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FITNESS OF *ALTERNARIA ALTERNATA* FIELD ISOLATES WITH MULTIPLE FUNGICIDES RESISTANCE

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FITNESS OF ALTERNARIA ALTERNATA FIELD ISOLATES WITH MULTIPLE FUNGICIDES RESISTANCE

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ABSTRACT

Field isolates of *Alternaria alternata* from peach were previously characterized for their sensitivity to succinate dehydrogenase (SDH) inhibitor fungicides and the underlying molecular basis of resistance was determined. In the present study we report that isolates resistant to the SDHI fungicide, boscalid, regardless of genotype, were also resistant to pyraclostrobin and thiophanate-methyl. Resistance to pyraclostrobin was due to the G143A mutation in the *cyt*b gene and resistance to thiophanate-methyl was due to a mutation of 167Y in the β-tubulin gene. Representatives of the two most commonly-isolated, SDHI resistance genotypes H277Y in *sdh* subunit B and H134R in *sdh* subunit C, as well as genotype D123E in *sdh* subunit D were selected for fitness evaluations. Genotypes H277Y and H134R suffered no fitness penalties based on mycelial growth on PDA, spore production in vitro, osmotic sensitivity, oxidative sensitivity, germination ability, or the ability to cause disease on peach fruit. Hypersensitivity to oxidative stress and weak sporulation was observed only in genotype D123E. No competitive advantage was detected for sensitive isolates over the course of five consecutive transfers on peach fruit when spores were mixed with genotypes H277Y or H134R. Results suggest that in the absence of fungicide pressure, *A. alternata* isolates resistant to MBC, QoI, and SDHI fungicides carrying the H277Y mutation in SDHB and the H134R mutation in SDHC may effectively compete with the boscalid-sensitive populations.

DEDICATION

I would like to dedicate this work to my family. Without their support, I am not able to step on a foreign land, and keep chasing my dream.

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CHAPTER ONE

The Fungal Pathogen *Alternaria alternata*

The fungus *Alternaria alternata* (Fr.) Keissl. is an ascomycete which belongs to the phylum Ascomycota, class Dothideomycetes, subclass Pleosporomycetidae, order Pleosporales, family Pleosporaceae (Rotem 1994). *A. alternata* is an important plant pathogen which can infect various plants species, causing both pre- and postharvest disease, symptoms include leaf spots, rots and blight. It is a well-known producer of host-selective toxins (HSTs) (Bottini, et al. 1981; Kohmoto, et al. 1991; Masunaka, et al. 2005). In the medical field, *A. alternata* is also recognized as one of the major causes of fungal allergies. Cloning, sequencing and heterologous expression of the genes encoding allergens of this fungus is done to understand fungal allergy and to develop new and improved methods of diagnosis and therapy (Achatz, et al. 1995; Salo, et al. 2006).

Nomenclature and systematics. Most *Alternaria* spp., including *A. alternata*, exhibit considerable morphological plasticity that is dependent upon cultural conditions of substrate, temperature, light, and humidity (Misaghi, et al. 1978). Metabolite profiles and growth characteristics can still be used to identify small-spored *Alternaria* spp. when isolates are cultured under defined conditions (Andersen, et al. 2001). In addition to morphological identification, molecular examinations such as RAPD-PCR analysis

provide more options to identify *Alternaria* spp. (Roberts, et al. 2000). In recent years, the nuclear rDNA internal transcribed spacer (ITS) has been widely used to identify fungal species. Sequence analysis revealed ITS can fully distinguish isolates in the *infectoria* species-group; however, ITS does not distinguish isolates in the *arborescens, tenuissima*, and *alternata* species-groups (Pryor and Michailides 2002). The development of molecular data sets based upon other genetic regions will likely be necessary to fully evaluate the utility of the ITS region in resolving relationships among *Alternaria* spp..

Biology, geographical distribution, and host range. *Alternaria alternata* is a ubiquitous necrotrophic pathogen found in a wide range of plants. On living plants it causes leaf and fruit spotting, fruit rots and other symptoms. Some of the most common diseases caused by species in this genus include citrus brown rot (Kohmoto, et al. 1991), black mold of tomato (da Motta and Soares 2000), leaf blotch of apple (Johnson, et al. 2000), black spot of strawberry (Maekawa, et al. 1984), black spot of Japanese pear (Nishimura, et al. 1978), and late blight of pistachio (Pryor and Michailides 2002). The disease cycle of *A. alternata* is similar to all other *Alternaria* species, the pathogen overwinters and survives on buried host debris as conidia, mycelium. Spores are produced abundantly during heavy dews or frequent rains, and are blown from infected debris or infected plants. The germinating spores penetrate susceptible plants directly or skin breaks. After establishing their colony, they produce more spores that are further spread by wind, splashing rains, etc. (Chełkowski and Visconti 1992).

A. alternata is able to produce several host specific toxins (HSTs) that assist the fungus in penetrating plant tissue. The toxins are only toxic to plants that are susceptible

to the toxin-producing pathogen. And if the mutation that causes the release of HSTs is reversed, the isolate will fail to infect the cultivar that it used to infect. In *A. alternata* Japanese pear pathotype, release of the AK-toxin (a type of HST) has been seen from virulent spores immediately after germination. It plays an important role in recognizing the susceptible host and causes dysfunction of the plasma membrane system (Chełkowski and Visconti 1992).

Alternaria Rot of Peach

The peach originated in China, where records of cultivation date back 3000 years. Many recent cultivars grown in the United States can be traced to a single cultivar called Chinese Cling, first grown in Marshallville, Georgia (Ogawa, et al. 1995). In the U.S., South Carolina is second only to California in peach production, the first in the eastern U.S. Many diseases, caused by a variety of organisms, are able to affect peach, including Brown rot, Green fruit rot, Peach scab, Peach leaf curl, Phytophthora root rot and others (Horton and Johnson 2002). Among those diseases, pre- and postharvest brown rot damages impact peach industry the most (Ogawa, et al. 1995).

Preharvest peach diseases caused by *A. alternata* have been reported only a few times. The first incidence was recorded in 1995 when *A. alternata* was isolated from circular pale brown spots on immature peach fruit. Necrotic spots were also observed on leaves and twigs. All tested isolates were pathogenic on peach but not on Japanese pear or apple (Inoue and Hideo 2000). Evidence also showed *A. alternata* can also cause peach core rot, characterized by black or gray mycelium in the pulp or stone of the fruit.

In Greece, core rot caused significant damage in the cultivar Fayette. Further research showed three highly toxic compounds (alternariol, alternariol mono methyl ether, and tenuazonic acid) were present in the fungal extraction. Evidence shows alternariol is lethal to unborn mice at levels of 200 mg/kg b.w. Mono methyl ether is cytotoxic, carcinogenic and mutagenic. Tenuazonic acid is toxic to chicken embryos and can cause hemorrhage and death in mice. Infected fruit poses a health risk to customers, necessitating the need to eliminate Alternaria rot of peach (Pose, et al. 2010; Thomidis, et al. 2007).

In the southeastern US, Alternaria rot is mainly observed postharvest in stone fruit production (Eckert and Ogawa 1988). However, it rarely causes problems and thus deliberate management recommendations against this pathogen is not featured in the Southeastern Peach Growers' Handbook or the 2013 Southeastern Peach, Nectarine and Plum Pest Management and Culture Guide (Horton, et al. 2013). Alternaria rot was a problem in the southeastern peach industry in 2013, and specialists and growers were unprepared. The outbreak caused economic loss in late-season peach varieties at three different locations (Yang, et al. 2015).

Alternaria rot of peach is characterized by circular, dry, firm, shallow lesions covered with dark, olive green to black surface mycelial growth. The infected tissue is brown, like that caused by *Monilinia* (Ogawa, et al. 1995; Yang, et al. 2015). *A. alternata* is saprophytic or weakly pathogenic on many plants. In spore trap studies, the concentration of *Alternaria* spores in the air increased during and after rain or foggy

conditions. On sweet cherries, the fungus is able to attack the dead or dying tissue of aborted fruit in the orchard (Ogawa, et al. 1995).

Brown Rot Control in peach orchard

Because the primary disease of peach is brown rot, the majority of disease management recommendations are targeted on this disease. Before effective fungicides were available, orchard sanitation was extremely important for controlling brown rot. Removing the decaying fruits, burying mummies and eradicating nearby wild host were common practices conducted by peach growers. However, these practices were not reliable for consistent, effective control (Zehr 1982).

Currently, chemical control plays the most important role in disease management of peach orchards. The introduction of methyl benzimidazole cabamates (MBCs), targeting β-tubulin, and sterol demethylation inhibitors (DMIs), targeting 14α-demethylase dramatically improved brown rot control in the 1970s and the 1980s. More recently, quinone outside inhibitors (QoIs), inhibiting electron transport in the mitochondrial respiratory chain at the bc1 complex, also known as complex III and succinate dehydrogenase inhibitors (SDHIs), inhibiting the enzyme succinate ubiquinone reductase, also known as complex II, were registered for brown rot management and rotated with MBCs and DMIs for brown rot control (Chen, et al. 2013a; Schnabel, et al. 2004).

Biological control is another control option that organic farmers are seeking. Many biological agents have been investigated for their potential for brown rot control

(Hong, et al. 1998). *Penicillium frequentans, P. purpurogenum,* and *Epicoccum nigrum* were investigated for control of twig blight of peach caused by *Monilinia laxa* in Spain (Cal, et al. 1990; Madrigal, et al. 1994). However these biocontrol agents have not been able to control stone fruit diseases sufficiently and therefore are not used commercially. Biological control may be more successfully used for postharvest disease control. *Pseudomonas corrugate, Epicoccum nigrum* and *Bacillus subtilis* were demonstrated to control the decay of stone fruit (Larena, et al. 2005; Pusey and Wilson 1984; Smilanick, et al. 1993).

Fungicide Resistance in *Alternaria alternata*

Single-site fungicides are effective and generally exhibit less risk to consumer, worker, and environment. However, their mode of action make them vulnerable to resistance development. A single change in the target enzyme may confer resistance because the fungicide can no longer bind. Fungicides kill fungi by damaging their cell membranes, inactivating critical enzymes or proteins, or by interfering with key processes such as energy production or respiration. Others impact specific metabolic pathways such as the production of sterols or chitin. For example, phenylamide fungicides bind to and inhibit the function of RNA polymerase in oomycetes, while the benzimidazole fungicides inhibit the formation of beta tubulin polymers used by cells during nuclear division. (McGrath 2004).

In the year 2000, QoI fungicides were introduced to California pistachio for controlling Alternaria disease. They inhibit mitochondrial respiration by binding to the

Qo site. Only four years later, azoxystrobin-resistant *A. alternata* isolates were detected in the field populations and amino acid change G143A in cytochrome b gene was confirmed to confer resistance to this chemical class (Ma, et al. 2003b). In addition to QoI fungicide, dicarboximides are effective in controlling Alternaria rot. However, an iprodione-resistant isolates were found in field populations from California pistachio. Although the mode of action and resistance mechanism is still unknown, the existence of iprodione resistance signals resistance development risks in *A. alternata* (Ma and Michailides 2004). New SDHI fungicides targeting succinate ubiquinone reductase (sdh) of the complex II in the respiration chain (Kuhn 1984) provide options for chemical control of Alternaria rot. But again, resistance arose quickly in both California pistachio orchards and southeastern peach orchards (Avenot and Michailides 2007; Yang, et al. 2015). Further research determined resistance to SDHIs was based on mutations in *sdh* gene sequences. Amino acid substitutions in the *sdh* genes, may alter the conformation of residue that could interact with SDHI fungicides, and impair the affinity of sdh with SDHI fungicides or diminish the space needed for fungicides binding (Avenot, et al. 2008a; Avenot, et al. 2009).

Fitness of *Alternaria alternata*

Fitness is a common currency in comparative biology. Without data on fitness, hypotheses about the adaptive significance of phenotypes or basic mechanisms of evolution, for example natural selection, remain speculative. Fitness can be defined as the survival and reproductive success of an allele, individual or group. In plant pathology, fitness is usually elucidated by focusing on single aspect of a complex fungal life cycle.

The fundamental biology of pathogenic and other fungi is the same, some approaches can be adopted by most plant pathogens (Pringle and Taylor 2002). Several fitness components are often conducted for estimating the fitness of an individual isolate, including mycelial growth on PDA, osmotic sensitivity, spore production in vitro, virulence, and sporulation (Bardas, et al. 2008). Development of resistance to fungicides classes are sometimes associated with fitness costs, (Bardas, et al. 2008; Markoglou, et al. 2006), but it is not always the case (Raposo, et al. 1995).

In *A. alternata,* fitness has been evaluated for iprodione-resistant and azoxystrobin-resistant isolates. Although artificially generated fungicide-resistant isolates are generally less fit than sensitive isolates, isolates collected in the field with resistance phenotypes are often not deprived of fitness. For iprodione-resistant field isolates, virulence and fitness of sensitive and resistant isolates overlap. Less, more, or similar virulence to sensitive isolates was detected in iprodione-resistant isolates (Biggs 1994). For azoxystrobin-resistant isolates, no significant fitness cost was found in any fitness component, while resistant isolates showed higher aggressiveness than sensitive isolates (Karaoglanidis, et al. 2011). The unaffected fitness suggests that *A. alternata* isolates resistant to QoI and dicarboximide fungicides may successfully compete with wildtype isolates in the field even in the absence of selection pressure.

CHAPTER TWO

Fitness and Competitive Ability of *Alternaria alternata* Field Isolates with Resistance to SDHI, QoI and MBC fungicides

THIS WORK HAS BEEN ACCEPTED FOR PUBLICATION BY PLANT DISEASE

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Introduction

Outbreaks of Alternaria rot caused by *A. alternata* (Fr.) Keissl. have been increasing in some South Carolina peach orchards due to the emergence and selection of *A. alternata* strains resistant to succinate dehydrogenase inhibitor (SDHI) fungicides (Yang, et al. 2015). Yield loss reached 60% in some late-season peach varieties (Yang, et al. 2015). SDHI fungicides have been applied routinely in combination with quinone outside inhibitor (QoI) fungicides since the year 2000 mainly for the control of brown rot of peach, which is caused by *Monilinia fructicola* (G. Winter) Honey. Repeated annual

applications appear to have unintentionally selected for resistance in this secondary plant pathogen. Resistance to SDHIs in *A. alternata* from peach was based on mutations in *sdh* gene sequences (Yang, et al. 2015). Resistant isolates revealed H277Y/R/L mutations in the *sdh*B gene, H134R and G79R mutations in the *sdh*C gene, and D123E and D133R mutations in the *sdh*D gene (Avenot, et al. 2008a; Avenot, et al. 2009; Yang, et al. 2015). *A. alternata* isolates from California pistachio were resistant to SDHI fungicides in an earlier study and the most prevalent genotypes were H277Y/R in the *sdh*B gene and H134R in the *sdh*C gene, (Avenot, et al. 2008a; Avenot, et al. 2009; Yang, et al. 2015).

Besides SDHI fungicides, methyl benzimidazole carbamates (MBC), demethylation inhibitor (DMI), and QoI fungicides have been used in rotation or mixture in peach orchards for summer disease control. To the best of our knowledge, *A. alternata* has reportedly been resistant to MBC fungicides (Eckert and Ogawa 1988; Thomidis, et al. 2009), but the mechanism of resistance is unknown. Among single-site fungicides, only SDHIs and QoIs are registered and reported to be effective against *A. alternata* isolates. The rotation or mixture of the above chemical classes in southeast peach orchards has already resulted in the selection of resistance in *Monilinia fructicola* (G. Winter) Honey, the causal organism of brown rot of stone fruits (Luo and Schnabel 2008; Ma, et al. 2003a) and in *Colletotrichum siamense*, one of the causal organisms of peach anthracnose (Hu, et al. 2015). For both pathogens an accumulation of resistance to two or more fungicides was observed. Whether resistance to multiple fungicides has developed in *A. alternata* isolates from peach is not known.

For effective resistance management, knowledge about the molecular mechanism and fitness of genotypes is essential (Karaoglanidis, et al. 2011). Fitness is defined as the survival and reproductive success of an allele, individual, or group (Pringle and Taylor 2002). If isolates carrying mutations in target genes have lower fitness than the boscalid-sensitive isolates, a decline in prevalence would be expected when removing the fungicide pressure. In contrast, if fitness cost is absent in resistant isolates, the resistant subpopulation would be expected to persist in the field even without fungicide selection pressure. Fitness of boscalid-resistant *A. alternata* isolates from pistachios was assessed previously, but it is unknown what genotype or genotypes were included in the study (Avenot and Michailides 2007). An in-depth analysis of prevalent SDHI-resistant genotypes is needed to assess their fitness and competitiveness.

The objectives of this study were to (i) determine whether *A. alternata* isolates resistant to SDHI fungicides have accumulated additional resistance to thiophanate-methyl and azoxystrobin, (ii) investigate the molecular mechanisms of this resistance, and (iii) conduct an in-depth analysis of key fitness components and competitiveness of resistant isolates.

Material and Methods:

Origin, collection, and identification of isolates. The *A. alternata* isolates used in this study were first characterized in a previous study (Yang, et al. 2015) for their sensitivity to four SDHI fungicides. In that study, details on origin, collection, and identification are reported. Briefly, isolates were collected in 2012 and 2013 from mature fruit of

commercial late-season peach varieties. Single-spore isolates were obtained and identified to species level based on ribosomal DNA sequences (Yang, et al. 2015).

For the present study 12 isolates with different nucleotide mutations in the *sdh* genes were selected to investigate fitness and competitiveness. Three sensitive isolates (Aa SE 12-17, Aa EY 12-1, Aa RR 13-5) with no mutations in *sdh* genes B, C, or D were included, as well as 3 isolates (Aa SE 12-10, Aa RR 13-28, Aa SE 12-13) with the H277Y mutation in the *sdh*B subunit, 3 isolates (Aa RR 13-26, Aa EY 12-6, Aa RR 13-36) with the H134R mutation in the *sdh*C subunit, and 3 isolates (Aa RR 13-21, Aa SE 12-14, Aa RR 12) with the D123E mutation in the *sdh*D subunit. These four genotypes were selected because they were the most common genotypes found in our collection of isolates from South Carolina. Our collection had one isolate sensitive to pyraclostrobin; this isolate was not included in this study because of its comparably slow mycelial growth. All mutations in *sdh* subunits conferred resistance to boscalid and penthiopyrad, but mutation H277Y also conferred resistance to fluxapyroxad and mutation H134R also conferred resistance to fluopyram and fluxapyroxad. Three sensitive isolates, three with mutation H277Y, and three with mutation H134R were selected to investigate competitiveness. Both mutations are associated with high resistance to boscalid. Isolates with mutation D123E had low to medium resistance to boscalid (Yang, et al. 2015) and were excluded due to our focus on high resistance and to keep the number of isolates in experiments at a manageable level.

Sensitivity to thiophanate-methyl and pyraclostrobin. Mycelial growth tests were conducted to estimate sensitivity to thiophanate-methyl and pyraclostrobin. Commercial

formulations of thiophanate-methyl (Topsin-M; Ceraxagri, King of Prussia, PA) and pyraclostrobin (Cabrio EG fungicide; BASF Corporation, Research Triangle Park, NC) were dissolved in distilled water to obtain a stock solution of 10 mg/ml. Salicylhydroxamic acid (SHAM) was dissolved in methanol at a concentration of 10 mg/ml. SHAM is often used as an alternative oxidative pathway inhibitor to prevent fungus from overcoming toxicity of a QoI fungicide (Pasche, et al. 2004). Fungicides were added to autoclaved yeast extract-bactopeptone-sodium acetate (YBA) cooled to 55℃. Three final concentrations of 0 (control), 5, and 100 µg/ml were used; at these discriminatory doses sensitive isolates were expected to only grow in the controls. SHAM was amended to the YBA medium at 100 µg /ml in control and pyraclostrobin-amended medium. For each isolate, three replicate plates were prepared. Isolates with low resistance to thiophanate methyl or azoxystrobin were expected to grow on a medium amended with 5 µg/ml but not on a medium amended with 100 µg/ml. Highly resistant isolates were expected to grow on medium amended with 100 µg/ml of the fungicides.

Molecular basis of resistance. Genomic DNA of the 12 *A. alternata* isolates chosen for this experiment (Aa RR 12, Aa RR 13-34, Aa RR 13-35, Aa RR 13-36, Aa SE 12-4, Aa SE 12-5, Aa SE 12-17, Aa EY 12-1, Aa RY 12-2, and Aa EY 12-5) was extracted using the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). These isolates represented different resistance phenotypes and had different geographical origins (Yang, et al. 2015). Pyraclostrobin-resistant isolates were from Ridge Spring, Monetta, and Chesnee in South Carolina. The sensitive isolate (Aa SE 12-3) was from Monetta. The cytochrome b gene fragment containing amino acid 143 was amplified and

sequenced with primer pair cytb2f (CTATGGATCTTACAGAGCAC) and CBR2 (AACAATATCTTGTCCAATTCATGG) (Ma, et al. 2003b; Vega, et al. 2012). PCR reactions were performed in 50µl volumes containing 50 ng DNA, ThermoPol Buffer (50 mM KCL, 1.5 mM $MgCl_2$, 10 mM Tris-HCL (pH 9.0)), dNTPs at 0.2 mM each (New England BioLabs, Inc., Beverly, MA), primers at 0.2 µm each and 1 U Taq DNA polymerase (New England Biolabs, Inc.,). PCR was carried out in a MyCycler Thermocycler (Bio-Rad Laboratories, Hercules, CA) with an initial pre-heat for 3 min at 95℃, followed by 34 cycles of denaturation at 94℃ for 40 s, annealing at 50℃ for 50 s and extension at 72℃ for 1 min, and terminated with a final extension at 72℃ for 10 min.

Primer pair btul1f (GTGCCGTCCTCATCGATCTC) and btu2r (AGTTGGGACAGCCA-TCATGT) were designed based on the NCBI submitted sequence (NCBI accession number 325514225) to amplify the *β*-tubulin gene. Two isolates (Aa EY 12-5, Aa RR 13-27) resistant to thiophanate-methyl were randomly chosen for sequencing. PCR reactions were performed as described above except the annealing temperature was increased to 55 ℃. PCR products were purified using ExoSAP-IT reagent (Affymetrix, Inc., Cleveland, OH) prior to sequencing at the Clemson University Genomics Institute, Clemson SC. The sequences were analyzed using BLAST-based algorithms. Both BLASTn and BLASTTx were performed to detect and annotate the nucleotide and protein sequences. Alignment of nucleotide and protein sequences was conducted using DNASTAR sequence analysis software (DNASTAR Inc., Madison, WI).

Fitness components. The following fitness components were determined for boscalid-resistant and boscalid-sensitive isolates: mycelial growth on PDA, spore production in vitro, osmotic sensitivity, oxidative sensitivity, germination ability, and disease severity. All experiments were repeated once.

Mycelial growth on PDA. Mycelium plugs of each isolate were transferred from 4-day-old PDA plates to the center of fresh PDA plates for growth measurement. The plates were incubated at 25 ℃ under continuous light. The colony diameter of each isolate was determined 3, 5, 7, and 10 days after inoculation. For each isolate, three plates were prepared.

Spore production in vitro. Isolates were cultured on PDA, V8 juice agar, and canned peach halves to determine sporulation in vitro. For PDA and V8 juice agar plates, mycelial plugs of each isolate were transferred to the center of the plates and incubated at 25℃ under continuous light for 14 days. We then added 5 ml of distilled water to each plate, dislodged the conidia with a cotton swab, and filtered the suspension through double-layered cheesecloth. Three plates were used for each isolate. Peach halves (from cans using light syrup) were placed in 400 ml Magenta boxes (one peach half per Magenta box), pit side facing down. Inoculation was conducted by pipetting 100 µl of a conidia suspension (10^5/ml) to the top of each peach half. Magenta boxes were covered with a lid, sealed with Parafilm and incubated at 25°C under continuous light for 7 days. The mycelia mass was then harvested, placed in a 50 ml tube, and suspended in 10 ml of sterile distilled water. After vortexing for 20 s, the conidial suspension was filtered through a double layer of cheesecloth. Two boxes were prepared for each isolate. The

spore concentration in the suspension was measured with the aid of a hemacytometer and expressed as number of conidia per milliliter water. Two peach halves were prepared for each isolate. Two droplets were counted for each plate or box.

Osmotic sensitivity. Osmotic sensitivity was determined by comparing the growth of isolates on PDA with growth on PDA-amended with 2% and 4% NaCl. Colony diameters were measured after 5 days at 25℃ under continuous light. Three replicate plates were prepared for each isolate.

Oxidative sensitivity. Paraquat was used as an indicator of oxidative stress (Avenot, et al. 2009). PDA was amended with a commercial formulation of paraquat dichloride (Gramoxone; Syngenta Crop Protection, Greensboro, NC) to a final concentration of 0, 90, and 300 µg/ml. Colony diameters were measured after 5 days at 25℃ under continuous light. For each isolate, three replicate plates were prepared.

Germination ability. A 50 ml spore suspensions of each isolate was harvested from V8 juice agar plates and spread onto water agar plates. The inoculated plates were incubated at 22℃ for 18 h and then the percentage of germinated spores per 100 conidia was recorded. A conidium was considered as germinated when the germ tube was longer than twice the length of the conidium. Three replicate plates were prepared for each isolate.

Disease severity. The mature peach fruit ‘Redglobe’ was harvested on 2 July 2014 and stored at 4℃ for no more than 2 weeks. Peach fruit were adjusted to room temperature overnight before inoculation, then washed with soap, immersed in 1% bleach for 1 min, and then rinsed with water to remove the bleach residue. Wetted paper towels were

placed on the bottom of a carbon-fiber tray containing 18 perforated pockets. Fruit were placed on 4-cm-diameter plastic rings in the pockets of the tray. After fruit were air dried, three plugs were removed with a cork borer from each fruit at three equidistant locations. Three same size mycelia plugs from the margin of 5-day-old PDA were placed on removed sites (Chen, et al. 2013c). There were three replicates per isolate, 3 fruit per replicate. After inoculation, the trays were wrapped by plastic bags to keep the relative humidity high, and incubated at 25℃ for 6 days. Lesion diameters were recorded after 6 days.

Stability of resistance to SDHI fungicides. EC_{50} values for boscalid, fluopyram, penthiopyrad, and fluxapyroxad were determined on YBA plates prior to and after transfers. The concentrations of boscalid, fluxapyroxad, and penthiopyrad were 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 μg/ml and the concentrations for fluopyram were 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 μg/ml. Mycelial plugs of each isolate were taken from the margin of 5-day-old colonies and transferred to PDA plates containing the different fungicide concentrations. A total of 10 transfers were conducted. Three replicate plates were prepared for each isolate and the experiment repeated once.

Competitiveness of isolates with different genotypes. We assessed the competitive ability between boscalid-resistant and boscalid-sensitive genotypes. A spore suspension of each resistant genotype was obtained from PDA plates as described above and mixed with a suspension of the sensitive phenotype at an initial ratio of 1:1 (H277Y:S; H134R:S). The final concentration of the initial conidia suspension was 3×10^4 spores/ml. Each genotype was represented by an equally proportioned (1:1:1) suspension of spores

of the three isolates with the same genotype. For example, H277Y was represented by a spore suspension of the resistant isolates Aa SE 12-10, Aa RR 13-28, and Aa SE 12-13 in equal proportions. This spore suspension was then mixed at a ratio of 1:1 with the spore suspension of equal proportions of the sensitive isolates Aa SE 12-17, Aa EY 12-1, and Aa RR 13-5. Control suspensions of each genotype were mixed with water to achieve the same dilution. Canned peach halves were inoculated as described above. Conidia from each fruit were harvested every 7 days and resuspended in 10 ml of water. A total of 100 µl of the conidia suspension was used to inoculate a new peach half. Three peach halves were inoculated for every mixture in each transfer. The experiment was conducted three times and each time terminated after five transfers. The spore concentrations were determined at the end of each cycle with the aid of a hemacytometer and expressed as number of conidia per milliliter of water. The percentage of spores resistant to boscalid in the spore suspension was calculated at the end of each cycle. A total of 400 µl of the spore suspension from each peach half were spread with an inoculation loop on 2 YBA plates (200 µl per plate) amended with 30 µg/ml boscalid. Plates were incubated for 16 h at 22°C and germination of 100 spores/plate was examined under a microscope. A conidium was considered germinated when the germ tube was longer than twice the length of the conidium. At that dose resistant isolates germinated and formed a germ tube but spores of sensitive isolates were inhibited.

Data analysis. In the experiments determining stability of resistance, EC_{50} values were calculated by regression analysis using dose-response curves. For experiments involving fitness components, data for 3 isolates of the same genotype were pooled. Brown-

Forsythe tests were conducted for each fitness component to ensure homogeneity between experiments. Mean values of each genotype group were compared using an LSD test at p =0.05. For the competitive ability study, parameters of ratio of resistant spores, transfer, experiment and transfer × experiment were fitted in a general linear model. Experiment and transfer × experiment were set as random effects. Analysis of variance (ANOVA) was conducted to test the significance of transfers. All statistical analyses were performed with JMP software (version 10; SAS, NC).

Results

Sensitivity to thiophanate-methyl and pyroclostrobin. The sensitivity of 63 *A. alternata* isolates from South Carolina to thiophanate-methyl and pyraclostrobin was determined. Relative growth at 5 µg/ml thiophanate-methyl ranged from 80.4% to 114.3%. Even at 100 µg/ml, none of the isolates were completely arrested in growth and relative growth ranged from 64.5 to 123.8% (Fig. 1). These data indicated that all isolates were resistant to thiophanate-methyl. For all but one isolate, relative growth at 5 and 100 µg/ml pyraclostrobin ranged from 51.6% to 95.2% and from 28.3% to 65.6%, respectively, indicating that most isolates were resistant to this fungicide. One isolate was pyraclostrobin-sensitive with relative growth of 12.5% and 0% at 5 and 100 µg/ml, respectively (Fig. 1).

Nucleotide sequence analysis of the cyt *b* and b-tubulin genes. The sensitivity to SDHI fungicides and underlying molecular basis of the isolates used in this study was determined previously (Yang, et al. 2015), but molecular mechanisms of resistance to

QoI fungicides and MBC fungicides were unknown. A single DNA fragment of the cyt *b* gene, 182 bp in length, was amplified and sequenced from all 12 isolates with primer pair cytb2f and CBR2. The nucleotide sequence matched the cyt *b* gene from *A. alternata* (GeneBank Accession No. AY263408.1). Comparison of the partial cyt *b* gene sequence of the pyraclostrobin-sensitive and the 11 remaining pyroclostrobin-resistant isolates collected from different locations revealed that resistant isolates had a single point mutation at position 428 that changed GGT into GCT. This mutation caused an amino acid change of glycine to alanine at codon 143 of the cyt *b* gene (G143A).

A single DNA fragment of 729 bp in length from two randomly-picked, thiophanate-methyl-resistant isolates was amplified and sequenced with primer pair btul1f and btu2r. The two sequences (available in GenBank, accessions KP640615 and KP640616) aligned perfectly with 25 other *A. alternata* β-tubulin gene sequences from GenBank and all sequences revealed the amino acid tyrosine at codon position 167. The F167Y mutation in the β-tubulin gene was confirmed to confer high resistance to benzimidazole compounds in other organisms like *Saccharomyces cerevisiae*, *Penicillum expansum*, and *Gibberella zeae* (Baraldi, et al. 2003; Li, et al. 1996; Qiu, et al. 2011). Our data indicated that this mutation is likely the reason *A. alternata* is resistant to thiophanate-methyl.

Fitness components. *Mycelial growth*. Isolates with mutation D123E in *sdh*D grew more rapidly after 3, 5, 7, and 10 days of incubation with mean values of 29.9, 51.3, 67.9, and 80.7 mm, respectively. Growth of boscalid-sensitive isolates isolates and isolates with the H134R or the H277Y mutation in *sdh*C and *sdh*B, respectively, was not significantly different from each other (Fig. 2).

Spore production in vitro. All genotypes produced spores on the three different substrates (including peach), but there were substrates-specific responses. The two independent experiments for each substrates were merged after verifying homogeneity of variance (P=0.07, P=0.82, and P=0.50, for V8 juice agar, PDA and peach, respectively). The sporulation ability of genotypes H277Y and H134R were similar and both genotypes outperformed D123E regardless of the substrate. Boscalid-sensitive isolates' sporulation was similar to genotype H277Y on two substrates, but produced significantly more spores than all other genotypes on PDA (P<0.001). Sporulation was greatest for all genotypes on V8 juice agar with mean values of 32.9×10^4 spores/ml compared to 3.5×10^4 and 13.8×10^4 spores/ml for peach and PDA, respectively (Table 1).

Osmotic sensitivity. Osmotic sensitivity was assessed on 2% and 4% NaCl-amended PDA. The two independent experiments were merged after verifying homogeneity of variance for 2% and 4% NaCl (P=0.64 and P=0.88, respectively). No significant difference in mycelial growth was observed between genotypes for both concentrations (P=0.55 and P=0.55, respectively) (Table 1).

Oxidative sensitivity. Oxidative stress was assessed on paraquat-amended PDA. Data from the two independent experiments were merged after verifying homogeneity of variance for 90 and 300 µg/ml concentrations of paraquat (P=0.78 and P=0.22, respectively). At the lower concentration of paraquat, the H277Y genotype grew the fastest (P<0.001) among all the genotypes, and genotype D123E was significantly impaired compared to the boscalid-sensitive isolates (P=0.01). At the higher dose the

same trend was observed but significant differences were only observed between the D123E genotype and the boscalid-sensitive isolates (P=0.02) (Table 1).

Germination rate. Although the Brown–Forsythe test showed variances between the two experiments were not equal, the data were combined after careful consideration due to the visual homogeny of the results. The germination rates of all genotypes ranged between 97.4 and 99.3, and there was no significant difference among genotypes (P=0.12) (Table 1).

Disease severity. Disease severity was assessed on peach fruit. The two experiments were merged after verifying homogeneity of variance (P=0.17). The H277Y genotype produced slight but significantly larger lesions than all other genotypes (*P*=0.01) (Table 1).

Stability of resistance to SDHI fungicides. After 10 transfers on non-amended PDA, resistance to penthiopyrad, boscalid, and fluxapyroxad remained stable for all resistant genotypes. Interestingly, a slight but significant increase in sensitivity to fluopyram was observed for the H277Y, H134R, and boscalid-sensitive isolates genotypes (Fig. 3).

Competitiveness of isolates with different genotypes. During the study all genotypes were transferred individually (in addition to the mixtures) and the viability of their inoculum was confirmed in the absence of competition with other genotypes. All genotype controls sporulated consistently on canned peaches throughout the study, indicating no detectable loss of saprophytic ability (data not shown). Regarding the experimental mixtures, no genotype was outcompeted over the course of this study. A

dynamic equilibrium of spores from both genotypes was observed for both mixtures (H277Y:S and H134R:S, H277Y was represented by a spore suspension of the resistant isolates Aa SE 12-10, Aa RR 13-28, and Aa SE 12-13 in equal proportions, S was represented by a spore suspension of the sensitive isolates Aa SE 12-17, Aa EY 12-1, and Aa RR 13-5 in equal proportions, H134R was represented by a spore suspension of the resistant isolates Aa RR 13-26, Aa EY 12-6, Aa RR 13-36 in equal proportions.) over the course of five generations. A general mixed linear model analysis revealed that transfers were not a significant factor in both mixture types (P=0.8 and P=0.08, respectively). Spore production of the H134R genotype initially increased over the boscalid-sensitive isolates at first but then stabilized at about a 60% prevalence. Spore production of the H277Y genotype, however, initially proportion decreased compared to the boscalid-sensitive isolates and then stabilized at about a 30% prevalence (Fig. 4).

Discussion

Resistance to multiple fungicides was confirmed in *A. alternata* isolates from peach characterized previously for their sensitivity to SDHI fungicides. The lack of efficacy of thiophanate-methyl and other thiophanates against *A. alternata* was noted as early as the 1970s (Eckert and Ogawa 1988; Thomidis, et al. 2009). It is therefore possible that *A. alternata* in North America had been resistant to thiophanate-methyl even prior to its introduction in the early 1970s. Regardless this study shows the fungus had sufficiently diversified and generated genotypes with resistance to QoI and SDHI fungicides. QoI fungicides were introduced as solo products in 1998 and then sold largely in a mixture with SDHI fungicides since 2002. Since then, the combination has been used routinely,

several times per year in South Carolina peach orchards. This is the first report of resistance to QoI and SDHI fungicides in *A. alternata* of peach on the east coast, indicating that selection of resistance to both fungicides at a level of economic relevance took little more than 10 years. In contrast, *A. alternata* resistant to pyraclostrobin and boscalid was found in pistachio orchards after only two years of application of the boscalid/pyraclostrobin mixture (Avenot, et al. 2008b). This more rapid emergence and selection of resistance may be a result of higher disease incidence and inoculum availability for selection; *A. alternata* is a major pathogen of pistachio but is generally of minor concern in peach orchards. Resistance to MBC, QoI and SDHI fungicides in the same field isolate has also been reported in *Botrytis cinerea* from the US and Europe (Fernández-Ortuño, et al. 2012; Weber 2011). Dual resistance to pyraclostrobin and boscalid was found in 7 of 59 *A. alternata* field isolates from California pistachio with a history of boscalid and pyraclostrobin treatments. Although it is not specifically stated by the authors, it is likely that the isolates were also resistant to thiophanate-methyl based on the above-mentioned inherent resistance trait to thiophanate fungicides (Avenot, et al. 2008b). In our study, the same resistance profile was detected in *A. alternata* field isolates from Southeast peach orchards with a much higher prevalence; 57 of 64 isolates with resistance to all three fungicides.

Tree fruit pathogens appear to be developing resistance to multiple fungicides at a fast pace. This may be due in part to the rotation and mixture practices of products belonging to different chemical classes of fungicides, the perennial nature of the host allowing continued selection pressure on its pathogen population, and the frequent

application of fungicides needed for sustained crop production. For example, field isolates of *Venturia inaequalis*, the causal agent of apple scab, were found to be resistant to dodine, kresoxim-methyl, myclobutanil, and thiophanate-methyl (Chapman, et al. 2011), and dodine, benzimidazoles, and DMI fungicides (Köller and Wilcox 2001). Under southeastern conditions, fungal pathogens thrive and *A. alternata* is now the third confirmed pathogen of peach in South Carolina to develop resistance to multiple fungicides. Field isolates of *M. fructicola*, the causal organism of peach brown rot, were reported to be resistant to the DMI propiconazole. MBC and thiophanate-methyl (Chen, et al. 2013b) as well as to propiconazole and the SDHI fungicide boscalid (Chen, et al. 2013c). More recently, field isolates of *Colletotrichum siamense*, causing anthracnose fruit rot on peach, were found to be resistant to thiophanate-methyl and azoxystrobin (Hu, et al. 2015). The observed acquisition of multiple resistances in field strains of peach pathogens is in line with selection pressure due to rotation or mixture of DMI, MBC, QoI, and SDHI fungicides for peach disease control.

The mechanisms of resistance to azoxystrobin and thiophanate-methyl in our isolates was based on point mutations in target genes, including G143A in cytochrome b and F167Y in β-tubulin. As opposed to quantitative resistance, which may be multigenic and which is characterized by a small decrease in population sensitivity over time, qualitative resistance is typically conferred by point mutations in genes encoding target enzymes and is characterized by an immediate and significant shift toward resistance. For example, quantitative resistance to the QoI fungicide azoxystrobin was observed in *Alternaria solani* isolates from potato fields throughout the midwestern United States. After three

years of applications the mean EC_{50} values of pathogen populations changed from 0.038 to 2.3 µg/ml (Pasche, et al. 2004). In contrast, qualitative resistance to the same chemical in *A. alternata* from California pistachios had EC_{50} values greater than 100 µg/ml (Ma, et al. 2003b). Our data also reflect qualitative resistance with EC_{50} values for both azoxystrobin and thiophanate methyl greater than 100 µg/ml. Prior to this study, *A. alternata* from pistachio had been reported to develop qualitative resistance to dicarboximides, QoIs, and SDHIs (Avenot and Michailides 2007; Ma, et al. 2003b; Ma and Michailides 2004).

Our genotypes resistant to three classes of fungicides only differed in their genetic basis of resistance to SDHI fungicides (Yang, et al. 2015). The most common SDHI resistance genotypes H277Y and H134R were subjected to fitness analysis. Fitness was evaluated in terms of both 'predicted' fitness (measurement of several components in individual isolates) and 'realized' fitness (competition between sensitive and resistant isolates) (Karaoglanidis, et al. 2011). Our investigation of fitness components revealed that genotypes H277Y and H134R didn't suffer obvious fitness penalties. For some parameters, including disease severity on peach and oxidative stress, genotype H277Y even showed greater fitness than the boscalid-sensitive isolates. This mutation also appears to be a common and resilient resistance determinant in other pathogens. For example, the equivalent mutation was widespread in boscalid-resistant *B. cinerea* populations from strawberry and apple (Veloukas, et al. 2011; Yin, et al. 2011) and was not shown to affect fitness (Kim and Xiao 2011; Veloukas, et al. 2014). *A. alternata* from pistachio with mutations in *sdh*B, *sdh*C, and *sdh*D genes showed a high sensitivity to

oxidative stress (Avenot, et al. 2009). In our study, hypersensitivity to oxidative stress and other fitness penalties were observed only for genotype D123E, suggesting that this mutation imposed a fitness cost. This may partially explain the lower prevalence of this genotype in the field compared to the H277Y and H134R genotypes. Our results of 'predicted' fitness based on results from the two commonly found resistant genotypes, H134R and H277Y, are largely consistent with findings for *A. alternata* isolates from California pistachios (Avenot and Michailides 2007). Both studies show minor fitness penalties for the resistant isolates suggesting that these genotypes probably compete successfully under field conditions.

Results of 'predicted' fitness are in in our study agree with 'realized' fitness. The competition analysis in the absence of fungicides indicated that genotypes H277Y and H134R successfully competed with boscalid-sensitive isolates strains. These results are consistent with a study that analyzed the boscalid-resistant H277Y-equivalent genotype in *Botrytis cinerea* (Lalève, et al. 2014). However, another study documented competitive weaknesses of boscalid-resistant *B. cinerea* strains of the H277Y-quivalent SDHI-genotype (Kim and Xiao 2011; Veloukas, et al. 2014). Depending on the pathogen studied, however, the same mutation may or may not be associated with fitness penalties (Karaoglanidis, et al. 2011; Ma and Uddin 2009; Rallos, et al. 2014).

The results of this study suggest that mixing or alternating chemical classes of fungicides for resistance management is now selecting for multifungicide resistance in primary and secondary pathogens of peach. While these resistant populations are still rarely observed in the southeast, independent emergence and movement from existing

locations will eventually spread resistant genotypes under current management strategies. The lack of a penalty in fitness or competitive ability among the most prevalent, boscalid-resistant *A. alternaria* genotypes implies these genotypes will likely remain in the population, even in the absence of selection pressure. This development justifies a review of current resistance management practices. Alternatives to sole reliance on single-site fungicides may become more important and may include the development and use of cultivars less susceptible to disease, the increased use of multi-site fungicides that do not select for resistance, stronger emphasis of cultural methods that decrease inoculum pressure, and further restriction of the number of applications of single-site fungicides per season.

Table 1. Sporulation, osmotic stress, oxidative stress, disease severity on detached fruit, and germination for pooled *Alternaria alternaria* isolates carrying different mutations in the succinate dehydrogenase genes.

Genotype[x]	No. isolates	Sporulation (× 10^4 spores/ml)			Relative growth (%)				Mean lesion diameter on peach (cm)	Germination (%)
					PDA+NaCl		PDA+Paraquat			
		V8	Peach	PDA	2%	4%	90 µg/ml	300 µg/ml		
H277Y	3	38.6 a[y]	4.8 a	12.1 b	68.1 a	58.6 a	103.2 a	74.6 a	1.39 a	98.7 ab
H134R	3	40.1 a	4.9 a	15.5 b	66.5 a	53.3 a	87.7 bc	60.3 ab	1.13 b	97.4 b
D123E	3	13.7 b	1.2 b	2.9 c	59.2 a	48.5 a	79.0 c	48.6 b	1.15 b	97.8 ab
No mutation	3	34.3 a	3 ab	25.3 a	60.6 a	47.6 a	92.1 b	60.5 ab	1.13 b	99.3 a

[x]Data of three isolates with the same mutation in SDH subunit were pooled. Mutation H277Y was represented by isolates AaSE12-10, AaRR13-28, and AaSE12-13; mutation H134R was represented by isolates AaRR13-26, AaEY12-6, and AaRR13-36; mutation D123E was represented by isolates AaRR13-21, AaSE12-14, and AaRR12; and wildtype was represented by sensitive isolates AaSE12-17, AaEY12-1, and AaRR13-5.

[y] Least significant difference (LSD) tests were conducted at P =0.05. Values next to same letters within columns are not significantly different.

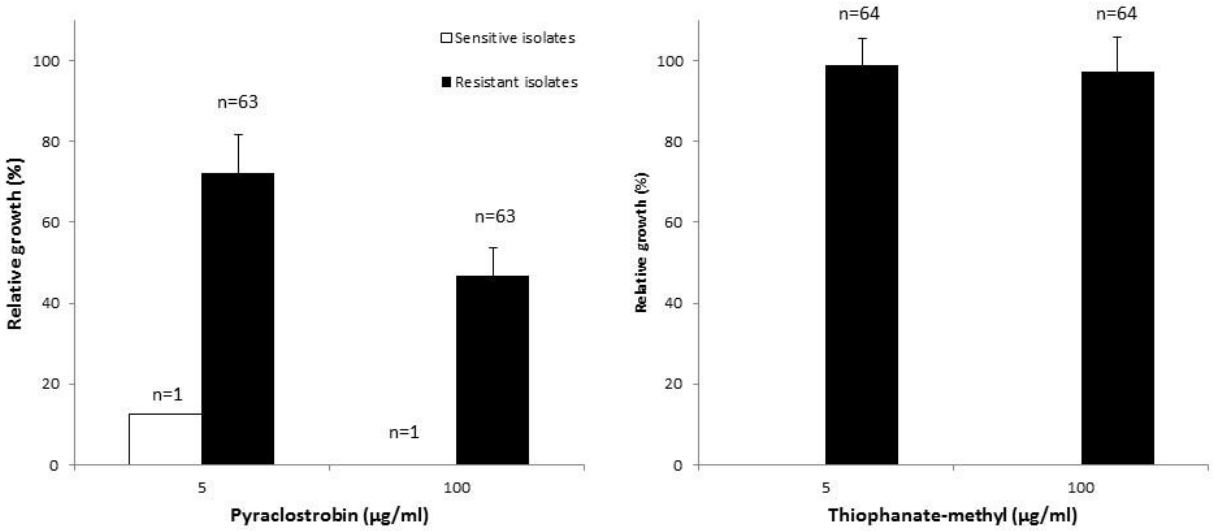

Fig. 2.1. Relative growth of 64 *A. alternata* isolates on agar plates containing 5 or 100 µg/ml pyraclostrobin or thiophanate-methyl. Error bars represent standard deviations.

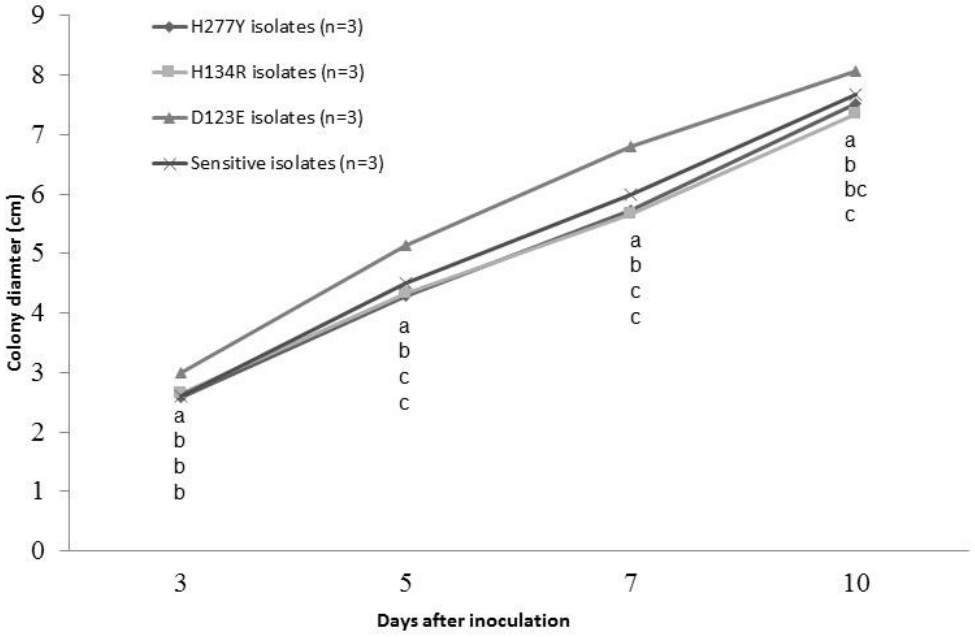

Fig. 2.2. Mycelial growth on PDA plates after 3, 5, 7, and 10 days incubation. Letters under data points are in order of isolate symbols. The same letters indicate no significant difference. Least significant difference (LSD) tests were conducted at *P*=0.05.

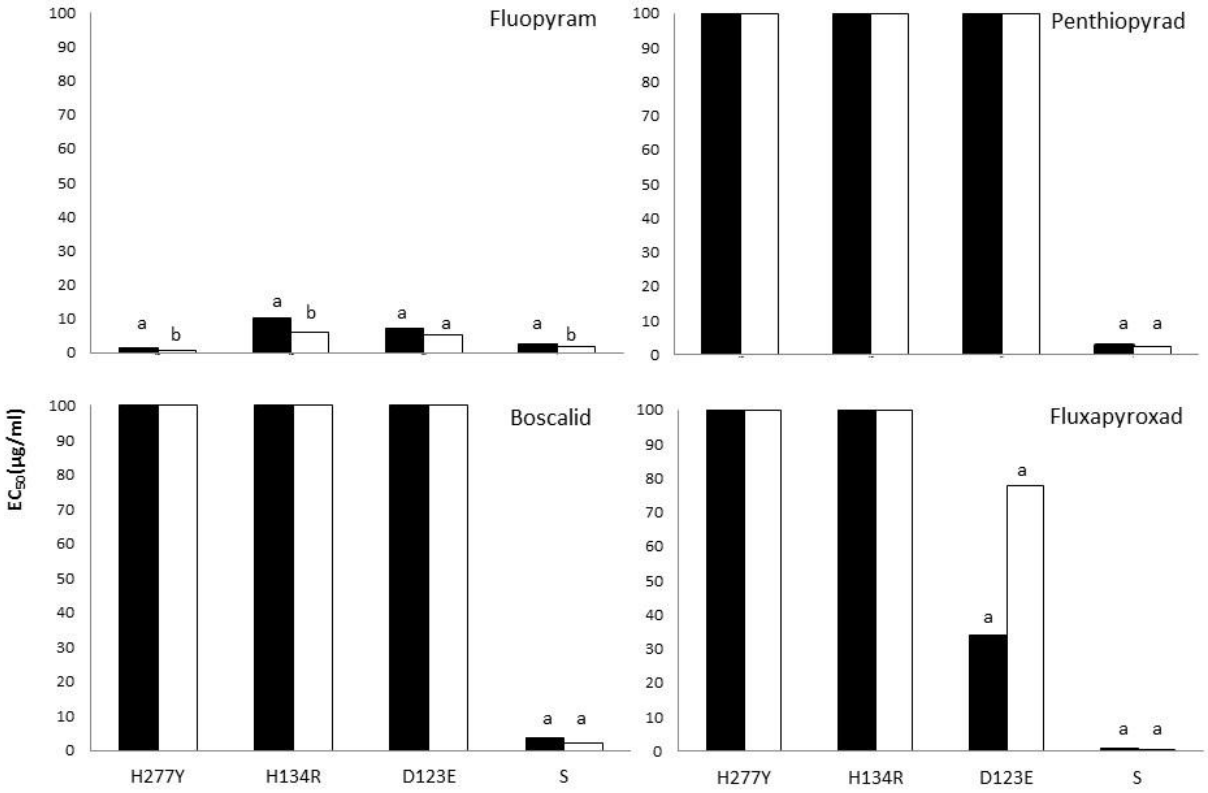

Fig. 2.3. EC_{50} values for SDHI fungicides before (black bars) and after (gray bars) 10 consecutive transfers on PDA medium. Mycelial plugs were transferred to a new PDA plate every 6 days. Data of three isolates with the same mutation were pooled. Same letters for a pair of bars indicate no significant difference (P=0.05)

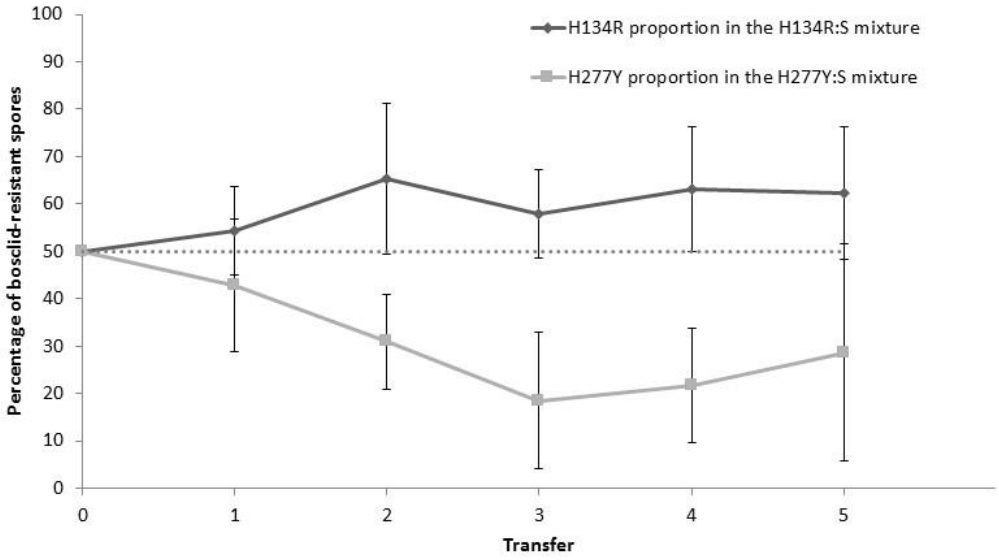

Fig. 2.4. Ratio of boscalid-resistant and boscalid-sensitive spores developing on canned peach fruit following five consecutive transfers. The dotted line at y = 50 is used as a reference line to improve visualization. Error bars represent the standard deviation of the percentage of boscalid-resistant spores in the mixture after each transfer

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