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ORIGINAL ARTICLE

Baseline sensitivity of *Colletotrichum acutatum* isolates from Brazilian strawberry fields to azoxystrobin, difenoconazole, and thiophanate-methyl

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Abstract

Anthracnose fruit rot, caused by *Colletotrichum acutatum*, is an important disease affecting strawberry fields in Brazil where fungicide applications are frequently needed for disease control. Isolates of *C. acutatum* were collected in conventional and organic strawberry fields in the states of São Paulo and Espírito Santo, Brazil, from 2013 to 2015. Sensitivity to azoxystrobin, difenoconazole and thiophanate-methyl was evaluated based on mycelial growth, spore germination, detached fruit assays and molecular characterization of genes targeted by these fungicides. The effective concentration needed to reduce mycelial growth by 50% (EC_{50}) was determined for 78 isolates. Mean EC_{50} values for isolates collected in organic fields were 0.44 and 0.10 μg/ml, and in conventional areas were 0.62 and 0.09 μg/ml for azoxystrobin and difenoconazole, respectively. Mean EC_{50} values, determined using spore germination test, for 43 isolates were 0.04 and 0.13 μg/ml for isolates from organic and conventional fields, respectively, for azoxystrobin. Azoxystrobin- and difenoconazole-resistant isolates were not observed. Populations of *C. acutatum* showed insensitivity rather than resistance to thiophanate-methyl and EC_{50} values could not be determined. Molecular analyses of the *cytb*, *cyp51* and *β-tub* genes did not reveal any of the most common point mutations associated with fungicide resistance.

Keywords *Fragaria x ananassa* · Anthracnose fruit rot · Fungicide sensitivity

Introduction

Anthracnose fruit rot (AFR), caused by *Colletotrichum acutatum* Simmonds (teleomorph *Glomerella acutata* J.C. Guerber & J.C. Correll), is an important strawberry disease in Brazil and worldwide, causing severe yield and economic losses (Domingues et al. 2001; Freeman 2008). AFR's most common symptoms are flower blight and fruit rot, but *C. acutatum* can also infect roots and crowns, causing anthracnose root and crown necrosis (Peres et al. 2005). AFR is favored by long wetness periods and temperatures above 20 °C, and disease symptoms are characterized by dark-brown, water-soaked lesions with pink or orange masses of spores on petioles, and green and ripe fruit (Mertely et al. 2017).

C. acutatum is usually introduced into fruit production fields by infected transplants from nurseries. Some transplants may show symptoms, but most of the time the pathogen occurs on asymptomatic leaves and petioles as appressoria or quiescent infections (Peres et al. 2005; Mertely et al. 2017). *C. acutatum* may also survive on alternative hosts and weeds and could be dispersed to strawberry plants (Freeman et al. 2001; Peres et al. 2005). Furthermore, appressorial formation and secondary conidiation of *C. acutatum* on asymptomatic tissues of strawberries may occur after exposure to flower extracts and these become sources of inoculum for flower and fruit infections (Leandro et al. 2003). *C. acutatum* is commonly spread at short distances by water splash from rainfall and overhead-irrigation, but also by contaminated equipment,

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farming and harvest operations (Freeman et al. 2001; Peres et al. 2005; Freeman 2008).

AFR of strawberry can be managed by using a combination of genetic, cultural and chemical control measures. The use of healthy propagation material and cultivars with moderate levels of resistance are the most important management tools to prevent occurrence of AFR in production fields (Freeman 2008; Seijo et al. 2008). Unfortunately, only highly susceptible cultivars are available for strawberry growers in Brazil (H. Costa, INCAPER, Domingos Martins, Brazil, personal communication). The disease can also be managed by using drip irrigation instead of overhead irrigation, removing diseased fruit, and using low tunnels in the fields (Freeman 2008). However, fungicide application is still the most effective management adopted by strawberry growers, mainly in conventional production systems (Domingues et al. 2001; Forcelini et al. 2016). Organic growers usually rely on the use of healthy transplants, management of weed, mulch and irrigation, crop rotation, application of plant extracts, and inorganic and biological fungicides (Carroll et al. 2016).

Single-site fungicides, *i.e.*, anilinopyrimidines (AP), demethylation inhibitors (DMI), dicarboximides (DC), methyl benzimidazole carbamates (MBC), and quinone-outside inhibitors (QoI), are registered for strawberry in Brazil (AGROFIT 2017). Although there is no active ingredient specifically labelled for the control of AFR in strawberry in Brazil (AGROFIT 2017), strawberry growers rely on the application of azoxystrobin (QoI), difenoconazole (DMI) and thiophanate-mehtyl (MBC) following the list of pesticides permitted in the 'Integrated Production of Strawberry' program in Brazil (Domingues et al. 2001, IDAF 2012, D. Juliato, strawberry grower, Valinhos, Brazil, personal communication).

QoI fungicides inhibit the production of ATP during mitochondrial respiration by blocking electron transport at the quinol-oxidizing (Qo) site of the cytochrome *b* gene complex (complex III) (Bartlett et al. 2002). Many different pathogens have lost sensitivity to this group of fungicides due to amino acid mutations in the cytochrome *b* (*cytb*) gene, at codons 129, 137, and 143 (Amiri et al. 2013; Forcelini et al. 2016;). The G143A mutation, leading to a change from glycine (G) to alanine (A) at codon 143, is responsible for high levels of resistance to QoIs. Moderate levels of resistance are associated with single-point mutations that lead to substitutions from phenylalanine (F) to leucine (L) and glycine (G) to arginine (R) at positions 129 (F129 L) and 137 (G137R), respectively (Bartlett et al. 2002).

DMI fungicides are inhibitors of sterol biosynthesis in membranes, especially ergosterol. These compounds are known to inhibit the cytochrome *b* P450-dependent oxidative demethylation of eburicol in the ergosterol biosynthetic pathway, leading to disruption of the membrane and electrolyte leakage (Ziogas and Malandrakis 2015). Resistance to DMIs is usually polygenic and related to three major mechanisms: point mutations in the *cyp51* (14α-demethylase) gene resulting in decreased affinity of fungicides, overexpression of *cyp51* gene during sterol formation, and decreased intracellular fungicide accumulation through an increased energy-dependent fungicide efflux (Ma and Michailides 2005).

MBC fungicides block nuclear division by binding to the beta-tubulin (*β-tub*) gene and preventing microtubule assembly, disrupting chromosome segregation (Davidse 1986). Substitutions of amino acids in the *β-tub* gene reduce binding and lead to fungicide resistance. Point mutations that lead to replacements of phenylalanine (F) by tyrosine (Y), glutamic acid (E) by alanine (A) or lysine (K) and phenylalanine (F) by tyrosine (Y) at codons 167 (F167Y), 198 (E198A/K) and 200 (F200Y) in the *β-tub* gene, respectively, are the most common positions associated with resistance to MBCs (Ma and Michailides 2005). However, resistance in *C. acutatum* from various fruit crops may be related to other mechanisms, as studies have shown that isolates have inherent resistance or were less sensitive to MBC (Chung et al. 2006; Nakaune and Nakano 2007).

The wide and indiscriminate use of fungicides in conventional strawberry production systems can lead to the emergence of fungicide-resistant mutants and put the management of diseases at risk. Despite the reports of resistant *Colletotrichum* spp. populations to different classes of fungicides in other crops (Avila-Adame et al. 2003), only recently, populations of *C. acutatum* resistant to QoI fungicides were reported in Florida strawberry fields (Forcelini et al. 2016).

Therefore, considering the historic context of resistance worldwide, monitoring shifts in sensitivity of strawberry pathogens such as *C. acutatum* is extremely important for the long-term management of the disease in Brazil. The objectives of this research were to (i) evaluate the *in vitro* and *in vivo* sensitivities to the fungicides azoxystrobin (QoI), difenoconazole (DMI) and thiophanate-methyl (MBC) in *C. acutatum* populations collected from both conventional and organic strawberry fields in Southeast Brazil and (ii) characterize isolates varying in the sensitivity to these fungicides molecularly.

Materials and methods

Isolation and culture of *C. acutatum* isolates

Seventy-eight isolates of *C. acutatum* were collected in conventional ($n = 56$) and organic ($n = 22$) strawberry fields in two Southeast Brazilian States: São Paulo and Espírito Santo in 2013, 2014 and 2015 (Table 1). Spores were harvested directly from sporulating lesions of symptomatic strawberry flowers and fruit and plated on water-agar (WA). A germinated single spore was transferred to potato-dextrose-agar (PDA;

Table 1 Origin, codes and total number of *Colletotrichum acutatum* isolates collected in conventional and organic strawberry fields in 2013, 2014, and 2015 in São Paulo and Espírito Santo States, Brazil

Year	System	County-State	Isolates code	Number of isolates
2013	Conventional	Jarinu- São Paulo	13–47 to 13–80	34
	Conventional	Valinhos- São Paulo	13–81	1
	Conventional	Piedade- São Paulo	13–82 to 13–90	9
	Conventional	Domingos Martins- Espírito Santo	13–91 and 13–92	2
	Organic	Atibaia- São Paulo	13–93 to 13–110	18
	Organic	Domingos Martins- Espírito Santo	13–111 to 13–112	2
	Organic	Irupi- Espírito Santo	13–113	1
2014	Conventional	Domingos Martins- Espírito Santo	14–46 and 14–47	2
	Organic	Domingos Martins- Espírito Santo	14–48	1
2015	Conventional	Valinhos- São Paulo	15–09 to 15–16	8
Total				78

Difco) and grown for 5 to 7 days at 23 °C under constant light. Isolates were stored dry on filter paper at −20 °C.

Fungicides

Commercial formulations of azoxystrobin (Abound Flowable or Amistar WG, Syngenta Crop Protection), difenoconazole (Score 250 EC, Syngenta Crop Protection) and thiophanate-methyl (Cercobin 700 WP, Ihara or Topsin 4.5 FL, UPI) were used in fungicide sensitivity trials. Fungicide stock suspensions or solutions were prepared in sterile distilled water. Fungicide sensitivity of *C. acutatum* isolates was determined based upon mycelial growth, spore germination and detached fruit assays.

Mycelial growth assay

Mycelial growth sensitivity to azoxystrobin (QoI) and difenoconazole (DMI) was evaluated using the spiral gradient dilution method (Amiri et al. 2013; Forcelini et al. 2016), whereas sensitivity to thiophanate-methyl (MBC) was characterized using discriminatory doses. In the spiral gradient dilution assays, inoculum was prepared according to the method described by Amiri et al. (2013) and Forcelini et al. (2016). Fifty microliters of azoxystrobin or difenoconazole stock suspensions at 930.4 and 1344.4 μg/ml, respectively, were applied exponentially to the surface of 150-mm-diameter PDA plates using the spiral plater (Autoplate 4000 and 5000 models, Spiral Biotech, Inc.). For azoxystrobin, the concentrations ranged from 0.04 to 7.2 μg/ml and for difenoconazole from 0.05 to 10.4 μg/ml. Application of inoculum strips and determination of the effective concentration needed to reduce mycelial growth by 50% (EC_{50}) were made according to Forcelini et al. (2016). Each fungicide-isolate combination was replicated three times and experiments were performed twice. Preliminary experiments were performed with some *C. acutatum* isolates with or without salicylhydroxamic acid (SHAM) in PDA amended with azoxystrobin and it was observed that the use of SHAM did not affect mycelial growth nor spore germination of the isolates, which was also reported by Forcelini et al. (2016). Therefore, SHAM was not included in the fungicide tests.

Since EC_{50} values for thiophanate-methyl could not be determined by the spiral gradient dilution method, sensitivity of *C. acutatum* isolates was characterized using discriminatory doses. Mycelial discs (5-mm-diameter) of *C. acutatum* isolates were transferred to plates containing PDA amended with thiophanate-methyl at 0, 1, 10 and 100 μg/ml. Three replications per isolate-concentration combination were used and experiments were performed twice. Plates were incubated at 23 °C under constant light for 5 days. The average colony diameter (two perpendicular measurements) was calculated and mycelium growth reduction (MGR) was expressed as MGR = ((C-FT)/C) × 100), in which, C is the colony diameter of the control treatment minus 5 mm and FT is the colony diameter of the fungicide treatment minus 5 mm. The results were expressed as percentage of mycelial growth reduction.

Spore germination assay

Sensitivity to azoxystrobin was also determined in a spore germination assay for 43 isolates (23 and 20 isolates from conventional and organic fields, respectively), since it is the most sensitive stage of fungal development to QoI fungicides and has been tested in other studies (Bartlett et al. 2002; Forcelini et al. 2016). Three aliquots (30 μl each) of *C. acutatum* spore suspensions (5×10^4 spores/ml) were placed on azoxystrobin-amended WA medium at 0, 0.01, 0.1, 1, 10 or 100 μg/ml and incubated at 23 °C under constant light for 9 h. Afterward, spore germination was paralyzed by the addition of 30 μl of lactoglycerol per each spore suspension aliquot. Three plates (replications) per isolate-concentration

combination were used and experiments were performed twice. Germinated spores were defined as those with a germ tube length greater than the spore length and the 100 spores first observed were counted. Germination inhibition (GI) was calculated by: GI = ((C-FT)/C) × 100), in which, C and FT are the spore germination of the control and fungicide treatments, respectively.

Fruit assay

The efficacy of commercial formulations of azoxystrobin, difenoconazole and thiophanate-methyl for control of the *C. acutatum* isolates 13-79C, 13-95O, 14-47C, 15-09C, and 15-10C (four and one from conventional [C] and organic [O] fields, respectively) with different EC_{50} values was characterized. Immature strawberry fruit from the cultivar 'Florida Radiance' harvested from experimental plots at the Gulf Coast Research and Education Center, in the University of Florida, Wimauma, FL, USA, were disinfested in 0.05% sodium hypochlorite for 2 min, and then rinsed twice in distilled water. Strawberries were placed in egg trays inside plastic boxes and sprayed with the fungicides azoxystrobin (80 μg/ml), difenoconazole (100 μg/ml) or thiophanate-methyl (490 μg/ml) at recommended field rates in Brazil, using a spray bottle. Non-treated controls were sprayed with sterile distilled water. After 24 h at room temperature, fruit were inoculated with 30 μl of a spore suspension of each isolate at 10^6 spores/ml, kept in sealed humid chambers, and evaluated for disease incidence and severity after 7 days. Six fruit per replication and three replications per isolate-fungicide combination were used and experiments were conducted twice. The frequency of infected fruit compared to the total inoculated fruit was used to estimate disease incidence, whereas disease severity was calculated as the percentage of visible fruit area with disease symptoms. Anthracnose fruit rot incidence and severity on fungicide-sprayed fruit were estimated compared to the non-treated control (water-sprayed).

Molecular analysis of species and cytb, cyp51 and β-tub gene sequences

Mycelia and spores of *C. acutatum* isolates grown on PDA for 7 days were collected and genomic DNA was extracted using the FastDNA kit (MP Biomedicals), according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed to classify the isolates into the *C. acutatum* or *C. gloeosporioides* complexes following procedures described by Ureña-Padilla et al. (2002).

Partial *cytb* gene was amplified using the primers C.gramcytb-bf1 and C.gramcytb-br1 (Table 1) (Forcelini et al. 2016) to investigate potential mutations at codons 129, 137 and 143 in the *cytb* gene of *C. acutatum* isolates. PCR was performed according to Forcelini et al. (2016). PCR products of nine isolates (six and three from conventional and organic fields, respectively) were purified with the ExoSAP-IT PCR purification kit (Affymetrix, Inc.), according to manufacturer's instructions, and sent to Genewiz, Inc., for sequencing in both directions. Sequences were assembled, translated and aligned using the MEGA version 6 and BioEdit version 7.2.5, and analyzed by BlastN against the GenBank database.

To characterize *cyp51* genes of *C. acutatum* associated with resistance to DMI fungicides, the sequenced *C. acutatum* genome (GenBank accession number LUXP01000000) (Han et al. 2016) was used to build a local blast database using BLAST+ (version 2.2.31). Searches for contigs containing CYP51-like sequences in this genome were performed using the BlastP algorithm in BLAST+ (version 2.2.31) with *C. gloeosporioides* CYP51B (GenBank accession number ELA23688) as the query sequence. If found, *cyp51* genes in the given contigs were further analyzed using the web interface of AUGUSTUS with *Botrytis cinerea* as the model organism for prediction. The predicted amino acid sequences were blasted against the GenBank database to verify the AUGUSTUS prediction results.

To determine the coding sequences of *cyp51* genes of *C. acutatum*, total RNA of the isolates 13-90C, 13-95O and 15-09C (two and one from conventional and organic fields, respectively) was extracted using the Qiagen RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies). After DNase treatment using the DNA-free kit (Ambion) to remove possible genomic DNA contamination from RNA samples, complementary DNA (cDNA) was synthesized using the SuperScript III first-Strand Synthesis System (Invitrogen) and oligo-dT_{20} following the instructions of the manufacturer. PCR reactions using cyp51A-F1/cyp51A-R1 or cyp51B-F2/cyp51B-R2 primers (Table 2) were performed in a 25-μl volume containing 2 μl of cDNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1× PCR buffer, 1.5 mM of $MgCl_2$, and 1 U of GoTaq G2 hot start *Taq* polymerase (Promega), based on the following conditions: 95 °C for 2 min, 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 3 min, with a final extension of 5 min at 72 °C. The resulting PCR products were then purified and sequenced, as described previously, using the same primers and additional internal primers (cyp51A-F3 and cyp51A-R3 for the *cyp51A* gene, and cyp51B-F3 and cyp51B-R3 for the *cyp51B* gene) (Table 2). In addition, the isolates 13-82C, 13-108O and 15-11C (two and one from conventional and organic fields, respectively) were sequenced to identify potential mutations in the *cyp51* genes associated with resistance to DMI fungicides as mentioned above.

To identify potential mutations associated with resistance to MBC fungicides, a portion of the *β-tub* gene was amplified using primers TB2 L and TB2R (Table) (Peres et al. 2004).

Table 2 Primers used for amplification of cytochrome *b* (*cytb*), cytochrome P450 sterol 14α-demethylase (*cyp51*), and beta-tubulin (*β-tub*) genes of *Colletotrichum acutatum*

Primer	Sequence (5′ to 3′)	Target	Reference
C.gramcytb-bf1	GAAGAGGTATGTACTACGGTTCATATAG	*Cytb*	Forcelini et al. 2016
C.gramcytb-br1	TAGCAGCTGGAGTTTGCATAG	*Cytb*	Forcelini et al. 2016
cyp51A-F1	CGACGGAGTTTTGGATATTTGC	cyp51A	This study
cyp51A-F3	TGGATTATATCAAGATGAGCCCTG	cyp51A	This study
cyp51A-R1	CTTGGTCAACTCAACTTTGCTA	cyp51A	This study
cyp51A-R3	GCCAAGATTCAGTATCTGCTCT	cyp51A	This study
cyp51B-F2	ATATTCGTCTTGTGCGTGCG	cyp51B	This study
cyp51B-F3	GACCACTCCCGTTTTTGGAC	cyp51B	This study
cyp51B-R2	TGGTGACCGTCTAGTCTGC	cyp51B	This study
cyp51B-R3	GCAGGCGAAGAGTCTCCTTG	cyp51B	This study
TB2 L	GYTTCCAGATYACCCACTCC	*β-tub*	Peres et al. 2004
TB2R	TGAGCTCAGGAACRCTGACG	*β-tub*	Peres et al. 2004

PCR was performed following the conditions used by Peres et al. (2004), except for the annealing temperature of 58 °C. The *β-tub* gene PCR products were purified using the ExoSAP-IT PCR purification kit (Affymetrix, Inc.), and seven isolates were sequenced to identify possible mutations at key points of the gene. Sequences were assembled, translated, aligned and analyzed as described above.

Data analysis

EC_{50} values for mycelial growth were determined using the Spiral Gradient Endpoint software (Förster et al. 2004). EC_{50} values for spore germination was calculated by linear regression between GI and the $\log_{10}$-transformed fungicide concentration (Pereira et al. 2017). Data from mycelial growth and spore germination assays from repeated experiments were combined after Levene's test indicated that variances were homogeneous. Analysis of variance (ANOVA) using PROC GLIMMIX was conducted to test the effects of production systems within experiments. Data from fruit assays were subjected to an ANOVA and means were separated using Fisher's least significance test ($p \leq 0.05$). Statistical analyses were performed using Statistica version 7.0 (Statsoft, Inc.) and SAS version 9.2 (SAS Institute, Inc.) software.

Results

Fungicide sensitivity assay

Mycelial growth EC_{50} values of *C. acutatum* isolates to azoxystrobin ranged from 0.07 to 4.16 μg/ml and the mean value was 0.59 μg/ml (Fig. 1). EC_{50} values of isolates collected in organic fields varied from 0.14 to 1.25 μg/ml and the average value was 0.44 μg/ml. In conventional fields, EC_{50} values ranged from 0.07 to 4.16 μg/ml with a mean value of

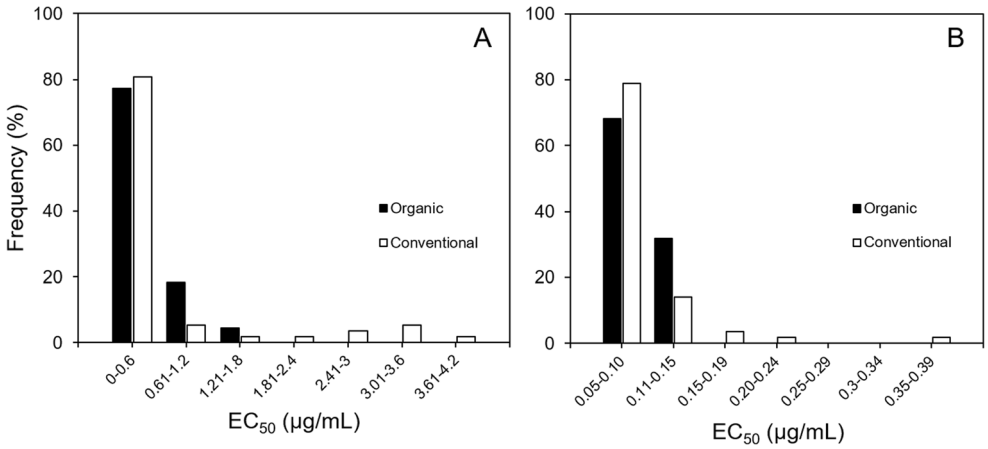

Fig. 1 EC_{50} (μg/ml) distribution of *Colletotrichum acutatum* isolates collected from organic (black bars) and conventional (white bars) strawberry fields to azoxystrobin (**a**) and difenoconazole (**b**), using the spiral gradient dilution method (mycelial growth assay)

0.62 μg/ml (Fig. 1). However, EC_{50} mean values were not significantly different ($p = 0.31$). EC_{50} values to difenoconazole varied from 0.05 to 0.39 μg/ml and the average value was 0.09 μg/ml (Fig. 1). EC_{50} values of isolates from organic fields ranged from 0.06 to 0.14 μg/ml, with a mean of 0.10 μg/ml. In conventional fields, the EC_{50} values varied from 0.05 to 0.39 μg/ml and a mean value of 0.09 μg/ml (Fig. 1). Differences between EC_{50} mean values from organic and conventional fields were not observed ($p = 0.84$). Mean mycelial growth across all *C. acutatum* isolates was reduced to about 32.0, 38.1 and 42.4% of the control at 1, 10 and 100 μg/ml of thiophanate-methyl, respectively (Table 3) and EC_{50} values could not be determined. None of the thiophanate-methyl concentrations used in our trials were able to inhibit completely the growth of *C. acutatum* isolates. Most of the isolates did not have their mycelial growth inhibited by 50% at 100 μg/ml and the mycelial growth reduction was the same with some isolates at the three concentrations used.

Spore germination assay

Azoxystrobin inhibited spore germination of the 43 *C. acutatum* isolates tested and the EC_{50} values varied from 0.01 to 0.98 μg/ml. These values were lower than those found in the mycelial growth assay, in which the same isolates had EC_{50} values ranging from 0.07 to 4.16 μg/ml. However, the relationship between EC_{50} values of spore germination and mycelial growth of isolates were significant and data were assessed by a linear regression ($R^2 = 0.87$, $p < 0.0001$). According to this analysis, EC_{50} values for mycelial growth assays were approximately four times greater than for the spore germination assays. Isolates collected in conventional and organic strawberry fields had mean EC_{50} values of 0.13 and 0.04 μg/ml, respectively, and they were not different ($p = 0.26$).

Fruit assay

AFR incidence and severity varied from 0 to 25% and from 0 to 8.4%, respectively, on fruit treated with field rates of azoxystrobin and inoculated with *C. acutatum* and differed from the non-treated control (Table 4). Disease incidence of difenoconazole-treated fruit differed among isolates, and ranged from 51.5 to 100% and did not differ from water-sprayed control. However, differences were not observed for severity, which varied from 12.2 to 34.5% and differed from the control (Table 4). AFR incidence values of fruit treated with thiophanate-methyl did not differ from the non-treated control and among isolates, and varied from 74.8 to 100%, whereas severity values were different from the control and among isolates and ranged from 25.2 to 86.7%, respectively (Table 4).

Molecular analyses

All 78 isolates were confirmed as belonging to the *C. acutatum* species complex. Nucleotide sequencing of part of the *cytb* gene of nine *C. acutatum* isolates did not reveal any amino acid substitutions at codons 129, 137 and 143 (Table S1). Isolates were compared to a *C. acutatum* sequence from GenBank (accession number KR349346). EC_{50} mean values of the sequenced isolates varied from 0.07 to 4.16 μg/ml and 0.01 to 0.98 μg/ml for the mycelial growth and spore germination assays, respectively, and none of them had any point mutations.

Blast queries using *C. gloeosporioides* CYP51B identified two proteins (g3608 and g9646) in contigs 6 (GenBank accession number LUXP01000006) and 14 (GenBank accession number LUXP01000014) from the sequenced *C. acutatum* genome, respectively, with an E-value of 0 for both sequences. The BlastP search against the GenBank database revealed that proteins g3608 and g9646, respectively, shared high identity with CYP51A and CYP51B of *Colletotrichum* species in the *C. acutatum* species complex (E-value = 0). The coding sequence of the *cyp51B* gene of the representative *C. acutatum* isolate 15–09 was confirmed by reverse transcription-PCR, and the completed sequence was deposited in GenBank under accession number MF662973. However, despite repeated attempts using RNA samples from different *C. acutatum* isolates and

Table 3 Effect of thiophanate-methyl on the mycelial growth of *Colletotrichum acutatum* isolates collected from conventional and organic strawberry fields 2013, 2014, and 2015 in São Paulo and Espírito Santo States, Brazil

Strawberry Production System	*n* isolates	Mycelial growth reduction (%)[y]		
		Thiophanate-methyl		
		1 μg/mL	10 μg/mL	100 μg/mL
Conventional	56	1.8–43.7	19.4–67.2	28.8–85.7
Organic	22	25.6–45.3	33.8–53.5	38.6–58.0
Total/Mean[z]	78	32.0	38.1	42.4

[y] Mycelial growth reduction (%) of *C. acutatum* isolates by thiophanate-methyl at 1, 10 and 100 μg/ml, using the discriminatory dose method. Data are the means of three replications for two experiments

[z] Total represents the total number of isolates and mean is the mean values of mycelial growth reduction of *C. acutatum* isolates

Table 4 Efficacy of azoxystrobin, difenoconazole and thiophanate-methyl on the control of anthracnose fruit rot (AFR) on strawberry fruit inoculated with *Colletotrichum acutatum* isolates with different levels of sensitivity to the tested fungicides

Fungicide[x]	Isolate[y]	EC_{50} (μg/mL)[z]	Anthracnose fruit rot[w] (%)	
			Incidence	Severity
Azoxystrobin (80 μg/ml)	13-79C	0.45	2.8* ab	0.6* b
	13-95O	0.67	0.0* b	0.0* b
	14-47C	0.28	25.0* a	8.4* a
	15-09C	0.07	8.4* ab	1.17* ab
	15-10C	0.15	5.8* ab	1.38* ab
Difenoconazole (100 μg/ml)	13-79C	0.14	51.5 ab	12.2* a
	13-95O	0.09	86.7 a	23.8* a
	14-47C	0.07	80.5 b	29.4* a
	15-09C	0.15	100.0 a	34.5* a
	15-10C	0.08	91.1 a	27.2* a
Thiophanate-methyl (490 μg/ml)	13-79C	–	86.2 a	29.6* c
	13-95O	–	74.8 a	25.2* c
	14-47C	–	83.3 a	43.9* bc
	15-09C	–	97.2 a	68.8* ab
	15-10C	–	100.0 a	86.7* a

[w] Anthracnose fruit rot (AFR) incidence and severity on fungicide-sprayed fruit were estimated compared to the non-treated control (water-sprayed). Data are the means of three replications for two experiments. Values, in columns, followed by the same letters for each fungicide separately are not significantly different at $p \leq 0.05$, as determined by analysis of variance and LSD tests. Means followed by (*) are different from the non-treated control, as determined by ANOVA at $p \leq 0.05$

[x] Fruit sprayed preventatively with azoxystrobin (80 μg/ml), difenoconazole (100 μg/ml) and thiophanate-methyl (490 μg/ml) were previously surface-disinfested and incubated for 24 h at room temperature. Strawberries were inoculated with 30 μL of *C. acutatum* spore suspensions at 10^6 spores/ml for each isolate. AFR incidence and severity were evaluated 7 days after incubation at 23 °C

[y] C and O represent the strawberry production system used in the fields from where the isolates were collected: conventional and organic, respectively

[z] Effective concentration (EC_{50}) of azoxystrobin and difenoconazole, in μg/ml, that inhibited mycelial growth of *C. acutatum* isolates by 50% using the spiral gradient dilution method

different commercial cDNA synthesis kits, the coding sequence of the *cyp51A* gene of *C. acutatum* was never successfully confirmed (GenBank accession number MF662972).

Newly designed cyp51A-F1/cyp51A-R1 primers specific for *cyp51A* amplified a 2100-bp band from six representative *C. acutatum* isolates (data not shown). DNA sequencing of the amplicons and sequence alignment revealed no substitutions at codons 136, 147, and 175 of the CYP51B protein (Table S2).

Nucleotide sequencing of a portion of the *β-tub* gene of seven *C. acutatum* isolates with different sensitivity responses to thiophanate-methyl did not reveal any substitutions at codons 167, 198 and 200 (Table S3). Isolate sequences were compared to *C. fioriniae* (*C. acutatum* species complex) sequence from GenBank (accession number XM_007601271.1).

Discussion

Although there is no active ingredient registered for the management of *Colletotrichum* spp. of strawberry in Brazil, azoxystrobin (QoI), difenoconazole (DMI) and thiophanate-methyl (MBC), registered for the control of other strawberry diseases, are frequently used by conventional growers to control Anthracnose fruit rot (AGROFIT 2017, IDAF 2012, D. Juliato; strawberry grower, Valinhos, Brazil, personal communication). Some of these fungicides are registered as solo products, whereas others come in pre-packaged mixtures (AGROFIT 2017). This study provides information on the sensitivity of *C. acutatum* isolates from conventional and organic strawberry fields of Southeast Brazil to single-site fungicides. Additionally, molecular characterization of target genes associated with fungicide resistance was also studied.

Isolates of *C. acutatum* resistant to azoxystrobin were not found in this study and the average EC_{50} value based upon mycelial growth was 0.59 μg/ml. In fact, it has been reported that *C. acutatum* populations collected from strawberry and citrus in Florida and never exposed to QoI fungicides had mean EC_{50} values of 0.3 and 0.4 μg/ml, respectively (Mondal et al. 2005; Forcelini et al. 2016). Isolates with the highest EC_{50} values in our studies (3.05 and 4.16 μg/ml) showed no mutations in the *cytb* gene. Pereira et al. (2017) also observed that isolates of *Monilinia fructicola* from

Brazilian stone fruits with reduced sensitivity to azoxystrobin had higher EC_{50} mean values which were not associated with any previously described mutations in the *cytb* gene. In Forcelini et al. (2016), moderately resistant isolates of *C. acutatum* from strawberry had EC_{50} values ranging from 31.4 to 37.0 μg/ml and possessed the F129 L mutation, whereas highly resistant populations had EC_{50} values higher than 100 μg/ml.

EC_{50} values determined in spore germination assays were lower than for mycelial growth as previously reported for *C. capsici* (Jin et al. 2009) and *Alternaria alternata* (Mondal et al. 2005). Since fungicidal activity of QoI fungicides is the inhibition of ATP production during mitochondrial respiration, spore germination is the most sensitive fungal development stage to these fungicides (Bartlett et al. 2002). In previous studies, spore germination assays were considered inadequate for evaluating *C. acutatum* sensitivity to QoI fungicides since spore germination of sensitive isolates was not inhibited even at high concentrations (Forcelini et al. 2016). This might have occurred due to the fungistatic rather than fungicidal activity of QoI (Inoue et al., 2012), since the colonies evaluated originated from single spores were counted 2 to 3 days after the spore suspension was placed on fungicide-amended media (Forcelini et al. 2016), whereas, in our studies, spore germination was evaluated after 9 h.

Field rates of commercial formulations of azoxystrobin completely inhibited the development of AFR on detached fruit inoculated with one of the *C. acutatum* isolates. Although at low disease incidence and severity, AFR symptoms were observed on fungicide-treated fruit inoculated with the other isolates. Our results differed from those of Forcelini et al. (2016), where azoxystrobin-treated fruit inoculated with sensitive isolates of *C. acutatum* did not develop AFR symptoms. The lack of complete control of AFR can be explained by the fungistatic activity of azoxystrobin (Inoue et al., 2012) and by the lower field rates used in Brazil (80 μg/ml) compared to Florida (117 to 302 μg/ml) (Whitaker et al. 2016; AGROFIT 2017). Moreover, the different sensitivity to azoxystrobin can be explained by the variability of the isolates, which belong to the complex *C. acutatum* that is comprised of at least 30 species (Bragança et al. 2016). However, AFR disease levels on fungicide-treated fruit was significantly lower than those in non-treated fruit.

In this study, difenoconazole-resistant isolates of *C. acutatum* were not found and the mean EC_{50} value based upon mycelial growth was 0.09 μg/ml. Similar results have been reported for populations of *C. acutatum* not exposed to difenoconazole in strawberry with mean EC_{50} values of 0.10 (Freeman et al. 1997). These results support the evidence that resistance to this group of fungicides is not present in the population from our studies since none of the isolates sequenced carried point mutations in the *cyp51B* gene associated with resistance to DMI fungicides.

Most fungi only possess one copy of the *cyp51* gene. However, some fungi have been found to have multiple *cyp51* copies, such as two copies (*cyp51A* and *cyp51B*) in *Magnaporthe oryzae* (Yan et al. 2011) and *Pyrenophora teres* f. sp. *teres* (Mair et al. 2016), and three copies (*cyp51A*, *cyp51B*, and *cyp51C*) in *Fusarium graminearum* (Liu et al. 2011). It remains unclear why some fungal species possess more than one *cyp51* gene, but it might be of great benefit to those fungi, allowing them to respond to the fluctuating requirements for ergosterol and conferring reduced sensitivity to DMI fungicides under fungicide selection pressure (Hawkins et al. 2014). Given that each mutation of *cyp51* gene may have a different degree of DMI resistance, it is worth noting that *cyp51B* is the essential and dominant *cyp51* gene for cell membrane integrity in filamentous fungi and therefore expressed constitutively, whereas *cyp51A* or *cyp51C* is inducible and only expressed at low levels under normal circumstances (Hawkins et al. 2014; Mair et al. 2016). This differential expression pattern of *cyp51* genes may explain why our multiple attempts to obtain the coding sequence of the *cyp51A* gene were unsuccessful.

Although *in vitro* studies revealed EC_{50} means as low as 0.09 μg/ml, field rates of commercial formulations of difenoconazole (100 μg/ml) did not completely inhibit AFR development on fungicide-treated fruit inoculated with *C. acutatum* isolates. However, disease severity differed from the non-treated control. In trials conducted at strawberry fields, Domingues et al. (2001) reported that difenoconazole was not able to inhibit completely flower blight caused by *C. acutatum*, with incidence varying from 25.8 to 64.5%. Freeman et al. (1997) observed that strawberry plants treated with difenoconazole and inoculated with *C. acutatum* isolates had an average mortality greater than 50%, whereas non-treated plants had 70 to 80% mortality. Similar levels of control were also observed in *C. capsici* in chili fruits, where inhibition of *in vitro* mycelial growth was achieved at 0.1 μg/ml, but disease incidence was reduced only by 58% on fruit treated with difenoconazole at 0.05% (Gopinath et al. 2006). The lack of AFR control in our trials may also be explained by the characteristics of DMI fungicides. Utture et al. (2011) found residues of difenoconazole within the outer part of pomegranate fruit, whereas azoxystrobin could penetrate the inner parts after foliar applications of these fungicides in the field, demonstrating the problem of basic activity of DMI fungicides.

C. acutatum isolates showed a divergent response regarding sensitivity to thiophanate-methyl and EC_{50} values could not be determined. In fact, some of the tested isolates showed same mycelial growth inhibition values for thiophanate-methyl at 1, 10 and 100 μg/ml, but none was higher than 50%. Similar results were found for *C. acutatum* from citrus tested with benomyl (another MBC fungicide), where colony area was reduced to about 25% of the control at 1 μg/ml, but higher concentrations did not further reduce growth and even

at 1000 μg/ml, mycelial growth was not completely inhibited (Peres et al. 2004). Similar observations occurred in the fruit assay, where isolates with different levels of sensitivity showed varied responses on the expression of symptoms on fruit treated with field rates of commercial formulations of thiophanate-methyl. Domingues et al. (2001) observed similar variations on the incidence of flower blight caused by *C. acutatum* in strawberry fields treated with MBC fungicides, which ranged from 32.6 to 85.5%.

Despite the low sensitivity to thiophanate-methyl, none of the sequenced isolates had the most common point mutations at codons 167 (F167Y), 198 (E198A/K) or 200 (F200Y) in the *β-tub* gene responsible for resistance to MBC fungicides (Ma and Michailides 2005). These mutations are commonly reported in resistant isolates of *C. gloeosporioides* of citrus and various fruits (Peres et al. 2004; Chung et al. 2006). In fact, differential sensitivity to benomyl has been useful to distinguish isolates from the *C. acutatum* and *C. gloeosporioides* complexes (Adaskaveg and Hartin 1997). Some studies report the *C. acutatum* response to benzimidazole fungicides as less sensitive or insensitive (Peres et al. 2004, Chung et al. 2006). The low inhibition levels of *C. acutatum* by thiophanate-methyl might be related to the findings of Nakaune and Nakano (2007), in which isolates showed inherent resistance to these fungicides. They reported that benzimidazole resistance of *C. acutatum* is not associated with amino acid substitutions in the *β-tub* gene responsible for high resistance in other pathogens, but resistance is caused by enhanced expression of *β-tub* 1 gene (*CaTUB1*) regulated by the protein *CaBEN1*. Laboratory mutants, with interrupted expression of *CaTUB1* and without any alteration on *β-tub* gene, had their mycelial growth completely inhibited at 0.5 and 10 μg/ml of benomyl and thiophanate-methyl, respectively, whereas wild-type isolates were not inhibited at 100 μg/ml of thiophanate-methyl (Nakaune and Nakano 2007). However, the mechanism by which *CaBEN1* enhances the expression of *CaTUB1* gene is still unclear.

Colletotrichum spp. have great genetic variability and the beta-tubulin is one of the genes used to classify *Colletotrichum* molecularly. Just the *C. acutatum* complex is comprised of 30 species, some recently described (Bragança et al. 2016). In our studies, isolates were simply classified as belonging to the complex *C. acutatum*. Further molecular studies would be needed to differentiate the isolates used in this study by these new species, which may help to explain the variations in sensitivity responses to thiophanate-methyl.

Resistance to azoxystrobin and difenoconazole was not detected in *C. acutatum* populations from strawberry fields of Southeast Brazil; however, some isolates with reduced sensitivity to azoxystrobin and higher EC_{50} values were observed. Although differences among isolates from organic and conventional fields were not significant, most of the isolates with greater EC_{50} values were recovered from conventional areas. This might indicate a shift towards resistance, despite the absence of mutations in the target genes, as already observed in *M. fructicola* isolates from peaches with reduced sensitivity to azoxystrobin (Pereira et al. 2017). Hence, this study will help in the monitoring of shifts in sensitivity to these fungicides in the future.

The insensitivity of *C. acutatum* populations to thiophanate-methyl suggests this fungicide should not be considered for use by growers to control AFR in strawberry fields. Moreover, this inherent resistance to MBC fungicides and the failure in control of the disease caused by *C. acutatum* has been reported in Brazilian strawberry fields since the 1990s (Tanaka et al. 1997). Furthermore, high frequencies of resistance to thiophanate-methyl (92.2%) in other pathogens targeted by MBCs, such as *Botrytis cinerea*, has already been reported in Brazil (Baggio et al. 2018). Despite these observations, Brazilian strawberry growers have been relying on the use of these fungicides for a long time, probably due to the limited number of active ingredients labelled for strawberry and the lack of products registered for the control of AFR (AGROFIT 2017).

Therefore, results of this study may be used to base the registration of azoxystrobin for the control of AFR in strawberry fields in Brazil along with discouraging the registration of thiophanate methyl. Moreover, despite the fact resistance was not found, this study provides a baseline for future monitoring of fungicide sensitivity shifts of *C. acutatum* populations from strawberry fields in Southeast Brazil**.** It is noteworthy to report that no resistance was found even though these fungicides are commonly used**.** Furthermore, for the first time, both *cyp51A* and *cyp51B* genes of *C. acutatum* were characterized by DNA sequencing with newly-designed primers, which will be of significant importance for future studies.

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