Fungicide Resistance Profiles in *Botrytis cinerea* from Strawberry Fields of Seven Southern U.S. States

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Abstract

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The sensitivity to seven chemical classes of fungicides was investigated in 1,810 *Botrytis cinerea* isolates collected from strawberry blossoms and fruit in 181 strawberry fields from seven southern states in the United States across 2 years. Ten isolates were examined from each field. Fungicide sensitivity assays were carried out based on visual assessment of diametrical mycelial growth after 4 days of incubation on media amended with discriminatory doses of fungicides in microtiter plates. Results of visual assessments were verified with selected isolates using a previously published germination assay and by inoculating representative isolates with resistant phenotypes on fungicide-sprayed fruit. The overall resistance frequencies of 750 isolates collected in 2012 for thiophanate-methyl, pyraclostrobin, boscalid, cyprodinil, fenhexamid, iprodione, and fludioxonil were 76, 42, 29, 27, 25,

3, and 1%, respectively. Frequencies of 1,060 isolates collected in 2013 were 85, 59, 5, 17, 26, 2, and 1%, respectively. Resistance to thiophanate-methyl and pyraclostrobin was found in virtually every location in both years, whereas resistance to iprodione and fludioxonil was rarely found. Resistant isolates were resistant to either one (23%), two (18%), three (19%), four (14%), five (3%), or six (0.1%) chemical classes of fungicides in 2012. In 2013, this distribution was 24, 29, 26, 8, 2, and 0.3%, respectively. Multifungicide-resistant isolates of *B. cinerea* were widespread in southern states and evidence suggests that the frequency of isolates with multifungicide resistance increased from 2012 to 2013. The data also show that fungicide resistance in *B. cinerea* was already present in blossoms, indicating that resistance management needs to be implemented early in the season.

Chemical control of gray mold of strawberry caused by *Botrytis cinerea* Pers. is essential to prevent pre- and postharvest fruit decay; however, resistance to multiple chemical classes of fungicides, including anilinopyrimidines (APs; cyprodinil and pyrimethanil), dicarboxamides (DCs; iprodione), hydroxylanilides (HAs; fenhexamid), methyl benzimidazole carbamates (MBCs; thiophanate-methyl), quinone outside inhibitors (QoIs; pyraclostrobin), or succinate dehydrogenase inhibitors (SDHIs; boscalid), was found recently in *B. cinerea* from strawberry fields in North Carolina and South Carolina (8–11,13,14). A significant proportion of isolates were resistant to thiophanate-methyl, pyraclostrobin, boscalid, and cyprodinil (8,9,11).

Multifungicide resistance in *B. cinerea* isolates is also well known in Florida, the largest strawberry production region on the U.S. east coast. In a study carried out between 2010 and 2012, 392 *B. cinerea* isolates were collected from Florida strawberry fields and evaluated for sensitivity to registered site-specific fungicides. The study documented widespread resistance to boscalid, pyraclostrobin, fenhexamid, cyprodinil, and pyrimethanil (1). Isolates resistant to two, three, and four fungicides from different chemical groups were also observed (1). Reports from other states are rare or focus on just one chemical class. For example, resistance to DCs in

B. cinerea was described on the west coast and in North Carolina in the 1990s (5,15) and Louisiana in 2000, 2002, and 2010 (29–31). Resistance to HAs, QoIs, and SDHIs has been documented only in California (12,22) and resistance to MBCs in *B. cinerea* from strawberry was reported from the west coast (15,22), North Carolina (5), and Louisiana (29,31).

Field resistance to phenylpyrroles fungicides (PPs, fludioxonil) in fungal plant pathogens is uncommon and has been reported in only a few studies. Resistance to fludioxonil has been described only in isolates from grapevines in Germany (18), from apple groves in Washington State (33), from a strawberry field in Virginia (7), and from blackberry fields in South Carolina (13). Some isolates with resistance to fludioxonil possessed fitness penalties (33), which may at least partly explain the low frequency of fludioxonil-resistant isolates in *B. cinerea* populations in the field (7,18).

In the southeastern United States, strawberry fields are sprayed weekly during the flowering period, which typically continues well into the production season, resulting in multiple fungicide treatments during the season. The appearance of gray mold despite applications of fungicides has been reported by strawberry growers in several states, especially during highly favorable conditions, enhancing concerns about the presence of fungicide-resistant isolates of *B. cinerea* in the region. Therefore, the objective of this study was to determine the prevalence and distribution of fungicide resistance in gray mold populations from strawberry fields of as many states as possible in the southern United States. A simple method to monitor resistance to seven classes of fungicides was developed based on visual assessment of mycelial growth and allowed screening of 1,810 isolates from 181 strawberry fields in seven states.

Materials and Methods

B. cinerea isolates recovered from storage and strawberry fields. In total, 63 single-conidia isolates of *B. cinerea* with 11

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defined resistance phenotypes and genotypes were recovered from storage to compare the germination assay published by Weber and Hahn (28) with the mycelial growth assay developed in this study for large-scale resistance monitoring. The isolates were collected previously from strawberry fruit with gray mold symptoms from different locations in North Carolina (Duplin, High Point, Mooresville, and Shelby) and South Carolina (Chesnee, Florence, Lexington, McBee, Monetta, and North Augusta). The sensitivities and molecular bases of resistance to APs, DCs, HAs, MBCs, PPs, QoIs, and SDHIs had been assessed (8,9,11,13,14). Single-conidia isolation was performed as previously described (8). For long-term storage, mycelium of each isolate was maintained at -80°C in 80% (vol/vol) glycerol (Fisher Scientific). For conidial production, all isolates were grown on potato dextrose agar medium (PDA; Difco Laboratories) in 9-cm-diameter petri dishes for 10 days at 22°C with 14-h intervals of fluorescent light and 10 h of darkness. Conidia were harvested by flooding the dish surface with 2 ml of sterile distilled water, and pipetting the suspension gently off the dish surface. Conidial suspensions were filtered through a 34- μm nylon mesh to remove fungal mycelium and adjusted to the desirable concentration for the *in vitro* (2 to 4×10^5 conidia/ml) or *in vivo* (10^6 conidia/ml) assay.

In 2012 and 2013, 1,810 bulk conidial isolates of *B. cinerea* were collected from 181 strawberry fields (10 isolates per field, designated a “sample” in this study) in seven states, including Arkansas (110 isolates), Florida (250 isolates), Georgia (90 isolates), Maryland (310 isolates), North Carolina (190 isolates), South Carolina (660 isolates), and Virginia (200 isolates) (Table 1). Isolates were collected from sporulating blossoms (660 isolates) and fruit (1,150 isolates). Most (77%) of the flower and fruit samples did not originate from the same farm. Blossoms and fruit samples from the same farms (23%) were not necessarily from the same field, which is why these datasets were considered to be not dependent. To obtain conidia from blossom samples, 20 to 40 strawberry blossoms with a degenerated, black torus (likely caused by freeze damage) were obtained from each strawberry field tested. After petals were removed, the blossoms were surface-sterilized with 10% bleach for 1 min, rinsed with sterile water for 1 min, and allowed to air dry for 5 min. Then, the blossoms were placed into 15-cm-diameter petri dishes containing sterile filter paper imbibed with 2 ml of sterile water. The blossoms were kept at 22°C for 2 to 4 days, after which many became symptomatic for gray mold. During the first 24 h, the dishes were kept in sealed plastic bags to keep the relative humidity at 98 to 100%. For fruit samples, 10 to 12 individual strawberry fruit with small (young) gray mold lesions were obtained from commercial fields; each fruit came from a different plant with at least five buffer plants between sampled plants. Conidia were collected using individually wrapped sterile cotton swabs (Fisher Scientific). The cotton tip was rubbed gently against the youngest area of sporulation (periphery of the lesion) of a fruit to capture conidia. The white cotton tip turned from pure white to lightly gray, indicating that sufficient conidia were collected; then, the swab was returned to its wrapper. Fungicide sensitivity of the bulk conidial isolates was defined using a novel mycelial growth assay.

Sensitivity of *B. cinerea* from strawberry to cyprodinil, iprodione, fenhexamid, thiophanate-methyl, fludioxonil, pyraclostrobin, and boscalid. The following active ingredients were obtained as formulated products: AP fungicide cyprodinil (Vangard WG fungicide; Syngenta Crop Protection), DC fungicide iprodione (Rovral 4 Flowable; Bayer Crop Sciences), HA fungicide fenhexamid (Elevate 50 WDG; Arysta Life Science), MBC fungicide thiophanate-methyl (Topsin M 70WP; Ceraxagri), PP fungicide fludioxonil (Scholar SC fungicide; Syngenta Crop Protection), QoI fungicide pyraclostrobin (Cabrio EG fungicide 20% W/W; BASF Corporation), and SDHI fungicide boscalid (Endura fungicide 70% W/W; BASF Corporation).

A single dose for each fungicide was determined on appropriate media that could be applied in mycelial growth assays to distinguish sensitive and resistant strains and that yielded results similar to the germination assay described by Weber and Hahn (28). Assays were conducted with 63 representative *B. cinerea* isolates with different fungicide-resistant phenotypes that were determined previously (8,9,11,13,14). For the conidia germination assay, a 40- μl drop of conidia suspension for each isolate and each fungicide concentration was streaked across the length of the corresponding medium to ensure an even distribution of conidia. Germination was assessed visually under a microscope (OLYMPUS BX41TF; Olympus Optical Co. Ltd.) at $\times 40$ magnification after 12 to 14 h (or 16 h in the case of cyprodinil and boscalid) of incubation at 22°C , as described previously (28). Isolates resistant (R) to boscalid were distinguished from sensitive (S) isolates in their ability to grow a germ tube at 50 $\mu\text{g/ml}$. Germination tubes of isolates moderately resistant (MR) and R to cyprodinil grew between 25 and 100% at 1 $\mu\text{g/ml}$ and up to 10% (MR isolates) or between 10 and 30% (R isolates) of the control at 25 $\mu\text{g/ml}$. Isolates R to fenhexamid had a fully grown germ tube at 1 and 50 $\mu\text{g/ml}$. Germ tubes of isolates S to fludioxonil grew 30 to 50% at 0.1 $\mu\text{g/ml}$ but were inhibited at 10 $\mu\text{g/ml}$; germ tubes of MR isolates grew more than 80% at 0.1 $\mu\text{g/ml}$ and 10 to 20% at 10 $\mu\text{g/ml}$. A new resistance category (low resistant [LR]) was assigned to isolates with germ tubes displaying growth of more than 80% at 0.1 $\mu\text{g/ml}$ but that were completely inhibited at 10 $\mu\text{g/ml}$. For iprodione, germ tubes of S isolates showed 10 to 25% residual growth at 5 $\mu\text{g/ml}$ but were completely inhibited at 50 $\mu\text{g/ml}$; germ tubes of MR isolates grew 50% at 5 $\mu\text{g/ml}$ and 20 to 30% at 50 $\mu\text{g/ml}$; isolates with a fully developed germ tube at 5 $\mu\text{g/ml}$ and a germ tube longer than 50% at 50 $\mu\text{g/ml}$ were categorized as R. The assay also distinguished isolates R to trifloxystrobin from S isolates at 10 $\mu\text{g/ml}$ (only germ tubes of R isolates developed). Finally, for thiophanate-methyl, a strong growth inhibition at 1 $\mu\text{g/ml}$ separated S from MR and R isolates whereas, at 100 $\mu\text{g/ml}$, MR isolates showed a markedly inhibited growth compared with the R isolates that showed a strong residual growth (28).

To find a suitable discriminatory dose for mycelial growth assays, three different concentrations were tested initially per fungicide. They were chosen to represent the range of discriminatory doses developed by Weber and Hahn (28) to distinguish S from R isolates in germination assays. Fenhexamid at 100, 50, and 25 $\mu\text{g/ml}$; fludioxonil at 2, 1, and 0.5 $\mu\text{g/ml}$; iprodione at 50, 25, and

Table 1. Number of *Botrytis cinerea* isolates collected from blossoms and fruit in seven different states in 2012 and 2013

State	Number of isolates					
	2012			2013		
	Blossoms	Fruit	Total	Blossoms	Fruit	Total
Arkansas	0	60	60	0	50	50
Florida	0	60	60	0	190	190
Georgia	0	40	40	10	40	50
Maryland	90	80	170	80	60	140
North Carolina	30	50	80	60	50	110
South Carolina	90	230	320	170	170	340
Virginia	20	0	20	110	70	180
Total	230	520	750	430	630	1,060

10 µg/ml; and thiophanate-methyl at 100, 50, and 25 µg/ml were tested in 1% malt extract agar (MEA) (28). Pyraclostrobin at 100, 10, and 0.1 µg/ml, plus the alternative oxidase inhibitor salicyl hydroxamic acid (SHAM) at 100 µg/ml, were added to 1% MEA (28). Cyprodinil at 20, 10, and 4 µg/ml was tested in two media; 0.5% sucrose agar (SA) and CzapeK-Dox agar medium (CzA): 2 g of NaNO₃, 0.5 g of KCl, 0.5 g of MgSO₄·7H₂O, 0.01 g of Fe₂SO₄, 1 g of K₂HPO₄, 0.01 g of ZnSO₄, 0.005 g of CuSO₄·5H₂O, 30 g of sucrose, and 15 g of agar in 1 liter of medium (1,22). Boscalid at 75, 50, and 25 µg/ml was tested in two media—0.5% yeast extract agar (YEA) and 1% yeast bacto acetate agar (YBA)—both media avoid the interference of sugars with the assay (26). Tests were conducted in wells (15 mm in diameter) of 24-well plates (6 by 4 wells, 12.5 by 8.5 by 2 cm; Thermo Fischer Scientific). Inoculated plates were incubated at 22°C for 4 days and diametric colony growth was visually assessed in each well: S for absence of growth, LR for less than 20% diametrical growth, MR for less than 50% but more than 20% diametrical growth, and R for more than 50% diametrical growth compared with the 15-mm well diameter. The experiment was repeated three times.

For large-scale resistance monitoring, two sterile 24-well plates named “A” and “B” were used to determine sensitivity of mycelial growth to various fungicides on selected media at a single discriminatory dose (Table 2). If held horizontally, six wells per row allowed for testing six isolates and four fungicides at one discriminatory dose per plate. Plate A contained the control treatment and three fungicide treatments (boscalid, cyprodinil, and fenhexamid) and plate B contained four fungicide treatments (fludioxonil, iprodione, pyraclostrobin, and thiophanate-methyl). Four 24-well plates were required (two plate As and two plate Bs) to test 10 isolates, leaving 2 of the 12 columns empty. Conidia from sporulating blossoms or from the cotton swabs (obtained from fruit) were transferred directly to the center of each well using a sterile wooden toothpick. Inoculated plates were incubated and assessed as described above.

Detached fruit assay. *B. cinerea* isolates GIK1, HP17, JEY2, NC12, SBY9, SBY36, and WLAND1 with various resistance phenotypes were assessed for their ability to produce disease on fruit sprayed with label rates of fungicides. All of these isolates had been evaluated in the conidial germination assay (28) and the mycelial growth assay (this study), and the molecular basis of fungicide resistance was characterized in previous studies (8,11,13,14). Resistance to boscalid, fenhexamid, pyraclostrobin, and thiophanate-methyl was based on single resistances (SRs) (i.e., point mutations in the corresponding target genes) (8,11,13,14). Commercially grown, mature but firm strawberry fruit were rinsed with sterile water three times for 30 s each and allowed to air dry. Then, they were placed into plastic boxes (8 strawberry fruit per box for each of the three replicates of each treatment). Fruit were sprayed 4 h prior to inoculation with Pristine (QoI fungicide pyraclostrobin and SDHI fungicide boscalid) at 3.0 g/liter, Scala SC (AP fungicide pyrimethanil) at 2.8 ml/liter, Elevate 50 WDG (HA fungicide fenhexamid) at 3.6 g/liter, Scholar SC (PP fungicide fludioxonil) at 2.5 ml/liter, Rovral 4 Flowable (DC fungicide iprodione) at 2.5

ml/liter, and Topsin M 70WP (MBC fungicide thiophanate-methyl) at 2.4 g/liter to runoff using a hand mister. These doses reflect the rate recommended for gray mold control according to the fungicide labels. The AP fungicide pyrimethanil was selected because cyprodinil was not available as a formulated, single-active-ingredient product. The seven *B. cinerea* isolates used for the detached fruit assays were equally S to AP fungicides pyrimethanil and cyprodinil in vitro (*data not shown*). Nontreated fruit were sprayed with sterile distilled water. When the fruit surface had dried off, each fruit was stabbed at one point to a depth of approximately 9.5 mm using a 26G3/8 9.5-mm beveled syringe tip (Becton Dickson & Co.). Immediately thereafter, the wounds were injected with a 30 µl-droplet of conidia suspension prepared in distilled sterile water (10⁶ conidia/ml) using the same type of syringe. Most of the conidia suspension formed a droplet on top of the wounded area. Nontreated strawberry fruit inoculated with sterile water were used as negative controls. After inoculation, the boxes were kept at 22°C for 4 days. During the first 24 h, the boxes were sealed with lids to keep the relative humidity at 98 to 100% and, after that, the boxes were slightly opened. Lesion diameters were measured after 4 days. The experiment was performed twice.

Data analysis. For the detached fruit assay, a statistical model related the experimental responses of the test to replication, isolate, fungicide treatment, and their combination. The method of least squares was used to estimate the model terms connected with the factors. Analysis of variance was used to test for a significant effect of factors on the response means. Whenever a factor was found to be significant, mean separation (using Student's *t* test) was used to further determine the nature of the effect of the factor on the response. All calculations were performed using the statistical package JMP (version 9.0.0; SAS Institute Inc.) and all tests were performed with $\alpha = 0.05$.

Results

Determination of discriminatory concentrations in mycelial growth assays. Resistance phenotypes of 63 isolates were determined using a previously described conidia germination assay (28) and a discriminatory concentration for each fungicide that allowed a similar categorization based on visual assessment of diametrical colony growth was determined (Table 2). Of the 63 *B. cinerea* isolates, 20 were S to all fungicides and 43 represented a total of 11 fungicide resistance profiles based on both assays (Table 3). Visual assessment of diametrical colony growth was carried out 4 days after inoculation and largely yielded phenotypic categorizations consistent with the germination assay. Resistance categorizations were identical in both assays for thiophanate-methyl, pyraclostrobin, fenhexamid, and iprodione, with discriminatory doses in the mycelial growth assay of 100, 10, 50, and 10 µg/ml, respectively. There were slight assessment differences between assays for boscalid, cyprodinil, and fludioxonil. Isolates characterized as R to boscalid in the conidia germination assay were MR in the mycelium growth assay at 75 µg/ml; isolates identified as MR and R to cyprodinil were indistinguishable in the mycelium growth assay, being categorized as R at 4 µg/ml; and isolates LR to fludioxonil

Table 2. Discriminatory concentrations and media used in spore germination and mycelial growth assays to monitor resistance in *Botrytis cinerea*^a

Fungicides	Spore germination assay		Mycelial growth assay	
	Conc (µg/ml)	Medium	Conc (µg/ml)	Medium
Boscalid	1, 50	0.5% YEA	75	1% YBA
Cyprodinil	1, 25	0.5% SA	4	CzA
Fenhexamid	1, 50	1% MEA	50	1% MEA
Fludioxonil	0.1, 10	1% MEA	0.5	1% MEA
Iprodione	5, 50	1% MEA	10	1% MEA
Pyraclostrobin	n/d	n/d	10	1% MEA + SHAM at 100 µg/ml
Trifloxystrobin	0.1, 10	1% MEA + SHAM at 100 µg/ml	n/d	n/d
Thiophanate-methyl	1, 100	1% MEA	100	1% MEA

^a Fungicide concentrations (Conc) and media were previously described by Weber and Hahn (30). CzA = CzapeK-Dox agar medium, MEA = malt extract agar, n/d = not determined, SA = sucrose agar, SHAM = the alternative oxidase inhibitor salicyl hydroxamic acid, YEA = yeast extract agar, and YBA = yeast bacto acetate agar.

were considered R in the mycelium growth assay at 0.5 µg/ml (Table 3). Diametrical colony growth was indistinguishable on 0.5% SA and CzA medium amended with cyprodinil at 4 µg/ml. However, distinction between S and R phenotypes was easier to assess visually on CzA medium because the mycelium was denser compared with 0.5% SA (*data not shown*). For the same reason, 1% YBA was chosen instead of 0.5% YEA amended with boscalid at 75 µg/ml (*data not shown*). Because mycelia of all 63 isolates filled the entire wells containing unamended CzA, 1% MEA, 0.5% SA, 0.5% YEA, or 1% YBA after 4 days of inoculation and because sensitivity assessment was not based on mycelium growth relative to the unamended control, we were able to reduce the number of unamended controls to one (CzA) medium for large-scale resistance monitoring.

Fungicide resistance monitoring. In 2012, 76, 42, 29, 27, 25, 3, and 1% of all isolates of *B. cinerea* (from blossoms and fruit) were MR and R to thiophanate-methyl, pyraclostrobin, boscalid, cyprodinil, fenhexamid, iprodione, and fludioxonil, respectively (Table 4). Resistance to thiophanate-methyl, pyraclostrobin, boscalid and cyprodinil was found in every state. Resistance to fenhexamid was found in all states except Georgia. No isolates R to iprodione were found in samples from Arkansas, Georgia, and

North Carolina. Resistance to fludioxonil was only found in Virginia. In 2013, 85, 59, 5, 17, 26, 2, and 1% of all isolates were R to thiophanate-methyl, pyraclostrobin, boscalid, cyprodinil, fenhexamid, iprodione, and fludioxonil, respectively. Isolates with resistance to thiophanate-methyl, pyraclostrobin, cyprodinil, and fenhexamid were found in every state. Resistance to boscalid was not found in Arkansas. Resistance to iprodione was found in all states but Georgia; resistance to fludioxonil was detected in Maryland, South Carolina, and Virginia (Table 4). The percentage of isolates R to thiophanate-methyl and pyraclostrobin increased in 2013 compared with the previous year but the percentage of isolates R to boscalid and cyprodinil decreased. No change was recorded for fenhexamid, iprodione, and fludioxonil. During the second year of monitoring, the AP fungicide pyrimethanil was included in the mycelial growth assay. All *B. cinerea* isolates R to cyprodinil were also R to pyrimethanil (*data not shown*).

In regard to blossom samples, isolates R to thiophanate-methyl and pyraclostrobin were found in fields of all states at high frequencies (Fig. 1). All fields from South Carolina and Virginia in both years and in Maryland in 2013 had two or more isolates per sample with resistance to thiophanate-methyl. In general, less than 50% of the fields from southern states had two or more isolates per

Table 3. Categorization of resistance phenotypes of *Botrytis cinerea* in spore germination and mycelial growth assays

N ^z	Phenotype ^w													
	Spore germination assay ^x							Mycelial growth assay ^y						
	Tm	Py	Bo	Cy	Fe	Ip	Fl	Tm	Py	Bo	Cy	Fe	Ip	Fl
20	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3	R	S	S	S	S	S	S	R	S	S	S	S	S	S
1	R	S	S	S	R	R	LR	R	S	S	S	R	R	R
2	R	R	S	S	S	S	S	R	R	S	S	S	S	S
1	R	R	R	S	S	MR	S	R	R	MR	S	S	MR	S
6	R	R	R	S	S	S	S	R	R	MR	S	S	S	S
5	R	R	R	S	R	S	S	R	R	MR	S	R	S	S
3	R	R	R	MR	S	MR	S	R	R	MR	R	S	MR	S
6	R	R	R	MR	S	S	S	R	R	MR	R	S	S	S
3	R	R	R	R	S	S	S	R	R	MR	R	S	S	S
9	R	R	R	MR	R	S	S	R	R	MR	R	R	S	S
4	R	R	R	R	R	S	S	R	R	MR	R	R	S	S

^wThiophanate-methyl (Tm), pyraclostrobin (Py), boscalid (Bo), cyprodinil (Cy), fenhexamid (Fe), iprodione (Ip), and fludioxonil (Fl). All resistant phenotypes are indicated in boldface for better visualization.

^xConidia germination assay was performed as previously described Weber and Hahn (30). S = sensitive, LR = low resistant, MR = moderately resistant, and R = resistant.

^ySpores from strawberry blossoms or fruit were transferred onto media with discriminatory doses of fungicides from seven different chemical classes. Visual assessment of diametrical growth relative to the 15-mm well diameter was carried out after 4 days of incubation at 22°C. S = absence of growth, LR = less than 20% diametrical growth, MR = up to 50% diametrical growth, and R = more than 50% diametrical growth.

^zNumber of isolates.

Table 4. Percentage of *Botrytis cinerea* isolates from blossoms and fruit moderately-resistant and resistant to 7 classes of fungicides and collected from 7 different states in 2012 and 2013

Fungicides ^y	Isolates (%)							
	Arkansas	Florida	Georgia	Maryland	North Carolina	South Carolina	Virginia	Total
2012 (n) ^z	(60)	(60)	(40)	(170)	(80)	(320)	(20)	(750)
Tm	90	95	65	42	50	88	100	76
Py	82	33	50	29	26	47	25	42
Bo	18	67	28	13	3	24	50	29
Cy	25	70	30	7	8	30	20	27
Fe	43	63	0	15	8	24	25	25
Ip	0	12	0	2	0	1	5	3
Fl	0	0	0	0	0	0	5	1
2013 (n) ^z	(50)	(190)	(50)	(140)	(110)	(340)	(180)	(1,060)
Tm	76	98	100	81	68	83	86	85
Py	64	44	80	71	50	50	54	59
Bo	0	4	2	14	2	4	7	5
Cy	8	16	26	33	8	15	16	17
Fe	30	58	26	15	8	20	23	26
Ip	2	1	0	2	1	5	2	2
Fl	0	0	0	1	0	4	2	1

^yTm = thiophanate-methyl, Py = pyraclostrobin, Bo = boscalid, Cy = cyprodinil, Fe = fenhexamid, Ip = iprodione, and Fl = fludioxonil.

^zNumbers in parenthesis = total number of *B. cinerea* isolates studied from blossoms and fruit each year.

field R to boscalid, cyprodinil, and fenhexamid (Fig. 1). With a few exceptions, the proportion of fields with two or more isolates R to boscalid, cyprodinil, and fenhexamid rose in all four southern states in 2013. Isolates R to fludioxonil and iprodione were rarely found in blossoms (Fig. 1). Only one field from Georgia was analyzed in 2013 and only resistance to thiophanate-methyl was found (*data not shown*).

In regard to fruit samples, resistance profiles largely resembled resistance profiles from blossoms (Fig. 2). Almost every sample collected from southern state fields had two or more isolates R to thiophanate-methyl. The overwhelming majority of samples collected in 2013 had two or more isolates R to pyraclostrobin whereas, in 2012, the frequency of fields with two or more isolates R to pyraclostrobin was only around 50% for four of the six states. With a few exceptions, less than 50% of the fields had two or more isolates per sample R to boscalid. Compared with boscalid, higher proportions of fields with two or more isolates per sample R to cyprodinil and fenhexamid were found. With one exception, the proportion of fields with two or more isolates R to fenhexamid, pyraclostrobin, and thiophanate-methyl (Maryland, South Carolina, and North Carolina only) rose in all five southern states sampled in 2013; the proportion of fields with isolates R to boscalid declined. The most fields with resistance to fenhexamid and cyprodinil were found in Florida (Fig. 2). Very few fields with isolates R to iprodione and fludioxonil were found in both experimental years. Fields with two or more isolates per sample R to iprodione were found in Maryland and South Carolina and fields with two or more isolates per sample R to fludioxonil were found in South Carolina in 2013.

Resistance to multiple fungicides in individual isolates. The majority of *B. cinerea* isolates from blossom and fruit samples possessed resistance to one or more fungicide classes. In 2012, 23, 18, 19, 14, 3, and 0.1% of the isolates were R to one (1 single

resistance; SR), two (2SR), three (3SR), four (4SR), five (5SR), and six (6SR) chemical classes of fungicides. In 2013, fewer isolates S to all chemical classes were detected, as well as a substantial decrease of 4SR isolates. The frequency of isolates with 2SR and 3SR increased (Fig. 3). The highest frequency of isolates with resistance to one or more fungicides was found in Florida and Virginia in 2012 and Georgia and Florida in 2013 (Fig. 4). Isolates with 4SR, 5SR, or 6SR phenotypes were found in all southern states in both years but the frequencies and distributions changed over the 2 years. In 2012, there was a rather uniform distribution of those phenotypes but, in 2013, the frequency of 4SR, 5SR, or 6SR phenotypes was clearly higher in Maryland, Virginia, South Carolina, and Florida compared with the other states (Fig. 4). The most dramatic reduction of isolates that were S to all fungicide classes was observed in Maryland and Georgia.

Detached fruit assay. Inoculation of selected isolates was conducted on sprayed and nonsprayed fruit to further validate our mycelial growth assay. Typical gray mold symptoms and signs developed on the fruit sprayed with distilled sterile water at the site of inoculation. Light-brown lesions developed quickly and conidia formed typically after 4 days of inoculation. Field rates of Pristine (pyraclostrobin plus boscalid), Scala SC (pyrimethanil), Elevate 50 WDG (fenhexamid), Scholar SC (fludioxonil), Rovral 4 Flowable (iprodione), and Topsin M 70WP (thiophanate-methyl) controlled S isolates but not the R ones (Table 5). The isolates considered R to a certain chemical (e.g., boscalid) in vitro also developed gray mold symptoms on nontreated fruit and fruit treated with the corresponding formulated product (e.g., Pristine). Only isolates SBY9 and WLAND1 produced smaller lesion sizes on strawberry fruit treated with Rovral 4 Flowable and Scholar SC, respectively, compared with the control fruit (Table 5).

Discussion

This study establishes that fungicide resistance in *B. cinerea* from strawberry fields is widespread in the southern United States.

Fig. 1. Origin and frequency of strawberry fields with blossom-derived *Botrytis cinerea* isolates sensitive or resistant to seven classes of fungicides collected in 2012 and 2013.

Fig. 2. Origin and frequency of strawberry fields with fruit-derived *Botrytis cinerea* isolates sensitive or resistant to seven classes of fungicides collected in 2012 and 2013.

Resistance to thiophanate-methyl and pyraclostrobin was found in virtually every field at high frequencies and resistance to fenhexamid, cyprodinil, and boscalid was commonly found in the majority of states in both years. MBC fungicides were introduced in the 1970s for the control of many diseases of strawberry. The intensive use of thiophanate-methyl during three decades, the high-risk character of *B. cinerea* for resistance development (19), and the high fitness of the MBC-R isolates (23) has contributed to a shift in the endemic population toward MBC resistance dominance. The high frequencies of thiophanate-methyl-resistant isolates obtained in our study (81%) were in agreement with previous observations in the United States and Spain (2,6,11,15,22,30). Taking into account the widespread occurrence of thiophanate-methyl resistance, this fungicide should not be used, or used with caution, for gray mold control in southern states for the foreseeable future.

Fig. 3. Frequency of single resistance phenotypes in *Botrytis cinerea* isolates collected in 2012 ($n = 750$) and 2013 ($n = 1,060$).

QoI and SDHI resistance in *B. cinerea* isolates was common in most states but, during the second year of monitoring, the frequencies of resistance were higher for pyraclostrobin and lower for boscalid in almost all states. This can be explained with practical aspects of gray mold control. Strawberry growers have been using the pyraclostrobin + boscalid combination formulated as Pristine 38WG frequently since 2003 and resistance to both active ingredients emerged. The phenomenon of dual resistance to QoIs and SDHIs has previously been reported for *B. cinerea* (1,3,8,20). Due to the aforementioned extension efforts, many producers from the eastern United States reduced considerably the number of Pristine 38WG applications in their fields (G. Schnabel, *personal communication*; N. A. Peres, *personal communication*). This could, in part, explain the significant reduction of boscalid-resistant isolates of *B. cinerea* in all states analyzed during the second year of monitoring. In general, isolates showing resistance to boscalid originated from fields extensively treated with this fungicide during several growing seasons (3). A recent study from French and German vineyards has shown that the continuous use of Pristine has increased the frequencies of boscalid-resistant isolates in the field from 2 to 26.7% in 4 years of monitoring (17). Resistance frequencies observed in our study for boscalid, with an average of 17%, were similar compared with previous studies from apple orchards in Washington State (32), berries in Germany and France (17), strawberry in Germany (18), and small fruit in Northern Germany (27), which had resistance frequencies of 19, 26.7, 18 and 21.5%, respectively. In these studies, Pristine was sprayed only once a year (32) or during very few growing seasons (18,27). The higher frequencies of resistance to the QoI fungicide pyraclostrobin during 2013 could be explained by the fact that pyraclostrobin and azoxystrobin were still used as solo products (as Cabrio and Abound, respectively) or in premixes such as azoxystrobin + propiconazole (QuiltXcel; Syngenta Crop Protection) for the control of other diseases in strawberry such as powdery mildew and anthracnose fruit rot. This could have helped to maintain and build-up QoI resistance in the gray mold pathogen. The overall frequen-

Fig. 4. Occurrence and prevalence of single resistances (SRs) in *Botrytis cinerea* isolates collected from blossoms and fruit in seven states during the 2012 and 2013 growing seasons.

cies of resistance observed for the QoIs in all states studied were 38 to 73%, which is in agreement with previous reports from strawberry fields in Greece (25) and the Carolinas (8) that reported resistance frequencies of 49 and 66.7%, respectively; and from small fruit in Germany with 76.1% of QoI-resistant isolates (27).

This study documents the presence of *B. cinerea* isolates R to cyprodinil and fenhexamid in all states surveyed. The highest percentages of R isolates for both fungicides, 60% for fenhexamid and 43% for cyprodinil, were obtained from commercial strawberry fields in Florida. In response to detection of resistance to QoI and SDHI fungicides, pyrimethanil, cyprodinil, and fenhexamid have been sprayed more frequently in Florida, increasing selection pressure to the latter fungicides (1). This trend has recently been documented in a follow-up study carried out in Florida, where fenhexamid resistance frequency increased from 2.9% in 2010 to 38.6% in 2012 (1). With the exception of Georgia and North Carolina, the overall frequencies of fenhexamid-resistant isolates obtained in other states was 15 to 40%, comparable with that observed in other studies from the Carolinas (17% frequency of fenhexamid-resistant isolates), California (38%), and Germany (45%) (14,22,27). Cyprodinil resistance frequencies in most states was comparable with previous studies carried out in strawberry fields from California and Northern Germany, with resistance frequencies of 28 and 25%, respectively (18,22), and from small-fruit or commercial vineyards in Germany (17,27).

Resistance to the DC fungicide iprodione and the PP fungicide fludioxonil was rarely observed. Since 1999, strawberry producers have not been using iprodione or have used it only occasionally due to its label-enforced restriction of one application prior to bloom, which could explain the low frequencies of iprodione-resistant isolates in this study. Other studies also failed to find high frequencies of iprodione-resistant isolates (13). Before label restrictions were implemented, some commercial fields in Oregon possessed 41% vinclozolin-resistant isolates (another DC fungicide; 15). Resistance to fludioxonil in fungal plant pathogens is rare and has been reported only in a very few studies (7,13,18,21,33), which makes this chemical class an attractive tool for resistance management programs. Nevertheless, the presence of fludioxonil-resistant isolates in Virginia, Maryland, and, especially South Carolina demonstrates the vulnerability of phenylpyrroles to resistance development. These fludioxonil-resistant isolates were also R to other site-specific fungicides, indicating that alternations and mixtures need to be carefully designed.

We found that, already during bloom, a significant portion of fields had two or more isolates per sample R to the seven classes of fungicides. This indicates that, at the beginning of the spray season, growers are already faced with fungicide resistance management challenges. However, we did not make an attempt to compare and relate the data from blossoms with the data from fruit for several reasons. For some states, including Arkansas, Florida, and

Georgia, we only collected fruit samples for both years. If blossoms and fruit samples were collected from the same states, they did not necessarily come from the same field or even farm. In future studies, the impact of spray programs on fungicide resistance profiles could be determined by sampling blossoms and fruit from the same field because most site-specific fungicides are applied from the beginning of bloom to harvest.

From 2012 to 2013, the number of isolates S to all fungicides (OSRs) declined, whereas the number of isolates with 1 or more SRs increased. The most dramatic increases were observed in isolates with 2SR and 3SR phenotypes. Most often, isolates were R to thiophanate-methyl (1SR), thiophanate-methyl + pyraclostrobin (2SR), thiophanate-methyl + pyraclostrobin + fenhexamid (3SR), thiophanate-methyl + pyraclostrobin + cyprodinil (3SR), and thiophanate-methyl + pyraclostrobin + boscalid (3SR). In contrast to common resistance management recommendations, rotations or mixtures of these chemical classes may not provide adequate control and will continue to select for multifungicide resistance. The overall frequency of *B. cinerea* isolates with resistance to two or more fungicides in the strawberry fields analyzed was 60%, which was similar to the 58% described from kiwi orchards in Greece (3) and 55% in French vineyards (16) but was lower than the 70% reported from strawberry fields in Florida (1). Populations with multifungicide resistance have built up over the last decade due to frequent and consistent applications of site-specific fungicides.

B. cinerea isolates that were characterized as MR and R to cyprodinil by Weber and Hahn (28) were indistinguishable in their resistance phenotype when using the mycelial growth assay developed in this study; MR and R isolates both showed more than 50% diametrical growth after 4 days of incubation. In a recent study, the distinction between these phenotypes was considered of little practical value because both phenotypes display comparable ability to infect wounded fruit pretreated with AP, DC, HA, MBC, PP, QoI, and SDHI fungicides (27). These practical observations were also confirmed in other studies from the Carolinas, where isolates MR and R to cyprodinil and iprodione were equally competitive in vitro and in vivo and were equally virulent on fruit treated with Scala SC and Rovral 4 Flowable (9,13).

Fungi other than the target fungus (fungal contaminants) can be problematic when field samples are directly transferred to complete, nonselective media. Contaminants were observed at very low frequencies and were easily distinguishable from *B. cinerea* based primarily on colony morphology after 4 days of incubation. Contaminants from blossom samples were rarely observed because blossoms were surface sterilized with 10% bleach and, in nearly all cases, the only fungus sporulating on sterilized blossoms after 2 to 4 days of incubation was *B. cinerea*. When conidia were directly transferred from strawberry fruit to the complete medium early in the season, contaminants were observed at very low frequencies

Table 5. In vitro and in vivo fungicide sensitivity assays of *Botrytis cinerea* isolates with defined fungicide resistant phenotypes on nontreated and fungicide treated fruit

Isolate	In vitro fungicide sensitivity assay ^y							In vivo fungicide sensitivity assay, lesion size (Ø in cm) ^z						
	Bo	Py	Cy	Fe	Fl	Ip	Tm	Control	Pristine	Scala	Elevate	Scholar	Rovral	Topsin-M
GIK1	S	S	S	S	S	S	S	3.1 b	0.0 a	0.2 a	0.1 a	0.0 a	0.0 a	0.0 a
HP17	S	S	S	S	S	S	S	2.8 ab	0.0 a	0.3 a	0.0 a	0.0 a	0.0 a	0.0 a
JEY2	MR	R	R	S	S	S	R	2.8 ab	2.7 c	2.5 b	0.0 a	0.0 a	0.0 a	2.9 cd
NC12	MR	R	R	R	S	S	R	2.6 a	2.1 b	2.7 b	3.3 b	0.0 a	0.0 a	2.6 bc
SBY9	MR	R	R	S	S	R	R	3.0 b	2.8 c	2.6 b	0.0 a	0.0 a	1.7 b	3.1 d
SBY36	MR	R	R	R	S	S	R	3.0 b	2.7 c	2.5 b	3.1 b	0.0 a	0.0 a	2.9 cd
WLAND1	S	S	S	R	R	R	R	2.7 ab	0.0 a	0.0 a	2.8 b	1.7 b	2.2 b	2.6 b

^y Resistance phenotypes of *B. cinerea* isolates based on mycelial growth assay. Boscalid (Bo), pyraclostrobin (Py), cyprodinil (Cy), fenhexamid (Fe), fludioxonil (Fl), iprodione (Ip), and thiophanate-methyl (Tm). Sensitive (S) = absence of growth, low resistant (LR) = less than 20% diametrical growth, moderately resistant (MR) = up to 50% diametrical growth, and resistant (R) = more than 50% diametrical growth relative to the 15-mm well diameter after 4 days of inoculation at 22°C.

^z Numbers in columns followed by the same letter are not significantly different at $\alpha = 0.05$, as determined by analysis of variance. Mean separation was conducted using Student's *t* test. Lesion diameters were measured 4 days after inoculation. Pristine (pyraclostrobin+boscalid), Scala (pyrimethanil), Elevate (fenhexamid), Scholar (fludioxonil), Rovral (iprodione), and Topsin-M (thiophanate-methyl). Ø = diameter.

(2%) but frequency increased (to about 5%) at the end of the season as more fungi accumulated over time in strawberry fields. The main fungal contaminants identified were *Cladosporium* spp., *Colletotrichum acutatum*, *Hypocrea lixii*, *Fusarium oxysporum*, and *Rhizopus microsporum* (data not shown). Contaminants were identified when a colony (i) did not originate at the point where the medium was touched with the toothpick, (ii) turned dark green (*Cladosporium* spp. and *H. lixii*) or slightly pink (*C. acutatum*) after 4 days of incubation, (iii) was faster growing than a typical *B. cinerea* colony and completely covered the well with a very dense mass of white mycelium after 4 days of incubation (*F. oxysporum*), (iv) or developed unusual amounts of aerial mycelium stretching to the underside of the plate lid (*Rhizopus* spp.).

The mycelial growth assay developed in this study was useful in quickly and reliably screening hundreds of isolates from commercial strawberry fields. Compared with the germination assay developed by Weber and Hahn (28), this method takes less time to conduct. The mycelial growth assay developed in this study can determine whether a *B. cinerea* isolate is S or R to seven chemical classes of fungicides in 6 to 8 days (for blossom samples) and 4 days (for fruit samples). In contrast, the germ tube assay described by Weber and Hahn (28) involved transfer of the conidia collected from the host to PDA plates and 10 days of incubation to obtain sufficient conidia for the assay (28). Although the time required for the germination assay can be significantly shortened if the conidia from disease tissue are directly used for testing (27), the method still requires a microscope. Whether the transfer of different numbers of conidia from the sporulating flower or the cotton swab to the medium results in different mycelial growth rates was not investigated in this study. However, given the generous incubation time (4 days), we would assume that a colony derived even from a single conidium would have enough time to develop into a colony that can be visually assessed. When conidia are directly taken from plant tissue for resistance testing, results can be obtained in a timely manner; however, there are some disadvantages. The conidia may have derived from a mixed infection of, for example, an S and an R strain. In that case, only the R strain would survive the fungicide treatment. Thus, the S proportion of the population could be underestimated if results were to be used to assess the prevalence of S and R strains.

The use of discriminatory doses allows for fast assessment of fungicide resistance frequencies in a population but there are also some tradeoffs to be considered. If resistance is of qualitative nature, typically caused by target gene alterations, the population consists of two discrete phenotypes that are clearly distinguishable with a single dose (4,24). However, when fungicide resistance is quantitative in nature, characterized by a gradual shift of the population toward reduced sensitivity, a clear differentiation between S and R categories is more difficult to observe and the use of a single discriminatory dose can be misleading. Resistance to cyprodinil and iprodione can be quantitative in nature, and even resistance to boscalid may increase gradually with different point mutations in the *sdh* gene complex conferring different levels of resistance. Therefore, it is possible that any given discriminatory dose may not detect lower levels of resistance that might still be of practical relevance and that resistance in general is being underestimated when using discriminatory doses. Therefore, it is important to verify discriminatory dose-based resistance assessments with actual field performance of fungicides. If disease control is dissatisfactory in a population that has assessed as sensitive in vitro, 50% effective concentration values should be determined and compared with baseline data to identify possible shifts of sensitivities due to new genotypes.

In conclusion, the monitoring data obtained in this study show widespread resistance to thiophanate-methyl, pyraclostrobin, fenhexamid, cyprodinil, and boscalid but the frequency of isolates R to iprodione and fludioxonil was still comparably low. Creative antiresistance management strategies must be developed and implemented to halt the emergence and further selection of multifungicide-resistant isolates.

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