very close to those that we determined for *P. digitatum* on PDA and AP media (i.e., 0.31 and 0.21 $\mu\text{g/ml}$, respectively). For *P. italicum*, with a mean EC_{50} value of

Fig. 1. Regression of effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) obtained using the spiral gradient dilution (SGD) method on those obtained using the agar dilution method. Data points for inhibition of mycelial growth of seven isolates of *Penicillium digitatum* are the means of three experiments. The slope and y intercept of the linear regression (solid line) are compared with a 1:1 relationship (dashed line).

Fig. 2. Frequency histograms of effective concentrations to inhibit mycelial growth of 67 isolates of *Penicillium digitatum* by 50% (EC_{50} values) for **A**, azoxystrobin, **B**, fludioxonil, and **C**, pyrimethanil as determined by the spiral gradient dilution method. Bar height indicates the total number of isolates within each bin, and bin width (0.109 for azoxystrobin, 0.182 for fludioxonil, 0.061 for pyrimethanil) was based on Scott's method (26).

0.041 µg/ml, however, pyrimethanil was found to be more toxic. Although a smaller number of isolates of *P. italicum* than of *P. digitatum* was evaluated in our study, a wider range in sensitivities against pyrimethanil was found for the former species. In comparisons of sensitivities of the fungi for the other two fungicides, the mean EC₅₀ value for azoxystrobin was lower for *P. digitatum*, whereas for fludioxonil it was lower for *P. italicum*. EC₅₀ values for conidial germination of *P. digitatum* in our study ranged from 0.863 to 1.608 µg/ml on PDA using 21 isolates. Smilanick et al. (28), using a single isolate of *P. digitatum* grown in a potato dextrose-based liquid medium that was adjusted to different pH values, reported EC₅₀ values between 0.2 and 0.4 µg pyrimethanil/ml for conidial germination. Fungicide sensitivities for conidial germination were not determined for *P. italicum*. Future monitoring of sensitivities in these pathogen populations will likely be based on the established fungicide sensitivities against mycelial growth. This is because visual assessment of fungal inhibition is less time-consuming for mycelial growth than for conidial germination, which is determined by microscopic observations.

Agar media were amended with formulated products of the fungicides evaluated, except for thiabendazole, which is used as technical grade for postharvest treatments of citrus fruit. In comparisons of EC₅₀ values for fludioxonil using either the formulated product Scholar 50WP or technical grade material, very similar values were obtained (data not shown). We preferred to use formulated materials in our studies because these are being utilized in fungicide applications in the packing-house. Although adjuvants in formulated products can potentially modify the activity of the fungicide or its diffusion rate in agar, toxicity values are never absolute because even the agar medium used might interfere with the activity of the fungicide (24).

To obtain a statistically representative sample size for determining population sensitivities, Leung et al. (20) developed the formula: $N = \log(1 - P) / \log(1 - F)$, where N is the sample size of the population and P is the probability of detecting an outlier at a frequency F . In our study, to detect at least one outlier in the population, a sample size of 67 was used for *P. digitatum*. This implies that 95.6% of all isolates will have EC₅₀ values for mycelial growth within the range that we described with 95% confidence (20). For conidial germination where we evaluated 21 isolates, 86.7% of all isolates will fall into the baseline range described with 95% confidence. Thirty-seven isolates of *P. italicum* were used for the baselines of fludioxonil and pyrimethanil, and 33 isolates were used for azoxystrobin. This implies that 92.2 and 91.3% of all isolates, respec-

tively, will have EC₅₀ values for mycelial growth within the described ranges with 95% confidence.

The baseline sensitivity of an organism is the range of sensitivities of a sample population before its widespread exposure to a particular chemical (16). Inherent to any population there is variation in the response due to evaluation methods used

Fig. 3. Frequency histograms of effective concentrations to inhibit mycelial growth of 37 isolates of *Penicillium italicum* by 50% (EC₅₀ values) for **A**, azoxystrobin, **B**, fludioxonil, and **C**, pyrimethanil as determined by the spiral gradient dilution method. Four isolates that had EC₅₀ values for azoxystrobin ≥ 0.772 µg/ml were not included in the histogram (see Results). Bar height indicates the total number of isolates within each bin, and bin width (0.155 for azoxystrobin, 0.096 for fludioxonil, 0.086 for pyrimethanil) was based on Scott's method (26).

and, more importantly, due to genetic differences among individuals. In our study, we found that four isolates of *P. italicum* had EC₅₀ values for azoxystrobin at least 25 times greater than the mean value for the remaining 33 isolates evaluated, although this fungus was never exposed to azoxystrobin or the QoI class of fungicides. These four isolates were verified to be *P. italicum* based on morphological and molecular comparisons (data not pre-

Fig. 4. Frequency histograms of effective concentrations to inhibit conidial germination of 21 isolates of *Penicillium digitatum* by 50% (EC₅₀ values) for **A**, azoxystrobin, **B**, fludioxonil, and **C**, pyrimethanil as determined by the spiral gradient dilution method. Bar height indicates the total number of isolates within each bin, and bin width (0.089 for azoxystrobin, 0.126 for fludioxonil, 0.177 for pyrimethanil) was based on Scott's method (26).

sented). Thus, these resistant isolates pre-existed in field populations of *P. italicum*, resulting in a preliminary estimate of resistance frequency of 10.8% based on the small number of isolates assessed. Resistant individuals most likely will also pre-exist for the other fungicides evaluated and in populations of *P. digitatum*, and they may be detected if larger numbers of isolates were evaluated. Therefore, additional

studies addressing these frequencies will provide information on the resistance potential for these fungicides. The four isolates of *P. italicum* less sensitive to azoxystrobin represent a distinct subpopulation of the baseline population. In future monitoring for changes in fungicide sensitivities in the populations, gradual or sudden shifts in sensitivity levels will be monitored for fungicides that exhibit quantitative or

qualitative resistance, respectively (16,31). Azoxystrobin has been reported to exhibit qualitative as well as quantitative resistance in *V. inaequalis* (17). By presenting the frequency distribution for the sensitive population of *P. italicum* in the histogram with multiple bins and by documenting a resistant subpopulation in our baseline, both types of resistance can be monitored in the future.

EC₅₀ values are the most precise sensitivity measures for fungus–fungicide interactions (3). Although there is no absolutely correct assay for determining in vitro fungicide toxicities, the mycelium-based agar dilution test has been the most commonly used method. Recently, the SGD method was developed as a single-plate assay (11). In our current study with *P. digitatum* and four fungicides, the SGD method was compared with the agar dilution method and was shown to be precise (CV = 4.9%) and accurate ($b = 1.87\%$). Thus, data obtained using the two methods were more similar than in our previous report where the SGD method was developed (11). This is probably because in this latter study nine fungicides and eight fungi were used. In our current study with only one fungus evaluated against three fungicides, less variability was observed as indicated by the high precision. Thus, the SGD method is an excellent alternative to traditional methods for quantification of fungicide toxicities and could have an important role in monitoring changes in fungicide sensitivity in populations of *P. digitatum* (29).

Early in vitro sensitivity studies with anilinopyrimidine fungicides indicated that the culture medium used influenced the inhibitory effect of these fungicides, and reversal of growth inhibition occurred by certain amino acids incorporated into a fully synthetic medium (21). This led to the suggestion that anilinopyrimidines are the first commercial fungicides that interfere with an amino acid biosynthesis pathway, most likely for methionine. As a result, the FRAC-AP committee suggested the use of a synthetic medium free of amino acids in monitoring and resistance risk assessment of *Botryotinia fuckeliana* to anilinopyrimidine fungicides (13,19). Although in our studies mean EC₅₀ values using the AP medium were significantly lower as compared to PDA, our baseline values were determined using PDA. This was done because of several reasons. 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 as opposed to 3 days on PDA). Thus, the difference in mean EC₅₀ values of 0.01 µg pyrimethanil/ml could possibly be due to a stress response of the fungus when grown in a nutrient poor medium. In addition, the natural substrate for *P. digitatum* is the citrus fruit, where amino acids, including methionine, are present. Furthermore, no significant differ-

Fig. 5. Scatter plots of effective concentrations to inhibit mycelial growth by 50% (EC₅₀ values) for imazalil plotted against those for **A**, azoxystrobin, **B**, fludioxonil, and **C**, pyrimethanil. Different symbols for the 38 isolates of *Penicillium digitatum* evaluated describe their sensitivity (sensitive or resistant) to imazalil (IM) and thiabendazole (TBZ). The coefficients of determination (r^2) and P values for the linear regressions are indicated.

ence was observed in percent mycelial growth inhibition at 0.35 µg pyrimethanil/ml, the concentration where growth of the fungus on PDA was inhibited by 99% (Table 3).

Because the alternative respiration pathway is known to interfere with the in vitro sensitivity to strobilurin fungicides in a range of fungi (2,33), the inhibitor of this pathway SHAM has been used extensively in vitro studies with strobilurins (22,30). In our mycelial growth studies with azoxystrobin, EC₅₀ values for *P. digitatum* and *P. italicum* were not significantly different using media with or without the addition of SHAM. Inhibition of growth for *P. digitatum* was significantly less (i.e., 83.5%), however, using the nonamended medium at a concentration of 0.25 µg azoxystrobin/ml as compared to the amended medium, where growth was inhibited by 99%. Mondal et al. (22) also showed that SHAM did not affect EC₅₀ values for mycelial growth of five citrus pathogens to the strobilurin fungicides azoxystrobin and pyraclostrobin. In our conidial germination studies, however, no inhibition of germination of *P. digitatum* was apparent when azoxystrobin was used alone. Thus, the addition of SHAM was required to determine EC₅₀ values similar to other studies where the effect of strobilurin fungicides on conidial germination was evaluated (30).

Reported sensitivities of baseline isolates of *P. digitatum* to imazalil range from 0.023 to 0.146 µg/ml, while for thiabendazole they range from 0.05 to 0.60 µg/ml (5). Based on this information, the discriminatory concentrations we used to characterize an isolate as resistant to imazalil and TBZ were 0.15 and 1 µg/ml, respectively. The isolates we used in this study can be assigned to four phenotypes based on their sensitivities to these two fungicides: 12 isolates were sensitive to both imazalil and TBZ, 13 isolates were resistant to both imazalil and TBZ, 11 isolates were resistant to imazalil and sensitive to TBZ, and 2 were sensitive to imazalil and resistant to TBZ. Because of the existence of single- as well as double-resistant isolates of *P. digitatum* to imazalil and TBZ, cross-sensitivity patterns to the new fungicides were studied for isolates sensitive or resistant to imazalil or TBZ. In our in vitro experiments, no evidence of multiple-resistance was evident. Statistical analyses showed no significant positive correlation between sensitivity to imazalil and the new fungicides. Thus, although the models in the statistical analyses were sometimes significant ($P < 0.1$), the coefficients of determination were very low in each of the regressions ($r^2 \leq 0.12$) (Fig. 5A to C). For *P. expansum*, others have shown that conidial germination and mycelial growth responses in the presence of fludioxonil also did not correlate with sensitivities to TBZ (9). For *P. italicum*, isolates

resistant to TBZ or imazalil were not found, and thus, cross resistance could not be evaluated. Development of baseline sensitivities and monitoring for changes in sensitivity are important antiresistance strategies (3). If shifts in sensitivity occur, rotations and mixtures between different chemical classes can be utilized before resistant populations cause crop losses. Thus, over 25 years after the introduction of imazalil in California packinghouses (14), the registration of azoxystrobin, fludioxonil, and pyrimethanil for postharvest use is of great importance for the citrus industry (1). In this study, baseline sensitivity data for these fungicides on citrus green mold and blue mold pathogens were established using the SGD method, a novel approach for determining 50% effective concentration values.

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