Fitness, Competitive Ability, and Mutation Stability of Isolates of *Colletotrichum acutatum* from Strawberry Resistant to QoI Fungicides

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ABSTRACT

Quinone-outside inhibitor (QoI) fungicides are used to manage anthracnose of strawberry, caused by *Colletotrichum acutatum*. However, selection for resistance to QoI fungicides was first reported in 2013 in Florida and, subsequently, in strawberry nurseries and production areas across the United States and Canada. *C. acutatum* resistance to QoIs is associated with the G143A point mutation in the cytochrome *b* gene. This mutation is known to be associated with field resistance even at high rates of QoI. In this study, we investigated the relative fitness and competitive ability of QoI-resistant and -sensitive *C. acutatum* isolates. A fitness comparison did not indicate any difference between resistant and sensitive isolates in aggressiveness, spore production, and mycelial growth at different temperatures. Additionally,

in the absence of selection pressure, resistant and sensitive isolates were equally competitive. Cultivation of QoI-resistant and QoI-sensitive isolates for four culture cycles in vitro in the absence of azoxystrobin showed that QoI resistance was stable. The observed lack of fitness penalties and stability of the G143A mutation in QoI-resistant *C. acutatum* populations suggest that the interruption and further reintroduction of QoI fungicides might not be an option for strawberry nurseries and fruit production areas. Further investigation of alternative chemical and nonchemical *C. acutatum* control practices, in addition to the integration of multisite fungicides, is needed to reduce the occurrence and distribution of QoI-resistant populations in strawberry fields.

Colletotrichum acutatum J. H. Simmonds is the causal agent of anthracnose on numerous plant species worldwide (USDA-ARS 2017). Anthracnose fruit rot (AFR), caused by species in the *C. acutatum* species complex, is one of the most important diseases in strawberry (*Fragaria × ananassa*) production areas, and disease incidence can exceed 50% if environmental conditions are favorable and disease control measures are not applied in time (Damm et al. 2012; Legard et al. 2003; Mertely et al. 2015, 2017a; Smith 1998). *C. acutatum* is known to infect fruit, flowers, crowns, roots, petioles, and leaves of strawberry plants. However, fruit rot and flower blight are most commonly observed in production fields (Legard et al. 2003; Smith 1998; Mertely et al. 2017b; Peres et al. 2005; Strand 2008). In Florida, *C. acutatum* inoculum does not overwinter in the soil or on plant debris (Howard et al. 1992; Smith 1998). Thus, transplants with quiescent infections of *C. acutatum* originating from nurseries are the main source of inoculum in annual production fields (Howard et al. 1992; Legard et al. 2003; Smith 1998).

Although cultural methods such as drip irrigation and use of resistant cultivars can reduce AFR incidence (Handley 2003; Legard et al. 2003; Seijo et al. 2012), growers still rely on frequent applications of single and multisite fungicides to diminish yield losses due to *C. acutatum* infection (Legard et al. 2003; Smith 1998; Strand 2008). Respiration-inhibitor fungicides such as the quinone-outside inhibitors (QoI) belong to Fungicide Resistance Action Committee code 11 (FRAC 2017). The strobilurin fungicides and one of its active ingredients (a.i.), azoxystrobin, represent a group within the QoI fungicides (Bartlett et al. 2002). These have broad-

spectrum activity against fungal species (Bartlett et al. 2002; Markoglou et al. 2006) and have been highly effective on AFR control since their registration (Legard et al. 2003). QoIs bind to the quinone-outside site of the cytochrome *b* (*cytb*) gene, which inhibits mitochondrial respiration. As a result, the fungus energy cycle is disrupted and ATP production ceases (Bartlett et al. 2002).

Resistance risk for QoIs is high (FRAC 2017) and increased frequency of selection pressure may select for fungicide-resistant populations (Brent and Hollomon 1998). QoI resistance was first reported in wheat powdery mildew in northern Germany in 1998 (Brent and Hollomon 1998). Shortly after, isolates resistant to QoI were detected in populations of several phytopathogenic fungi (Bartlett et al. 2002) such as *Alternaria* spp. in pistachio (Ma et al. 2003), *Venturia inaequalis* in apple (Lesniak et al. 2011), *C. graminicola* in turf (Avila-Adame et al. 2003), and *Botrytis cinerea* in strawberry (Amiri et al. 2013). Resistance was discovered to be a result of an amino acid change from glycine to alanine at position 143 (G143A) of the *cytb* gene. The G143A mutation confers high levels of resistance to the QoI fungicides and is the most commonly found mutation among resistant isolates (Gisi et al. 2002; Sierotzki et al. 2000). Two other mutations, a substitution of phenylalanine by leucine at codon 129 (F129L) and the replacement of glycine by arginine at position 137 (G137R), have also been reported to be associated with moderate levels of resistance (Bartlett et al. 2002; Pasche et al. 2004; Sierotzki et al. 2007).

Even though *C. acutatum* is a major pathogen causing anthracnose in a wide range of hosts, cross-resistance to QoI fungicides was first reported in strawberry in 2016 by Forcelini et al. (2016) (USDA-ARS 2017). Resistant isolates were found in Florida during the 2013–14 season, after strawberry growers experienced an AFR outbreak despite QoI treatment prior to planting. At the time, resistance was detected in isolates obtained from infected crowns, roots, and fruit. However, during the 2016 season, resistant *C. acutatum* isolates were recovered in nurseries and production fields across the United States and Canada (Forcelini and Peres 2016). Resistance was associated with either the G143A or F129L mutations in the *cytb* gene (Forcelini et al. 2016), which conferred complete or moderate resistance levels, respectively. The loss of QoI efficiency to control *C. acutatum* has left

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strawberry growers relying mainly on the use of multisite fungicides such as Captan to manage AFR (Legard et al. 2003). However, multisite fungicides must be applied frequently and at much higher rates than the single site fungicides such as the QoI, leading to consumer concerns about pesticide residues.

Mutations associated with QoI resistance may come with a fitness cost in the absence of fungicide selection pressure. Fitness can be defined as the survival and reproductive success of an allele, individual, or group (Pringle and Taylor 2002). Several parameters have been measured to determine fitness inherent to the G143A and F129L mutations in fungal pathogens (Ma and Michailides 2005). Veloukas et al. (2014) observed that, in the absence of QoI, mycelial growth, sclerotial production, aggressiveness, and spore production of *B. cinerea* isolates with the G143A mutation were similar to those values obtained from sensitive isolates. Similar results were obtained in laboratory-generated *Magnaporthe grisea* mutants (Avila-Adame and Köller 2003). Fitness of G143A mutants of *M. grisea* was not compromised and resistance remained stable during four consecutive disease cycles on nontreated barley leaves (Avila-Adame and Köller 2003). Moreover, competition between resistant and sensitive isolates has many implications for the evolution of fungicide resistance in fungal populations and, subsequently, for resistance management programs (Parnell et al. 2005; Peever and Milgroom 1995). Several studies have shown that QoI-resistant isolates were similarly competitive or even outcompeted sensitive isolates when selection pressure was removed (Karaoglanidis et al. 2011; Klosowski et al. 2016). However, very little is known about the fitness and competitive ability of resistant isolates of *C. acutatum* and the stability of the G143A mutation.

Because *C. acutatum* resistance to QoI fungicides was reported for the first time in 2016 (Forcelini et al. 2016), there is a knowledge gap concerning the effects of mutations in the *cytb* gene on the fitness of *C. acutatum*. Investigation of the fitness of resistant mutants is instrumental in elucidating the behavior of such isolates in the absence of selection pressure, to predict sensitivity shifts in strawberry field populations, and to optimize current AFR management programs. Thus, the objectives of this study were to (i) develop a molecular assay to quickly identify the G143A mutation in *C. acutatum* populations, (ii) evaluate fitness parameters of isolates with different levels of sensitivity to QoI fungicides, (iii) determine the competitive ability of QoI-resistant and -sensitive isolates in the absence of fungicide selection pressure, and (iv) investigate the stability of the G143A mutation in QoI-resistant isolates on nonamended media.

MATERIALS AND METHODS

Fungal isolates. In total, 24 *C. acutatum* isolates collected from Florida and California production fields with or without a QoI spray history over a 20-year period were analyzed in this study. Isolates were previously studied and categorized according to their sensitivity level to QoI fungicides (Forcelini et al. 2016). When exposed to azoxystrobin, isolates whose mycelial growth was inhibited at 3 µg/ml were considered sensitive (S). Isolates that were not inhibited at 3 µg/ml but inhibited when exposed to 100 µg/ml were called moderately resistant (MR). Completely resistant (CR) isolates were not inhibited at 100 µg/ml (Forcelini et al. 2016). Sensitive *C. acutatum* isolates that formed colonies with pink coloration were considered sensitive chromogenic (SC). Partial sequences of the *cytb* gene of one S (2-179), one MR (14-141), and one CR (13-466) isolate have been deposited in GenBank under accession numbers MF784585, MF784586, and MF784587, respectively. Isolates were maintained in glycerol at -80°C or stored dry on filter paper in a sterile envelope in an airtight container with Drierite (W. A. Hammond Drierite Company, Ltd., Xenia, OH) at -20°C until isolate revival.

Fungicide preparation. The commercial formulation of azoxystrobin (Abound 2.08F, 22.9% a.i.; Syngenta Crop Protection, Greensboro, NC) was used in the competitive ability and mutation

stability experiments. Azoxystrobin was diluted in sterile deionized water to prepare a stock suspension at 388.4 µg a.i./ml. A previous study where sensitivity of *C. acutatum* of strawberry to QoI fungicides was tested showed that these isolates did not develop an alternative respiration pathway; therefore, the addition of salicylhydroxamic acid to the culture media was not required (Forcelini et al. 2016).

Genotyping of *C. acutatum* isolates. A polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) assay was developed to confirm the presence or absence of the G143A mutation in the *cytb* gene of *C. acutatum*. PCR with *cytb*-specific primers were performed for 24 *C. acutatum* isolates (8 S, 4 SC, 2 MR, and 10 CR) according to Forcelini et al. (2016) (Supplementary Fig. S1). Amplified products were subjected to restriction enzyme digestion with the *AluI* restriction enzyme (New England Biolabs, Ipswich, MA), which recognizes the AG|CT site. Digestion reactions were performed at 37°C for 1 h in a total volume of 20 µl containing 5 µl of PCR products, 1× CutSmart Buffer, and 5 U of *AluI* restriction enzyme. Digested products were separated in a 2% agarose gel containing GelRed (Biotium, Fremont, CA) in 1× Tris-acetate-EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) at 50 V for 2 h.

Fitness components. Mycelial growth at different temperatures, aggressiveness, and spore production were determined to evaluate the fitness of MR and CR isolates compared with S and SC isolates. All experiments were conducted twice.

Mycelial growth. Mycelial plugs (6 mm in diameter) of 24 isolates were removed using a cork-borer from 4-day-old cultures grown on potato dextrose agar (PDA) (Difco Laboratories, Livonia, MI) in 60-mm-diameter Petri dishes at room temperature under continuous light. Each fresh PDA plate received one mycelial plug that was placed upside down so that fungal mycelia had direct contact with the surface of the medium. Plates were sealed and incubated at room temperature under continuous light at 4, 22, and 30°C for 3 days. Two radial measurements of mycelial growth were taken from each colony. Three plates per isolate-temperature combination were used.

Aggressiveness and spore production. Ten isolates (three S, one SC, two MR, and four CR) were evaluated for their aggressiveness and spore production in a fungicide-free environment. Actively sporulating 7-day-old colonies grown on PDA (Oxoid Ltd., Basingstoke, UK) were gently scraped with a flame-sterilized glass rod into sterile water. The resulting conidial suspensions were filtered through sterile double-layered cheesecloth and then adjusted to 1×10^6 conidia/ml for each isolate using a hemocytometer. Immature fruit of cultivar Florida Radiance that had receptacles with chlorophyll were harvested from experimental plots at the University of Florida-Gulf Coast Research and Education Center. Fruit of similar size were surface disinfested in 0.7% sodium hypochlorite for 6 min, rinsed four times with sterile deionized water, and subsequently air dried under a fume hood. Three replications of three fruit per isolate were inoculated with 20 µl of the conidial suspension. Fruit were incubated at 24°C for 7 days and aggressiveness was evaluated as disease severity (percent affected fruit area). Plugs (6 mm in diameter) were removed from the periphery of the decayed tissue, placed in 50-ml conical tubes with 10 ml of sterile water, and then vortexed to dislodge conidia from fruit tissue. The number of conidia produced was determined with the aid of a hemocytometer. Average conidial production of three replicates was determined for each of the 10 isolates.

Competitive ability. The competitive ability of one S (02-179) and one CR (13-466) isolate was evaluated in a fungicide-free environment. Immature fruit and spore suspensions were prepared for inoculation as mentioned above. Inoculum suspensions of S and CR isolates were mixed in the following CR/S ratios: 100:0, 80:20, 50:50, 20:80, 0:100, and 0:0. Three fruit per replication were inoculated with 20 µl of each of the conidial suspensions. Seven days after inoculation and incubation at 24°C, spores were recovered from the periphery of the decayed tissue, as mentioned above. Spore suspensions of each replication were adjusted to 1×10^3 conidia/ml and 100 µl were seeded on three general isolation

(GI) plates (PDA amended with streptomycin at 0.1 g/liter and ampicillin at 0.25 g/liter) with or without azoxystrobin at 100 µg/ml. The number of colony-forming units (CFU) was counted 2 days after seeding and the difference of CFU between GI with and GI without azoxystrobin was determined for each treatment.

Fig. 1. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay for detection of the G143A mutation in the cytochrome *b* (*cytb*) gene of *Colletotrichum acutatum*. PCR products amplified from *C. acutatum* isolates using *cytb*-specific primers (Forcelini et al. 2016) were digested with *AluI* and separated on a 2% agarose gel stained with GelRed. Lane M = 100-bp DNA Step Ladder. Isolates 13-459 and 14-692 displaying two bands after PCR-RFLP analysis were confirmed to possess the G143A mutation in the *cytb* gene. Undigested PCR products of isolates 02-179 and 14-692 are also shown.

TABLE 1. Radial mycelial growth of *Colletotrichum acutatum* isolates with different quinone-oxidase inhibitor sensitivity phenotypes when exposed to different temperatures

Isolate	Phenotype ^z	Average mycelial growth (mm) ^y	
		22°C	30°C
95-61	S	5.58 d	4.25 e
98-1	S	6.08 d	5.54 c
02-179	S	5.83 d	4.58 e
02-180	S	5.91 d	3.92 e
3-2	S	6.00 d	4.38 e
11-119	S	7.58 b	4.54 e
14-57	S	6.41 c	4.42 e
16-31	S	5.87 d	4.75 d
13-10	SC	8.79 a	6.58 a
13-11	SC	9.29 a	6.92 a
13-73	SC	8.12 b	6.04 b
13-486	SC	9.00 a	7.04 a
13-494	MR	7.37 b	5.21 c
14-141	MR	7.62 b	4.83 d
13-140	CR	7.45 b	4.79 d
13-459	CR	6.87 c	4.71 d
13-465	CR	6.50 c	4.46 e
13-466	CR	7.42 b	4.88 d
13-473	CR	7.45 b	4.63 e
14-58	CR	7.08 b	4.63 e
14-61	CR	7.16 b	5.00 d
14-687	CR	7.08 b	4.92 d
14-692	CR	7.33 b	4.50 e
16-200	CR	7.42 b	4.38 e

^y Values are mean mycelial growth of isolates from two trials. Numbers in each column followed by the same letter are not statistically different according to Scott-Knott's cluster analysis at *P* = 0.05.

^z S = sensitive, SC = sensitive chromogenic, MR = moderately resistant, and CR = completely resistant to azoxystrobin.

Mutation stability. Two CR isolates (13-466 and 13-473) were used to determine whether the G143A mutation was stable after four transfer cycles in a fungicide-free medium (PDA). An S isolate (02-179) was also included to verify that, if they occurred, reduction in germination, mycelial growth, or sporulation were due to the genotype and not to the specific isolate. The effective concentration of azoxystrobin at which 50% inhibition of mycelial growth occurred (EC₅₀) was determined for each isolate before the first transfer (T0) and after the last transfer (T4). Isolates were exposed to two ranges of azoxystrobin concentrations, 0.01 to 3 µg/ml and 1 to 100 µg/ml, which were applied to PDA plates using the spiral gradient dilution method, as previously described by Amiri et al. (2013). Mycelial growth was measured 72 h after seeding, and the EC₅₀ value was determined at the point within the fungicide gradient where mycelial growth was reduced by 50% compared with the control (PDA). Three plates per isolate were used as a repetition in each of the four transfer cycles. For each azoxystrobin concentration range, three plates per isolate were used to determine the EC₅₀ values. The experiment was performed twice.

Data analysis. Data for mycelial growth were analyzed using a two-way analysis of variance (ANOVA) using PROC GLIMMIX in SAS (version 9.4; SAS Institute Inc., Cary, NC). Sensitivity group (S, SC, MR, and CR) or isolate, temperature, and their interaction were considered as fixed effects and replication and interaction among replication, temperature, and sensitivity group or isolate as random effects to inflate standard errors. Scott-Knott cluster analysis was used to assess the differences among treatments in the mycelial growth experiment. Data for disease severity and spore production were analyzed using a generalized linear mixed-model approach in PROC GLIMMIX with a β -binomial distribution (logit-link function) and a Poisson distribution (log-link function), respectively. In generalized linear mixed models, sensitivity group or isolate was considered a fixed effect and replication and the interaction between replication and the fixed effect were considered random effects. Fisher's protected least significant difference at the 5% level was used to test differences among sensitivity groups or isolates. In the competition experiments, a one-way block ANOVA testing for linear contrasts between differences in CFU on fungicide-amended medium compared with controls and inoculum ratios was performed using PROC GLIMMIX to determine whether a competitive advantage existed in QoI-resistant or wild-type populations.

RESULTS

Molecular basis of resistance. Restriction enzyme analysis of the partial *cytb* gene of *C. acutatum* isolates identified an extra *AluI* digestion site at the position of 143 (G143A) in QoI-resistant populations. To further test this finding, we developed a PCR-RFLP assay and showed that *AluI* digestion of the 517-bp PCR products, amplified from QoI-sensitive isolates of *C. acutatum*, generated six fragments of 6, 27, 30, 45, 204, and 205 bp in size (Fig. 1). In contrast, seven fragments, including an exclusive fragment of 144 bp in size derived from the 204-bp fragment, were produced from PCR products of QoI-resistant isolates with the G143A mutation after *AluI* digestion (Fig. 1), which confirms the identification of an extra *AluI* site created by the G143A mutation in the *cytb* gene of *C. acutatum*.

Fitness parameters. A representative group of *C. acutatum* isolates with different fungicide sensitivity levels was assessed in vitro (mycelial growth) or in vivo (aggressiveness and conidial production) to evaluate three fitness components.

Mycelial growth. Mycelial growth rates were significantly different across the three temperatures tested (*P* < 0.05). No fungal growth was observed at 4°C. Mycelial growth was greater at 22°C and then decreased at 30°C. Within each temperature, the growth rates were significantly different among isolates, with SC isolates showing greater growth than the other phenotypes (Table 1). At 22°C, mycelial growth in the S group after incubation for 3 days ranged from 5.58 to 7.58 mm whereas, in the MR and CR groups, growth ranged from 7.37 to 7.62 and 6.5 to 7.45 mm, respectively. In the

SC group, mycelial growth was more pronounced, going from 8.1 to 9.3 mm. When isolates were exposed to 30°C, mycelial growth ranged from 3.92 to 5.54 mm for isolates in the S group, and from 4.83 to 5.2 and 4.38 to 5 mm for isolates in the MR and CR groups, respectively. Mycelial growth of SC isolates ranged from 6.04 to 7.04 mm.

Aggressiveness of resistant and sensitive isolates. Some degree of variability between the two experiments was found, with disease severity of isolates ranging from 26 to 45% in the first trial and from 2 to 26% in the second trial. However, the ability to cause decay on detached fruit was not significantly different among sensitivity groups ($P = 0.6941$) or isolates ($P = 0.5414$) (Fig. 2).

Conidial production. Similarly, the mean conidial production among sensitivity groups or isolates was not significantly different

($P = 0.7413$ and 0.6022 , respectively) (Fig. 3), though some variation was observed. Among S isolates, *C. acutatum* isolate 14-57 produced relatively more conidia (3.2×10^6 conidia/ml) than isolates 98-1 and 02-179 did (approximately 1.5×10^6 conidia/ml) (Fig. 3). A similar pattern was also observed in the CR group, with isolate 13-473 producing 1.2×10^6 conidia/ml, whereas isolates 13-466 and 14-61 produced approximately 3×10^6 conidia/ml (Fig. 3).

Competitive ability of QoI-resistant and -sensitive isolates.

A linear contrast analysis showed a strong linear relationship between differences in CFU and inoculum ratios ($P > 0.05$) (Fig. 4). The mean fungal colonies recovered from detached fruit inoculated with the CR/S ratio of 100:0 was -1.4 , and no fungal colonies from the CR/S ratio of 0:100 were formed on the fungicide-amended

Fig. 2. Anthracnose fruit rot severity on detached fruit inoculated with isolates of *Colletotrichum acutatum* sensitive (S), sensitive chromogenic (SC), moderately resistant (MR), or completely resistant (CR) to quinone-oxidase inhibitors. Vertical bars represent the mean of disease severity $\pm$ standard error of the mean of two independent experiments.

Fig. 3. Number of spores produced on detached fruit inoculated with isolates of *Colletotrichum acutatum* sensitive (S), sensitive chromogenic (SC), moderately resistant (MR), or completely resistant (CR) to quinone-oxidase inhibitors. Vertical bars represent the mean of the number of spores recovered $\pm$ standard error of the mean of two independent experiments.

media (Fig. 4), because conidial germination of all S isolates was inhibited under that condition. The number of differences in CFU increased with the decrease of the CR/S ratios. At the ratios of 80:20 and 50:50, mean differences in CFU were 6.3 and 28.8, respectively; and, at 20:80, the number was 49.3 (Fig. 4).

Stability of the G143A mutation. After four consecutive transfers on fungicide-free PDA, both CR and S *C. acutatum* isolates maintained their initial fungicide sensitivity levels. The EC₅₀ values of the two CR isolates obtained before the first transfer (T0) and after the last transfer (T4) for azoxystrobin remained higher than 100 µg/ml. In contrast, mycelial growth of the QoI-sensitive isolate was completely inhibited when exposed to the higher dose range (1 to 100 µg/ml) of azoxystrobin. When exposed to azoxystrobin at 0.01 to 3 µg/ml, the EC₅₀ values of the S isolate obtained before T0 and after T4 fluctuated slightly in the first trial. T0 and T4 EC₅₀ values were 0.101 and 0.082 µg/ml, respectively, for the first trial, whereas they were 0.062 (T0) and 0.061 (T4) µg/ml in the second trial.

DISCUSSION

Fungal isolates harboring mutations that decrease sensitivity to fungicides may suffer fitness penalties and may compete poorly with sensitive counterparts when fungicides are withdrawn (Chen et al. 2016; Ma and Uddin 2009). Moreover, the ability of fungal cells to retain the QoI-resistant phenotype indicates the stability of mitochondrial DNA (mtDNA) mutations. A greater understanding of these fitness components of resistant populations will provide the basis for decisions on whether a fungicide should be reintroduced to the field where fungicide resistance has been reported as well as for the refinement of resistance management strategies for AFR of strawberry. Given the recent emergence of QoI resistance in *C. acutatum* from strawberry production fields in Florida (Forcelini et al. 2016), fitness, competitive ability, and mutation stability of QoI-resistant *C. acutatum* isolates were investigated in the current study to provide such information.

Strawberry *C. acutatum* isolates that had either the F129L or the G143A mutation in the *cytb* gene did not show any obvious fitness

costs in the evaluated parameters in this study compared with sensitive isolates. Based on tests of mycelial growth, *C. acutatum* isolates with different levels of sensitivity to azoxystrobin showed similar growth rates after being exposed to 22 and 30°C for 3 days, although no growth was observed at 4°C. This was expected because *C. acutatum* from strawberry requires temperatures above 5°C for infection (Wilson et al. 1990). Mycelial growth was significantly higher at 22°C and then decreased at 30°C. This finding is in agreement with that reported by Wilson et al. (1990), who found that the optimal temperature for growth of *C. acutatum* was approximately 25°C and mycelial growth sharply declined at 30°C. Within each temperature, differences were observed between isolates of different phenotypes. At 22°C, growth of the majority of S, SC, MR, and CR isolates was not significantly different from one another. Similar studies have shown that mycelial growth of sensitive and resistant (G143A) *Alternaria alternata* isolates from pistachio were not significantly different at 22°C (Karaoglanidis et al. 2011). However at 30°C, differences between growth of mycelia of SC and S, MR, and CR isolates were more pronounced, and isolates 13-10, 13-11, and 13-486 (SC) grew faster than any other phenotype. It is not clear why SC isolates had higher growth rates because only a few studies have been published on the cultural features of chromogenic isolates and their ability to produce pink and red pigments (Baxter et al. 1983; Gunnell and Gubler 1992). Based on previous molecular studies of genetic diversity in chromogenic *C. acutatum*, we suggest that increased mycelial growth of SC isolates compared with S, MR, and CR isolates may be due to genetic polymorphism (Denoyes-Rothan et al. 2003; Gunnell and Gubler 1992). Further investigation of chromogenic isolates is needed for characterization and better understanding of their cultural characteristics.

Isolates with different sensitivity levels to QoI did not differ in aggressiveness and were able to cause similar levels of fruit decay and sporulate on immature fruit. The ability to infect and form lesions on fruit was expected because isolates were recovered from plant tissue. However, a potential difference in aggressiveness in a fungicide-free environment had not yet been investigated within

Fig. 4. Difference in CFU on azoxystrobin-amended media compared with nonamended controls. Fungal colonies were recovered from anthracnose fruit rot lesions on strawberry inoculated with different ratios of quinone-oxidoreductase inhibitor (QoI)-resistant (R) and sensitive (S) isolates of *Colletotrichum acutatum*. Circles represent the mean of differences in CFU from two independent experiments, and error bars represent the standard error of the mean.

these isolates. *B. cinerea* isolates resistant to multiple chemical classes, including the QoI, were as aggressive as sensitive isolates on apple (Chen et al. 2016), while G143A mutants of *M. oryzae* from perennial ryegrass were less aggressive than wild-type isolates (Ma and Uddin 2009). In contrast, Karaoglanidis et al. (2011) observed that *A. alternata* isolates that had the G143A mutation and were resistant to the QoI were more aggressive than sensitive isolates on nontreated pistachio.

Similarly, conidial production of S, SC, MR, and CR isolates evaluated in the study was not significantly different among sensitivity groups. However, some variation within each group was found, which, rather than fitness penalties, could be attributed to the genetic background of individual isolates. It is also possible that isolates within each sensitivity group may have different latent periods with respect to conidial production. If true, the amount of conidia recovered with the time frame of this study (7 days after inoculation) would be different.

Competition experiments conducted on detached immature fruit inoculated with different ratios of CR and S isolates in the absence of a selection pressure showed constant competition between isolates. The CR isolate may have been slightly favored when present in higher or equal frequencies than the S isolate, explaining the increase in CR growth at the end of the experiment. However, this increase was not significant. Such results suggest that, when selection pressure is absent, mutated and wild-type isolates will compete similarly for nutrients and, consequently, will have similar chances of surviving and reproducing (fitness). This is in agreement with the results from our fitness component studies, where no difference in fitness was found between mutated and wild-type isolates. Thus, based on our results, the removal of QoI fungicides from strawberry disease management programs may not result in the decrease of QoI-resistant populations if they are present in mixture with nonmutated populations. In studies where the competitive ability of G143A resistant mutants was investigated, contrasting results have also been reported. When QoIs were withdrawn, G143A resistant mutants of *A. alternata* and *Erysiphe necator* tended to persist at high frequencies when mixed with wild-type isolates (Karaoglanidis et al. 2011; Rallos et al. 2014). In contrast, Ma and Uddin (2009), and Veloukas et al. (2014) observed an increase in spore production by wild-type isolates of *M. oryzae* and *B. cinerea*, respectively, in comparison with the mutated isolate.

The persistence of resistance of G143A mutant isolates was observed after four cycles on nonamended media. An increase in QoI sensitivity was not observed after QoI-resistant isolates were cultured in the absence of selection pressure. With these results, we suggest that mitochondria of mutant *C. acutatum* isolates are homoplasmic; that is, they only contain mutated mtDNA and are able to persist in the environment by transmitting mutations into the next generation (Klosowski et al. 2016). This was observed in several studies, where G143A mutation was shown to be stable in QoI-free environments (Avila-Adame and Köller 2003; Chen et al. 2016; Ma and Uddin 2009; Rallos et al. 2014). Some examples are the detection of QoI-resistant *E. necator* isolates in a vineyard 4 years after the last QoI spray (Rallos et al. 2014). In addition, in vivo and in vitro experiments confirmed that resistance of *M. grisea* and *M. oryzae* isolates, respectively, harboring the G143A mutation remained stable during consecutive disease cycles (Avila-Adame and Köller 2003; Ma and Uddin 2009). In contrast, inhibition of mycelial growth of QoI-sensitive isolates was achieved even though EC₅₀ values slightly varied from T0 to T4.

In this study, we developed a PCR-RFLP technique for reliable detection of QoI-resistant *C. acutatum* isolates with the G143A mutation. This molecular assay was based on an extra *AluI* digestion site created by the mutation at position 143 (GGT to GCT), as has been observed in most plant pathogens such as *B. cinerea* and *Cercospora sojina* (Banno et al. 2009; Standish et al. 2015). In spite of the lack of ability to detect other mutations (F129L and G137R) in the *cytb* gene causing decreased sensitivity, this technique will

enable a prompt identification of QoI-resistant *C. acutatum* isolates in any investigation and in diagnostic facilities and will also be valuable for monitoring resistant populations of *C. acutatum* in the field, particularly for those with complete resistance to QoI fungicides.

The lack of a clear fitness cost in QoI-resistant *C. acutatum* populations from strawberry reported in our study may have a significant impact on the current disease management program. Because resistant populations are already present at high frequencies in some locations, the removal of QoI fungicides for management of strawberry diseases is suggested to halt selection of resistant isolates. Moreover, the G143A mutation has been widely shown to be stable and persistent in the absence of selection pressure and, therefore, the reintroduction of QoI would not be recommended. As a result, where resistance is present, strawberry growers can no longer rely on the use of QoI fungicides as one of their disease management strategies to effectively control *C. acutatum*, in addition to other strawberry diseases where QoI resistance has been reported such as gray mold (Amiri et al. 2013) and leather rot (Seijo et al. 2016). The use of QoI in mixtures or in alternation with products with different modes of action may not be effective either when *C. acutatum* QoI-resistant isolates are present at high frequencies and might induce selection for multifungicide-resistant isolates (Hu et al. 2016). Deployment of chemical products with different modes of action in strawberry nurseries and fruit production fields should be investigated as an antiresistance strategy, as well as nonchemical control alternatives such as heat and ultraviolet light treatments, (Buchner 1991; Janisiewicz et al. 2016; Keck et al. 1995; Suthaparan et al. 2016). The use of heat in an aerated steam chamber has been shown to reduce or even eliminate resistant *C. acutatum* inoculum in strawberry transplants (N.-Y. Wang, W. W. Turechek, and N. A. Peres, unpublished data). Overall, understanding fitness costs of pathogens in response to selection pressure might help us to improve disease management programs that aim to extend the effective life of chemical products.

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