Resistance in Strawberry Isolates of *Colletotrichum acutatum* from Florida to Quinone-Outside Inhibitor Fungicides

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Abstract

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Anthraxnose fruit rot of strawberry, caused by *Colletotrichum acutatum*, is a major disease in Florida and frequent quinone-outside inhibitor (QoI) fungicide applications are needed for disease control. From 1994 to 2014, 181 *C. acutatum* isolates were collected from multiple strawberry fields in Florida with or without QoI spray history. Sensitivity to azoxystrobin and pyraclostrobin was tested based upon mycelial growth and germ tube elongation inhibition. Mean effective concentration where growth was reduced by 50% (EC₅₀) values for isolates collected prior to 2013 based upon mycelial growth were 0.22 and 0.013 µg/ml and upon germ tube elongation were 0.57 and 0.03 µg/ml for azoxystrobin and pyraclostrobin,

respectively. Mycelial growth and germ tube elongation of 48 isolates collected in 2013 and 2014 were not inhibited with azoxystrobin at 3 µg/ml and pyraclostrobin at 0.110 µg/ml. A fungicide discriminatory dose assay indicated that 43 of the 48 isolates had EC₅₀ values higher than 100 and 10 µg/ml for azoxystrobin and pyraclostrobin, respectively. Azoxystrobin and pyraclostrobin sprayed preventively on strawberry fruit inoculated with *C. acutatum* failed to control resistant isolates. Sequencing of the *cytochrome b* gene of sensitive and resistant isolates showed that QoI-resistant isolates contained either G143A or F129L amino acid substitutions.

Anthraxnose fruit rot (AFR) and anthracnose root and crown necrosis (ARN), caused by *Colletotrichum acutatum* J. H. Simmonds, are major diseases of strawberry (*Fragaria × ananassa*) in Florida and in the southeastern United States, where weather conditions are favorable for disease development (Legard et al. 2003; Maas 1998; Mertely and Peres 2012a). Flower blight and fruit rot are the main symptoms of AFR (Maas 1998; Peres et al. 2005). However, when transplants are infected in the nursery, root and crown necrosis can occur (Peres and MacKenzie 2013; Peres et al. 2005).

Strawberry is cultivated as an annual crop in Florida and *C. acutatum* does not overwinter in the soil (Ureña-Padilla et al. 2001). The source of primary inoculum is nursery transplants carrying quiescent infections (Howard et al. 1992). After plant establishment, under long wetness periods and temperatures above 20°C, secondary conidia are produced and become the source of inoculum for flower and fruit infections (Leandro et al. 2001; Maas 1998; Mertely and Peres 2012a; Peres et al. 2005). Characteristic AFR symptoms on infected fruit are dark, sunken lesions with orange mucilage containing conidia. *C. acutatum* has been shown to successfully infect fruit at all maturity stages; however, red mature fruit are most susceptible (Mertely and Peres 2012a; Wilson et al. 1990). Strawberry flowers are also extremely susceptible to *C. acutatum* and the main symptom of AFR consists of brown lesions on infected petals (Mertely and Peres 2012a). Under severe epidemics, *C. acutatum* can cause root necrosis. Not much information is available about the spread of inoculum from aboveground strawberry tissue to the roots. However, it has been suggested that roots may become contaminated by farming operations during digging of transplants and packing in nurseries (Mertely and Peres 2012b; Peres et al. 2005; Ureña-Padilla et al. 2001). After plant establishment, when overhead irrigation is withdrawn, strawberry transplants with *C. acutatum*-infected roots fail to establish and collapse. Infected young roots display brown lesions, whereas

older roots are brown or black and decayed (Freeman and Katan 1997; Mertely and Peres 2012b).

The use of disease-free transplants is the most important control method to prevent AFR and ARN disease outbreaks in production fields (Mertely and Peres 2012a,b; Peres 2015). ARN control starts prior to planting. Some growers in Florida treat bareroot transplants by dipping them in suspensions of single-site mode-of-action fungicides to kill inoculum present on root and crown tissue (Daugovich et al. 2009; Mertely and Peres 2012b; Mertely et al. 2010). After plant establishment, when weather conditions are less favorable for *C. acutatum* development, low label rates of multisite mode-of-action fungicides such as Captan are recommended to prevent AFR (Mertely and Peres 2012a). Because petioles and leaves may also harbor *C. acutatum* inoculum, these frequent applications of multisite fungicides are essential to prevent AFR outbreaks when strawberry flowers and fruit are developing. Rates and frequencies of application of these compounds increase toward the end of the season, when inoculum builds up and plants are exposed to prolonged wetness periods and high temperatures (Maas 1998; MacKenzie and Peres 2012; Wilson et al. 1990). When AFR symptoms are present, single-site fungicides such as azoxystrobin and pyraclostrobin are recommended as tank mixtures with multisite fungicides (Mertely and Peres 2012a; Turechek et al. 2006).

Respiration-inhibitor (RI) fungicides such as the quinone-outside inhibitors (QoI) are the most commonly used single-site fungicides for control of anthracnose (Mertely and Peres 2012a,b; Turechek et al. 2006). One of the most common groups within the QoI are the strobilurin fungicides, and two of its active ingredients (a.i.), azoxystrobin and pyraclostrobin, have been used intensely on strawberry since their release (Bartlett et al. 2002; Mertely and Peres 2012a). These compounds inhibit ATP production during mitochondrial respiration by binding to the Qo center on the *cytochrome b* (*cytb*) gene (Bartlett et al. 2002; Sauter et al. 1999; Ypema and Gold 1999). Some fungi have been shown to develop an alternative respiration pathway in vitro and are able to grow on amended media. This alternative pathway can result in false positive resistant isolates. Consequently, salicylhydroxamic acid (SHAM) is incorporated into the medium when testing QoI fungicides in in vitro assays because it blocks the alternative respiration pathway (Wood and Hollomon 2003). The addition of SHAM to azoxystrobin-amended media

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increased mycelial growth and respiration inhibition of *Sclerotinia sclerotiorum* compared with when azoxystrobin was used alone (Duan et al. 2012). However, in vitro studies evaluating sensitivity of *C. acutatum* of citrus (Mondal et al. 2005) and *C. graminicola* of turf (Avila-Adame et al. 2003) to RI fungicides have shown that SHAM did not increase mycelial inhibition and suggests that these pathogens do not utilize an alternative respiration pathway. Currently, no information is available on the effects of SHAM on *C. acutatum* mycelial growth and respiration inhibition when conducting in vitro testes with RI fungicides.

QoI fungicides have been categorized as code group 11 by the Fungicide Resistance Action Committee (FRAC 2016), which has a high risk of resistance development. Numerous applications of QoI fungicides can lead to selection of isolates resistant to these products. QoI resistance has already been reported in many pathogens such as *C. graminicola* in turf (Avila-Adame et al. 2003), *Venturia inaequalis* on apple (Lesniak et al. 2011), *Podosphaera fusca* (synonym *P. xanthii*) and *Pseudoperonospora cubensis* on cucumber (Ishii et al. 2001), *Botrytis cinerea* on strawberry (Amiri et al. 2013), and different *Alternaria* spp. on pistachio (Ma et al. 2003). However, QoI resistance has not yet been reported in *C. acutatum* isolates from strawberry despite frequent use or, to the best of our knowledge, in *C. acutatum* affecting any other crop (Peres et al. 2009).

Resistance to QoI fungicides can occur as a result of point mutations in the *cytB* gene. Three amino acid substitutions (i.e., G143A, F129L, and G137A) have been reported to confer different levels of QoI resistance (Gisi et al. 2002; Sierotzki et al. 2007). The G143A mutation results in the substitution of glycine (G) by alanine (A) at position 143. This mutation confers a high level of resistance and is the most commonly found in resistant isolates (Gisi et al. 2002). When the G143A mutation is present, disease control usually fails even at high fungicide rates. The two other mutations, a replacement of phenylalanine (F) by leucine (L) at position 129 (F129L) and a substitution of glycine by arginine at position 137 (G137R), are linked with moderate levels of resistance (Bartlett et al. 2002; Pasche et al. 2004; Sierotzki et al. 2007). Fungal isolates with these mutations are usually controlled when exposed to field rates of QoI fungicides.

During the 2013–14 Florida strawberry season, growers experienced anthracnose control failure after treating transplants with QoI fungicides before planting. *C. acutatum* was isolated from plants with root and crown rot. The objectives of this study were to (i) determine the sensitivity to azoxystrobin and pyraclostrobin of *C. acutatum* isolates collected from Florida strawberry fields in a 20-year period, (ii) evaluate the efficacy of azoxystrobin and pyraclostrobin field rates to control recently collected *C. acutatum* isolates, and (iii) characterize the molecular mechanism of resistance in QoI-resistant isolates.

Materials and Methods

Collection, isolation, and storage of *C. acutatum* isolates. In total, 181 *C. acutatum* isolates (Table 1) were collected from 1994 to 2014 from anthracnose-affected strawberry plants from commercial fields (148 isolates) in Florida and the research fields (33 isolates) of the University of Florida Institute of Food and Agricultural Sciences Gulf Coast Research and Education Center (GCREC; Dover and Balm, FL). Isolates were collected by harvesting spores directly from sporulating lesions or from tissues surface disinfested with ethanol and sodium hypochlorite, and plated on potato dextrose agar (PDA) medium amended with streptomycin at 0.1 g/liter and ampicillin at 0.25 g/liter. *C. acutatum* isolates identified by cultural morphology and conidial shape and size were single spored on PDA and stored dry on filter paper at -20°C or in glycerol at -80°C . In all, 23 isolates collected from 1994 to 1999 that had not been exposed to QoI fungicides formed the baseline population. The other 154 isolates collected after the commercial release of QoI products represented the QoI-sprayed population.

Fungicides. The commercial formulations of azoxystrobin (Abound 2.08F, 22.4% a.i.; Syngenta Crop Protection) and pyraclostrobin (Cabrio EC, 20% a.i.; BASF) were used for fungicide sensitivity evaluations. Fungicides were diluted in sterile deionized water to prepare stock

suspensions of a.i. at 388.4 and 14.23 $\mu\text{g/ml}$ for azoxystrobin and pyraclostrobin, respectively. For azoxystrobin, the concentrations evaluated ranged from 0.01 to 3 $\mu\text{g/ml}$ and for pyraclostrobin from 0.001 to 0.110 $\mu\text{g/ml}$. For additional trials, increased concentrations of azoxystrobin (1 to 100 $\mu\text{g/ml}$) and pyraclostrobin (0.110 to 10 $\mu\text{g/ml}$) were used to test mycelial growth inhibition of seven *C. acutatum* isolates. SHAM at 100 $\mu\text{g/ml}$ was added to QoI-amended PDA to evaluate sensitivity of mycelium, spore, and germ tube.

Fungicide sensitivity evaluation. Mycelium growth and germ tube elongation were evaluated to determine *C. acutatum* sensitivity to QoI fungicides using the spiral gradient dilution method previously described by Förster et al. (2004).

Mycelium growth. For isolates collected between 1994 and 2011, cellophane strips were used to seed the medium (Förster et al. 2004) whereas, for isolates from 2013 and 2014, PDA + agar (PDAA) strips were used according to Amiri et al. (2013). Medium (50 ml) containing 39 g of PDA amended with 5 g of agar per liter to harden the medium was poured into 150-mm-diameter petri dishes to produce strips. Seeding of the medium with fungal isolates and cutting of the strips followed the method described by Amiri et al. (2013).

Each fungicide suspension (50 μl) was applied in a continuous spiral concentration gradient to the surface of 150-mm plates with PDA using an Autoplate 4000 (Spiral Biotech, Inc.). Plates were incubated for 2 to 4 h at room temperature to allow fungicide to diffuse through the agar. Fungicide-amended plates were seeded across the fungicide concentration gradient with cellophane or PDAA strips covered with mycelium. Strips of each isolate were applied in duplicate and two isolates were tested per plate. Of the 181 isolates, 12 (1 baseline and 11 QoI-sprayed isolates) were tested in SHAM-amended medium. Three replicate petri dishes were seeded for each isolate–fungicide combination and control (PDA) and then incubated at 24°C under constant light. Mycelium growth was measured after 72 h. The point within the fungicide gradient where mycelial growth was reduced by 50% as compared with control plates (50% effective concentration [EC_{50}]) was marked and distance to the plate center was used to determine the EC_{50} values using the Spiral Gradient Endpoint Software (Spiral Biotech, Inc.). Each experiment was conducted twice.

Mycelial growth of 54 of the 181 isolates was also tested using discriminatory doses of azoxystrobin and pyraclostrobin. One 6-mm-diameter plug from the edge of 7-day-old colonies from each isolate was transferred to 60-mm petri dishes containing PDA (control), PDA + azoxystrobin (100 $\mu\text{g/ml}$), or PDA + pyraclostrobin (10 $\mu\text{g/ml}$). Mycelial growth was measured after 3 days of incubation at 24°C . Three plates per fungicide–isolate and two for control–isolate combinations were used. The experiment was repeated once.

Conidial germination. Three baseline isolates 95-61C, 96-90, and 96-95 were selected to evaluate the effect of azoxystrobin on spore germination. Conidial suspensions containing 100 spores were seeded to PDA plates amended with azoxystrobin at 0, 0.01, 0.1, 1, 10, or 100 $\mu\text{g/ml}$ with or without SHAM. Three 150-mm-diameter petri dishes were seeded for each isolate–fungicide concentration and germinated conidia were determined by the number of single colonies after 2 to 3 days of incubation at 24°C .

Germ tube elongation. Germ tube elongation sensitivity to azoxystrobin and pyraclostrobin was also tested for 1 baseline isolate (95-61C) and 11 QoI-exposed isolates using the spiral gradient dilution method. After plates were divided into six sections, 7 μl of a 10^3 conidia/ml suspension was seeded in duplicate across the concentration gradient with a multichannel pipette. Three isolates were evaluated per plate and two replicate plates were used for each fungicide, fungicide + SHAM, or control. Plates were incubated at room temperature (approximately 23°C) and germ tube elongation was evaluated microscopically. The germ tube length of 10 spores/replicate of each of the three control plates was measured and averaged. The EC_{50} value was determined at the fungicide gradient point where most of the germinated spores had germ tubes that measured half the length of the average germ tube on the control plates.

Inoculum preparation. Isolates were transferred from glycerol or filter paper to 100-mm-diameter plates containing PDA, and grown for 4 to 7 days at 24°C under constant light. Conidia were harvested by flooding plates with sterile deionized water and scraping the fungal colonies with a glass rod. Conidial suspensions were then filtered through a sterile double layer of cheesecloth and adjusted to 10⁶ conidia/ml with a hemocytometer. Suspensions were prepared the day of the experiment and kept at 4°C.

Fruit assay. The efficacy of the commercial formulations of azoxystrobin and pyraclostrobin to control sensitive and resistant isolates on strawberry fruit was tested. Four resistant and eight sensitive isolates were selected. Three of the eight sensitive isolates were chromogenic and produced dark purple fungal colonies. Immature fruit of 'Strawberry Festival' that had receptacles with chlorophyll were harvested from experimental plots at the GCREC. In the laboratory, fruit were selected for uniformity and surface disinfested in 0.7% sodium hypochlorite for 6 min, then rinsed twice with sterile deionized water. Four fruit per isolate were placed in egg cartons inside clean plastic boxes according to replication and treatment and were surface dried inside the hood for 20 min. To maintain humidity inside the boxes, 100 ml of deionized water was placed under the egg cartons.

Fruit were sprayed with field rates of Abound (1.24 µg/ml) or Cabrio (0.001 µg/ml) using a spray bottle. Control fruit were sprayed with autoclaved deionized water. Fruit were incubated in closed boxes at room temperature for 24 h before inoculation. Two sets of isolates (95-61, 13-73, 13-459, 13-483, 14-57, and 14-58; and 3-2,

8-11, 13-10, 13-139, 13-486, and 13-488) were tested on two different dates. A 5-µl droplet of a 10⁶ conidia/ml suspension of each *C. acutatum* isolate was placed on the upper part of each fruit. Fruit were incubated in closed boxes at room temperature for 5 more days and then assessed for AFR incidence and severity. Disease incidence was estimated as the percentage of infected fruit and disease severity as the percentage of fruit area with AFR symptoms. Four replicates of four fruit per isolate were used for each fungicide treatment. Seventy-two fruit were sprayed with water only as controls to determine whether the fruit were infected prior to this test. The experiment was repeated once.

Analysis of *cytB* gene sequences from sensitive and resistant *C. acutatum* isolates. Fungal colonies of 11 QoI-resistant and 7 QoI-sensitive *C. acutatum* isolates were grown on PDA media for 7 days. Mycelia were then harvested and DNA was extracted using the FastDNA Kit (MP Biomedicals). Highly conserved regions of the *cytB* gene of *C. graminicola* (GenBank accession number AY285743.1) were used to design primers C.gramcytB-bf1 5'-GAAGAGGTATGTAC TACGGTTCATATAG-3' and C.gramcytB-br1 5'-TAGCAGCTGGAG TTTGCATAG-3' using Primer3Plus (<http://primer3plus.com>). Polymerase chain reaction (PCR) amplification was performed using the following reaction conditions: initial DNA denaturation at 95°C for 3 min; followed by 34 cycles of denaturation 95°C for 40 s, annealing at 60°C for 50 s, and extension at 72°C for 1 min; and final extension at 72°C for 5 min. Amplification products were assessed by electrophoresis in a 1% agarose gel with 1X TAE buffer (0.04 M Tris-acetate,

Table 1. Season, tissue of isolation, number of isolates tested, and effective concentration where growth was reduced by 50% (EC₅₀) values for sensitive *Colletotrichum acutatum* isolates from strawberry used in this study

Season	Tissue	Tested ^b	Mean EC ₅₀ (µg/ml) ^a		Sensitive ^c	Resistant ^d
			Azoxystrobin	Pyraclostrobin		
1994–95	Fruit	2	0.220	0.015	2	0
1995–96	Fruit	4	0.588	0.035	4	0
1996–97	Crown	1	0.467	0.024	1	0
	Fruit	3	0.299	0.012	3	0
1997–98	Crown	2	0.799	0.026	2	0
	Fruit	1	0.417	0.027	2	0
	Petiole	3	0.174	0.013	3	0
	Unknown	2	0.332	0.010	2	0
1998–99	Crown	2	0.162	0.023	2	0
	Fruit	1	0.126	0.011	1	0
	Petiole	2	0.156	0.012	2	0
2001–02	Crown	2	0.131	0.007	2	0
	Fruit	2	0.113	0.005	2	0
	Petiole	1	0.093	0.004	1	0
	Root	6	0.104	0.005	6	0
2002–03	Crown	6	0.142	0.011	6	0
	Fruit	1	0.220	0.009	1	0
	Leaf bud	1	0.330	0.010	1	0
	Petiole	3	0.252	0.010	3	0
	Root	1	0.245	0.011	1	0
2003–04	Crown	8	0.145	0.011	8	0
	Petiole	1	0.112	0.012	1	0
2006–07	Fruit	10	0.151	0.013	10	0
2007–08	Fruit	20	0.121	0.016	20	0
2009–10	Fruit	17	0.051	0.010	17	0
2010–11	Fruit	22	0.277	0.007	22	0
2012–13	Fruit	10	0.635	0.055	3	7
2013–14	Crown	11	–	–	0	11
	Fruit	9	–	–	0	9
	Petiole	1	0.055	0.042	1	0
	Root	19	0.473	0.047	5	14
	Fruit	4	–	–	0	4
2014–15	Root	3	–	–	0	3

^a Mean EC₅₀ values are based on mycelial growth assay; – indicates that isolates were not inhibited with azoxystrobin and pyraclostrobin at 3 and 0.110 µg/ml, respectively.

^b Number of isolates tested.

^c Number of sensitive isolates.

^d Number of resistant isolates.

0.0001 M EDTA buffer) + 2.5 μ l of ethidium bromide. PCR products were purified according to the USB ExoSAP-IT PCR Product Cleanup Product (Affymetrix) instructions, then sequenced by Genewiz Inc. Alignment of sequences was conducted using the BioEdit program (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Data analysis. Analysis of variance (ANOVA) using PROC GLM was conducted to test the effects of treatments and repetitions within experiments. Data from mycelial growth and germ tube elongation assays were combined after ANOVA determined no significant differences. All statistical analysis were conducted using the SAS software (version 9.4; SAS Institute Inc.).

Results

Fungicide sensitivity assay. *Mycelial growth.* EC₅₀ values for the mycelial growth assay were not significantly different between repetitions for azoxystrobin ($P = 0.1183$) and pyraclostrobin ($P = 0.8125$). Three QoI sensitivity levels were observed among the 181 *C. acutatum* isolates. Average EC₅₀ values for isolates collected between 1994 and 2011 was 0.22 μ g/ml for azoxystrobin and 0.012 μ g/ml for pyraclostrobin (Table 1). For isolates collected from 1994 to 1999, prior to the registration of azoxystrobin, the average EC₅₀ value was 0.30 μ g/ml. For those collected after azoxystrobin registration until 2011, the mean EC₅₀ value was 0.15 μ g/ml. Average EC₅₀ values for pyraclostrobin were 0.014 and 0.011 μ g/ml for isolates collected before 2000 and 2011, respectively (Table 1). For isolates collected in 2013 and 2014, 10 isolates had EC₅₀ values that ranged from 0.055 to 1.625 μ g/ml and from 0.017 to 0.092 μ g/ml for azoxystrobin and pyraclostrobin, respectively, and were considered sensitive. For the other 48 isolates collected after 2012, azoxystrobin and pyraclostrobin concentrations of 3 and 0.110 μ g/ml, respectively, were not sufficient to inhibit mycelial growth (Table 1). Average EC₅₀ values for the seven isolates exposed to concentrations of azoxystrobin and pyraclostrobin at 1 to 100 and 0.110 to 10 μ g/ml, respectively, was 28.38 μ g/ml (azoxystrobin) and 1.43 μ g/ml (pyraclostrobin).

Of the 54 isolates tested at discriminatory doses of azoxystrobin and pyraclostrobin at 100 and 10 μ g/ml, respectively, 43 isolates were inhibited <50% when compared with the control treatment. Mycelial growth of the other 11 *C. acutatum* isolates was inhibited. Of the 11 isolates that were inhibited, 2 had not been exposed to strobilurin fungicides, whereas the other 9 isolates (collected in 2013 to 2014) had been exposed to strobilurin fungicides.

Isolates showing mycelial growth inhibition at azoxystrobin and pyraclostrobin concentrations up to 3 and 0.11 μ g/ml, respectively, were considered QoI sensitive. Isolates that were not inhibited at the aforementioned concentrations but were inhibited when exposed to azoxystrobin at 100 μ g/ml and pyraclostrobin at 10 μ g/ml were considered moderately resistant. Isolates that had EC₅₀ values greater than 100 and 10 μ g/ml for azoxystrobin and pyraclostrobin, respectively, were designated completely resistant. All isolates collected from fruit and crown tissues during the 2013–14 and 2014–15 seasons were resistant. In all, 14 of the 19 root isolates collected during the 2013–14 season and all of the root isolates collected during the 2014–15 season were also resistant. In addition, of the 48 resistant isolates, the majority ($n = 33$) of them came from transplants obtained from three of the six nurseries sampled (data not shown).

Spore germination and germ tube elongation. *C. acutatum* spore germination was not significantly affected by the addition of SHAM at 100 μ g/ml to fungicide-amended media. The percentage of germinated conidia ranged from 46 to 71% on the control plates without the addition of SHAM and from 54 to 78% with the addition of SHAM. Germination of *C. acutatum* spores was not inhibited by azoxystrobin at concentrations up to 100 μ g/ml but growth rate of germ tubes was inhibited compared with the control treatments. Of the 12 isolates tested, 7 had EC₅₀ values higher than 3 μ g/ml for azoxystrobin and 0.110 μ g/ml for pyraclostrobin and were considered resistant to both fungicides. The other five isolates had EC₅₀ values that ranged from 0.08 to 1.2 μ g/ml for azoxystrobin and from 0.009 to 0.07 μ g/ml for pyraclostrobin and were considered sensitive, including baseline isolate 95-61C (Fig. 1). The lowest germ tube elongation

mean EC₅₀ values for both QoI were observed for isolate 14-57 collected from petiole tissue, whereas the highest EC₅₀ value among the sensitive isolates was from fruit isolate 13-73 collected in 2013. Mycelial growth and germ tube elongation results were similar for the SHAM-amended media. EC₅₀ for mycelial growth and germ tube elongation were not significantly different for each of the 12 isolates ($P = 0.4201$).

Detached-fruit assay. Field rates of commercial formulations of azoxystrobin and pyraclostrobin failed to control anthracnose on fruit inoculated with QoI-resistant *C. acutatum* isolates. In the first trial, disease incidence on fruit treated with azoxystrobin and pyraclostrobin ranged from 43.7 to 87.5% and from 56.2 to 100%, respectively. Disease incidence in the second trial ranged from 25 to 62.5% (azoxystrobin) and from 31.2 to 100% (pyraclostrobin) (Table 2). Fruit inoculated with sensitive isolates were controlled by field rates. AFR symptoms were observed in the nontreated control when inoculated with 9 of the 12 tested isolates. Interestingly, disease incidence and severity was null or low (6.25% incidence and 1.2% severity) on fruit inoculated with the three chromogenic *C. acutatum* isolates (13-10, 13-73, and 13-486).

Analysis of *cytB* gene sequences from sensitive and resistant *C. acutatum* isolates. Nucleotide sequencing of part of the *cytB* gene of 18 *C. acutatum* isolates revealed two substitutions from phenylalanine (F) by leucine (L) at codon 129 and from glycine (G) to alanine (A) at codon 143 in QoI-resistant isolates. Of the 11 isolates sequenced, 2 isolates showed the F129 L mutation (13-494 and 14-141) whereas 7 exhibited the G143A mutation (Fig. 2). These isolates were all collected in 2013 or 2014. No mutation was observed in any of the QoI-sensitive isolates.

Discussion

In Florida strawberry fields, azoxystrobin and pyraclostrobin are frequently used to control *C. acutatum*. In this study, we observed a decrease in sensitivity and a shift toward resistance of *C. acutatum* isolates collected in 2013 and 2014 from isolates collected previously. To our knowledge, this is the first report of QoI resistance in *C. acutatum* on strawberry or any other crop affected by this pathogen.

All isolates collected from 1994 to 2011 exhibited sensitivity to azoxystrobin and pyraclostrobin. Mean EC₅₀ values of isolates not exposed to QoI fungicides based upon mycelial growth were 0.30 and 0.014 μ g/ml for azoxystrobin and pyraclostrobin, respectively. Similar findings have been reported for isolates with no previous QoI exposure in *Alternaria* spp. on pistachio, *C. graminicola* on turf, and *C. acutatum* on citrus (Avila-Adame et al. 2003; Ma et al. 2003; Mondal et al. 2005). For isolates collected after the release of QoI fungicides until 2011, mean EC₅₀ values were 0.15 μ g/ml for azoxystrobin and 0.011 μ g/ml for pyraclostrobin. EC₅₀ values determined upon mycelial growth and germ tube elongation assays for sensitive isolates of *C. acutatum* were lower for pyraclostrobin than azoxystrobin. This difference has been observed for many other pathogens such as *Guignardia citricarpa*, *Alternaria alternata*, *Diaporthe citri*, *Mycosphaerella citri*, and *C. acutatum* from citrus (Hincapié et al. 2014; Mondal et al. 2005).

A majority of *C. acutatum* isolates collected from the 2012–13, 2013–14, and 2014–15 seasons were not inhibited by pyraclostrobin and azoxystrobin at 0.110 and 3 μ g/ml, respectively, and were considered resistant. When exposed to concentrations of pyraclostrobin at up to 10 μ g/ml and azoxystrobin at up to 100 μ g/ml, most but not all of the isolates were still not inhibited. Mycelial growth of *C. acutatum* isolates with EC₅₀ values between 3 and 100 μ g/ml for azoxystrobin and 0.110 and 10 μ g/ml for pyraclostrobin were considered moderately resistant; and those with EC₅₀ values higher than 100 and 10 μ g/ml for azoxystrobin and pyraclostrobin, respectively, were considered completely resistant. A similar level of resistance was found in a study testing field azoxystrobin-resistant isolates of *B. cinerea*; of 184 isolates, 7 grew on media amended with azoxystrobin at 50 or 100 mg/liter (Jiang et al. 2009). More recently, 86.5% of *B. cinerea* isolates from Florida had a mean EC₅₀ value for pyraclostrobin higher than 347 μ g/ml based upon mycelial growth using the spiral gradient method (Amiri et al. 2013). Interestingly,

B. cinerea isolates tested by Amiri et al. (2013) were collected from the same region from which our tested *C. acutatum* isolates were collected.

Although QoI fungicides act by blocking the pathway required for fungal ATP production, disrupting energy supply, many plant-pathogenic fungi avoid the toxic effects of RI fungicides by developing an alternative respiration pathway in vitro. Thus, the addition of SHAM into the fungicide-amended media is to prevent a false-positive result when evaluating the sensitivity of a certain fungi to RI fungicides (Wood and Hollomon 2003). In our study, the addition of SHAM to fungicide-amended media had no effect on *C. acutatum* spore germination and germ tube elongation. Our findings concur with studies evaluating sensitive *C. acutatum* isolates of citrus (Mondal et al. 2005) and *C. coccodes* of potato (Aqeel 2007) and resistant *C. graminicola* isolates from turf (Avila-Adame et al. 2003). These studies found that SHAM had no effect on the activity of strobilurin fungicides and concluded that some *Colletotrichum* spp. do not

develop the alternative respiration pathway and cannot overcome QoI sensitivity (Aqeel 2007; Avila-Adame et al. 2003; Mondal et al. 2005). Likewise, Hincapie et al. (2014) observed no significant increase in sensitivity in mycelial growth and spore germination of *G. citricarpa* exposed to azoxystrobin + SHAM. Because SHAM had no effect on spore germination and mycelial growth of *C. acutatum* obtained from strawberry and citrus, we suggest that SHAM may not be necessary when evaluating QoI sensitivity in *C. acutatum*.

Interestingly, spore germination of sensitive *C. acutatum* isolates was not inhibited by azoxystrobin even though the growth rate was reduced. Spore germination of *C. acutatum* from citrus was also not highly inhibited by benomyl (Peres et al. 2004). However, germ tubes of sensitive isolates had reduced elongation on azoxystrobin- and pyraclostrobin-amended media, whereas germ tube length of resistant isolates was similar to the control. Our results suggest that either *C. acutatum* germ tube elongation or mycelial growth assays are adequate to evaluate QoI sensitivity but spore germination assays are inadequate.

Fig. 1. Relationship between effective doses of **A**, azoxystrobin and **B**, pyraclostrobin to reduce 50% (EC₅₀) of germ tube elongation (GTE) and mycelial growth (MG) for six sensitive isolates of *Colletotrichum acutatum* from strawberry.

Table 2. Efficacy of azoxystrobin and pyraclostrobin in controlling anthracnose fruit rot on strawberry fruit inoculated with *Colletotrichum acutatum* isolates with different levels of sensitivity

Fungicide, isolate	EC ₅₀ (µg/ml) ^a	Phenotype ^b	Anthracnose fruit rot (%)			
			Experiment 1		Experiment 2	
			Incidence	Severity	Incidence	Severity
Azoxystrobin						
95-61	0.140	S	0	0	0	0
03-2	0.196	S	0	0	0	0
08-11	0.134	S	0	0	0	0
13-10	0.419	S	0	0	0	0
13-73	1.010	S	0	0	0	0
13-483	0.150	S	0	0	0	0
13-486	1.625	S	0	0	0	0
14-57	0.055	S	0	0	0	0
13-139	>3	R	43.7	3.1	25	3.2
13-459	>3	R	87.5	11.2	43.7	5.6
13-488	>3	R	75	6.9	37.5	5.2
14-58	>3	R	68.7	6.8	62.5	4.8
Pyraclostrobin						
95-61	0.014	S	0	0	0	0
03-2	0.011	S	0	0	0	0
08-11	0.013	S	0	0	0	0
13-10	0.035	S	0	0	0	0
13-73	0.081	S	0	0	25	1.2
13-483	0.037	S	0	0	0	0
13-486	0.066	S	0	0	0	0
14-57	0.041	S	0	0	0	0
13-139	>0.110	R	56.2	4.1	31.2	5.3
13-459	>0.110	R	68.7	7.8	62.5	5.6
13-488	>0.110	R	62.5	4.5	37.5	3.7
14-58	>0.110	R	100	9	100	9.8

^a EC₅₀ = effective concentration where growth was reduced by 50%.

^b S = sensitive *C. acutatum* isolates. Mycelial growth EC₅₀ values were <3 µg/ml and <0.110 µg/ml for azoxystrobin and pyraclostrobin, respectively. R = resistant *C. acutatum* isolates. Mycelial growth EC₅₀ values were ≥3 µg/ml and ≥0.110 µg/ml for azoxystrobin and pyraclostrobin, respectively.

Florida strawberry growers have considered QoI fungicides a powerful tool against AFR and ARN and have made repeated use of them since their release (Mertely and Peres 2012a,b; Turechek et al. 2006). In our study, field rates of azoxystrobin and pyraclostrobin failed to control AFR on strawberry fruit inoculated with resistant *C. acutatum* isolates but successfully controlled sensitive isolates. Control failures of ARN were observed by some growers in Florida during the 2013–14 season and were confirmed in an experiment where infected transplants were dipped in azoxystrobin (Mertely and Peres 2014). Disease control failures of azoxystrobin have been reported with *C. graminicola* and *B. cinerea* (Avila-Adame et al. 2003; Jiang et al. 2009). Avila-Adame et al. (2003) observed that, on turf previously exposed to frequent azoxystrobin applications, the same fungicide failed to control resistant isolates of *C. graminicola*, and Jiang et al. (2009) reported normal disease development on cucumber leaves treated with azoxystrobin at 100 µg/ml and inoculated with resistant *B. cinerea* isolates.

In our study, resistance to azoxystrobin and pyraclostrobin in *C. acutatum* was conferred by two point mutations in the *cytb* gene. For the 18 *C. acutatum* isolates sequenced, *cytb* sequences indicated the presence of the F129L and G143A amino acid substitutions. The occurrence of the F129L point mutation has been linked to moderate levels of resistance and disease control can be achieved, but only with higher fungicide concentrations than that used to control sensitive isolates (Bartlett et al. 2002; Pasche et al. 2004; Sierotzki et al. 2007). Based upon mycelial growth, the two *C. acutatum* isolates containing the F129L mutation (13-494 and 14-141) had EC₅₀ values of 36.96 and 31.41 µg/ml for azoxystrobin and 1.22 and 2.46 µg/ml for pyraclostrobin, respectively. These values ranged between the EC₅₀ values of sensitive isolates and isolates having the G143A mutation. The occurrence of this mutation has been documented in studies with strobilurin-resistant isolates of *Pyricularia grisea* from ryegrass turf (Kim et al. 2003) and *A. solani* from potato (Pasche and Gudmestad 2008) but no information on *C. acutatum* has yet been reported. The glycine-to-alanine exchange at position 143 was found in the other nine QoI-resistant *C. acutatum* isolates. The EC₅₀ values of these isolates for pyraclostrobin and azoxystrobin are higher than 10 and 100 µg/ml, respectively. This suggests that these isolates are completely resistant to both QoI fungicides and cannot be controlled even at high concentrations. The G143A point mutation has been previously reported as the main mutation involved in practical resistance of many fungal pathogens to QoI-fungicides (Fernández-Ortuño et al. 2012; Lesniak et al. 2011; Ma et al. 2003; Miles et al. 2012),

including *C. graminicola* (Avila-Adame et al. 2003). Similar to our results, Ma et al. (2003) reported the presence of the G143A point mutation in isolates of *Alternaria* spp. from pistachio with EC₅₀ values for azoxystrobin higher than 100 µg/ml, based upon conidial germination. Likewise, in strawberry fields previously sprayed with pyraclostrobin, Fernández-Ortuño et al. (2012) identified a high frequency of *B. cinerea* isolates that had the G143A substitution.

Resistance in strawberry isolates of *C. acutatum* from Florida to QoI fungicides is reported herein for the first time. We found that AFR and ARN control failures observed during the 2013–14 season were due to QoI resistance caused by two point mutations in the *cytb* gene in QoI-resistant isolates of *C. acutatum*. It is likely that resistant *C. acutatum* populations were carried on transplants that originated from strawberry production nurseries because the majority of the resistant isolates were recovered from infected roots and crowns soon after planting. The fact that the majority of resistant isolates originated from only three nurseries leads us to believe that resistance is still not widespread and it is linked to plant source. Our findings should have significant implications for disease management because azoxystrobin and pyraclostrobin have been the main single-site fungicides used to control AFR and ARN. Shifts toward resistance to QoI and other single-site fungicides have also been reported for *B. cinerea*, resulting in limited chemical resources available for control of the two major diseases affecting strawberry in Florida (Amiri et al. 2013). QoI resistance in *C. acutatum* appears to be limited to Florida and not yet widespread; however, this situation warrants immediate changes in AFR and ARN management strategies to limit selection and prevent spread of QoI-resistant populations. Furthermore, management strategies should aim to integrate disease management across the nursery and fruit production systems, and research on alternative nonchemical suppressive methods is needed.

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98-1 (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51
02-159A (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51
13-73 (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51
02-153 (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 50
14-57 (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51
13-494 (R)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51
14-87 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 50
13-473 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 51
13-479 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 51
13-466 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 52
13-475 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 53
13-471 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 51
13-468 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 52
14-61 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 52
14-141 (R)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 49
13-472 (R)	AMMGIG-FLGYVLPYGQMSL	AATVITNLISAIP-I 51
02-160 (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51
02-179 (S)	AMMGIG-FLGYVLPYGQMSL	GATVITNLISAIP-I 51

Fig. 2. Amino acid sequence alignment of the partial *cytochrome b* gene for *Colletotrichum acutatum* isolates from strawberry. A mutation at codon 129 from phenylalanine to leucine was observed for moderately resistant isolates 13-494 and 14-141. A mutation at codon 143 from glycine to alanine was observed for completely resistant isolates 13-466, 13-468, 13-471, 13-472, 13-473, 13-475, 13-479, 14-61, and 14-87. Isolates 98-1, 02-153, 02-159, 02-160, 02-179, 13-73, and 14-57 were sensitive and had no point mutation.

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