Fitness and Competitive Ability of *Botrytis cinerea* Isolates with Resistance to Multiple Chemical Classes of Fungicides

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ABSTRACT

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Resistance to multiple chemical classes of fungicides in *Botrytis cinerea* isolates from eastern United States strawberry fields is common and strategies to control them are needed. In this study, we compared fitness and competitive ability of eight sensitive isolates (S), eight isolates resistant to five or six chemical classes of fungicides but not to phenylpyrroles (5CCR), and eight isolates resistant to six or seven chemical classes including phenylpyrroles (6CCR/MDR1h). The latter included the MDR1h phenotype due to overexpression of *atrB* based on $\Delta 497V/L$ in *mrr1*. The 6CCR/MDR1h

isolates grew more slowly at 4°C on potato dextrose agar, and both 5CCR and 6CCR/MDR1h isolates were hypersensitive to osmotic stress compared with S isolates. In contrast, no differences were found in oxidative sensitivity, aggressiveness, and spore production in vivo, and sclerotia production and viability in vitro. In competition experiments, the 5CCR and 6CCR/MDR1h isolates were both outcompeted by S isolates and 6CCR/MDR1h isolates were outcompeted by 5CCR isolates in the absence of fungicide pressure. Under selective pressure of a fludioxonil/pyraclostrobin rotation, the 6CCR/MDR1h isolates dominated after coinoculation with 5CCR and S isolates. The competitive disadvantage of 5CCR and especially 6CCR/MDR1h isolates suggest that, in the absence of fungicide selection pressure, S isolates may reduce inoculum potential of multifungicide-resistant isolates under field conditions.

Botrytis cinerea Pers. (teleomorph *Botryotinia fuckeliana*) is the causal agent of gray mold of more than 200 plant species and represents a high risk for development of resistance to fungicides. Although cultural methods such as appropriate plant spacing and fertilization can reduce disease incidence, fungicide applications are still the key to disease management. Site-specific fungicides used for gray mold control belong to Fungicide Resistance Action Committee (FRAC) codes 1, 2, 7, 9, 11, 12, and 17, representing methyl benzimidazole carbamates, dicarboximides, succinate dehydrogenase inhibitors (SDHI), anilinopyrimidines, quinone outside inhibitors, phenylpyrroles, and sterol biosynthesis inhibitor class III fungicide hydroxylanilide, respectively. Multisite inhibitors such as captan and thiram (FRAC codes M3 and M4, respectively) are also frequently used alone or in tank mixtures with site-specific fungicides.

Botrytis cinerea isolates with resistance to multiple fungicides are found in commercial strawberry and blackberry fields of Germany (Hahn et al. 2014; Leroch et al. 2013; Weber 2011), the United States (Amiri et al. 2012; Fernández-Ortuño et al. 2014, 2015; Li et al. 2014a), Israel (Elad et al. 1992), Spain (Raposo et al. 1996), and Chile (Latorre and Torres 2012), as well as from kiwi fields in Greece (Bardas et al. 2010). In South Carolina and other eastern states, *B. cinerea* isolates from strawberry fields with widespread resistance to five fungicides of different chemical classes (5CCR), including boscalid, pyraclostrobin, fenhexamid, cyprodinil, and pyrimethanil, have been reported (Amiri et al. 2013; Fernández-Ortuño et al. 2015). Resistance to multiple FRAC groups is mostly conferred via the accumulation of point mutations in target genes over time (Li et al. 2014a). However, *B. cinerea* populations with a single mechanism conferring resistance to multiple fungicides were identified previously. Resistance was based on increased activity of drug efflux pumps, designated the multidrug-

resistant (MDR) phenotype. MDR isolates were reported in French and German vineyards (Kretschmer et al. 2009; Leroch et al. 2011) and small fruit fields in Virginia and South Carolina (Fernández-Ortuño et al. 2015). MDR1 isolates overexpress the ATP transporter gene *atrB*, which makes them partially resistant to fludioxonil, cyprodinil, and toltafate (Kretschmer et al. 2009). A variant of the MDR1 phenotype, MDR1h, was subsequently identified in German strawberry fields (Leroch et al. 2013). Compared with MDR1 isolates, the expression of *atrB* was higher in MDR1h isolates and resulted in higher levels of resistance to fludioxonil and cyprodinil (Fernández-Ortuño et al. 2015). The MDR1h phenotype was recently confirmed in the United States in isolates from blackberry in Georgia and isolates from strawberry in Connecticut (Fernández-Ortuño et al. 2015) but very little is known about these isolates with regard to fitness and competitiveness. Such information will help assess the establishment and dynamics of fungicide resistance frequencies in the pathogen population (Chin et al. 2001; Parnell et al. 2005).

In a previous study, a limited number of fitness characteristics were investigated for three MDR1 and three MDR1h phenotypes. Data showed that multifungicide-resistant MDR1h isolates were not impaired in their ability to grow on multiple different media at different temperatures but may be significantly more sensitive to salt stress (Fernández-Ortuño et al. 2015). The objective of this study was to more thoroughly investigate fitness parameters with more isolates and to determine competitiveness of eight sensitive isolates (S isolates), eight isolates resistant to five or six chemical classes of fungicides from different chemical classes except the phenylpyrroles (5CCR isolates), and eight isolates resistant to at least six chemical classes including the phenylpyrroles (6CCR/MDR1h isolates).

MATERIALS AND METHODS

Fungal isolates. In total, 24 *B. cinerea* isolates from Maryland, North Carolina, South Carolina, Michigan, Georgia, and Connecticut in the United States and from Canada were analyzed in this study (Table 1). Three different fungicide resistance phenotypes were

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represented, including eight isolates sensitive to seven chemical classes of fungicides (S), eight isolates resistant to at least five chemical classes of fungicides (5CCR), and eight isolates resistant to at least six chemical classes of fungicides including the phenylpyrroles (6CCR/MDR1h). The 5CCR isolates were resistant to iprodione, thiophanate-methyl, pyraclostrobin, fenhexamid, and boscalid but not to fludioxonil. Three of the eight 5CCR isolates were also resistant to cyprodinil. The 6CCR/MDR1h isolates were resistant to cyprodinil, iprodione, fludioxonil, pyraclostrobin, fenhexamid, and boscalid. Four of the eight 6CCR/MDR1h isolates were also resistant to thiophanate-methyl (Table 1). Resistance to the phenylpyrrole fludioxonil was due to MDR1h, as described above. Single-spore isolation was performed as previously described (Fernández-Ortuño et al. 2012). All isolates originated from either strawberry fruit or strawberry blossoms. They were collected from commercial fields between 2011 and 2014 and stored on dried filter papers at -20°C (data not shown).

Genotyping of the isolates. DNA was extracted as described previously (Chi et al. 2009). The partial β -tubulin and cytochrome *b* (*cyt**b*) genes were amplified using the primer pair TUB-F1/TUB-R1 and Qo13ext/Qo14ext with polymerase chain reaction (PCR) conditions previously described by Banno et al. (2008) and Leroux et al. (2010), respectively. The complete two-component histidine kinase gene (*bos1*) was amplified with five primer pairs (BF1/BR1, BF2/BR2, BF3/BR3, BF4/BR4, and BF5/BR5) following published protocols (Ma et al. 2007). The entire 3-keto reductase gene (*erg27*) was amplified and sequenced with primer pair *erg27*Beg/*erg27*End (Fillinger et al. 2008). The *sdhB* gene was amplified using the primer pair IpBcBeg/IpBcEnd2, as described previously (Leroux et al. 2010). The *mrr1* gene was amplified from 6CCR/MDR1h isolates with primers *mrr1*_atg/TF1-2_new for the upstream fragment and TF1-3_new/TF1-4 for the downstream fragment (Kretschmer et al. 2009; Leroux et al. 2013).

PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH) following the manufacturer's instructions and Sanger sequencing was conducted at the Clemson University Genomics Institute, Clemson, SC.

Fitness components. The following fitness components were determined: (i) mycelial growth in vitro, (ii) osmotic sensitivity in vitro, (iii) oxidative sensitivity in vitro, (iv) aggressiveness and spore production in vivo, and (v) sclerotial production and viability in vitro. All experiments were conducted twice.

To investigate mycelial growth rates, mycelial plugs (5 mm in diameter) were removed from the margins of 3-day-old cultures actively growing on potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) in petri dishes (90 mm). Plugs were placed upside down at the center of fresh PDA plates. The plates were incubated at 4, 22, and 30°C in the dark for 4 days. For each isolate, three replicate dishes were used. The diameter of each colony was measured after 4 days.

When microorganisms are placed into a hypertonic environment where the concentration of solute is higher outside the cell, water is lost from the cell, resulting in dehydration, shrinkage of the plasma membrane, and eventual death. To investigate the ability to withstand plasmolysis by exposure to NaCl-amended medium, mycelial plugs were taken from the margin of 3-day-old colonies and placed upside down at the center of PDA medium amended with 2, 4, or 6% NaCl. For each isolate, three replicate plates were used. After 4 days of incubation at 22°C in the dark, colony diameters were measured.

Generation of reactive oxygen species (ROS) occurs at the host–fungus interface during *B. cinerea* infections (Prins et al. 2000; Schouten et al. 2002). ROS play a major role in fungus–plant interactions as signaling components in both partners (Heller and Tudzynski 2011). Thus, oxidative stress adaptation is essential for pathogenicity. To measure the oxidative sensitivity, mycelial growth was measured at 22°C on PDA amended with 100 μM menadione, paraquat at 100 $\mu\text{g}/\text{ml}$, or 10 mM H_2O_2 (Giesbert et al. 2012; Temme and Tudzynski 2009). Three petri dishes per set of conditions were inoculated with agar plugs containing 3-day-old mycelium (5 mm in diameter). Colony diameters were measured after 4 days of incubation at 22°C in the dark.

Aggressiveness and spore production were determined on detached organic apple fruit ('Gala'). Apple fruit of similar size and commercial maturity were disinfected by immersion in 1%

TABLE 1. Fungicide resistance phenotype, origin of isolation, and presence or absence of associated point mutations in the class III histidine kinase (*bos1*), β -tubulin, cytochrome *b* (*cyt**b*), partial 3-keto reductase (*erg27*), and succinate dehydrogenase iron-sulfur protein (*sdhB*)

		Resistance phenotype and amino acid substitutions in target proteins or transcription factors ^z													
Isolates	Location	AP		DC		MBC		PP		QoI		SBI		SDHI	
		Cy	Unknown	Ip	<i>bos1</i>	Tm	β -tubulin	Fl	<i>mrr1</i>	Py	<i>cyt</i> <i>b</i>	Fe	<i>erg27</i>	Bo	<i>sdhB</i>
BC14-76-9	Maryland	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
BC11NC4	North Carolina	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
BC14-101-1	Michigan	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
BC14-101-3	Michigan	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
KC20	North Carolina	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
Flor5	South Carolina	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
BC11WIC6	South Carolina	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
BC11HP9	North Carolina	S	n/a	S	None	S	None	S	None	S	None	S	None	S	None
BC14-77-7	Georgia	S	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412S	R	H272R
BC14-77-5	Georgia	S	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412S	R	H272R
BC14-105-4	Michigan	S	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412S	R	H272R
BC14-105-5	Michigan	S	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412S	R	H272R
BC14-77-8	Georgia	S	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412S	R	H272R
BC14-96-7	Canada	R	n/a	R	I365S	R	E198A	S	None	R	G143A	R	F412S	R	H272Y
BC11NC7	North Carolina	R	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412C	R	H272R
BC11MOD11	South Carolina	R	n/a	R	I365N	R	E198A	S	None	R	G143A	R	F412S	R	H272Y
BC14-106-3-14	Connecticut	R	n/a	R	I365N	R	F200Y	R	MDR1h	R	G143A	R	T63I	R	H272R
BC14-106-9-14	Connecticut	R	n/a	R	I365S	S	None	R	MDR1h	R	G143A	R	F412S	R	H272R
BC14-106-7-14	Connecticut	R	n/a	R	I365N	R	F200Y	R	MDR1h	R	G143A	R	F412S	R	H272R
BC14-107-10	Connecticut	R	n/a	R	I365N	S	None	R	MDR1h	R	G143A	R	F412S	R	H272R
BC14-107-2	Connecticut	R	n/a	R	I365S	S	None	R	MDR1h	R	G143A	R	F412S	R	H272R
BC14-107-8	Connecticut	R	n/a	R	I365S	R	F200Y	R	MDR1h	R	G143A	R	F412S	R	H272R
BC14-106-8-14	Connecticut	R	n/a	R	I365N	R	None	R	MDR1h	R	G143A	R	F412S	R	H272R
BC-14-106-14	Connecticut	R	n/a	R	I365S	S	None	R	MDR1h	R	G143A	R	F412S	R	H272R

^z Abbreviations: AP = anilino-pyrimidines, DC = dicarboximides, MBC = methyl benzimidazolecarbamates, PP = phenylpyrroles, QoI = quinone outside inhibitors, SBI = sterol biosynthesis inhibitors, SDHI = succinate dehydrogenase inhibitors, Cy = cyprodinil, Ip = iprodione, Tm = thiophanate-methyl, Fl = fludioxonil, Py = pyraclostrobin, Fe = fenhexamid, Bo = boscalid, S = sensitive, R = resistant, and n/a = not applicable.

(vol/vol) sodium hypochlorite solution for 5 min, rinsed three times in sterile deionized water, and subsequently air dried. Spores were produced on PDA after 10 days of incubation at 22°C under continuous light. Spore suspensions were prepared in Gamborg Minimal Medium (3 g of Gamborg B5 basal salt mixtures, 1.36 g of KH_2PO_4 , and 9.9 g of glucose per liter of medium) and adjusted to 5×10^5 spores/ml with the aid of a hemocytometer. A 20- μl droplet of the conidial suspension was placed with three equidistant points on apple fruit. A 0.9-mm-diameter needle was used to make a wound on the fruit to an approximate depth of 4 mm below the epidermis. Inoculated fruit were placed in boxes, covered with a plastic bag to keep the relative humidity approximately 100%, and maintained at room temperature at approximately 20°C. Lesion diameters were measured after 5 days. For each isolate, three replicate apple fruit were inoculated. To determine spore production, a piece of decayed tissue (8 by 8 by 2 cm) was cut from the fruit with a sterile scalpel 10 days after inoculation and placed in a sandwich box (18 by 18 by 10 cm) with two layers of moist paper towels on the bottom of the plate and open lid to maintain high relative humidity. After 3 days of incubation, conidia were rinsed off the decayed tissue with 10 ml of sterile water by sterile cotton swabs. The suspension was transferred to 50-ml tubes with 10 glass beads and vortexed for 1 min. The concentration of conidia was determined using a hemacytometer. Mean conidia production of three replicates was determined for each isolate.

Sclerotia are a compact mass of hardened fungal mycelium that enable *B. cinerea* to survive environmental extremes. They are also involved in sexual reproduction. For determination of sclerotia production in vitro and assessment of sclerotia viability under different storage conditions, PDA plates inoculated with agar plugs containing actively growing mycelium were incubated in the dark at 4 and 22°C for 6 and 10 weeks. The number of sclerotia formed in each plate was counted visually. For each isolate, three replicates were used. Sclerotia were harvested from PDA medium after 4 weeks of incubation in the dark. They were sterilized by immersing for 1 min in 70% (vol/vol) ethanol, rinsed twice in sterile water, and dried with sterile filter paper (Mosbach et al. 2011). The sclerotia were stored in 1.5-ml tubes at 4 and 22°C. Three replicate tubes were prepared per isolate and storage temperature, with 10 sclerotia in each tube. After 1 and 3 months, viability was determined by calculating the percentage of sclerotia that had germinated after 72 h of incubation on PDA in the dark.

Stability of resistance to fludioxonil and other fungicides in 6CCR/MDR1h isolates. The stability of resistance to fludioxonil in five 6CCR/MDR1h isolates (BC14-106-3-14, BC14-106-9-14, BC14-107-8, BC106-8-14, and BC106-6-14) was determined by culturing the isolates on untreated, organically grown apple halves (Gala). The spores generated at each of four transfers were harvested into Gamborg Minimal Medium and used to reinoculate new apple halves. Droplets (20 μl) containing 5×10^5 conidia/ml were placed at six equidistant points on the surface of each apple half. Three inoculated apple halves were prepared for each isolate and halves were placed in an enclosed plastic container (18 by 18 by 10 cm) and incubated at room temperature. After 7 days, lids were removed and halves were incubated for another 7 days to facilitate sporulation. Before the first and after the last transfer, the effective inhibitory dose at which 50% inhibition occurs (EC_{50} in micrograms per milliliter) was determined for fludioxonil using a germ tube elongation assay. Briefly, aliquots of 100 μl of conidial suspensions at 10^5 spores/ml were plated on petri dishes with malt extract agar (MEA) amended with fludioxonil at 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 $\mu\text{g/ml}$. After 16 h of incubation at 22°C in the dark, 70% ethanol was sprayed onto the surface of all plates to stop fungal growth. The germination rate and germ tube length were determined for 50 spores/plate, with three plates per treatment under an Olympus BX41 microscope, using a micrometer. The five 6CCR/MDR1h isolates were also transferred individually onto fludioxonil-treated apple halves. Apple halves were immersed for 1 min in fludioxonil suspension (Scholar; Syngenta Crop Sciences, Raleigh,

NC) at 100 $\mu\text{g/ml}$. Inoculation and data collection were carried out as described above.

The response of 6CCR/MDR1h isolates to six other fungicides at discriminatory doses was also determined before the first and after the fourth transfer, as described previously (Fernández-Ortuño et al. 2014). Briefly, Czapek-Dox agar medium with cyprodinil (Vanguard WG fungicide; Syngenta Crop Protection, Raleigh, NC) at 4 $\mu\text{g/ml}$, fenhexamid (Elevate 50 WDG; Arysta Life Science, New York) at 50 $\mu\text{g/ml}$, iprodione (Rovral 4 Flowable; Bayer Crop Sciences, Morrisville, NC) at 10 $\mu\text{g/ml}$, pyraclostrobin (Cabrio EG fungicide 20% W/W; BASF Corporation, Ludwigshafen, Germany) at 10 $\mu\text{g/ml}$, or thiophanate-methyl (Topsin M 70WP; United Phosphorus, Inc., Cheshire, UK) at 100 $\mu\text{g/ml}$, and 1% yeast bacto acetate agar with boscalid (Endura fungicide 70% [wt/wt]; BASF Corporation) at 75 $\mu\text{g/ml}$ were used to assess sensitivity in the form of mycelial growth to fungicides described as above.

Competition experiments. Experimental design. Five S isolates (BC14-101-1, BC14-101-3, Flor5, BC11WIC6, and BC11HP9), five 5CCR isolates (BC14-77-7, BC14-77-5, BC14-96-7, BC11NC7, and BC11MOD11), and five 6CCR/MDR1h isolates (BC14-106-3-14, BC14-106-9-14, BC14-107-8, BC106-8-14, and BC106-6-14) were used in the competition experiments. Although all isolates originated from strawberry fruit, apple fruit were chosen to avoid the more frequent contamination issues when working with strawberry. All isolates had similar growth rates and sporulation ability on apple fruit (data not shown). Each of the fungicide resistance phenotypes (S, 5CCR, and 6CCR/MDR1h) were represented by five isolates with equal spore proportions (designated phenotype mixture). Competition experiments were conducted with and without fungicide exposure. Conidial suspensions were prepared with mixtures of the S, 5CCR, and 6CCR/MDR1h isolates using different combinations and conidia ratios at 5×10^5 conidia/ml. Experiments without fungicide exposure were conducted with mixtures of 6CCR/MDR1h and S isolates, 5CCR isolates and S isolates, and 6CCR/MDR1h and 5CCR isolates at a ratio of 1:1. Experiments with mixtures of 6CCR/MDR1h and S isolates were also examined at ratios of 3:1 and 9:1. Experiments with fungicide exposure were conducted with mixtures of S, 5CCR, and 6CCR/MDR1h at a ratio of 79:20:1, respectively.

Fungicide exposure consisted of rotating fludioxonil and pyraclostrobin starting with fludioxonil at the first transfer. Six fruit half-replicates were used for each combination. Each phenotype was transferred separately to examine viability separately over the course of the experiment. The experiments were terminated after four transfers and repeated twice.

Artificial inoculation of apple fruit. For the competition test in the absence of fungicides, organic apple fruit (Gala) were prepared, wounded, inoculated, and incubated as described above in the “Aggressiveness and spore production” section. After 10 days of incubation at room temperature, the symptomatic fruit were longitudinally cut into halves and incubated for another 3 days in open-lid containers to induce sporulation on the cut surface. Apple halves were used for all experiments using fungicide treatments instead of the intact apple, which reduced variability between replicates. Apple halves were immersed for 1 min in a fludioxonil suspension (commercial grade) at 100 $\mu\text{g a.i./ml}$ prior to the first and third transfer and in a pyraclostrobin suspension at 100 $\mu\text{g a.i./ml}$ prior to the second and fourth transfer. Conidia produced on each apple half were collected by scraping the sporulating area with premoistened sterile cotton swabs and transferred into a 15-ml tube containing 10 ml of Gamborg Minimal Medium and used for determination of phenotype frequency after each transfer.

Determination of 6CCR/MDR1h and 5CCR frequencies. To distinguish 6CCR/MDR1h conidia from 5CCR and S conidia, 100 μl of spore suspension (1.0×10^5 conidia/ml) was transferred to MEA in petri dishes amended with fludioxonil at 0.3 $\mu\text{g/ml}$, the dose at which only 6CCR/MDR1h but not S and 5CCR isolates germinated. To distinguish 5CCR conidia from S conidia, spores were transferred to 1% MEA amended with pyraclostrobin at

10 µg/ml and alternative oxidase inhibitor salicylhydroxamic acid at 100 µg/ml. At the dose of pyraclostrobin at 10 µg/ml, only 5CCR but not S conidia germinated. Six replicate plates were investigated for each treatment. The plates were incubated for 16 h in the dark. At least 100 conidia/plate were examined for germination microscopically.

Data analysis. Student's *t* test for equality of variance was conducted to determine whether two experimental runs could be combined. If there was no significant ($P > 0.05$) difference between runs, pooled data were used for analysis of variance. Fisher's protected least significant difference test was used to assess the differences of fitness components and the stability of resistance to fludioxonil in 6CCR/MDR1h isolates at $P = 0.05$. In the competition test, data from the two replicates were merged. The frequencies of observed resistant isolates were compared with the initial frequencies using the χ^2 test. All the statistical analysis tests were performed using SPSS Software (release 13.0; SPSS Inc., Chicago).

RESULTS

Molecular basis of resistance. None of the isolates sensitive to iprodione, thiophanate-methyl, pyraclostrobin, fenhexamid, and boscalid had sequence variations in target genes. The eight 5CCR isolates revealed at least three different genotypes with mutations I365 N/S in *bos1*, E198A in β -tubulin, G143A in *cytB*, F412S/C in *erg27*, and H272R/Y in *sdhB* (Table 1), and none of them were resistant to fludioxonil. The eight isolates resistant to fludioxonil were identified to be MDR1h due to Δ 497V/L in the *mrr1* gene. These isolates consisted of different genotypes with mutations I365 N/S in *bos1*, either no mutation or the F200Y mutation in β -tubulin, G143A in *cytB*, F412S or T63I in *erg27*, and H272R in *sdhB*. Because resistance to fungicides other than fludioxonil and cyprodinil (unknown) was based on point mutations in target genes, resistance was at the same qualitative level for both isolate groups.

Fitness components. Mycelial growth. Mycelial growth rate between S isolates and 6CCR/MDR1h isolates was not different at all temperatures examined, with one exception. The 6CCR/MDR1h isolates grew significantly more slowly ($P < 0.05$) compared with the S isolates and 5CCR isolates at 4°C but no significant differences in mycelial growth rate were observed among the phenotypes at 22 and 30°C (Table 2).

Sensitivity to osmotic and oxidative stress. The 5CCR and 6CCR/MDR1h isolates were significantly more susceptible ($P < 0.05$) to salt stress compared with the S isolates regardless of the NaCl dose, except for the 6CCR/MDR1h isolates at 6% NaCl (Table 2). No significant difference in the ability to cope with oxidative stress was observed among the S, 5CCR, and 6CCR/MDR1h isolates (Table 2).

Determination of lesion size, conidia and sclerotia production, and sclerotia viability. All isolates used in the study were pathogenic on wounded apple. Aggressiveness was estimated by lesion size and sporulation ability. S, 5CCR, and 6CCR/MDR1h isolates did not differ significantly in the ability to cause decay and sporulation on apple fruit (Table 3). Likewise, no significant differences were

observed among S, 5CCR, and 6CCR/MDR1h isolates in their ability to produce sclerotia ($P > 0.05$) and the ability of sclerotia to survive for up to 3 months at different temperatures ($P < 0.05$) (Table 4). However, significant decrease of sclerotia production was found for 6CCR/MDR1h isolates when incubation temperature changed from 22 to 4°C. For the 5CCR and S isolates, the change of temperature didn't have a significant effect on the production of sclerotia (Table 5).

Stability of resistance to fludioxonil and other fungicides. After four transfers on both untreated and fludioxonil-treated apple halves, all 6CCR/MDR1h isolates were still able to germinate with fludioxonil at 1 µg/ml. The EC₅₀ values obtained before the first (T0) and after the last (T4) transfer for 6CCR/MDR1h isolates grown on nonfungicide-treated fruit fluctuated slightly; however, the mean values were not statistically different ($P = 0.05$) (Table 6). Likewise, the mean resistance factors for T0 and T4 were not significantly different. The mean EC₅₀ values and resistance factor values of 6CCR/MDR1h isolates between T0 and T4 were also not significantly different ($P < 0.05$) (Table 6). All 6CCR/MDR1h isolates maintained their ability to grow on medium with discriminatory doses of cyprodinil, iprodione, fludioxonil, pyraclostrobin, fenhexamid, and boscalid fungicides, indicating stability of resistance to these fungicides as well (data not shown).

Competition test. Over the course of the competition experiment, the viability of each phenotype mixture was monitored separately throughout the four transfers. At the fourth transfer, all isolates produced similar-sized lesions and produced similar amount of spores compared with the first transfer (Table 7), indicating no loss in aggressiveness.

In competition with S isolates, the 5CCR isolates steadily were outcompeted over the four transfers. By the fourth cycle, the proportion of 5CCR isolates had dropped to less than 8% (Fig. 1A).

The 6CCR/MDR1h isolates failed to compete effectively with S or 5CCR isolates (Fig. 1B and C). A sharp decline ($P < 0.05$) of the 6CCR/MDR1h isolate frequency was observed even after the first transfer. At the end of the fourth cycle in mixtures with S and 5CCR

TABLE 3. Lesion size and sporulation of *Botrytis cinerea* isolates with different fungicide resistance phenotypes on detached apple fruit^w

Phenotype ^x	Lesion size ^y	Sporulation ^z
S	20.2 ± 5.2 a	5.8 ± 3.0 a
5CCR	23.0 ± 5.8 a	7.0 ± 3.0 a
6CCR/MDR1h	22.8 ± 4.8 a	7.7 ± 2.3 a

^w Values are means ± standard error from the average of eight isolates for each phenotype group. Shown are combined data from two biological replicates. Numbers in each column followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

^x S = sensitive to all the fungicides tested; 5CCR = resistant to at least five fungicides of different chemical classes, with the exception of fludioxonil; and 6CCR/MDR1h = resistant to fludioxonil and other fungicides of different chemical classes.

^y Mycelial growth was measured after 5 days of incubation.

^z Number of spores ($\times 10^3$) per square centimeter of lesion.

TABLE 2. Radial mycelial growth, osmotic sensitivity, and oxidative sensitivity of *Botrytis cinerea* isolates with different fungicide resistance phenotypes^v

Phenotype ^z	Mycelial growth ^w			Osmotic sensitivity ^x			Oxidative sensitivity ^y		
	4°C	22°C	30°C	6%	4%	2%	Menadione	Paraquat	H ₂ O ₂
S	6.2 ± 0.5 a	24.5 ± 1.4 a	16.5 ± 1.4 a	9.2 ± 1.8 a	19.0 ± 0.7 a	25.0 ± 1.1 a	15.9 ± 2.5 a	19.1 ± 0.9 a	14.5 ± 0.4 a
5CCR	5.6 ± 0.4 a	24.4 ± 1.2 a	14.5 ± 2.4 a	7.7 ± 1.2 b	16.5 ± 1.9 b	20.3 ± 6.9 b	15.1 ± 1.8 a	17.1 ± 1.6 a	14.6 ± 0.5 a
6CCR/MDR1h	5.0 ± 0.7 b	23.1 ± 2.3 a	12.3 ± 4.6 a	7.8 ± 1.5 ab	16.5 ± 1.4 b	22.7 ± 2.0 b	16.6 ± 3.8 a	16.9 ± 1.2 a	15.7 ± 2.7 a

^v Values are mean mycelial growth ± standard error from the average of eight isolates for each phenotype group. Shown are combined data of two biological replicates. Numbers in each column followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

^w Mycelial growth was measured after 4 days of incubation on potato dextrose agar.

^x Mean colony diameter in the presence of NaCl at 22°C.

^y Mean colony diameter in the presence of menadione (100 µM), paraquat (100 µg/ml), and 10 mM H₂O₂ at 22°C.

^z S = sensitive to all the fungicides tested; 5CCR = resistant to at least five fungicides of different chemical classes, with the exception of fludioxonil; and 6CCR/MDR1h = resistant to fludioxonil and other fungicides of different chemical classes.

isolates, the 6CCR/MDR1h isolates had almost disappeared and were found at frequencies of 0.20 and 1.05%, respectively (Fig. 1B and C). When the 6CCR/MDR1h isolates and S isolates were mixed at ratios of 3:1 and 9:1, the frequency of 6CCR/MDR1h isolates decreased gradually to nearly 0% in both cases, despite the initial disadvantage in spores being outnumbered. After only three transfers, the spores of 6CCR/MDR1h isolates were found at frequencies of 1.25 and 4.49%, respectively (Fig. 2).

As expected, when the competition assay was conducted on apple halves treated with label rates of fludioxonil and pyraclostrobin fungicides, the frequency of 6CCR/MDR1h isolates increased sharply after the first transfer from 1 to 97% (Fig. 3).

DISCUSSION

B. cinerea is a pathogen at high risk for fungicide resistance development due to its high genetic variability (Leroux et al. 2002), abundance of sporulation, short generation time (Petsikos-Panayotarou et al. 2003), and wide host range (Yourman et al. 2001). Resistance to three or more fungicide classes is commonly found in *B. cinerea* from conventional strawberry fields (Bardas et al. 2010; Fernández-Ortuño et al. 2015; Leroch et al. 2013) and isolates with resistance to five

and more classes of fungicides have emerged in the United States. Management of these isolates will be challenging because isolates resistant to multiple classes of fungicides may not be controlled when alternating or mixing compounds belonging to these different classes of fungicides. More emphasis must be given to the reduction of inoculum to reduce selection pressure. One way to reduce inoculum of multifungicide-resistant isolates would be to eliminate selection pressure in hopes that reduced fitness and competitiveness will favor wild-type strains and reduce the proportion of multifungicide-resistant isolates in the population.

In this study, phenotypes S, 5CCR, and 6CCR/MDR1h were examined. The isolates representing each phenotype were purposely chosen to originate from different locations so that they would not be genetically identical to avoid genotypic bias. The S isolates came from Maryland, North Carolina, Michigan, and South Carolina and were collected in two different years. The 5CCR isolates were collected from Georgia, Michigan, North Carolina, South Carolina, and Canada and represented at least three different genotypes based on target gene variations. Although all 6CCR/MDR1h isolates were from Connecticut, they had unique compositions of mutations in target genes and at least five different genotypes based on target gene modifications were represented. For

TABLE 4. Sclerotia production and viability (%) of *Botrytis cinerea* isolates with different fungicide resistance phenotypes

Phenotype ^z	Sclerotia production ^x				Sclerotia viability (%) ^y			
	4°C		22°C		4°C		22°C	
	6 weeks	10 weeks	6 weeks	10 weeks	1 month	3 month	1 month	3 month
S	38.0 ± 50.5 a	41.2 ± 55.1 a	60.7 ± 18.8 a	62.1 ± 18.8 a	90.9 a	94.7 a	56.0 a	65.3 a
5CCR	53.0 ± 41.0 a	54.2 ± 41.8 a	47.9 ± 57.6 a	56.9 ± 56.8 a	100 a	100 a	80.0 a	97.5 a
6CCR/MDR1h	23.4 ± 27.3 a	37.3 ± 43.0 a	71.8 ± 44.1 a	76.1 ± 48.5 a	92.0 a	100 a	95.5 a	87.5 a

^x Number of sclerotia per plate after 6 and 10 weeks of incubation on potato dextrose agar medium at two different temperatures. Mean values of groups followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

^y Percent sclerotia that had germinated after 72 h of incubation. Statistical analyses were performed independently for each set of conditions by the χ^2 test. Mean values of groups followed by the same letter are not significantly different at $P = 0.05$.

^z S = sensitive to all the fungicides tested; 5CCR = resistant to at least five fungicides of different chemical classes, with the exception of phenylpyrroles; and 6CCR/MDR1h = resistant to fludioxonil and other fungicides of different chemical classes.

TABLE 5. Influence of temperature (Temp) on sclerotia production of *Botrytis cinerea* isolates with different fungicide resistance phenotypes

Temp	Sclerotia production ^z					
	6 weeks			10 weeks		
	S	5CCR	6CCR/MDR1h	S	5CCR	6CCR/MDR1h
22°C	60.7 ± 18.8 a	47.9 ± 57.6 a	71.8 ± 44.1 a	62.1 ± 18.8 a	56.9 ± 56.8 a	76.1 ± 48.5 a
4°C	38.0 ± 50.5 a	53.0 ± 41.0 a	23.4 ± 27.3 b	41.2 ± 55.1 a	54.2 ± 41.8 a	37.3 ± 43.0 b

^z Number of sclerotia per plate after 6 and 10 weeks of incubation on potato dextrose agar medium at two different temperatures. Mean values of groups followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$. S = sensitive to all the fungicides tested; 5CCR = resistant to at least five fungicides of different chemical classes, with the exception of phenylpyrroles; and 6CCR/MDR1h = resistant to fludioxonil and other fungicides of different chemical classes.

TABLE 6. Effective inhibitory dose at which 50% inhibition occurs (EC_{50}) values and resistance factors obtained before and after transfer of 6CCR/MDR1h isolates on apple with and without fludioxonil treatment^x

Isolates	EC_{50} (µg/ml)			Resistance factor ^y		
	T0	T4	TF4	T0	T4	TF4
BC106-6-14	0.1	0.1	0.2	9.9	6.9	14.8
BC106-8-14	0.3	0.1	0.3	22.0	9.8	22.9
BC14-107-8	0.2	0.2	0.3	14.5	13.7	29.3
BC14-106-9-14	0.1	0.2	0.4	12.4	16.8	31.7
BC14-106-3-14	0.2	0.1	0.1	13.2	7.0	5.0
Mean ^z	0.2 ± 0.1 a	0.1 ± 0.1 a	0.2 ± 0.1 a	14.4 ± 4.6 a	10.8 ± 4.4 a	20.7 ± 11.0 a

^x T0 = prior to first transfer on untreated apple fruit, T4 = after four transfers on fungicide-free apple, and TF4 = after four transfers on fludioxonil (100 µg/ml)-treated apple.

^y Resistant factor = EC_{50} value of the 6CCR/MDR1h isolates/mean EC_{50} value of sensitive isolates (BC14-101-1, BC14-101-3, Flor5, BC11WIC6, and BC11HP9).

^z Mean EC_{50} and resistance factor values for 6CCR/MDR1h isolates ± standard error. Mean values within rows, for EC_{50} and resistance factor separately, followed by the same letter are not significantly ($P = 0.05$) different according to Fisher's protected least significant difference test.

example, isolate BC14-106-3-14 had mutations I365N, F200Y, G143A, T63I, and H272Y in *bos1*, β -*tubulin*, *cytb*, *erg27*, and *sdhB*, respectively, whereas isolate BC14-106-9-14 had I365S, no mutation, G143A, F412S, and H272R, respectively. This choice of isolates representing a phenotype avoided the risk of using clones as isolate replicates but the potential high diversity within a phenotype may make direct comparisons challenging. We tried to keep the variability at a minimum, however, by selecting nearly identical numbers of group S isolates in each phenotype group. S and 5CCR isolates each consisted of seven group S isolates (out of eight each), and all 6CCR/MDR1h isolates were group S isolates based on *mrr1* gene sequence alignments (Leroch et al. 2013).

When compared with the S isolates, hypersensitivity to osmotic stress was the only fitness penalty found in the 5CCR isolates. Increased sensitivity to salt stress was also reported in lab mutants or field isolates with resistance to iprodione in *Botrytis* spp. (Yan et al. 2010), *Neurospora crassa* (Fujimura et al. 2003), *Rhizoctonia cerealis* (Hamada et al. 2011), and *Alternaria* spp. (Lim et al. 2003). Hypersensitivity to salt stress in those resistant isolates may have been related to altered cell wall composition (Beever 1983; Ma et al. 2001). Thus, the 5CCR isolates may not successfully compete in nature with S isolates. This hypothesis was supported by our competition assays. Mixtures of S and 5CCR isolates resulted in a predominance of the S isolates over time. A similar competitive dominance of S isolates compared with isolates resistant to SDHI fungicides was observed in competition experiments conducted on apple (Veloukas et al. 2014).

Resistance of 5CCR isolates was solely based on point mutations in target genes. Which mutation or mutation combination may have been responsible for the observed fitness cost was not determined in this study. In previous studies, isolates possessing only *cytb* G143A substitution did not show fitness costs (Veloukas et al. 2014) but SDHI-resistant isolates with H272Y/R/L, N230I, or P225F *sdhB* substitutions showed reduced fitness compared with S isolates (Veloukas et al. 2014). Similarly, laboratory fenhexamid-resistant mutants possessing *erg27* F412S/I/V mutations grew more slowly than the wild-type strain and displayed variations in sclerotia and conidia production (Billard et al. 2012; De Guido et al. 2007; Saito et al. 2014; Ziogas et al. 2003). Several studies showed that fludioxonil and anilino-pyrimidine resistance was associated with fitness costs, specifically an increase of osmotic sensitivity (Fujimura et al. 2003; Li and Xiao 2008; Li et al. 2014b; Malandrakis et al. 2013; Markoglou et al. 2008; Zhao et al. 2010). In our study, both 5CCR and 6CCR/MDR1h isolates were more susceptible to salt and osmotic stress, which may have been a consequence of the mutations I365 N/S found in *bos1* of all isolates representing both phenotypes. This is consistent with earlier studies, where site-directed mutagenesis of the *bos1* gene was applied to validate mutations including I365S, and mutants experienced reduced fitness (Fillinger et al. 2012). This increased sensitivity to osmotic stress may be due to a linkage of genes conferring iprodione and fludioxonil resistance involved in the

osmoregulation pathway if resistance is based on target gene mutations (Fujimura et al. 2003; Yan et al. 2010). Hypersensitivity to osmotic stress has been reported for MDR1h isolates but these isolates also possessed the I365N/S mutation (Fernández-Ortuño et al. 2015). Our study shows no direct link between the MDR1h phenotype and osmotic stress.

The MDR1h phenotype is based on enhanced activity of ATP-dependent efflux transporters. Increased energy demand for active fungicide efflux may impair fitness (Buchenauer 1987). Our study shows that the 6CCR/MDR1h isolates but not 5CCR isolates had lower mycelial growth rates at 4°C (Table 2). Thus, it is possible that these isolates are outcompeted during cooler field days such as in the late autumn, winter, and early spring, when *B. cinerea* remains on decayed foliage and fruit and develops into sclerotia (Billard et al. 2012). Sclerotia production of 6CCR/MDR1h isolates was drastically lower at 4°C (Table 5) compared with 22°C, which further supports a potential weakness in overwintering ability of MDR1h isolates compared with S and 5CCR isolates. Our study is in contrast to a previous study (Fernández-Ortuño et al. 2015) showing significantly more sclerotia produced for MDR1h isolates

TABLE 7. Sporulation of *Botrytis cinerea* isolates with different fungicide resistance phenotypes on apple fruit over four consecutive transfers

Phenotype ^a	First transfer	Second transfer	Third transfer	Fourth transfer
S	6.3 ± 2.0 a	5.4 ± 1.4 a	7.2 ± 2.8 a	6.6 ± 1.2 a
5CCR	6.9 ± 1.3 a	7.3 ± 2.4 a	6.8 ± 2.6 a	6.3 ± 1.8 a
6CCR/MDR1h	7.2 ± 3.3 a	6.8 ± 2.5 a	7.5 ± 2.5 a	6.5 ± 1.1 a

^a Number of spores ($\times 10^3$) per square centimeter of lesion produced on apple fruit. S = sensitive to all the fungicides tested; 5CCR = resistant to at least five fungicides of different chemical classes, with the exception of fludioxonil; and 6CCR/MDR1h = resistant to fludioxonil and other fungicides of different chemical classes.

Fig. 1. Competition at 1:1 spore ratio between A, 5CCR and S isolates; B, 6CCR/MDR1h and S isolates; C, 6CCR/MDR1h and 5CCR isolates. S = sensitive to cyprodinil, iprodione, thiophanate-methyl, fludioxonil, pyraclostrobin, fenhexamid, and boscalid; 5CCR = resistant to at least five fungicides of different chemical classes, with the exception of fludioxonil; 6CCR/MDR1h = resistant to fludioxonil and other fungicides of different chemical classes; an asterisk (*) indicates significant differences compared with the initial value according to the χ^2 test at $P = 0.05$.

compared with S isolates at 11°C on minimal medium (MM) and intermediate medium (IM). The difference of the two studies may be explained by the limited number of isolates (three for each phenotype) used in the previous study.

Fludioxonil resistance in *B. cinerea* was stable under the experimental conditions in our study. The 6CCR/MDR1h isolates still germinated with fludioxonil at 1 µg/ml, which is characteristic for MDR1h but not MDR1 isolates (Fernández-Ortuño et al. 2015), and isolates retained resistance after transfers on treated and untreated apple halves. The EC₅₀ values for fludioxonil of 6CCR/MDR1h isolates in our study ranged from 0.11 to 0.25 µg/ml at T₀, which were lower than the values (1.7 to 3.1 µg/ml) reported previously (Fernández-Ortuño et al. 2015). The authors used different methods for EC₅₀ determination, which may explain the differences. In our study, germ tube elongation was used, instead of microtiter tests which reflect inhibition of early mycelial growth rather than inhibition of germ tube elongation. Still, the ranges of resistance factors of S and 6CCR/MDR1h isolates were consistent with what was reported in other studies (Fernández-Ortuño et al. 2015; Hahn et al. 2014).

The detached apple assays in this study showed that S isolates not only were more competitive in 1:1 mixture with 6CCR/MDR1h isolates but they also outcompeted 6CCR/MDR1h isolates when initially outnumbered by 9:1 in the absence of fungicide selection. We also showed a competitive advantage of 5CCR isolates when mixed with 6CCR/MDR1h isolates, which indicated yet another level of competitive disadvantage of 6CCR/MDR1h isolates. Whether the isolates originally obtained from strawberry would behave differently on the original host compared with apple was not investigated in this study. Competitive disadvantages were also observed in isolates of *Saccharomyces cerevisiae* exhibiting overexpression of ABC transporters (Anderson et al. 2003). The slight differences in fitness data between 5CCR, 6CCR/MDR1h, and S isolates may not be enough to explain the dramatic competitive disadvantage observed in this study. However, it is possible that, during the stepwise accumulation of resistance (Li et al. 2014a), mutations elsewhere in the genome were obtained that compromised competitiveness but the effects were not identified in our fitness assays. Also, the ATP-dependent increased activity of *atrB* and the associated increased energy requirement

may have contributed to the observed fitness penalties and resulting competitive disadvantage of 6CCR/MDR1h isolates over 5CCR isolates. Whether MDR1 isolates reveal penalties similar to 6CCR/MDR1h isolates is not known and was not examined in this study but it is reasonable to speculate that MDR1 isolates also carry a fitness cost due to almost identical mechanisms of resistance to fludioxonil due to *atrB* overexpression.

In contrast, when the competition test was conducted in the presence of fungicides, a rapid increase of 6CCR/MDR1h isolates was observed. In order to better simulate observations in the field that suggest that the MDR1h phenotype is still at low (equal or less than 10%) frequencies (Fernández-Ortuño et al. 2014), we reduced the portion of MDR1h in our inoculum substantially. Also, we used fungicide rotations (fludioxonil and pyraclostrobin rotations in our transfers) to avoid one-sided selection. A rapid increase of 6CCR/MDR1h isolates was observed from 1% before the initial transfer to 97% after the first transfer. The ratio of 6CCR/MDR1h isolates remained high in subsequent transfers. Fungicide treatments reduced 5CCR and S isolates to less than 3% in subsequent transfers. They may have been completely eradicated but that was not investigated in this study to this detail. This suggests that applications of fludioxonil in the field will select for 6CCR/MDR1h isolates and lead to changes in ratios of genotypes among the resistant population. Most growers on the U.S. east coast have been using fludioxonil regularly in rotation with other fungicides over the last 3 years but the occurrence of isolates resistant to fludioxonil has been at a steady 10% or less based on extensive resistance monitoring (data not shown). This lack of increasing frequency of fludioxonil resistance is likely a function of successful chemical rotations as well as the here-described penalty in fitness and competitive ability.

The observed fitness and competitive penalty of 5CCR and especially 6CCR/MDR1h isolates has important implications for disease management, in that this phenomenon may be exploited to reduce their proportion in the population. A simple reduction of the number of applications per season or the use of nonselective fungicides such as biologicals and multisite fungicides such as captan

Fig. 2. Competition in vivo between 6CCR/MDR1h and sensitive (S) isolates with initial spore ratios of 3:1 and 9:1. Vertical lines indicate the standard error of the mean and an asterisk (*) indicates significant differences compared with the initial value according to the χ^2 test at $P = 0.05$.

Fig. 3. Competition in vivo among 6CCR/MDR1h, 5CCR, and S isolates coinoculated on fludioxonil-treated apple fruit with initial spore ratios of 1:20:79, respectively. S = sensitive to fungicides; 5CCR = resistant to at least five fungicides of different chemical classes but not to fludioxonil; and 6CCR/MDR1h = resistant to fludioxonil and at least five other fungicides of different chemical classes. Vertical lines indicate the standard error of the mean and an asterisk (*) indicates significant differences compared with the initial value according to the χ^2 test at $P = 0.05$.

and thiram (Legard et al. 2001; Wilcox and Seem 1994) may help reduce the proportion of MDR1h isolates in the pathogen population. Both captan and thiram are effective against MDR1 isolates based on EC₅₀ values of approximately 1 µg/ml (Leroux and Walker 2013). Investigations not part of this study confirmed no loss in efficacy of captan and thiram against 6CCR/MDR1h isolates for captan (EC₅₀ value 0.06 µg/ml) and thiram (EC₅₀ value 0.02 µg/ml) (data not shown). Efforts to implement weather-based decision support systems to reduce the number of applications per season are already made in the southeastern states (MacKenzie et al. 2012; Pavan et al. 2011).

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