Phenotypic Characterization of Multifungicide Resistance in *Botrytis cinerea* Isolates from Strawberry Fields in Florida

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Abstract

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Chemical control has always been essential for the management of gray mold, caused by *Botrytis cinerea*, to ensure sustainable strawberry production. However, lack of knowledge about actual resistance development may have disastrous consequences and lead to severe epidemics such as the one that affected several strawberry fields in 2012 in Florida. In this study, we tested 392 isolates collected from Florida strawberry fields between 2010 and 2012 for their sensitivity to boscalid (Bosc), a succinate dehydrogenase inhibitor (SdhI); pyraclostrobin, a quinone outside inhibitor (QoI); boscalid + pyraclostrobin (Pristine); fenhexamid, a hydroxylanilide (Hyd); pyrimethanil and cyprodinil, anilinopyrimidines; fludioxonil, a phenylpyrrole; and fludioxonil + cyprodinil (Switch). The respective resistance frequencies for boscalid, pyraclostrobin, Pristine, fenhexamid, cyprodinil, and pyrimethanil were 85.4, 86.5, 86.0, 44.4, 52.7, and 59.5%. Overall, 17.8 and 19.8% of isolates showed reduced sensitivity to fludioxonil and

Switch, respectively. All fungicides sprayed preventively on detached strawberry fruit failed to control isolates with high levels of resistance to each fungicide except for fludioxonil and Switch. Four phenotypes with multifungicide resistance (MFR) were detected in *B. cinerea* populations from Florida. Isolates resistant to one fungicide (FR1), two (MFR2), three (MFR3), and four (MFR4) fungicides from different chemical groups represented 5.9, 28.6, 41.8, and 23.7% of the total resistant population, respectively. The MFR3 isolates were predominant and contained two subpopulations, the Bosc-QoI-AP^R isolates (56.5%) and the Bosc-QoI-Hyd^R isolates (40.6%). In addition to reporting on very highly resistant populations to boscalid and QoI fungicides, we show evidence for a widespread multifungicide resistance to *B. cinerea* that warrants immediate implementation of novel management strategies to impede the development of more resistant populations.

Gray mold, also known as Botrytis fruit rot, caused by *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana*), represents a major threat for strawberry production in Florida, the second largest production region in the United States, as well as in many other growing areas worldwide. The fungus infects flowers but is more damaging to fruits pre- and postharvest, especially under favorable conditions, i.e., temperatures around 20°C and prolonged periods of high humidity. Such conditions are commonly encountered in Florida during the production period between November and March. Quiescent infections start on young strawberry leaves early in the season, and conidia produced on necrotic tissues serve as the primary inoculum source. Conidia are dispersed mainly by wind and insects to infect flowers. Infections usually remain quiescent in immature fruit until conditions become favorable for disease development (7,8,32).

Current strategies used to control gray mold in strawberry rely on preventive applications of fungicides from multiple chemical groups. Besides the multi-site fungicides captan and thiram, compounds from five different chemical groups, i.e., succinate dehydrogenase inhibitors (SdhI), quinone outside inhibitors (QoI, suppression), hydroxylanilides (Hyd), anilinopyrimidines (AP), and phenylpyrroles (PP) are registered for gray mold control in Florida (38) and worldwide. In Florida, a standard disease management program consists of weekly applications of the multi-site fungicides throughout the season and single-site fungicides such as fenhexamid, pyrimethanil, pyraclostrobin + boscalid (Pristine), and

cyprodinil + fludioxonil (Switch) during the main peak bloom periods, which vary depending on the cultivar (33). In addition to pyraclostrobin, a component of Pristine and also available as a single product (Cabrio), other QoIs such as azoxystrobin and trifloxystrobin represent the mainstay of anthracnose fruit rot (AFR) (caused by *Colletotrichum acutatum*) control in Florida strawberry fields (34,38). The total number of applications of QoI fungicides for AFR control and suppression of gray mold is limited to five per season in Florida. Although not yet investigated in strawberry fields in Florida, populations of *B. cinerea* resistant to the QoIs and boscalid are widespread in strawberry fields as well as in many other crops worldwide (12,19,28,39,41).

The anilinopyrimidines (pyrimethanil and cyprodinil) and phenylpyrroles (fludioxonil) are commonly used to protect the flowers against gray mold during the peak bloom period. Pyrimethanil is registered as a single fungicide (Scala), whereas cyprodinil is combined with fludioxonil in a pre-packaged mixture (Switch). The APs act on fungi by inhibiting the secretion of hydrolytic enzymes essential during infection as well as by targeting the synthesis of methionine and other amino acids (16,30). Populations of *B. cinerea* resistant to pyrimethanil and cyprodinil have been already reported in French vineyards (27) and vegetable fields in Greece (35). Fludioxonil, the sole PP registered for gray mold management, is very effective against conidial germination and mycelial growth. Most likely, fludioxonil causes an increase in glycerol accumulation in the fungal cells as a result of its interference with the signal transduction pathway (36). Laboratory and field *B. cinerea* mutants showing reduced sensitivity to fludioxonil have been selected (17,27,43); however, resistance to this fungicide has not been yet reported in the field.

The hydroxylanilide fenhexamid has a different mode of action than the previously mentioned fungicides. It is a protectant and locally systemic fungicide proven to reduce colonization of flowers by *B. cinerea* and to enhance control of gray mold in strawberries (25,26,32). Fenhexamid targets the 3-keto reductase (3-KR), one of the three microsomal enzymes involved in the inhibition of the C4-demethylation complex (9,22). Resistance to fenhexamid in *B.*

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary figure is available online.

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cinerea has been reported in French and Chilean vineyards (2,10,13) and in a single case from different vegetables in Greece (35). Resistance to fenhexamid in *B. cinerea* has been linked to a point mutation at codon 412 in the *erg27* gene encoding for the 3-KR enzyme (13). This mutation was found to be deleterious to mutants carrying it in comparison to the wild-type isolates under laboratory conditions (6). The extent to which these isolates can persist in the field and cause control failures is unknown.

A severe gray mold epidemic affected many strawberry fields in Florida in February 2012 and caused high yield losses. Such situations warrant detailed information regarding the occurrence of *B. cinerea* populations resistant to the most common fungicides in use to provide scientifically based recommendations and to avoid a total control failure. Therefore, this study was intended to (i) determine the level of in vitro and in vivo sensitivities to quinone outside inhibitors, succinate dehydrogenase inhibitors, hydroxyanilides, phenylpyrroles, and anilino-pyrimidines in *B. cinerea* populations collected from commercial strawberry fields in Florida, and (ii) investigate the multifungicide resistance and cross-resistance patterns.

Materials and Methods

Fungal isolates. A total of 392 *B. cinerea* isolates collected over a 3-year period between 2010 and 2012 from commercial strawberry fields were used in this study (Table 1). Isolates were collected from nine fields in Hillsborough County, where the majority of strawberries are grown in Florida, and from one field in Citrus County. Single-spore isolates were grown on malt yeast agar (MYA) medium for 1 week and stored in 20% glycerol at -80°C . Conidial suspensions for in vitro and in vivo experiments were prepared from new cultures grown from the stock for 7 days at

23°C on 2% malt extract agar for each new experiment. The conidia were scraped from the surface of the culture, suspended in 15 ml of sterile distilled water containing 0.05% Tween 80, and filtered through cheesecloth. The concentration was determined using a hemacytometer and adjusted to a final concentration of 10^6 conidia/ml.

Fungicides. All isolates were tested for their sensitivity to boscalid (Endura, BASF Crop Protection, Research Triangle Park, NC), pyraclostrobin (Cabrio EC, BASF), pyrimethanil (Scala SC, Bayer CropScience, Research Triangle Park, NC), cyprodinil (Vanguard WG, Syngenta Crop Protection, Greensboro, NC), fludioxonil (Medallion WP, Syngenta), and fenhexamid (Elevate WDG, Arysta LifeScience, Cary, NC), whereas 150 isolates were tested for boscalid + pyraclostrobin (Pristine, BASF) and cyprodinil + fludioxonil (Switch WG, Syngenta). These fungicides are the most commonly used to control gray mold in Florida. The characteristics of the fungicides and the concentrations used are summarized in Table 2. For simplicity, the mixtures of boscalid + pyraclostrobin and cyprodinil + fludioxonil are referred to as Pristine and Switch, respectively, throughout the manuscript. Stock solutions of the fungicides were prepared in 10 ml of sterile distilled water and stored at 4°C . Salicylhydroxamic acid (SHAM) was added at 100 $\mu\text{g/ml}$ to autoclaved media to test for sensitivity to pyraclostrobin and Pristine.

Media. The sensitivity to cyprodinil and pyrimethanil was evaluated using mycelial growth inhibition tests on a minimal Czapek-Dox agar medium (CzA) containing 2 g NaNO_3 , 0.5 g KCl, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g Fe_2SO_4 , 1 g K_2HPO_4 , 0.01 g ZnSO_4 , 0.005 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 30 g sucrose, and 12 g agar in 1.0 liter of distilled water (29). The sensitivity to fludioxonil and Switch was determined on CzA amended with 5 g/liter yeast extract. The sensitivity

Table 1. Origin of *Botrytis cinerea* isolates used in this study

County	City	Location ^z	Number of isolates ^y			
			<i>n</i> total	2010	2011	2012
Hillsborough	Balm	GC	70	17	10	43
	Balm	BBY	22	0	0	22
	Balm	ASB	22	0	0	22
	Dover	PC	50	6	12	32
	Dover	SS	61	8	13	40
	Dover	AN	27	5	14	8
	Dover	BI	31	5	10	16
	Dover	ASD	23	0	0	23
	Plant City	FY	57	5	13	39
Citrus	Floral City	FS	29	0	0	29
Total			392	46	72	274

^y Total number of isolates per location and the number of isolates collected per location in each year.

^z Abbreviation code corresponding to each surveyed strawberry field.

Table 2. Characteristics, concentration, ranges, and recommended field rates of fungicides used in this study

Active ingredient (a.i.)	Trade name	% a.i.	Chemical group ^y	Stock solutions a.i. ($\mu\text{g/ml}$) ^w	Range ($\mu\text{g/ml}$) ^x	Recommended field rate/liter
Boscalid	Endura	70.0	SdhI	880.0 ^y 56,900.0 ^y	0.05 to 5 3.63 to >688	0.86 g
Pyraclostrobin	Cabrio	20.0	QoI	187.0 ^y 61,100.0 ^y	0.01 to 1 3.5 to >711	1.09 g
Boscalid + pyraclostrobin ^z	Pristine	25.2 + 12.8	SdhI + QoI	57,000.0	0.73 to 137.6/1.11 to 225.11	1.79 g
Pyrimethanil	Scala SC	54.6	AP	28,548.7	<0.79 to 200	1.4 ml
Cyprodinil	Vanguard WG	75.0	AP	15,023.5	<0.69 to 100	0.7 g
Fludioxonil	Medallion WP	50.0	PP	78.1	0.005 to 0.5	2.5 g
Fludioxonil + cyprodinil ^z	Switch WG	25.0 + 37.5	AP + PP	152.6 (62.5 + 90.1)	0.004 to 0.4/0.006 to 0.6	1.05 g
Fenhexamid	Elevate WDG	50.0	Hyd	84.2 ^y 16,834.1 ^y	0.004 to 0.66 0.75 to 132.3	1.8 g

^y QoI, SdhI, Hyd, PP, and AP indicate quinone-outside inhibitors, succinate dehydrogenase inhibitor, hydroxyanilide, phenylpyrroles, and anilino-pyrimidines, respectively.

^w Concentrations of the active ingredient predetermined by the spiral gradient endpoint (SGE).

^x Radial concentration range from the center to the perimeter of the plate.

^y Lower and higher concentrations were used to test for low and high resistance to each fungicide, respectively.

^z Sensitivity to Pristine and Switch was determined on 150 isolates.

to pyraclostrobin and fenhexamid was evaluated on potato dextrose agar (PDA) using a mycelial growth inhibition test because the latter was found to be accurate in characterizing resistant from sensitive isolates (3,19,21,29). The sensitivity to boscalid was assessed using conidial germination tests on water agar (WA) based on results from previous studies (3,42). Media were prepared at least 12 h before each experiment as explained below.

In vitro phenotyping of isolates. The sensitivity of isolates was determined using the spiral gradient dilution method developed previously by Förster et al. (15) except that cellophane strips were replaced with agar strips to simplify the inoculation step. To produce the agar strips, 70 to 80 ml of MYA containing 20 g/liter of agar was poured into 15-cm-diameter petri dishes to obtain a 4- to 5-mm thick layer of agar. The MYA contained 20 g of agar instead of 12 g/liter to harden the medium and make the strips easier to manipulate for plate inoculation. The medium was amended with 0.5 g/liter of streptomycin and 0.2 g/liter ampicillin after autoclaving. For fast and more efficient production of inoculum strips, we designed an agar slicer (Supplementary Fig. 1) to produce strips with the exact dimensions required for the SGD method. Sixteen agar strips (9 cm long), obtained by pressing the agar slicer into the MYA medium, were cut perpendicularly into 32 strips (4.5 cm long and 6 mm wide) using a sterile scalpel. One milliliter of a conidial suspension (10^6 conidia/ml) from each isolate prepared as described above was spread onto the sliced agar using a glass spreader. The plates were sealed with Parafilm and incubated for 48 h at 23°C and used for fungicide sensitivity tests as described below.

For fungicides tested using mycelial growth inhibition, 50 µl of each fungicide stock solution at the concentrations indicated in Table 2 were applied spirally on 15-cm-diameter plates containing the corresponding medium using the spiral plater (Spiral System, Autoplate 4000 model, Spiral Biotech, Norwood, MA). A duplicate mycelium-covered agar strip (from each isolate) was applied radially across the fungicide gradient dilution on each plate allowing testing two isolates per plate. Three replicate plates (six strips per isolate) were used for each fungicide. To verify that the spiral gradient was applied accurately by the spiral plater, a sensitive isolate (03-37) was tested in each experiment in addition to the test isolates. Plates were sealed in plastic bags, incubated at 23°C, and mycelium growth was assessed after 48 h.

To test for sensitivity to boscalid, WA plates (15 cm diameter) were divided into thirds with a marker, and seven 10-µl drops of a conidial suspension at 10^6 conidia/ml were placed in duplicate per third along the concentration gradient using a multichannel pipette. Three isolates were tested per plate. Conidial germination was

evaluated under a microscope after 16 to 24 h of incubation at 23°C to determine a point along the fungicide gradient at which 50% of conidia had germinated. Only conidia with a germ tube twice as long as their diameters were considered germinated. Two replicate plates were used for each isolate. Mycelial growth and conidial germination inhibition tests were conducted twice.

Fruit assay. The fungicides tested in this study were evaluated for their ability to control isolates with different levels of resistance on strawberry fruit. Four to five isolates with different EC_{50} values to the different fungicides tested were selected. Fruit (cv. Camarosa) that had never been sprayed with fungicides were harvested before commercial maturity (white-pink stage) from experimental plots at the Gulf Coast Research and Education Center (GCREC), Wimauma, FL. Fruit were washed in 0.05% sodium hypochlorite for 3 min, rinsed twice with sterile distilled water, and allowed to dry under a laminar flow hood. Subsequently, fruit were placed in carbon-fiber trays, punctured using a 5-mm cork-borer to a depth of 4 mm and immediately sprayed with Scala (a.i. pyrimethanil), Endura (a.i. boscalid), Cabrio (a.i. pyraclostrobin), Vanguard (a.i. cyprodinil), Medallion (a.i. fludioxonil), Switch (a.i. cyprodinil + fludioxonil), or Elevate (a.i. fenhexamid) at field rates shown in Table 2 using a disposable spray gun (Aerove, Gardnerville, NV). Control fruit were sprayed with sterile water. Fruit were incubated for 24 h at room temperature, and a 40-µl droplet of a conidial suspension of *B. cinerea* isolates at 10^6 conidia/ml was placed on each wound. Inoculated fruit were incubated in sealed humidity chambers for 4 to 6 days, and incidence of gray mold was determined as the frequency of fruit showing disease in comparison to the water-sprayed control fruit. The disease severity (DS) was estimated using the formula $DS = (\text{lesion diameter in control fruit} - \text{lesion diameter in fungicide-treated fruit}) / \text{lesion diameter in control fruit} \times 100$. Six replicate fruit were used for each isolate–fungicide combination, and the experiment was conducted twice.

Data analysis. Data from in vitro and in vivo sensitivity tests from repeated experiments were combined after homogeneity of variance between experiments was tested using Bartlett's test. The EC_{50} value of each isolate to the mixtures Pristine (boscalid + pyraclostrobin) and Switch (cyprodinil + fludioxonil) was determined as the sum of the two EC_{50} values determined for each active ingredient separately. Interaction between the two active ingredients of each mixture was calculated based on EC_{50} values of mycelial growth using the Abbot formula (1) as described previously (3). Pearson correlation coefficients were calculated to determine cross-resistance between different fungicides. Gray mold incidence data from fruit assays were subjected to an ANOVA, and means

Table 3. Summary of sensitivity to multiple fungicides of *Botrytis cinerea* isolates from strawberry fields in Florida

Fungicide	Range EC_{50} (µg/ml) ^x	RF ^y	Resistance frequency (%) ^z	Characterization and frequency of resistant phenotypes ^w							
				LR/RS		MR		HR		VHR	
				EC_{50}	%	EC_{50}	%	EC_{50}	%	EC_{50}	%
Boscalid	0.42–>688	>1612.0	85.4	<5	0.0	5 to <50	19.0	≥50 to <500	39.0	>500	42.0
Pyraclostrobin	0.03–>711	>20866.0	86.5	<5	0.0	...	0.0	5 to <500	59.5	>500	40.5
Pristine	0.24–>117	>487.5	86.0	...	0.0	...	0.0	50 to <100	42.0	≥100	58.0
Pyrimethanil	<0.79–125	158.2	59.5	1 to <5	43.5	≥5 to 10	31.8	>10	24.7	...	0.0
Cyprodinil	0.85–84.8	99.8	52.7	1 to <5	56.1	≥5 to 10	22.7	>10	21.2	...	0.0
Fludioxonil	0.01–0.20	20.0	17.8	≥0.1	100.0	...	0.0	...	0.0	...	0.0
Switch	0.03–0.63	35.8	19.8	≥0.1	100.0	...	0.0	...	0.0	...	0.0
Fenhexamid	0.06–132.3	2205	44.4	...	0.0	1.46 to <10	34.8	>10	65.2	...	0.0

^w Isolates were characterized as LR/RS = low/reduced sensitivity, MR = moderately resistant, HR = highly resistant, VHR = very highly resistant based on the EC_{50} value range indicated in the column corresponding to each phenotype.

^x Mean effective concentrations inhibiting 50% of mycelial growth and conidial germination after 48 and 24 h, respectively ($n = 392$ except for Pristine and Switch for which 150 isolates were tested for each fungicide).

^y Resistance factor (RF) was calculated for each fungicide by dividing highest EC_{50} value by the lowest EC_{50} value.

^z Resistance frequency (reduced sensitivity [RS] for fludioxonil and Switch) was estimated based on a discriminatory EC_{50} obtained from the correlation between the discriminatory EC_{50} in vitro and the EC_{50} value of isolates causing gray mold on strawberry fruit when sprayed at the recommended field rate. Discriminatory dose of 5 µg/ml was used for boscalid, pyraclostrobin, pyrimethanil, and cyprodinil, 10 µg/ml for fenhexamid, and 0.1 µg/ml for fludioxonil (and Switch).

were separated using Fisher's LSD test. All statistical analyses were performed using the SAS software (Version 9.2, SAS Institute Inc., Cary, NC).

Results

In vitro sensitivity of *B. cinerea* isolates. EC₅₀ values were the highest for pyraclostrobin and boscalid and ranged from 0.03 to >711 µg/ml and from 0.42 to >688 µg/ml, respectively (Table 3), resulting in respective mean EC₅₀ values of >347.47 and >268.14 µg/ml (data not shown). Corresponding resistance factors (RF) were >20,866 and >1,612, respectively. EC₅₀ values for Pristine ranged from 0.24 to >177 µg/ml with a RF > 487.5. The RF (2205) for fenhexamid was the highest after pyraclostrobin among all tested fungicides, and EC₅₀ values ranged from 0.06 to 132.31 µg/ml (Table 3).

The mean EC₅₀ values for the APs pyrimethanil and cyprodinil were 20.79 and 10.21 µg/ml, respectively (data not shown), and the EC₅₀ values ranged from <0.79 to 125 µg/ml and from 0.85 to 84.8 µg/ml, respectively. Respective RFs were 158.2 and 99.8 (Table 3). The EC₅₀ values for fludioxonil ranged between 0.01 and 0.20 µg/ml, which resulted in the lowest RF (20) among all fungicides tested in this study. The mean EC₅₀ value for Switch was three times higher than the mean EC₅₀ value for fludioxonil (data not shown). The RF for Switch was 2 times higher than fludioxonil and 3 times lower than the RF of cyprodinil. Neither an additive nor a synergistic interaction was observed between cyprodinil and fludioxonil in the mixture Switch since calculated interaction ratios were less than 0.5 for all isolates (data not shown).

Resistance phenotyping. Overall, 339 (86.5%) and 335 (85.4%) isolates were resistant to pyraclostrobin (Pyr^R) and boscalid (Bosc^R), respectively (Table 3). Two resistant phenotypes: high (Pyr^{HR}, EC₅₀ from 5 to 500 µg/ml) and very high (Pyr^{VHR}, EC₅₀ > 500 µg/ml) level were distinguished for pyraclostrobin with respective frequencies of 59.5 and 40.5% (Table 3). For boscalid, a third phenotype, moderately resistant (Bosc^{MR}, EC₅₀ from 5 to 50 µg/ml), was distinguished in addition to Bosc^{HR} (EC₅₀ from 50 to 500 µg/ml) and Bosc^{VHR} (EC₅₀ > 500 µg/ml) phenotypes accounting for 19, 39, and 42% of the resistant isolates, respectively.

For the two APs pyrimethanil and cyprodinil, the overall resistance frequencies were 59.5 and 52.7%, respectively. Overall, a majority of isolates showed a low (EC₅₀ from 1 to 5 µg/ml) or moderate (EC₅₀ from 5 to 10 µg/ml) resistance, whereas 24.7 and 21.2% of isolates were characterized as highly resistant (AP^{HR}, EC₅₀ > 10 µg/ml) to the two fungicides, respectively (Table 3). On the other hand, a majority of isolates (78%) had an EC₅₀ value ≤ 0.04 µg/ml for fludioxonil and 17.8% had an EC₅₀ ≥ 0.1 µg/ml (Table 3), whereas the frequency of isolates showing reduced sensitivity to Switch (EC₅₀ ≥ 0.1 µg/ml) was 19.8% (Table 3). The frequency of isolates resistant to fenhexamid was 44.4% with a majority of 65.2% of Fenh^R isolates being highly resistant (Fenh^{HR}, EC₅₀ > 10 µg/ml).

Cross-resistance. The highest correlations were observed between Pristine and boscalid ($r = 0.9506$), Pristine and pyraclostrobin ($r = 0.8898$, $P < 0.001$) (data not shown), as well as between boscalid and pyraclostrobin ($r = 0.7954$, $P < 0.001$) (Table 4). An intermediate positive correlation ($r = 0.537$, $P < 0.001$) was

observed between the two APs cyprodinil and pyrimethanil. The correlations observed between pyrimethanil and both boscalid and pyraclostrobin were stronger in comparison to the correlation with cyprodinil. Significant negative cross-resistance was observed between pyraclostrobin and fludioxonil, whereas correlations observed between the other fungicides were not significant. A low positive significant correlation was observed between Switch and fludioxonil ($r = 0.234$) in contrast to Switch and cyprodinil ($r = 0.175$, data not shown).

Multifungicide resistance (MFR) patterns. The overall frequencies of isolates resistant to one fungicide (FR1) decreased from 21.6% in 2010 to 3.4% in 2012 (Fig. 1A). Meanwhile, the frequency of isolates showing resistance to four fungicides (MFR4) increased from 0 to 23.7%. The isolates showing resistance to three (MFR3) and two (MFR2) fungicides, respectively, were predominant every year (Fig. 1A). Among the FR1 isolates, 43.5, 26.1, 26.1, and 4.3% of isolates were resistant to Bosc, QoI, Hyd, and AP fungicides, respectively (Fig. 1B). In the MFR2 phenotype, 87.4% of the isolates were Bosc-QoI^R, whereas a low frequency included Bosc-Hyd^R (5.4%), QoI-AP^R (2.7%), QoI-Hyd^R (2.7%), or Bosc-AP^R (1.8%) isolates. The MFR3 phenotype included the Bosc-QoI-AP^R, the Bosc-QoI-Hyd^R, the Bosc-Hyd-AP^R, and the QoI-Hyd-AP^R isolates with respective frequencies of 56.5, 40.5, 1.8, and 1.2%, whereas the MFR4 phenotype included the Bosc-QoI-Hyd-AP^R isolates (Fig. 1B).

Among isolates collected in 2010, the FR1 and MFR2 isolates were more frequent in all surveyed locations with the exception of the GC field, where MFR3 isolates were more abundant than MFR2 isolates (Fig. 2). An increase in the frequency of MFR3 isolates was observed in all locations in 2011, and this phenotype was predominant except for the PC, BI, and FY locations. MFR4 isolates were absent from all locations in 2010 but were present in three and five locations in 2011 and 2012, respectively (Fig. 2). The highest frequencies of MFR4 isolates were 32 and 50% and were recorded in the SS and BI locations, respectively, in 2012.

Fruit assay. All sensitive isolates were controlled by the recommended field rate of each fungicide (Table 5). Isolates characterized as highly resistant (HR) and very highly resistant (VHR) to boscalid and pyraclostrobin caused 50 to 100% disease incidence on fruit sprayed preventively with either fungicide. Isolates with EC₅₀ values lower than 5 µg/ml for pyrimethanil, cyprodinil, and fenhexamid inoculated on strawberry fruit were controlled with the field rates of the respective fungicides applied preventively (Table 5). Gray mold incidence and severity were ≥ 50% in all cases, and more specifically, for pyrimethanil (>66%) and cyprodinil (>83.3%) when fruit were inoculated with isolates exhibiting EC₅₀ values higher than those mentioned above. Only Fenh^{HR} isolates were not controlled by fenhexamid. All isolates inoculated on fruit were controlled by fludioxonil (Medallion) and cyprodinil + fludioxonil (Switch) regardless of their EC₅₀ value to these two fungicides (data not shown).

Discussion

Populations of *B. cinerea* very highly resistant (EC₅₀ > 500 µg/ml) to boscalid and pyraclostrobin, two components of a common fungicide used continuously in Florida strawberry fields since

Table 4. Cross-resistance patterns between fungicides in *Botrytis cinerea* isolates from strawberry fields in Florida

Fungicide	Fungicide									
	Boscalid		Pyraclostrobin		Cyprodinil		Fludioxonil		Switch	
	r^z	P	r	P	r	P	r	P	r	P
Boscalid	...	...	0.7954	<0.001	0.1061	0.334	-0.1951	0.073	0.0097	0.929
Pyraclostrobin	0.7954	<0.001	...	...	0.0836	0.448	-0.3161	0.003	-0.0475	0.665
Pyrimethanil	0.3053	0.004	0.3546	<0.001	0.537	<0.001	-0.09	0.407	0.117	0.285
Cyprodinil	0.1961	0.034	0.1836	0.048	...	...	0.175	0.108	0.153	0.160
Fludioxonil	-0.1951	0.073	-0.3161	0.003	0.175	0.108	...	...	0.234	0.030
Fenhexamid	-0.2388	0.027	-0.1461	0.182	0.010	0.922	0.063	0.563	0.027	0.801

^z Pearson correlation coefficients were calculated by log₁₀ EC₅₀ values of 150 isolates collected in 2010 and 2012.

2004, were reported in this study. Consequently, even higher rates of these fungicides will not control the large portion of *Bosc*^{VHR} (42%) or *Pyra*^{VHR} (40.5%) isolates. The high resistance frequencies (>80%) observed for the QoIs in Florida agree with previous reports from strawberry fields in Japan (18), Greece (37), Germany (40), and the Carolinas (12), but not with lower resistance frequencies from apples, grapes, and multiple other crops (19,21,28,41). Resistance frequency observed for boscalid in Florida is equivalent to that reported from strawberry fields in the Carolinas (12) and Greece (39), but much higher compared to those reported from apple orchards in Washington State, vegetable fields in Greece, and berry farms in Northern Germany, where boscalid has been sprayed once or twice a season only and resistance frequencies as low as 20% have been reported (35,40–42). A different genetic background of *B. cinerea* populations from the southeastern U.S. strawberry fields coupled with more intensive spray programs could have influenced the selection for higher resistance frequencies in this region. The positive cross-resistance observed herein between boscalid and pyraclostrobin confirms similar previous reports in vitro (4,19). This may also suggest a role for pyraclostrobin and the other QoIs, which have been sprayed intensively in Florida since 1998 to control other major diseases such as powdery mildew and anthracnose fruit rot, in selecting for *Bosc*^R-populations in the field. It is likely that the small fraction of isolates resistant to boscalid but sensitive to pyraclostrobin carry an intron, reported to impede resistance development to the QoI, after codon 143 of the *cytb* gene (19,28). The lack of variability in phenotypes of QoI^R isolates in contrast to *Bosc*^R isolates from Florida suggests that a single mutation in the cytochrome *b* gene is likely responsible for QoI resistance development, whereas multiple point mutations in the succinate dehydrogenase *SDH*

gene may be involved in resistance to boscalid as reported previously (28,37,39,41,42).

Pyrimethanil (Scala) and cyprodinil, a component of Switch, have been regularly sprayed in Florida strawberry fields between 2004 and 2012, during which resistant populations were selected and became widespread. Resistance frequencies for the two APs were similar to those reported previously from grapes in Switzerland (5) and from vegetable crops in Greece (35). Overall, the AP^S and the AP^{LR} phenotypes were more frequent than the AP^{MR} and the AP^{HR} phenotypes in Florida. The fact that only isolates from the latter two phenotypes were not controlled on detached fruit sprayed with the recommended field rates implies that these two fungicides could still control a large portion of isolates in the field if appropriate spray programs are implemented to reduce the risk of selecting more resistant isolates. Interestingly, and in accordance with previous reports (14,24,35), about 7% of isolates resistant to pyrimethanil were sensitive to cyprodinil. This may explain, in part, that the correlation ($r = 0.537$) between these two fungicides is not as high as it is usually expected to be between active ingredients from the same chemical group. The lower intrinsic activity of pyrimethanil may result from differences in the up-take and detoxification of the two APs by the fungus. Pyrimethanil was registered before cyprodinil (in Switch) for gray mold control in Florida. However, a single application of Switch, containing cyprodinil, per year was found to be enough to create a shift toward reduced sensitivity to cyprodinil, and the risk was doubled with two applications per year (5).

There was no clear evidence for a shift toward resistance to fludioxonil in isolates of *B. cinerea* from strawberry. All RS isolates ($EC_{50} \geq 0.1 \mu\text{g/ml}$) to fludioxonil or Switch were controlled by both fungicides on detached fruit. It is obvious that fludioxonil

Fig. 1. A, Overall frequency of multifungicide resistance (MFR) phenotypes and **B**, frequency distribution of MFR phenotypes observed in *Botrytis cinerea* populations across 10 strawberry fields in Florida ($n = 392$) between 2010 and 2012. FR1 indicates resistance to a single fungicide, whereas MFR2, MFR3, and MFR4 indicate isolates that are resistant to two, three, and four fungicides from different chemical groups, respectively. QoI, SdhI, Hyd, and AP indicate quinone-oxidoreductase inhibitor, succinate dehydrogenase inhibitor, hydroxynilamide, and anilinopyrimidine, respectively.

is the most active fungicide available currently for gray mold management. However, under weather conditions conducive to gray mold such as those that occurred in February 2012 in Florida, the activity of fludioxonil may not be sufficient to control severe epidemics, especially in fields where the fungicide is not sprayed at bloom, a key growth stage for gray mold control on strawberry. Field resistance to fludioxonil has been reported to date only in *Penicillium digitatum* (20), likely due to the complexity of the biochemical and molecular mechanisms involved in its activity (44) and the fitness penalties associated with reduced sensitivity to fludioxonil. Nonetheless, limited applications of fludioxonil, the sole active ingredient to which no resistance is apparent, and continuous monitoring of its resistance are highly recommended to avoid an unexpected shift toward field resistance. Neither additive nor synergistic effects were observed between the two active ingredients in Switch and Pristine as well. This raises a critical question about the impact of co-formulated fungicides on selecting for multiple resistant populations, especially for “high risk” pathogens such as *B. cinerea*, since no beneficial effect of the mixtures has been evidenced in this or in previous studies (21). The same conclusions also may be valid for the new SdhI-fluxapyroxad that

is expected to be co-formulated with pyraclostrobin in the new fungicide Merivon (BASF Crop Protection). Knowing the widespread distribution of QoI^R populations in Florida and elsewhere, it is crucial to determine if resistance to the QoIs would hasten resistance development to the new SdhIs.

Applications of fenhexamid over the peak bloom period in the 1999 and 2000 seasons in experimental plots in Florida significantly improved gray mold control and increased marketable yield (26). After a decade of use, populations resistant to fenhexamid have emerged, and a quick shift toward resistance has occurred in the last 2 years as evidenced herein. Following the decrease in the efficacy of boscalid and QoIs, fenhexamid has been used more frequently in recent years in Florida, which increased the resistance frequency from 2.9% in 2010 to 38.6% in 2012. The two phenotypes Fenh^{MR} (EC₅₀ from 1 to <10 µg/ml) and Fenh^{HR} (EC₅₀ ≥ 10 µg/ml) reported herein agree with the phenotypes reported in previous studies (11,13,40). Based on the results from this study, fenhexamid will not control Fenh^{HR} isolates in the field unless resistant isolates fail to survive under field conditions as was documented recently under controlled conditions (6). In contrast to the other fungicides surveyed in this study, resistance to fenhexamid

Fig. 2. Temporal change in frequency of multifungicide resistance (MFR) phenotypes observed in *Botrytis cinerea* isolates ($n = 296$) collected between 2010 and 2012 from six strawberry fields (GC, PC, SS, AN, BI, FY) in Florida. FR1 indicates resistance to a single fungicide, whereas MFR2, MFR3, and MFR4 indicate isolates that are resistant to two, three, and four fungicides, respectively.

was not found in all locations, presumably because of different spray programs used among locations. Therefore, additional precautions should be taken such as determining the site-specific resistance profiles for more appropriate spray recommendations. The best rotation scenarios would include fenhexamid with fludioxonil and the new SdhIs fluopyram, penthiopyrad, and fluxapyroxad that are expected to be registered soon for gray mold management.

Four MFR phenotypes were detected in *B. cinerea* populations in Florida. The FR1 isolates diminished in frequency and were progressively replaced by the MFR2 and MFR3 isolates. It is clear from these results that the SdhI-boscalid, the QoIs, and the APs would not control a large portion of isolates present in Florida fields. The predominance of Bosc-QoI^R isolates (87.4%) among the MFR2 isolates as evidenced by the strong cross-resistance observed between these two fungicides is certainly a result of a simultaneous selection of QoI-Bosc resistant populations following 8 years of Pristine use in Florida, especially since QoI^R isolates were found to be more fitted to develop resistance to the SdhIs (4,19). Moreover, although the SdhIs and QoIs target the mitochondrial complexes II and III, respectively, both complexes are involved in electron transfer and are structurally interconnected. Knowing the history of resistance of *B. cinerea* to benzimidazoles (BZI) in Florida (N. Peres, *personal observation*) and the low frequency of FR1 isolates found in this study, it is likely that Bosc-QoI^R have been selected from a population already resistant to certain fungicides such as the BZIs. A predominant population (MFR3) seems to have evolved from the MFR2 population and split into two subpopulations. The frequent use of the APs since 2003 in Florida has selected for the predominant MFR3 population Bosc-QoI-AP^R (57%) in comparison to the Bosc-QoI-Hyd^R isolates (41%). This may reflect, in part, the stronger resistance correlation observed between the two APs and the QoIs and boscalid compared to the Hyd. Recently, a MFR4 population (23%), which would be controlled by fludioxonil only, has been selected across almost all locations surveyed as a consequence of the advent of resistance to fenhexamid (A. Amiri, S. M. Heath, and N. A. Peres, *unpublished*). It is interesting to note that the APs

seem to exercise a much stronger selection pressure compared to the Hyd, since the latter was in use several years before the APs. While the molecular mechanism involved in resistance to the APs is still unknown, the resistance to the Hyd has been linked to a point mutation in the *erg27* gene encoding the 3-ketoreductase enzyme (13; A. Amiri, S. M. Heath, and N. A. Peres, *unpublished*). The resistance to fenhexamid does not seem to fit the traditional qualitative resistance development usually associated with single point mutations as was the case for the BZIs and the SdhIs. Therefore, the presence of another mechanism of resistance that may hamper selection for Hyd^R populations is unknown but possible. In a comparable study in French and German vineyards, three resistant phenotypes, MDR1 (multidrug resistance), MDR2, and MDR3, involving positive cross-resistance between unrelated fungicides, have been characterized in *B. cinerea* populations (23,31). The MDR1 showed a reduced sensitivity to PP, AP, and the dicarboxamide (Dic) iprodione, the MDR2 showed reduced sensitivity to AP, Hyd, and Dic, whereas the MDR3 isolates, resistant to most fungicides, are thought to be a genetic combination of the MDR1 and MDR2 (23). An overexpression of a major facilitator superfamily transporter gene *mfsM2* and mutations occurring within its promoter have been suggested to cause the MDR2 isolates to become resistant to the three chemical classes mentioned previously (23,31). In our study, a majority of isolates (20) that showed reduced sensitivity to fludioxonil were also MFR4 but were all controlled by fludioxonil on detached fruits. Because of the control failure that growers experienced in Florida in 2012 and the widespread resistance observed in the present study to most fungicides, fludioxonil will be a key constituent of gray mold management programs in the years to come. Therefore, determining the impact of rotating fludioxonil with other fungicides on selecting for resistance, especially among the predominant MFR3 and MFR4 isolates, and the ability of MFR isolates to survive and evolve to more resistant populations under field conditions would help in developing more appropriate spray programs.

The overall frequency of MFR populations in Florida strawberry fields without considering the Bosc-QoI^R phenotypes was 56.5% of

Table 5. Efficacy of fungicides in controlling gray mold on strawberry fruit inoculated with *Botrytis cinerea* isolates with different levels of sensitivity

Fungicide ^a	Isolate	EC ₅₀ (µg/ml)	Phenotype ^z	Gray mold ^x (%)	
				Incidence	Severity
Pyraclostrobin	05-31	0.17	S	0.0 a	0.0
	05-29	0.59	S	0.0 a	0.0
	10-38	6.25	HR	100 b	100
	11-37	123.31	HR	100 b	70.3
	10-43	606.12	VHR	100 b	82.2
Boscalid	05-29	0.89	S	0.0 a	0.0
	11-26	1.70	MR	0.0 a	0.0
	07-66	13.3	MR	0.0 a	0.0
	11-50	326.41	HR	52.8 b	50.7
	10-35	562.01	VHR	100 c	87.2
Pyrimethanil	07-64	<1.79	S	0.0 a	0.0
	08-99	2.79	S	0.0 a	0.0
	10-34	6.10	MR	66.7 b	68.2
	10-33	26.9	HR	100 c	98.2
Cyprodinil	08-96	2.70	S	0.0 a	0.0
	05-251	3.20	S	0.0 a	0.0
	07-65	6.30	MR	83.3 b	83.2
	10-34	13.57	HR	100.0 b	91.3
Fenhexamid	05-25	0.12	S	0.0 a	0.0
	08-98	1.56	MR	0.0 a	0.0
	10-34	10.75	HR	50.0 b	68.9
	11-48	12.54	HR	75.0 b	92.4

^x Gray mold incidence was estimated in comparison to the nontreated control. Data are the means of six values across two experimental runs. Gray mold incidence values followed by the same letters for each fungicide separately are not significantly different based on ANOVA and LSD test at $P \leq 0.05$.

^y Fruit never sprayed with any fungicide were surface-sterilized and wounded near the calix area. Fruit were sprayed preventively with pyraclostrobin (Cabrio), boscalid (Endura), cyprodinil (Vanguard), and fenhexamid (Elevate) at 1.09, 0.86, 0.7, and 1.8 g/liter, respectively. Pyrimethanil was applied as Scala at 1.4 ml/liter. Fruit were incubated overnight at room temperature and inoculated with a 40-µl droplet of conidial suspension at 10^6 conidia/ml from each isolate. Incidence and severity of gray mold was evaluated after 4 to 5 days of incubation at 24°C. Data for fludioxonil and Switch are not shown.

^z S, MR, and HR indicate sensitive, moderately resistant, and highly resistant, respectively.

the total population, equivalent to 55% of multiple resistant populations found in French vineyards (23) but higher than 35 to 38% reported in German vineyards (23) and kiwi orchards in Greece (4). However, adding the Bosc-Qol^R phenotypes increases the frequency of MFR to 70% of the total population in Florida. To the best of our knowledge, applications of the same fungicide have been limited to one per year in most European vineyards several years ago, whereas some fungicides are still sprayed four to six times a year in Florida. The overall fungicide resistance situation in Florida may be worsened by several factors. First, strawberry production in Florida is intensive and with up to 90% of the fields situated within a 50-km perimeter around Plant City and Dover. This may increase the risk of cross-contamination and gene flow between fields where different spray regimes are observed. Moreover, it is possible that new populations of *B. cinerea* are being introduced every year to Florida via the strawberry plantlets shipped from different nurseries in North America. It is, however, unknown if part of the potential new inoculum is already resistant to certain fungicides commonly used in nurseries. Ultimately, it is possible that MFR isolates are being spread when strawberries carrying latent infections are shipped from Florida to other regions.

Our data substantiate widespread resistance to multiple fungicides in *B. cinerea* on strawberries and explain in part the recent gray mold epidemic that has spread across Florida fields. This new situation requires drastic behavioral changes in gray mold management including changes in fungicide spray regulations and recommendations. Continuous research and monitoring efforts will be necessary in the future to evaluate the risks associated with such widespread resistance as well as developing new strategies to avoid the selection of more highly resistant populations.

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