Genotypic and Phenotypic Variations in *Botrytis* spp. Isolates from Single Strawberry Flowers

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Abstract

Gray mold, caused by *Botrytis* spp., is among the most devastating diseases affecting strawberry worldwide. The great diversity present in the pathogen enhances its ability to survive and adapt in the field. In this study, we explored the genotypic and phenotypic diversity present in single strawberry flowers. In total, 192 isolates were collected from 19 flowers and four farms, and 9 to 12 isolates were collected from each flower. Forty-two haplotypes were found using microsatellite fragment analysis. Multiple haplotypes of two different *Botrytis* spp. (*Botrytis cinerea* and *B. fragariae*) were found in 12 flowers. In the remaining seven flowers, the single-spore isolates examined were of identical haplotypes. In three flowers, the two *Botrytis* spp. were found to coexist. Isolates were either sensitive (zero chemical class resistance) or resistant to one, two, three, four, or five chemical classes of fungicides. Resistance to multiple fungicides was commonly observed in both

species but resistance to boscalid and penthiopyrad was only found in *B. cinerea* isolates. Resistance to cyprodinil was found in *B. fragariae* for the first time in the United States. Each haplotype was generally linked to a single resistance profile; however, a single resistance profile often was represented by multiple haplotypes. Isolates from the same flower of multiple haplotypes were largely identical in resistance profiles. This study is a first detailed investigation of genotypic diversity combined with phenotypic analysis of *Botrytis* spp. at the single-tissue level. It demonstrates that high genotypic and phenotypic diversity is present not only within fields but also in individual blossoms as well. This information is important for understanding the epidemiology of *Botrytis* and also has implications for fungicide resistance management, particularly related to resistance monitoring practices.

Gray mold, caused by *Botrytis* spp., of commercial strawberry fruit is initiated through flower infections (Bristow et al. 1986; Jarvis 1962; Powelson 1960). Inoculum for these flower infections in the form of conidia is produced from overwintering sclerotia and plant debris (Williamson et al. 2007) but the fungus can also enter the field in the form of latent infections in nursery stock (Sutton 1998). Open flowers are most vulnerable to infection (Jarvis and Borecka 1968), which is favored by temperatures between 15 and 25°C and moisture in the form of either high humidity or extended leaf wetness (Bulger et al. 1987; Wilcox and Seem 1994). Following penetration, the fungus may remain quiescent in the flower organs (Bristow et al. 1986; Powelson 1960). When fruit matures, the colonization of the tissue may continue, and subsequent sporulation will provide inoculum for secondary infections (Bristow et al. 1986; Powelson 1960).

Strawberry flowers may be infected with multiple species of *Botrytis*, including *Botrytis cinerea*, *B. fragariae* (Rupp et al. 2017), *B. caroliniana* (Li et al. 2012), *B. mali* (Dowling and Schnabel 2017), and *B. pseudocinerea* (Leroch et al. 2013; Plesken et al. 2015a). In addition to *Botrytis* spp., *Amphobotrys ricini* has also been reported to cause gray mold of strawberry in Florida (Amiri et al. 2016). *B. cinerea* and *B. fragariae* are the main pathogens infecting strawberry flowers; however, although *B. cinerea* does not appear to have tissue preferences, the other species appear to prefer flowers and vegetative tissues (Dowling et al. 2017a; Rupp et al. 2017). *B. fragariae* showed poor colonization of inoculated non-host plant tissues such as tomato leaves, gerbera flowers, and apple fruit and invaded strawberry tissues with lower efficiency than *B. cinerea* under laboratory conditions (Rupp et al. 2017).

There appear to be differences with regard to fungicide sensitivity between the two species. The majority of *B. fragariae* isolates collected in Germany and the United States displayed resistance to single or multiple chemical classes of fungicides, and mutations conferring resistance in *B. fragariae* were found to be largely identical to those of *B. cinerea* (Rupp et al. 2017). However, there were some marked differences between German and U.S. *B. fragariae* isolates in their resistance profiles. No resistance to fenhexamid was found in Germany (Rupp et al. 2017) whereas about 10% of *B. fragariae* collected displayed resistance to fenhexamid in the United States, cyprodinil resistance was commonly observed in German isolates but was absent in U.S. isolates, and iprodione resistance in Germany was more frequently observed in *B. fragariae* (Dowling et al. 2017b). Interestingly, no resistance to boscalid was found in *B. fragariae* in either country (Dowling et al. 2017b; Rupp et al. 2017). Clearly, both species have the ability to develop resistance to multiple chemical classes of fungicides.

The different species involved in flower infections and the unique resistance profiles of isolates within and among species warrant a detailed assessment of flower colonization. Whether individual strawberry flowers can be infected by multiple species or even multiple isolates of the same species is unknown. Sexton et al. (2006) showed multiple haplotypes in both petal- and stem-infecting *Sclerotinia sclerotiorum* populations within single plants but whether multiple haplotypes were found within a single petal was unclear. López-Villavicencio et al. (2007) detected 2.33 genotypes, on average, of *Microbotryum violaceum* per *Silene latifolia* plant. Although up to nine different genotypes were found in the same plant, flower buds only revealed a single genotype (López-Villavicencio et al. 2007). Detailed knowledge about strawberry flower colonization would help in assessing fungicide resistance profiles during location-specific monitoring, especially when flowers are used as the source of fungal isolation (Schnabel et al. 2015). Thus, the objective of the present study was to investigate whether different isolates of the same *Botrytis* sp. and even different species can coexist on single flowers. Isolates were fingerprinted using fungicide resistance profiles and polymorphic microsatellite markers (Fournier et al. 2002).

Materials and Methods

Collection of *B. cinerea* isolates from strawberry fields. In total, 192 single-spore isolates were obtained from 19 strawberry flowers

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collected from four different farms during the 2015–16 strawberry season. The number of isolates collected per flower, the number of flowers, and the number and locations of strawberry farms are shown in Supplementary Table S1. Flowers from the same location were collected from different plants at least 5 m apart. Four to five strawberry flowers with black tori were collected from each of the four strawberry farms and processed as described previously (Fernández-Ortuño et al. 2014). Briefly, flower petals were removed, dipped into 10% bleach solution (Clorox, Oakland, CA) for 1 min, and rinsed with sterilized water for another minute. Flowers were then transferred to 150-mm Petri dishes and incubated at room temperature to induce *Botrytis* isolates. For single-spore isolation, conidia were scraped from the sporulating area on the flower using a sterile inoculation loop and suspended in 1 ml of sterile distilled water. The conidial suspension (50 μ l) was spread on 1.5% (wt/vol) water agar plates and incubated at 22°C overnight. In all, 9 to 12 germinated conidia from one flower were transferred onto potato dextrose agar (PDA) plates (Difco Laboratories, Sparks, NJ). All isolates were stored in the form of dried mycelium on filter paper (Whatman Inc., Maidstone, UK) at –20°C, as previously described (Hu et al. 2011).

DNA extraction and genotyping. Each of the 192 single-spore isolates of *B. cinerea* formed mycelial colonies on PDA plates at 22°C. Aerial mycelia and conidia were used to extract genomic DNA according to Chi et al. (2009). Six of the nine microsatellite markers (Bc1, Bc2, Bc3, Bc4, Bc7, and Bc10) developed by Fournier et al. (2002) were used to generate genotypic profiles. The fluorescent probe 5' FAM was used to label the forward primers and a 5'GTTT "pigtail" was added to the reverse primers (Brownstein et al. 1996). Polymerase chain reactions (PCR) were conducted using AccuPower HotStart PCR PreMix (Bioneer Corporation, Daejeon, Republic of Korea), according to the manufacturer's instructions. A touchdown PCR was performed as described previously (Everhart and Scherm 2015). Specifically, amplification conditions were 95°C for 2 min; 10 cycles of 95°C for 20 s, 60°C for 20 s (decreased by 1°C for each cycle), and 72°C for 30 s; followed by 30 cycles of 95°C for 20 s, 50°C for 20 s, and 72°C for 20 s. Fragments derived from microsatellite primers were analyzed as described previously (Dowling et al. 2016). Statistical differences in haploid genetic diversity, number of different alleles, or number of effective alleles between populations was determined using JMP 12 Pro (SAS Institute, Inc., Cary, NC) at $\alpha = 0.05$.

Species identification. *Botrytis* spp., including *B. cinerea*, *B. fragariae*, and *B. mali*, have been found affecting strawberry on the east coast of the United States (Dowling and Schnabel 2017; Rupp et al. 2017). In this study, a previously developed PCR-based method (Dowling et al. 2017a) was used to identify *Botrytis* isolates collected from strawberry flowers. We assumed that different species likely had different haplotypes; therefore, only one representative of each haplotype detected was screened for species identification. Primers CFM_NEP2, C_NEP2, F_NEP2, and M_NEP2 were mixed with a DNA template in AccuPower PCR PreMix (Bioneer Corporation). The amplification program began with an initial denaturation step of 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. This was followed by a final extension step of 72°C for 5 min. Gel electrophoresis of PCR products was performed on 1 or 1.5% agarose gels run at 110 V for 20 to 30 min.

Determination of resistance profiles. Resistance profiles were determined as described previously (Hu et al. 2016b). Briefly, formulated

products of cyprodinil, fludioxonil, fenhexamid, iprodione, thiophanate-methyl, boscalid, fluopyram, penthiopyrad, and isofetamid were tested at discriminatory doses of 4, 0.5, 50, 10, 100, 75, 10, 5, and 5 μ g/ml, respectively. An isolate's resistance profile is defined as a combination of phenotypes either resistant or sensitive to the tested active ingredients.

Sensitivity assays were conducted on 24-well, fungicide-amended plates. To obtain conidia, the isolates were grown in the dark on PDA at room temperature for 4 days and for another 4 days under light conditions (24-h cycle of 80 μ mol/m²/s). Conidia were then collected using a cotton swab and transferred to the center of fungicide-treated and control wells using the tip of a sterile wooden toothpick. For each isolate, a fresh toothpick was used. Inoculated plates were incubated at 22°C for 4 days and resistance was visually assessed in each well, as described previously (Hu et al. 2016b). The presence or absence of growth on fungicide-amended media distinguished resistant from sensitive isolates, respectively. Sensitivity tests for each isolate and fungicide combination were conducted once. However, when multiple resistance profiles were detected in the same flowers, the tests were repeated to confirm.

Results

Genetic variation in *Botrytis* isolates collected from the same flower and from different flowers and locations. The 192 *Botrytis* isolates were genotyped with six microsatellite markers. All six microsatellite loci were polymorphic, with an average of 5.67 alleles per locus and 3.80 effective alleles per locus (Table 1). A genotype accumulation curve was generated to determine whether enough loci were used to distinguish isolates. The curve was sigmoidal and began to plateau at five loci, indicating that six microsatellite markers used in the analysis were sufficient to resolve isolates from each other (data not shown).

Private alleles in single-farm populations (farm populations) ranged from 0 to 5, with farms A and B having the most ($n = 5$) private alleles (Table 1). Genetic diversity is a measure of allelic variation, and no significant difference was observed in haploid genetic diversity between farm populations ($P > 0.05$). The average number of alleles per locus indicates the potential for diversity in the population. There was no significant difference in the number of alleles and the number of effective alleles between farm populations ($P > 0.05$). Analysis of molecular variance (AMOVA) was used to calculate pairwise Φ_{PT} values to investigate the genetic differentiation among populations (Fig. 1A). The results indicated that the Φ_{PT} values were significantly different from 0 between farm populations (Fig. 1A).

In total, 25 haplotypes, designated H1 to H25, and 17 unique haplotypes (haplotypes that only occurred once), designated UH1 to UH17, were detected in the 192 isolates examined. In all, 12 of 19 flowers contained isolates with at least two haplotypes, whereas the remaining 7 flowers contained isolates with a single haplotype (Fig. 2; Table 2). The 10 same-flower (SF) isolates of flower A5 had the highest number ($n = 7$) of haplotypes (Table 2). Farms A, B, C, and D contained 12, 15, 8, and 8 *Botrytis* haplotypes, respectively. Haplotypes from different farms were unique, except for haplotype H20, which was detected in farms B (located in South Carolina) and C (located in Maryland) (Table 2).

Table 1. Microsatellite variation at six polymorphic loci in single-spore *Botrytis* isolates from 19 flowers from four different farms

Population	Number of isolates ^a	Alleles per locus ^b	Effective alleles per locus ^b	Private alleles ^a	Haploid diversity ^b
Farm A	53	2.83	2.03	5	0.49
Farm B	50	3.83	3.07	5	0.70
Farm C	49	2.33	2.05	0	0.44
Farm D	40	2.33	1.90	2	0.45
Pooled	192	5.67	3.80	-	0.72

^a Nonclone-corrected data.

^b Clone-corrected data.

Fig. 1. Pairwise analysis of molecular variance in clone-corrected populations of *Botrytis* isolates. Analyses were conducted based on **A**, farm populations and **B**, resistance phenotype populations. Asterisks denote significant Φ_{PT} values compared with 1,000 randomizations.

Species identification of *Botrytis* isolates from single strawberry flowers. Of the 42 haplotypes identified to the species level, 13 were *B. fragariae*, 2 could not be identified due to lack of bands, and the remaining 27 were *B. cinerea* (Table 2). *B. cinerea* and *B. fragariae* coexisted in flowers A4, A5, and D3. Unidentified isolates were only present in flowers B5 and D2 (Table 2).

Genetic population analysis was also conducted for isolates separated by species. No significant differences were found in the number of alleles and haploid genetic diversity between species populations but differences were detected in the number of effective alleles ($P = 0.483$) (Supplementary Table S2). AMOVA analysis showed that *B. cinerea* and *B. fragariae* populations were different ($P < 0.05$), supporting the separation of the two species. Index of association (I_A) describes linkage disequilibrium and is utilized to indicate sexual out-crossing. If values are not significantly different from zero, then there is likely a high linkage disequilibrium, suggesting that the population is undergoing sexual recombination. I_A values for *B. cinerea* and *B. fragariae* were both significantly different from zero, suggesting both species were lacking in sexual recombination.

Resistance profiles of *Botrytis* isolates from single strawberry flowers. Sensitivity to nine active ingredients representing six chemical classes of fungicides was determined, including anilinopyrimidines (cyprodinil), phenylpyrroles (fludioxonil), hydroxanilides (fenhexamid), dicarboxamides (iprodione), methyl benzimidazole carbamates (thiophanate-methyl), and succinate dehydrogenase inhibitors (SDHI; boscalid, fluopyram, penthiopyrad, and isofetamid). In total, 12 different resistance profiles (RP) were detected for the 192 *Botrytis* isolates used in this study. Isolates were either sensitive (zero chemical class resistance [CCCR]) (0CCR) or resistant to one (1CCR), two (2CCR), three (3CCR), four (4CCR), or five chemical classes (5CCR) of fungicides (Table 3). Only isolates of *B. cinerea* revealed phenotypes that were sensitive to all fungicides or resistant to five chemical classes of fungicides, indicating a wider range of phenotypic diversity as compared with *B. fragariae*. Isolates of the latter species were 1CCR to 4CCR but did not reveal 0CCR or 5CCR phenotypes (Table 3). Resistance to cyprodinil and thiophanate-methyl was most commonly observed in both species (Fig. 3), whereas resistance to fludioxonil and certain SDHI fungicides (fluopyram, penthiopyrad, and isofetamid) were least frequent or nonexistent (Table 3). Frequency of resistance was higher to cyprodinil, fenhexamid, and iprodione in *B. cinerea*, whereas resistance to fludioxonil and thiophanate-methyl was more frequently observed in *B. fragariae* (Fig. 3). Resistance to boscalid and penthiopyrad was only found in *B. cinerea* isolates (Table 3).

The flowers contained isolates with more variation in haplotype than resistance phenotype (Fig. 2). SF isolates had identical resistance profiles, with the exception of flowers A4, A5, and B5 (Table 2). Isolates from flower B5 had the broadest ($n = 5$) resistance profiles; isolates from flowers A4 and A5 both had two resistance profiles (Table 2). Interestingly, within SF isolates, the number of haplotypes was the same or greater than the number of resistance profiles (Table 2).

Fig. 2. Frequency (%) of strawberry flowers containing *Botrytis* spp. isolates with single or multiple phenotypes or haplotypes.

The overwhelming majority of haplotypes was linked to a single resistance profile. Haplotypes H5, H7, H21, and H22 were linked to multiple resistance profiles and each haplotype was found in three, two, three, and two different profiles, respectively (Table 2). The overwhelming majority (11 of 12) of resistance profiles were represented by more than one haplotype (Fig. 4). RP11 consisted of nine haplotypes, leading the number of haplotypes found in a single resistance profile. A single haplotype was only found in profile RP12, which was represented by only three isolates (Fig. 4; Table 2).

In addition to analyzing *Botrytis* populations by farm and by species, we determined the diversity of the 192 isolates by resistance

Table 2. Phenotypes and genotypes of single-spore *Botrytis* isolates from individual flowers

Farm	Flower	Resistance profiles (RP)		Multilocus genotypes	
		Designation	N ^a	Haplotypes ^b	N ^a
A	A1	RP8	10	BcH24	10
	A2	RP2	9	BcH5	1
	...	...	...	BcH6	7
	...	...	...	BcUH7	1
	A3	RP1	12	BcH5	12
	A4	RP1	11	BcH1	11
	...	RP3	1	BfUH10	1
	A5	RP5	8	BfH3	2
	...	RP3	2	BfH4	2
	...	...	...	BcH5	2
	...	...	...	BfUH2	1
	...	...	...	BfUH6	1
	...	...	...	BfUH8	1
	...	...	...	BfUH9	1
B	B1	RP11	10	BcH9	7
	...	...	...	BcH15	2
	...	...	...	BcUH3	1
	B2	RP10	10	BcH25	8
	...	...	...	BcUH16	1
	...	...	...	BcUH17	1
	B3	RP11	10	BcH10	8
	...	...	...	BcH11	2
	B4	RP11	10	BcH20	10
	B5	RP4	1	BcH7	4
	...	RP6	1	BcH14	2
	...	RP7	1	BcUH11	1
	...	RP9	4	UnUH12	1
	...	RP12	3	BcUH13	1
	...	...	...	BcUH14	1
C	C1	RP9	10	BcH16	10
	C2	RP9	10	BcH12	10
	C3	RP8	10	BcH2	8
	...	...	...	BcH8	2
	C4	RP11	10	BcH20	8
	...	...	...	BcUH4	1
	...	...	...	BcUH5	1
	C5	RP6	9	BcH13	9
	D1	RP4	9	BfH21	5
	...	...	...	BfH23	2
D	...	...	...	BfH17	2
	D2	RP3	11	UnH18	5
	...	...	...	BfH19	3
	...	...	...	BfH21	1
	...	...	...	BfUH1	1
	...	...	...	BfUH15	1
	D3	RP7	10	BfH21	6
	...	...	...	BcH22	4
	D4	RP11	10	BcH22	10

^a Number of isolates.

^b BcH and BfH indicate specific haplotypes found in *B. cinerea* and *B. fragariae*, respectively; BcUH and BfUH indicate unique haplotypes found in *B. cinerea* and *B. fragariae*, respectively; and UnH and UnUH indicate haplotypes found in *Botrytis* isolates of unidentified (Un) species.

phenotype group. The grouping was done to ensure a sufficient number of isolates suitable for analysis in each resistance profile. One group was represented by 0CCR/1CCR/2CCR, one by 3CCR/4CCR, and one by 5CCR (Supplementary Table S3). Between those groups, there were no significant differences ($P > 0.05$) in genetic diversity, number of alleles, or number of effective alleles. Significant differences were found, however, using genetic differentiation analysis (Φ_{PT} values). Significant differences in Φ_{PT} values were found among farm populations (Fig. 1A) as well as between phenotype groups 3CCR/4CCR and 5CCR (Fig. 1B).

Discussion

Previous studies have detected high diversity in *Botrytis* populations present in single fields (Fournier and Giraud 2008) but this is a detailed exploration of the diversity that can be found in tissues of a single plant infected by *Botrytis* spp. Genetic diversity was generally high among SF isolates as well as in isolates from different flowers and farms. Kerssies et al. (1997) investigated 29 isolates from greenhouses and found only 2 identical markers of 70 random amplified polymorphic DNA markers scored. Giraud et al. (1997) studied 259 isolates from grape and identified 134 haplotypes using restriction fragment length polymorphism markers. The most common haplotypes accounted for only 5% of the isolates. In that study, every isolate from the same plant was a different haplotype. Moreover, multiple haplotypes were obtained from a single grape. These data indicate high diversity between isolates from the same location or even the same host plant. In accordance with these results, our study identified multiple SF haplotypes (based on an SF sample size of 9 to 12 isolates) in 12 of 19 single flowers collected from commercial fields, which confirms that multiple haplotypes can reside in the same plant organ. The present study also shows that *B. cinerea* and *B. fragariae* can coexist in the same flower, which further increased the diversity of haplotypes in SF isolates.

The existence of multiple SF haplotypes with varying phenotypes complicates fungicide sensitivity screening processes that use bulk conidia instead of single-spore isolates (Schnabel et al. 2015; Weber and Hahn 2011). Location-specific resistance profiling has been conducted in the southeastern United States and California to identify the most effective spray programs for gray mold control in strawberry fields (Schnabel et al. 2015). For this assay, bulk conidia were transferred directly from sporulating, surface-sterilized flowers to medium containing discriminatory doses of fungicides. Based on the findings of the present study, conidia from a single flower can consist of multiple species and haplotypes, with potentially multiple fungicide-resistant phenotypes. Analysis of bulk conidia has advantages and disadvantages. The major advantage is that most or perhaps all fungicide resistance phenotypes are identified. For example, if there are two SF isolates, one resistant to fungicide A and the other to fungicide B, then both resistance problems will be detected. The disadvantage is that the assay does not

reveal whether two isolates are involved, each with a different resistance phenotype, or one isolate with both phenotypes. In the latter scenario, rotations of fungicides A and B would lead to fast selection of dual resistance due to “selection by association” (Hu et al. 2016a). Other large-scale monitoring programs to assess fungicide resistance in *Botrytis* spp. are based on germ tube development Weber and Hahn (2011). Similar to the assay described above, bulk conidia obtained from specific tissues

Fig. 3. Frequency (%) of *Botrytis cinerea* and *B. fragariae* isolates with resistance to six chemical classes of fungicides collected from strawberry blossoms.

Fig. 4. Number of haplotypes found in each resistance profile of *Botrytis* isolates collected from strawberry blossoms.

Table 3. Number of chemical class resistances (CCR) and resistance profiles (RP) of 192 *Botrytis* isolates collected from four farms and 19 strawberry flowers^a

RP ^b	Species	AP	PP	SBI	DC	MBC	SDHI			
		Cyp	Flu	Fen	Ipr	Thi	Bos	Fluo	Pen	Iso
RP1 (0CCR)	<i>Botrytis cinerea</i>	S	S	S	S	S	S	S	S	S
RP2 (1CCR)	<i>B. cinerea</i>	S	S	R	S	S	S	S	S	S
RP3 (1CCR)	<i>B. cinerea</i> , <i>B. fragariae</i>	S	S	S	S	R	S	S	S	S
RP4 (2CCR)	<i>B. fragariae</i>	R	S	S	S	R	S	S	S	S
RP5 (3CCR)	<i>B. fragariae</i>	R	R	S	S	R	S	S	S	S
RP6 (3CCR)	<i>B. cinerea</i>	R	S	S	R	R	S	S	S	S
RP7 (4CCR)	<i>B. cinerea</i> , <i>B. fragariae</i>	R	S	R	R	R	S	S	S	S
RP8 (4CCR)	<i>B. cinerea</i>	R	S	R	S	R	R	S	S	S
RP9 (4CCR)	<i>B. cinerea</i>	R	S	S	R	R	R	S	S	S
RP10 (5CCR)	<i>B. cinerea</i>	R	R	S	R	R	R	S	S	S
RP11 (5CCR)	<i>B. cinerea</i>	R	S	R	R	R	R	S	S	S
RP12 (5CCR)	<i>B. cinerea</i>	R	S	R	R	R	R	S	R	S

^a Abbreviations. Fungicide chemical class name: anilinopyrimidines (AP), phenylpyrroles (PP), SBI class III (SBI), dicarboximides (DC), methyl benzimidazole carbamates (MBC), and succinate dehydrogenase inhibitors (SDHI); Cyp= cyprodinil, Flu= fludioxonil, Fen= fenhexamid, Ipr= iprodione, Thi= thiophanate-methyl, Bos= boscalid, Fluo= fluopyram, Pen= penythiopyrad, and Iso= isofetamid; and S = sensitive and R = resistant.

^b *Botrytis* isolates sensitive (0CCR) or resistant to one, two, three, four, or five chemical classes of fungicides (1CCR to 5CCR), respectively.

(flowers, leaves, or fruit) are transferred in aliquots onto fungicide-amended medium and germination or length of germ tubes are assessed after 12 to 16 h of incubation. Similar to the mycelial-based assessment, this method would not reveal whether a single isolate or multiple isolates were involved in conferring resistance to multiple fungicides. Furthermore, the limited number of spores examined for each fungicide (20 to 30 conidia) may not capture conidia of phenotypes that are underrepresented in the bulk sample in form of low conidia frequency. However, in contrast to the mycelial growth assay described above, this method does allow for an estimate of frequency of resistance to each fungicide. Both methods still have practical value despite the shortcomings described here, in that they allow for large-scale resistance monitoring.

Multiple *Botrytis* spp. were commonly found coexisting in the same locations. A German study showed that at least two species among the *B. calthae*, *B. cinerea*, and *B. cinerea* group S and *B. pseudocinerea* species complex were found together in *Caltha palustris* plants grown at all four investigated locations (Plesken et al. 2015b). Rupp et al. (2017) has also shown *B. cinerea* and *B. fragariae* or *B. cinerea*, *B. fragariae*, and *B. pseudocinerea* living in sympatry in German strawberry fields. In the United States, *B. caroliniana* and *B. cinerea* were found coexisting in commercial blackberry fields (Li et al. 2012). In this study, we showed that multiple *Botrytis* spp. (i.e., *B. cinerea* and *B. fragariae*) can coexist not only in the same field or plant but on the same plant organ (flower), suggesting that gray mold on individual strawberry flowers can be a result of multiple infections. The enormous genetic diversity of *Botrytis* populations in fields, single plants, and even tissues (such as flowers) demands a rethinking of current resistance management programs. Rather than rotating or mixing Fungicide Resistance Action Committee codes with single-site modes of action, the risk of control failure due to resistance selection may be substantially reduced if nonselective products such as multisite fungicides or biologicals are primarily used.

In many cases, the number of haplotypes and number of fungicide resistance phenotypes in single flowers was equal (e.g., C1 and C2; Table 2), but, often, more haplotypes than fungicide resistance phenotypes (e.g., C3 and C4; Table 2) were found. How do we explain the lack of resistance phenotypes in flowers when multiple genotypes are present? Depending on the grower, strawberry may or may not be sprayed with fungicides between planting in October and bloom in March to April of the following year (using South Carolina plasticulture calendar as an example). According to growers' spray records, two farms (farms C and D) were only sprayed once prior to bloom with captan, a broad-spectrum fungicide that reduces the pathogen population size without selecting for resistance. Therefore, the pathogen population of the experimental fields was not subjected to selection pressure by any of the fungicides evaluated in this study. The most straightforward explanation for the question raised above is that a single flower was infected by multiple haplotypes, many with identical resistance profiles. Perhaps these haplotypes went through a selection process on the nursery level and then were established in the crop (Schnabel et al. 2015), or they were present in the field as a result of fungicide applications in previous seasons. However, it is also possible that a single haplotype infected the flower and then genetically diversified within the flower tissue. The ability of single isolates to implement genetic changes within a short period of time may be based on heterokaryosis (Büttner et al. 1994; Van Der Vlugt-Bergmans et al. 1993) and has been reported in previous studies. For example, genetic changes within clonal derivatives of *B. cinerea* were observed readily, some of which even led to the acquisition of a new resistance phenotype (Yourman et al. 2000). Interactions between the pathogen and the host, UV radiation, and other field conditions may also play a role in changing the fungal genome. No spray records were available for the other two farms included in this study. However, it is common practice for southeastern growers to use multisite fungicides such as thiram and captan at least a couple of times after fall planting and before row covers are used for frost protection during the winter.

In this study, *B. cinerea* isolates with multifungicide resistance had different genetic backgrounds based on microsatellite fragment analysis and, thus, likely originated from different ancestors. Isolates were collected from farms at multiple geographic locations that used different nurseries to obtain the planting stock. Each nursery

probably uses its own set of tools to manage diseases during nursery stock production, including nursery-specific fungicide programs. Therefore, we would expect isolates from different nurseries to exhibit unique haplotypes and fungicide resistance phenotypes. As expected, genetic differentiation analysis revealed that farm populations were genetically different from each other, with diverse fungicide resistance phenotypes (Fig. 1A). Moreover, many isolates from each location were resistant to multiple fungicide classes. This is in agreement with a previous study which showed that *B. cinerea* isolates with resistance to fungicides from seven chemical classes can emerge independently (Fernández-Ortuño et al. 2015). Rupp et al. (2017) also revealed high haplotype diversity in *B. fragariae* isolates with multifungicide resistance.

In conclusion, this detailed analysis of *Botrytis* isolates from single flowers revealed phenotypic and genotypic diversity. Knowing the extent of diversity present in a single tissue is important for future epidemiological studies, because current sampling and single-spore isolation techniques may ignore the diversity contained within single tissues while focusing on field-wide diversity. Understanding the extent of diversity within a single tissue can also improve our methods of *Botrytis* fungicide resistance management and monitoring.

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