Mutations in the Membrane-Anchored SdhC Subunit Affect Fitness and Sensitivity to Succinate Dehydrogenase Inhibitors in *Botrytis cinerea* Populations from Multiple Hosts

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ABSTRACT

Succinate dehydrogenase inhibitors (SDHIs) are an essential group of fungicides for managing gray mold, caused by *Botrytis cinerea*, in numerous crops. Resistance to boscalid, an early-generation SDHI, is widespread worldwide and was linked to mutations in the iron-sulfur protein encoding the SdhB subunit of the SDH complex. Herein, we report on four simultaneous dependent mutations at codons 85 (G85A), 93 (I93V), 158 (M158V), and 168 (V168I) of the membrane-anchored SdhC subunit of *B. cinerea*. Isolates without and with mutations in SdhC were referred to as C[−] and C⁺ genotypes, respectively. The C⁺ genotype was found in all the five surveyed hosts from different U.S. regions but its frequency was higher, 25 to 40%, in the tree fruit isolates compared with 12 to 25% in the small fruit populations. The four SdhC mutations were found in isolates without mutations in SdhB or with mutations known to confer resistance to the SDHIs in SdhB. However, the frequency of C⁺ isolates was significantly higher in the SdhB wild-type isolates, which suggests that SDHI sprays may have played a role in selecting for the

C[−] over the C⁺ genotype. Field C⁺ isolates exhibited reduced sensitivity to fluopyram and increased sensitivity to boscalid and penthiopyrad in vitro and on detached fruit. Homology modeling confirmed the positioning of the four mutations in the ubiquinone-binding pocket. The SdhC^{G85A} is found in the proximal ubiquinone binding site and SdhC^{M158V} is positioned in the iron sulfur protein interface next to the [3Fe-4S] cluster, whereas SdhC^{I93V} is positioned next to the heme *b* with vital functions in the SDH enzyme. Beside the differential sensitivity to the SDHIs, these mutations caused a significant fitness cost in the C⁺ isolates including sporulation and increased sensitivity to reactive oxygen species. The presence of *Botrytis* populations differentially sensitive to the SDHIs suggests increased risks for resistance development but also opens up new perspective for future gray mold management using different SDHI fungicides.

Keywords: disease control and pest management, mycology

Botrytis cinerea Pers. (teleomorph *Botryotinia fuckeliana*) is a ubiquitous fungal plant pathogen causing gray mold on various fruit, vegetable, and ornamental crops (Elad et al. 2004; Williamson et al. 2007). The pathogen is difficult to control because of its extensive host range, genetic diversity, and mode of reproduction (Elad et al. 2004; Williamson et al. 2007). Recent findings indicated that *Botrytis cinerea* may suppress plant immunity by hijacking host defense systems (Weiberg et al. 2013), which may limit the use of any potential resistant cultivars. Currently, chemical control remains the most effective method for gray mold management. Fungicides from multiple chemical classes and different modes of action have been developed to control gray mold on various crops. However, continuous selective pressure resulted in populations of *B. cinerea* increasingly resistant to multiple fungicides simultaneously (Amiri et al. 2013; Bardas et al. 2010; Fernández-Ortuño et al. 2014; Leroch et al. 2012; Leroux et al. 1999; Myresiotis et al. 2007). For example, resistance to fungicides from six chemical classes has been reported in *B. cinerea* populations from strawberry fields across the eastern United States (Amiri et al. 2013; Fernández-Ortuño et al. 2014). In pome fruit, lower resistance frequencies of approximately 20% have been reported from the U.S. Pacific

Northwest (Kim and Xiao 2010; Yin et al. 2011), most likely due to lower fungicide input compared with small fruit.

The carboxamides, or succinate dehydrogenase inhibitors (SDHIs), constitute one of the largest chemical groups used in agriculture and the most fast-growing class of fungicides, with at least seven new products registered since 2012 (FRAC 2018). The SDHIs target complex II, also known as succinate dehydrogenase (SDH), one of the four complexes of the respiratory chain of enzymes of the mitochondrial inner membrane. SDH is an oxidoreductase enzyme that catalyzes the oxidation of succinate to fumarate with the reduction of quinone to quinol (Cecchini 2003; Hägerhäll 1997; Horsefield et al. 2004; Lancaster 2002). The enzyme consists of two hydrophilic larger polypeptides, the flavoprotein (SdhA, approximately 60 to 70 kDa) and the iron-sulfur protein (SdhB, approximately 27 kDa) which contains three iron sulfur clusters (i.e., [2Fe-2S] next to SdhA, [4Fe-4S] in the center of SdhB, and [3Fe-4S] located next to the membrane interface) (Cecchini 2003; Lancaster 2002). These two subunits are anchored to the mitochondrial membrane by two smaller hydrophobic polypeptides—SdhC (approximately 15 kDa), encoding the cytochrome *b*₅₆₀, and SdhD (approximately 9 kDa)—in addition to a heme *b* (*H_b*) interfaced between the SdhC and SdhD subunits (Cecchini 2003; Lancaster 2002). Amino acid sequences of the SdhA and SdhB subunits are highly conserved among species in contrast to the SdhC and SdhD subunits. The ubiquinone-binding pocket (*Q_p*), involving conserved amino acids from SdhB (W164 and H207), SdhC (S27), and SdhD (W83) in *Escherichia coli* (Cecchini 2003; Horsefield et al. 2006), equivalent to SdhB (W229 and H272), SdhC (S84), and SdhD (W144) in *B. cinerea*, is of a particular importance. By binding to the residues forming the *Q_p*, the SDHIs interact with ubiquinone reduction which, ultimately, alters the fungal respiration pathway.

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Resistance to carboxin, flutolanil, and boscalid, the first SDHs used in agriculture, was linked to multiple mutations in three of the four SDH subunits (i.e., SdhB, SdhC, and SdhD). Among the 14 pathogens in which resistance to the aforementioned SDHs has been reported thus far, 11, 9, and 6 mutations were found in the SdhC, SdhB, and SdhD subunits, respectively (Amiri et al. 2014; Avenot et al. 2008, 2009; Fraaije et al. 2012; Leroux et al. 2010; Scalliet et al. 2012; Shima et al. 2009; Sierotzki and Scalliet 2013; Stammler et al. 2008). In *B. cinerea* field isolates resistant to boscalid, seven mutations at five codons of SdhB and at one codon in SdhD have been reported (Amiri et al. 2014; Leroux et al. 2010; Veloukas et al. 2011). The two most frequent mutations in *B. cinerea* occur at codon 272 (SdhB^{H272R/Y}) located near the highly conserved [3Fe-4S] iron-sulfur cluster of the SdhB subunit (Amiri et al. 2014; De Miccolis Angelini et al. 2010; Leroux et al. 2010; Veloukas et al. 2011; Yin et al. 2011). Less frequent mutations have also been reported at codons 272 (SdhB^{H272L}), 225 (SdhB^{P225F}), and 230 (SdhB^{N230I}). The highest numbers of mutations conferring resistance to the SDHs have been reported in SdhC of several fungal species such as *Alternaria alternata* and *A. solani* (Fairchild et al. 2012; Fraaije et al. 2012), *Aspergillus oryzae* (Shima et al. 2009), *Coprinus cinereus* (Ito et al. 2004), and *Mycosphaerella graminicola* (Fraaije et al. 2012; Scalliet et al. 2012) but none was reported thus far in the SdhC of *B. cinerea*.

Numerous studies documented various sensitivity and resistance phenotypes in *B. cinerea* isolates with or without mutations in the SdhB subunit (Amiri et al. 2014; Fernández-Ortuño et al. 2012; Leroux et al. 2010; Miyamoto et al. 2010; Veloukas et al. 2011; Weber and Hahn 2011). For example, several wild-type isolates from strawberry fields in Florida (Amiri et al. 2014), the Carolinas (Fernández-Ortuño et al. 2012), and Germany (Weber and Hahn 2011) were characterized as highly sensitive (HS) to boscalid compared with other sensitive (S) isolates. The HS isolates were not able to germinate on a sublethal dose used for characterization, in contrast to the S isolates. In *Botrytis* populations from Florida strawberry fields, 15% of isolates did not have a mutation in SdhB but were moderately resistant to boscalid (Amiri et al. 2014). Moreover, 10% of the SdhB^{H272Y} *B. cinerea* mutants, known to be HS to fluopyram, showed reduced sensitivity to fluopyram (Amiri et al. 2014), whereas *C. cassicola* isolates moderately resistant to boscalid were highly resistant to fluopyram (Ishii et al. 2011; Miyamoto et al. 2010). In addition to the possible variable intrinsic sensitivities between isolates and differential affinity of SDHs to the target sites, additional resistance mechanisms could cause such discrepancies in sensitivity levels.

Following a study on resistance to numerous SDHs in *B. cinerea* from Florida strawberry fields (Amiri et al. 2014), sequencing of the SdhC subunit revealed that some isolates with or without mutations in SdhB also carried four concurrent mutations in SdhC. Herein, concurrent mutations refer to the simultaneous presence of the three other mutations if the first one is observed. The isolates without or with mutations in the SdhC are referred to as C⁻ and C⁺, respectively. In the this study, we (i) determined the different mutations occurring in the SdhB subunit from a large *Botrytis* population; (ii) investigated the distribution of C⁻ and C⁺ genotypes in five different hosts and geographic U.S. regions; (iii) evaluated the sensitivity of C⁻ and C⁺ field isolates to three SDHs (boscalid, penthiopyrad, and fluopyram) and to fludioxonil, cyprodinil, and fenhexamid; and (iv) evaluated the effect of the SdhC mutations on several fitness parameters in *B. cinerea* isolates.

MATERIALS AND METHODS

Botrytis isolates. In all, 558 *Botrytis* spp. isolates collected from 40 different locations (fields and orchards) in Florida, South Carolina, North Carolina, Washington, and Oregon were used to investigate the presence of mutations in the SdhC subunit. Isolates were collected from five different hosts that included apple, cherry, blueberry, pear, and strawberry (Table 1). The SDHI boscalid,

premixed with pyraclostrobin (Pristine; BASF, Research Triangle Park, NC, U.S.A.), has been used regularly since 2004 in all fields and orchards surveyed. Florida strawberry fields surveyed in 2013 and 2014 have been sprayed with penthiopyrad and fluopyram since 2012. Thirty baseline isolates from strawberry fields in the Carolinas, characterized previously as HS or S to boscalid (Fernández-Ortuño et al. 2012), were used in this study. Blueberry isolates were obtained from flowers and fruit collected in 2013 and 2014 from five commercial fields in Central Florida. Isolates from apple, cherry, and pear were obtained from rotten fruit collected from packinghouses in Washington State (apple and cherry) and Oregon (pear only) between 2000 and 2011. In all, 40 and 120 isolates from strawberry were used for sequencing and to assess sensitivity to four SDHs, respectively, as described below. Thirty-two strawberry isolates from different genotypes were used to evaluate the fitness parameters and seven apple isolates were used to assess efficacy of three SDHs on detached apples. All 588 isolates were screened for the presence of *B. cinerea*, *B. pseudocinerea*, *Botrytis* group S, and MDRh1 phenotype, as described previously (Amiri et al. 2018; Fournier et al. 2003; Leroux et al. 2012).

Characterization of mutations in the SDH subunits from Botrytis field isolates and SDH homology modeling. DNA was extracted from 7- to 10-day-old conidia and mutations in the SdhB were characterized as described previously (Amiri et al. 2014). Fragments including the SdhC and SdhD subunits were amplified from 10 sensitive isolates and 30 isolates from strawberry, with different levels of resistance to boscalid, using the primer sets shown in Table 2. PCR assays were performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, U.S.A.) using an initial denaturation at 95°C for 3 min; followed by 35 cycles of 94°C for 40s, 55°C for 40s, and 72°C for 1 min; and a final extension at 72°C for 5 min. Resulting PCR products were purified and used for sequencing. Four mutations were found at codons 85, 93, 158, and 168 of SdhC in some isolates harboring or not harboring mutations in the SdhB subunit. A unique *BsmI* restriction site (GTCTCN/) was detected at codon 85 of SdhC of isolates not carrying the four mutations (C⁻). Therefore, a PCR restriction fragment length polymorphism (RFLP) assay was developed to detect the substitution at the SdhC^{G85} amino acid. The primer pair SdhC-F2/SdhC-R2 (Table 2) was used to amplify a 850-bp fragment of SdhC using the same parameters described above. Resulting PCR products were digested with the *BsmI* enzyme (New England BioLabs) and separated on a 2% agarose gel. The PCR-RFLP was used to characterize the C⁻ from C⁺ genotype in 558 *B. cinerea* isolates from five hosts.

The three-dimensional structure of the partial SDH protein of *B. cinerea* was modeled based on a structure template of the SDH protein from the *Sus scrofa* (porcine) SDH complex II (Protein Database Bank PDB, accession number 3AE2) with OH-phenylbenzamide at the Q-binding site using the PyMOL 0.99rc6 program (DeLano Scientific LLC, San Francisco, CA, U.S.A.) to represent the mutations observed in the SdhC subunit of *Botrytis* isolates.

Fungicide sensitivity assays in isolates from the C⁻ and C⁺ genotypes. The sensitivity of 120 isolates, i.e., 70 C⁻ isolates (no mutations in SdhC) and 50 C⁺ isolates (with mutations in SdhC)—was evaluated to fluopyram (Luna Privilege; Bayer Crop Science, Research Triangle Park, NC, U.S.A.), penthiopyrad (Fontelis; Dupont Crop Protection, Wilmington, DE, U.S.A.), boscalid (Endura; BASF), and carboxin (Santa Cruz Biotech., Santa Cruz, CA, U.S.A.) using a conidial germination and germ-tube elongation assay on yeast-peptone-acetate agar (YBA) medium (Miyamoto et al. 2010; Stammler et al. 2008). Autoclaved YBA was amended with boscalid, fluopyram, carboxin, or penthiopyrad at 0.0, 0.1, 0.5, 1.0, 5.0, 10 and 50 µg/ml and poured into 15-cm Petri dishes. The plates were divided into 60 squares (external bottom side of the plate), allowing testing for 60 isolates simultaneously on each plate. Two 7-µl droplets from a conidial suspension at 10⁵ conidia/ml were placed inside each square for each isolate. Two replicate plates were used for each 60-isolate subsample and the test was repeated twice.

Germination (%) and germ tube elongation were evaluated microscopically after 16 h incubation at $21 \pm 1^\circ\text{C}$. Rating was conducted as described previously (Fernández-Ortuño et al. 2014, Weber and Hahn 2011) and values were used to calculate mean effective concentrations of the fungicide to inhibit 50% germination and growth tube elongation (EC_{50}) values. Sensitivity of the C⁻ and C⁺ isolates to cyprodinil (Vangard WG; Syngenta Crop Protection, Greensboro, NC, U.S.A.), fludioxonil (Medallion WP; Syngenta), and fenhexamid (Elevate 50 WDG; Arysta, Cary, NC, U.S.A.) was evaluated as described previously (Fernández-Ortuño et al. 2014).

Efficacy of SDHIs against C⁻ and C⁺ isolates on detached fruit. The efficacy of three SDHIs in controlling gray mold was evaluated for seven *B. cinerea* isolates with or without mutations in the SdhB and SdhC subunits; that is, three C⁻ (Bc769, Bc8001, and Bc4737) and four C⁺ (Bc807, Bc1462, Bc7425, and Bc9513) isolates isolated from rotten apple fruit. Efficacy tests were conducted on detached organically grown apple (Golden Delicious) prepared and inoculated as described previously (Ali and Amiri 2018). Fruit were wounded and preventively sprayed with the recommended label or equivalent rates of fluopyram (Luna Privilege) or penthiopyrad (Fontelis) at 531 and 1,877 $\mu\text{l/liter}$, respectively, or with boscalid (Endura) at 0.86 g/liter. Control fruit were sprayed with sterile water. Eight replicate fruit were used for each isolate–fungicide combination and the experiment was done in triplicate. Fruit were incubated in sterile boxes in a saturated atmosphere at $21 \pm 1^\circ\text{C}$ and gray mold incidence and lesion diameter were recorded after 4 and 8 days.

Effect of mutations in the SdhC subunit on fitness of *B. cinerea*. In all, 32 isolates collected from strawberry, including 16 isolates from each of C⁻ and C⁺ genotypes and 8 isolates from each of the four SdhB genotypes (WT-H272, R272, Y272, and I230) per SdhC genotype (4 isolates for each SdhB–SdhC genotype), were used to study potential fitness penalties caused by the mutations in SdhC. Parameters included growth, sporulation, and sclerotia production on artificial media and on fruit as well as sensitivity to osmotic stress and reactive oxygen species (ROS). To test for mycelial growth, two 5-mm-diameter mycelial plugs, taken from the edge of a growing colony of each isolate, were placed upside down on a potato dextrose agar (PDA) plate. Radial growth was measured after 5 days of incubation at 22°C and four plates were used for each isolate. Production of sclerotia was evaluated after 40 days incubation on the same plates used for the mycelial growth assay. Sporulation in vitro was evaluated on malt yeast agar (MYA) plates inoculated with mycelial plugs as described above, using four plates for each isolate.

After 10 days incubation, 15 ml of sterile distilled water were added to each plate, and conidia were carefully scraped from the MYA surface, filtered through cheesecloth, and counted using a hemacytometer. Sporulation on fruit was evaluated on detached blackberry fruit prepared and inoculated as described previously (Amiri et al. 2014). After 10 days incubation at 22°C , conidia were scraped from the fruit surface using a sterile cotton swab and resuspended in 1 ml of sterile distilled water. Spores were counted as described for sporulation on MYA. The sensitivity to osmotic stress was evaluated on PDA amended with NaCl at 60 g/liter as described above for mycelium growth tests and growth was measured after 10 days. Sensitivity to ROS was evaluated on PDA amended with 10, 20, and 40 mM paraquat dichloride (Gramoxone SL, 30.1% a.i.; Syngenta Crop Protection) to induce ROS formation. Two mycelial plugs were placed upside down on each plate and five plates were used for each isolate. Radial growth was measured after 10 days incubation at 22°C . All tests were conducted twice.

Data analysis. Data from in vitro sensitivity tests from repeated experiments were combined after homogeneity of variance between experiments was tested using Bartlett's test. A χ^2 analysis was conducted to assess the relationship between the mutations in SdhC with the sensitivity level (EC_{50}) to the SDHIs. Analysis of variance and mean separations using Fisher's least significant difference were carried out to determine the effect of single SdhC substitutions on the sensitivity to SDHIs and the six fitness parameters. All statistical analyses were performed with JMP statistical software (SAS institute Inc., Cary, NC, U.S.A.).

RESULTS

The H272R substitution in the SdhB subunit is pre-dominant in all populations. All 558 isolates tested in this study were characterized as *B. cinerea* because neither *B. pseudocinerea* nor *Botrytis* group S were detected. Of these 558 isolates, 28.6% (160 isolates) were characterized as the wild type (SdhB^{WT}), with no mutation in the SdhB subunit, whereas 45.7% (255 isolates) carried the SdhB^{H272R} mutation (Fig. 1A). Overall, 19.3, 7.2, and 2.2% of isolates carried the SdhB^{H272Y}, SdhB^{N230I}, and SdhB^{P225F} mutations, respectively (Fig. 1A).

When grouped by host, isolates from apple, cherry, and pear had a higher frequency of the SdhB^{WT} isolates, with 56, 58 and 51%, respectively (Fig. 1B), whereas the strawberry population had the lowest SdhB^{WT} frequency (17%). The frequency of isolates with the SdhB^{H272R} mutation ranged from 26% in apple to 51.4% in strawberry,

TABLE 1. Origin, numbers, and characteristics of *Botrytis* isolates used in this study

Host	Isolates (n)	Locations(n)	Geographic origin	Year of collection			
				Before 2005	2008–2011	2013	2014
Apple	65	10	Washington	17	48	...	...
Blueberry	80	5	Florida	...	...	40	40
Cherry	12	2	Washington	12	...	...	...
Pear	39	7	Washington (20) and Oregon (19)	7	32	...	...
Strawberry	362	16	Florida(332), North Carolina (11), South Carolina (19)	61	80	122	99
Total	558	...	...	97	160	162	139

TABLE 2. Primers used for sequencing the SdhB, SdhC, and SdhD subunits of *Botrytis cinerea*

Gene	Primer name	Sequence (5'–3')	Expected size (bp)	Reference
<i>SdhB</i>	Bc.SdhB-F1	AAGGTATCTGCGGCAGTTGT	850	This study
	IpBecEnd2	CTCATCAAGCCCCCTCATTGATATC	...	Leroux et al. (2010)
<i>SdhC</i>	SdhC1	ATCACGTGCCAGATTTCCCTT	1,100	...
	SdhC5	AGGTGGGATCTTGGGGTTAT	...	Leroux et al. (2010)
	SdhC-F2	AACCGATACCTTCCGCATCA	851	...
	SdhC-R2	CCCCTCCCAACCTTAACCTA	...	This study
	SdhD1-F	TGATGACCTTGACGTGACT	950	...
<i>SdhD</i>	SdhD2-R	AGCAGCATGTGTTTGACAGC	...	Leroux et al. (2010)

whereas the SdhB^{H272Y} mutation was found at 19, 13, and 12% in strawberry, pear, and apple, respectively, and was lower than 10% in blueberry and cherry (Fig. 1B). Overall, the SdhB^{N230I} and SdhB^{P225F} mutations were found at frequencies ≤ 10% in apple, blueberry, and

Fig. 1. A, Overall frequency of mutations in the SdhB subunit of 558 *Botrytis cinerea* isolates surveyed in this study and B, their distribution by host. Numbers in parentheses indicate the number of isolates screened from each host and WT stands for wild type. Mutation characterization was conducted as described previously (Amiri et al. 2014).

strawberry and were absent from the cherry and pear populations. No mutations were found in the SdhD subunit (data not shown).

Four simultaneous mutations are detected in the SdhC subunit of SDHI-exposed and nonexposed populations of *B. cinerea*. Sequencing the SdhC subunit of 40 field isolates revealed four simultaneous mutations in SdhC of some isolates (Fig. 2). A glycine to alanine mutation (SdhC^{G85A}) occurred at codon 85 in the proximal quinone binding (*U_Q*) site, adjacent to the well-conserved serine (Ser84) known to directly interact with the SDHs. Another mutation from isoleucine to valine (SdhC^{I93V}) occurred at codon 93 positioned near a proximal heme binding site (*H_b*), whereas the third mutation from methionine to valine (SdhC^{M158V}) occurred at codon 158 at the interface between the [3Fe-4S] cluster in SdhB and *U_Q*. The fourth mutation, valine to isoleucine (SdhC^{V168I}) at codon 168, was in a domain with unknown function in the SdhD interface binding site. Docking modeling studies showed that the four amino acids at which mutations occurred in *B. cinerea* are part of the *Q_P* (Fig. 3). The positioning of SdhC^{G85} in the *U_Q* site and the C^{M158} near the [3Fe-4S] cluster of SdhB are of a major importance for SDHs docking and sensitivity.

The isolates without and with mutations in the SdhC were referred to as C⁻ and C⁺ genotypes, respectively, and a PCR-RFLP assay was developed to characterize them. A *BsmI* digestion of the 850-bp *SdhC* fragment amplified with the primer set SdhC-F2/SdhC-R2 generated two fragments of 550 and 350 bp in isolates with a glycine at codon 85 (SdhC^{G85}), whereas DNA from C⁺ isolates with an alanine at codon 85 (SdhC^{A85}) remained undigested (Fig. 4).

Among the 558 *B. cinerea* isolates screened using the PCR-RFLP assay, 415 (74.4%) were from the C⁻ genotype versus 143 isolates (25.6%) from the C⁺ genotype. The latter was present in all hosts but at higher frequencies in apple (40%), pear (31%), strawberry (25%), and cherry (25%) compared with 12.5% in blueberry (Fig. 5A).

The C⁺ genotype was detected in both SdhB^{WT} and SdhB field mutants; however, it was more frequent (56%) in the SdhB^{WT} isolates (Fig. 5B). Interestingly, a majority of *B. cinerea* isolates which were S to boscalid from tree fruit in addition to the isolates HS to boscalid from the Carolinas were from the C⁺ genotype. Comparatively, the isolates with mutations in the SdhB contained lower frequencies of C⁺ isolates, with the SdhB^{H272R}, SdhB^{N230I}, and SdhB^{H272Y} mutants showing 23.1, 17.5, and 14.1% of the C⁺ genotype, respectively, whereas all of the 11 SdhB^{P225F} mutants were from the C⁻ genotype (Fig. 5B). The frequency of C⁺ genotypes decreased slightly from 40% in isolates collected before 2004 (prior

Isolate	Phenotype	SdhB			SdhC				SdhC Genotype
		225	230	272	85	93	158	168	
05-30	HS	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
11-26	HS	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
05-31	HS	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
12-457	HS	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
10-36	MR	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
11-67	MR	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
12-316	R	STSCPSYWWNSEE	LYRCY [*] TILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
12-255	R	STSCPSYWW [*] I [*] SEE	LYRCHTILNCSRTCP		83 MSALN [*] ITGCVLSGG		150 LRHLAWDV [*] GKTFKNATVIKTGWT		C ⁺
11-63	S	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
08-98	S	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
05-28	S	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
07-65	HR	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
08-100	HR	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
11-04	HR	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
10-31	HR	STSCPSYWWNSEE	LYRCY [*] TILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
10-34	HR	STSCPSYWWNSEE	LYRCY [*] TILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
12-450	HR	STSCPSYWW [*] I [*] SEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻
12-374	HR	STSCPSYWWNSEE	LYRCHTILNCSRTCP		83 MSGLN [*] ITGCVLSGG		150 LRHLAWDMGKTFKNATVVKTGWT		C ⁻

Fig. 2. Alignment of deduced amino acid sequences of the SdhB (left) and SdhC (right) sequences of *Botrytis cinerea* isolates showing different levels of sensitivity to boscalid. In all, 40 strains were sequenced but only 18 are shown here. Residues involved in resistance to succinate dehydrogenase inhibitors (SDHIs) in both subunits are shaded. Residues in regular and bold fonts represent nonmutated and mutated amino acids, respectively. Asterisks indicate amino acids involved in SDHI binding. HS, S, MR, R, and HR indicate that the isolate is highly sensitive, sensitive, moderately resistant, resistant, or highly resistant, respectively, to boscalid.

to SDHI registration) to 37 and 36% in isolates collected in the 2005-to-2009 and 2010-to-2014 periods, respectively (data not shown).

Botrytis C⁻ and C⁺ field isolates exhibit different sensitivities to multiple fungicides. The mean EC₅₀ value of the SdhB^{WT}-C⁺ isolates was significantly lower for boscalid ($\chi^2 = 6.28$, $P = 0.0037$), penthiopyrad ($\chi^2 = 4.52$, $P = 0.023$), and carboxin ($\chi^2 = 3.75$, $P = 0.041$) compared with the SdhB^{WT}-C⁻ isolates (Table 3). Similarly, the SdhB^{H272R/Y} and SdhB^{N230I} isolates from the C⁺ genotype were significantly more sensitive to all SDHIs, except to fluopyram. In contrast, all SdhB mutants from the C⁺ genotype had higher mean EC₅₀ values for fluopyram than the C⁻ isolates, including the SdhB^{WT} isolates ($\chi^2 = 3.02$, $P = 0.033$) and SdhB^{H272Y} ($\chi^2 = 4.39$, $P = 0.0360$). The mean EC₅₀ value of the SdhB^{N230I}-C⁺ mutants for fluopyram was higher but not significantly different from those of the SdhB^{N230I}-C⁻ genotype (Table 3). Ultimately, the C⁺ isolates had slightly higher but not significantly different EC₅₀ values for fludioxonil and fenhexamid compared with the C⁻ isolates, whereas the two genotypes had similar sensitivities to cyprodinil (data not shown).

Mutations in the SdhC subunit affect sporulation, sclerotia production, and sensitivity to osmotic stress and ROS. The results from fitness parameter studies provide evidence for fitness penalty in C⁺ isolates (Table 4). Mycelial growth on PDA was not affected by the SdhC mutations because growth was not significantly different, except for the SdhB^{WT} isolates. However, the C⁺ isolates were more sensitive to osmotic stress and ROS, especially the SdhB^{Y272} and B^{I230} mutants from the C⁺ genotype (Table 4). Sporulation was the most severely affected fitness parameter in C⁺ isolates on MYA (Fig. 6A and B), whereas C⁺ isolates failed to sporulate on detached fruit after 10 days at 22°C (Table 4; Fig. 6C and D). Sclerotia production on PDA after 40 days at 22°C was lower in C⁺ isolates in all SdhB genotypes and differences were significant in the SdhB^{WT}, SdhB^{H272Y}, and SdhB^{N230I} mutants (Table 4).

SDHI fungicides differentially reduce gray mold incidence of C⁺ isolates on detached apple fruit. Boscalid and penthiopyrad applied preventively at the recommended field rate fully controlled the SdhB^{WT}-C⁺ isolates but not the SdhB^{WT}-C⁻ isolate after 6 days, whereas fluopyram did not completely inhibit the SdhB^{WT}-C⁺ isolates (Table 5). Similarly, gray mold incidence and severity were higher in fruit treated with boscalid and penthiopyrad (in contrast to fluopyram) and inoculated with the SdhB^{H272R/Y}-C⁻ mutants compared with the SdhB^{H272R/Y}-C⁺ mutants (Table 5). After 4 days of incubation at room temperature,

penthiopyrad controlled the SdhB^{WT}-C⁺ but not the SdhB^{WT}-C⁻ mutant (Fig. 6F) and an opposite efficacy was seen for fluopyram on detached apple fruit (Fig. 6G). There was no difference in gray mold incidence and severity when the SdhB^{N230I}-C⁻ and SdhB^{N230I}-C⁺ were inoculated regardless of the SDHI used (data not shown).

DISCUSSION

Previous studies have linked SDHI resistance in *B. cinerea* to multiple mutations in the SdhB subunit (Amiri et al. 2014; Fernández-Ortuño et al. 2012; Leroux et al. 2010; Stammler et al. 2008, 2011; Veloukas et al. 2011, 2013; Weber and Hahn 2011). The potential higher risk for *B. cinerea* to develop resistance to the SDHIs is reinforced by the high polymorphism in the membrane-anchored SdhC subunit reported in this study. Herein, we report for the first time on four simultaneous mutations in the SdhC subunit defining two coexisting *Botrytis* genotypes, C⁻ and C⁺. The C⁺ genotype was found in all hosts investigated and was widespread in U.S. small-fruit fields and tree-fruit orchards. Our results indicate that the C⁺ isolates do not correspond to *B. pseudocinerea* (Fournier et al. 2003) or *Botrytis* group S (Leroch et al. 2012), because all C⁺ isolates were characterized as *B. cinerea*. The mutations confer different sensitivity levels to the FRAC 7 fungicides; that is, a higher sensitivity to the pyridine (boscalid), pyrazole (penthiopyrad), and oxathiin (carboxin) carboxamides and reduced sensitivity to the benzamides (fluopyram). Moreover, these mutations clearly

Fig. 4. Gel electrophoresis (2% agarose) of nondigested (1 to 9) and *BsmI*-digested (10 to 18) SdhC PCR fragments amplified using the primer set SdhC-F2/SdhC-R2. Lanes 10 to 13 show isolates with mutations in SdhC (C⁺), with the SdhC^{G85A} mutation at codon 85 (also at codons 93, 158 and 168); lanes 14 to 18 show isolates without mutations (C⁻) at codon 85. M = molecular marker.

Fig. 3. **A**, Side view and **B**, opposite-side view of *Botrytis cinerea* succinate dehydrogenase (SDH) complex II modeled based on *Sus scrofa* (porcine) (Protein Database Bank PDB, accession number 3AE2) with OH-phenyl-benzamide at Q-binding site (U_Q) to demonstrate the locations of the four SdhC mutations in *Botrytis cinerea*. SdhC^{G85}, SdhC^{I93}, SdhC^{M158}, and SdhC^{V168} residues salmon, gray, orange, and cyan spheres, respectively. The connecting part of helix 1 is shown in pink (visible in **A**) and a loop between M158 and V168 is shown in beige (visible in **B**). The heme *b* and the U_Q site are shown in red and orange sticks, respectively. SdhB (partial), SdhC, and SdhD are shown in gray, green, and blue helices, respectively, the [3Fe-4S] cluster is shown in golden and reddish spheres within the SdhB.

affected the fitness abilities of the C⁺ *B. cinerea* isolates and likely their ability to outcompete and survive in field conditions. The positioning of the four mutations most likely caused an alteration in the SDH structure which reduced or increased its affinity to the different SDHIs and, therefore, led to the different sensitivity levels observed herein and previously (Amiri et al. 2014; Fairchild et al. 2012; Fernández-Ortuño et al. 2012; Ishii et al. 2011; Leroux et al. 2010; Stammler et al. 2008).

Fig. 5. Overall distribution of C⁻ and C⁺ genotypes among **A**, hosts and **B**, SdhB genotypes in 558 *Botrytis cinerea* isolates screened using the PCR restriction fragment length polymorphism assay. C⁻ and C⁺ refer to isolates without and with the four mutations in the SdhC subunit, respectively.

Homology modeling studies showed the four mutations in the C⁺ isolates, to be part of the quinone-binding pocket. Three residues at which mutations occurred (SdhC^{G85}, SdhC^{I93}, and SdhC^{M158}) are all part of domains known to be vital in interaction with ubiquinone inhibitors and the activity of the SDH complex. SdhC^{G85A} has major importance because it is located in the U_Q site, which includes SdhC^{Ser84}, known to form a hydrogen bond with the O4-carbonyl oxygen of the quinone molecule and to interact directly with the SDHs (Maklashina and Cecchini 2010; Ruprecht et al. 2009; Scalliet et al. 2012; Tran et al. 2006). SdhC^{I93V} is at the downstream start of the H_b site, which is primordial for the assembly and stability of the SDH complex (Haegerhaell et al. 1995; Nakamura et al. 1996). SdhC^{M158V} is positioned in the iron-sulfur protein interface close to U_Q and within the vicinity of the [3Fe-4S] cluster actively involved in electron transfer. Although homology modeling conducted in this and in a previous study (Glättli et al. 2009) has confirmed the positioning of residues important for SDHI sensitivity, it is important to characterize the actual SDH enzyme of *Botrytis* spp. because the low SdhC conservation between species would not allow definitive conclusions on the role of the different mutations found in the SdhC subunit.

The role of residues near the well-conserved SdhC^{Ser84} in sensitivity or resistance of the C⁺ *B. cinerea* isolates to the SDHIs is supported by recent findings. In an effort to evaluate risks of fungicide resistance development in *M. graminicola*, mutants with amino acid substitutions at 10 different residues in SdhC have been selected by different SDHIs in laboratory conditions (Scalliet et al. 2012). Interestingly, an alanine substitution by a valine (SdhC^{A84V}), equivalent to SdhC^{G85A} in *B. cinerea*, was the most selected by fluopyram (Scalliet et al. 2012). These mutants were highly resistant to fluopyram, whereas their sensitivity to boscalid was only slightly reduced. Moreover, in *B. calthae*, a specific pathogen of the wet woodland plant *Caltha palustris* (Hennebert and Groves 1963), isolates with significantly reduced sensitivity to fluopyram and boscalid had the SdhC^{G85A} and SdhC^{I93V} mutations in the SdhC (Plesken et al. 2014), similar to the ones found in the *B. cinerea* C⁺ isolates. The authors also found two other mutations at codons 133 (I to F) and 138 (S to T) but did not indicate if the latter occurred simultaneously. Further evidence supporting our findings consists of a serine-to-proline substitution at codon 73 of SdhC which conferred moderate resistance and reduced sensitivity to boscalid and fluopyram in *C. cassicola* (Ishii et al. 2011; Miyamoto et al. 2010). Interestingly, SdhC^{S73} of *C. cassicola* aligned with SdhC^{A84} of *M. graminicola* and SdhC^{G85} of *B. cinerea* (data not shown).

Although a direct effect on SDHI affinity with docking sites is the most likely mechanism, the positioning of SdhC^{M158} may have altered the electronic properties of the [3Fe-4S] cluster and the U_Q

TABLE 3. Sensitivity of *Botrytis cinerea* isolates with and without mutations in the SdhC subunit to four succinate dehydrogenase inhibitors

Genotype ^a			Boscalid			Penthiopyrad			Carboxin			Fluopyram		
SdhB	SdhC	n	EC ₅₀ ^b	χ ^{2c}	VF/RF ^d	EC ₅₀	χ ²	VF/RF	EC ₅₀	χ ²	VF/RF	EC ₅₀	χ ²	VF/RF
WT	C ⁻	16	0.35	6.28**	60	0.89	4.52*	78	1.89	3.75*	12	0.47	3.02*	78
	C ⁺	20	0.17	...	78	0.11	...	55	0.75	...	3	0.76	...	154
H272R	C ⁻	23	4.51	15.46**	451	0.61	11.21**	61	5.00	15.41**	23	0.46	3.58*	46
	C ⁺	18	1.31	...	131	0.13	...	13	2.26	...	9	1.31	...	131
H272Y	C ⁻	21	3.99	4.76*	399	1.74	13.45**	174	7.69	4.47*	18	0.11	4.39*	11
	C ⁺	6	1.36	...	136	0.10	...	10	3.15	...	8	0.39	...	35
N230I	C ⁻	10	4.75	11.79**	475	1.05	4.57*	105	4.60	4.87*	12	1.74	0.32	174
	C ⁺	6	0.94	...	94	0.79	...	79	2.35	...	9	1.86	...	186

^a Genotypes indicate isolates without mutations in SdhB (wild type [WT]) or SdhC (C⁻) or with mutations in SdhB (CH272R/Y and N230I) and SdhC (C⁺).
^b EC₅₀ = mean effective concentrations in micrograms per milliliter of the fungicide to inhibit 50% germination and growth tube elongation.
^c Values within the same row for each SdhB (B) genotype followed with an *, **, and *** differ significantly, very significantly, and very highly significantly, respectively, based on the χ² test.
^d VF and VR indicate variation factors and resistance factors, respectively, used to estimate sensitivity variation in WT and mutated isolates, respectively. VF for each genotype was calculated by dividing the highest EC₅₀ value by the lowest EC₅₀ value in the WT isolates, whereas RF for each genotype was calculated by dividing the highest EC₅₀ value within each SdhB genotype by the lowest EC₅₀ value in the WT isolates.

TABLE 4. Evaluation of six fitness parameters in *Botrytis cinerea* isolates with and without mutations in the SdhC subunit

Genotype ^a		Growth (mm) on ^b			Sporulation ($\times 10^5$) ^c		
SdhB	SdhC	PDA	NaCl (5%)	Paraquat (20 mM)	MYA	Blackberry	Sclerotia ^d
WT	C ⁻	39.8 $\pm$ 4.1	13.1 $\pm$ 3.2	21.3 $\pm$ 3.1	25.3 $\pm$ 16.2	129.6 $\pm$ 84.5	78.1 $\pm$ 5.8
	C ⁺	22.7 $\pm$ 5.2*	11.5 $\pm$ 8.0	17.2 $\pm$ 5.3	3.6 $\pm$ 8.9*	0.0*	57.1 $\pm$ 4.2*
H272R	C ⁻	39.4 $\pm$ 2.0	10.5 $\pm$ 2.5	21.5 $\pm$ 4.1	44.2 $\pm$ 44.3	98.1 $\pm$ 94.3	47.2 $\pm$ 6.2
	C ⁺	37.9 $\pm$ 3.2	07.5 $\pm$ 1.1	14.3 $\pm$ 2.5*	29.8 $\pm$ 35.9*	0.0*	58.5 $\pm$ 5.6
H272Y	C ⁻	39.4 $\pm$ 2.1	20.2 $\pm$ 1.2	11.8 $\pm$ 2.9	46.9 $\pm$ 59.4	78.4 $\pm$ 51.0	65.6 $\pm$ 4.2
	C ⁺	38.9 $\pm$ 2.4	12.4 $\pm$ 2.1*	6.1 $\pm$ 1.2*	36.6 $\pm$ 42.7*	0.0*	37.3 $\pm$ 2.7*
N230I	C ⁻	41.0 $\pm$ 1.1	15.1 $\pm$ 2.1	10.9 $\pm$ 3.1	58.1 $\pm$ 38.6	132.1 $\pm$ 42.8	21.0 $\pm$ 3.4
	C ⁺	39.5 $\pm$ 1.0	6.8 $\pm$ 1.1*	3.2 $\pm$ 1.9*	46.8 $\pm$ 45.6*	0.0*	0.0*

^a Genotypes indicate isolates without mutations in SdhB (wild type [WT]) or SdhC (C⁻) or with mutations in SdhB (CH272R/Y and N230I) and SdhC (C⁺).
^b Mean growth after 5 days of incubation on potato dextrose (PDA), 10 days on 6% NaCl, and 20 mM Paraquat at 22°C. Data are means of 64 values from four isolates within each genotype, four replicate plates per isolate across two experimental runs.
^c Number of spores $\times 10^5$ after 10 days at 22°C expressed as the mean from four isolates within each genotype, four replicate plates or fruit per isolate across two experimental runs. MYA = malt yeast agar.
^d Sclerotia production on PDA. Number of sclerotia produced on PDA after 40 days incubation at 22°C. Data are means of 64 values from four isolates within each genotype, four replicates. An asterisk indicates significant difference in values within the same column and each genotype, separately, based on *t* test at *P* $\leq$ 0.05.

Fig. 6. Example of sporulation of *Botrytis cinerea* isolates from C⁻ and C⁺ genotypes on **A** and **B**, malt yeast agar and **C** and **D**, blackberry fruit after 10 days of incubation at 22°C. Gray mold lesions on Golden Delicious apple **E**, nontreated or treated with **F**, penthiopyrad and **G**, fluopyram and inoculated with C⁻ (Bc8001) and C⁺ (Bc7425) isolates after 4 days of incubation at 22°C.

TABLE 5. Efficacy of three succinate dehydrogenase inhibitor fungicides to control *Botrytis cinerea* isolates from different SdhC genotypes on detached apple fruit^a

Isolate	Genotype		Boscalid			Penthiopyrad			Fluopyram		
	SdhB	SdhC	EC ₅₀	Incid	Les	EC ₅₀	Incid	Les	EC ₅₀	Incid	Les
Bc769	WT (H272-N230)	C ⁻	0.4	12.8	12.0	1.14	8.0	12.0	1.57	0.0	0.0
Bc807	WT (H272-N230)	C ⁺	0.1	0.0	0.0	0.34	0.0	0.0	0.36	45.0	8.8
Bc1462	WT (H272-N230)	C ⁺	0.2	0.0	0.0	0.32	0.0	0.0	0.73	55.0	10.3
Bc8001	H272R	C ⁻	4.0	100.0	22.6	2.02	12.5	8.6	0.38	0.0	0.0
Bc7425	H272R	C ⁺	1.5	40.0	6.8	0.74	0.0	0.0	2.88	60.0	9.1
Bc4737	H272Y	C ⁻	3.5	80.0	29.8	6.06	100.0	28.4	0.16	0.0	0.0
Bc9513	H272Y	C ⁺	1.2	60.0	9.7	5.51	40.0	14.1	0.91	45.0	10.8

^a EC₅₀ = mean effective concentrations of the fungicide to inhibit 50% germination and growth tube elongation, Incid = mean gray mold incidence (percent) after 6 days of incubation at 22°C from eight replicate fruit across two experimental runs for each isolate within each genotype, and Les = mean lesion in millimeters after 6 days of incubation at 22°C from eight replicate fruit across two experimental runs for each isolate within each genotype.

site or favored electron transfer to *H_b*, as reported recently with other mutations in *E. coli* (Anderson et al. 2014; Ruprecht et al. 2011). On the other hand, the SdhC¹⁹³ residue is located next to *H_b*, which has been studied extensively and is thought to play a role in SDH assembly and stability (Hägerhäll 1997; Lancaster 2002; Lemarie and Grimm 2009). In fact, mutations in *H_b* or its vicinity have not been found to affect SDH activity or SDHI sensitivity (Anderson et al. 2014; Oyedotun et al. 2004; Tran et al. 2006; Yankovskaya et al. 2003). However, mutations in SdhC and *H_b* have been reported to cause ROS production and accumulation of succinate, known to cause hypoxia leading to ageing in different organisms (Friden et al. 1987; Ishii et al. 1998; Szeto et al. 2007). These studies corroborate our findings on mutation in *H_b* causing increased ROS sensitivity as seen in C⁺ isolates of *B. cinerea*. Finally, the SdhC^{V168I} mutation occurred at a position with an unknown, apparent functional activity.

The presence of two *Botrytis* SdhC genotypes differentially sensitive to SDHIs and other fungicides in the field indicates different risks for resistance development and may shed some light on future gray mold management strategies. Because only boscalid has been used extensively in regions where the isolates have been collected, it is still unclear how the C⁻ and C⁺ populations have been selected. However, the predominance of the C⁺ genotype in the SdhB^{WT} isolates (i.e., those HS to boscalid from the Carolinas and those from tree fruit, which are less sprayed than strawberry) suggests a gradual negative selection pressure caused by boscalid on the C⁺ genotype. In *M. graminicola*, boscalid selected preferentially for the SdhB^{H267Y} mutations (equivalent to SdhB^{H272Y} in *B. cinerea*), whereas fluopyram selected predominantly (60%) for mutants carrying the SdhC^{A84V/I} mutations (equivalent to SdhC^{G85A} in *B. cinerea*) (Scalliet et al. 2012). In contrast, fluopyram selected significantly less for isolates with mutations at the well-conserved residues SdhC^{S84} (3%) and C^{R88} (18%) (Scalliet et al. 2012). In addition to a higher potential for fluopyram to select more SdhC mutants, the reduced sensitivity of the B^{H272Y} isolates from the C⁺ group to the benzamides may put in jeopardy all SDHIs because of cross-resistance to all SDHIs in these mutants.

Although the effect of the four dependent mutations on SDHIs sensitivity was variable, we found evidence for a strong fitness penalty due to these mutations on the ability of the C⁺ isolates to undergo severe selection pressure conditions compared with the C⁻ isolates. Overall, the C⁺ isolates were less fit to sporulate and produce sclerotia, which limits their spread and survival under normal field conditions. Moreover, the C⁺ isolates were more sensitive to water stress and ROS, which lowers their ability to survive under harsh conditions. This fitness cost combined with a negative selection pressure by most SDHIs, except fluopyram, may explain why the C⁺ genotypes are less frequent in the field. Our results on fitness costs due to the reported mutations corroborate previous findings on strong effects of mutations in SdhC and *H_b* of *Bacillus subtilis*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* (Friden et al.

1987; Ishii et al. 1998; Szeto et al. 2007) on their lifespan and fitness due to increased ROS production and accumulation of succinate, known to cause hypoxia and accelerated ageing.

We present evidence for increased risks for selecting populations resistant to the newly developed SDHIs in the gray mold fungus. The presence of multiple sites conferring reduced sensitivity to three major SDHIs from the pyrazole and pyridine groups in the C⁻ population, together with other mutations conferring reduced sensitivity to the benzamides in the C⁺ population, can rapidly select for populations highly resistant to all SDHIs. This will significantly limit the role of SDHIs in future rotation programs aimed to manage gray mold. Future research should focus on studying the functional role of the four mutations, characterizing the genetic relatedness between the C⁻ and C⁺ populations, and evaluating the impact of spray regimes on selection and spread of such genotypes. Our findings emphasize that antiresistance strategies should be implemented at introduction of new fungicides rather than after resistance is observed, a time at which strategies aimed to slow selection may be too late to be effective.

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