Sensitivity of *Colletotrichum* Species, Including *C. fioriniae* and *C. nymphaeae*, from Peach to Demethylation Inhibitor Fungicides

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Abstract

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Few fungicides are effective against anthracnose, caused by *Colletotrichum* spp., and emerging resistance makes the search for chemical alternatives more relevant. Isolates of the *Colletotrichum acutatum* species complex were collected from South Carolina and Georgia peach orchards and phylogenetic analysis of the combined internal transcribed spacer region, glyceraldehyde-3-phosphate dehydrogenase, and β -tubulin gene sequences separated the isolates into *C. nymphaeae* and *C. fioriniae*. The sensitivity of these and three other previously reported *Colletotrichum* spp. from peach, including *C. fructicola*, *C. siamense*, and *C. truncatum*, to demethylation inhibitor (DMI) fungicides difenoconazole, propiconazole, tebuconazole, metconazole, flutriafol, and fenbuconazole was determined based upon mycelial growth inhibition. *C. truncatum* was

resistant to tebuconazole, metconazole, flutriafol, and fenbuconazole and *C. nymphaeae* was resistant to flutriafol and fenbuconazole based on 50% effective concentration (EC_{50}) values $>100 \mu\text{g/ml}$. *C. fructicola* and *C. siamense* were sensitive to all DMI fungicides (EC_{50} values of 0.2 to $13.1 \mu\text{g/ml}$). *C. fioriniae* subgroup 2 isolates were less sensitive to DMI fungicides (EC_{50} values of 0.5 to $16.2 \mu\text{g/ml}$) compared with *C. fioriniae* subgroup 1 (EC_{50} values of 0.03 to $2.1 \mu\text{g/ml}$). Difenoconazole and propiconazole provided the best control efficacy in vitro to all five species, with EC_{50} values of 0.2 to $2.7 \mu\text{g/ml}$. Tebuconazole and metconazole were effective against all *Colletotrichum* spp., except for *C. truncatum*. The strong in vitro activity of some DMI fungicides against *Colletotrichum* spp. may be exploited for improved anthracnose disease management of peach.

Anthracnose of peach is mainly caused by *Colletotrichum acutatum* J. H. Simmonds and *C. gloeosporioides* (Penz.) Penz. & Sacc. (Bernstein et al. 1995; Kim and Hong 2008; Ramsey et al. 1951; Schnabel et al. 2006). Recently, a third species, *C. truncatum*, was also reported to cause peach anthracnose in South Carolina (Grabke et al. 2015). Numerous studies have reported *C. gloeosporioides* and *C. acutatum* to be species complexes (Damm et al. 2012; Weir et al. 2012) but, for peach, only subspecies of *C. gloeosporioides* were recently delimited (Hu et al. 2015a). Anthracnose often occurs during high rainfall and warm temperatures (Ogawa et al. 1995). Symptoms include sunken and firm lesions with circular rings.

C. acutatum and *C. gloeosporioides* are morphologically very similar but there are some characteristic differences. Conidia of *C. acutatum* have acute ends (Simmonds 1965), while those of *C. gloeosporioides* have rounded ends (Wharton and Diéguez-Urbeondo 2004). Several other characteristics such as culture morphology, conidium size, mycelial growth rate, optimum growth temperature, and sensitivity to methyl benzimidazole carbamates (MBC) fungicides also can separate them (Adaskaveg 2000; Peres et al. 2004). However, morphological features may change during repeated subculture, conidial shapes may vary, and fungicide sensitivity can change. Therefore, taxonomical and phenotypical methods have to be used with caution (Damm et al. 2012; Weir et al. 2012) and supported by molecular characterizations (Weir et al. 2012).

Recently, the *C. acutatum* species complex was separated using multilocus molecular phylogenetic analysis and 31 species were accepted to be members (Damm et al. 2012). *C. gloeosporioides* was distinguished into 22 species using similar tools (Weir et al. 2012). Among them are two species, *C. fructicola* and *C. siamense*,

identified recently to cause anthracnose of peach in South Carolina (Hu et al. 2015b).

Chemical management of anthracnose is a challenge because few chemical classes are effective. Apart from multisite fungicides such as captan, the most commonly used chemical classes for anthracnose control are the benzimidazoles (against *C. gloeosporioides*) and quinone outside inhibitor fungicides. However, their widespread use over recent decades has led to resistance problems with both chemical classes of fungicides (Hu et al. 2015a; Wong and Midland 2004; Young et al. 2010). Sterol demethylation-inhibitor fungicides (DMI) are widely used against fungal plant pathogens due to their broad-spectrum activity, more favorable toxicity profile compared with many protectants, and postinfection activity (Szkolnik 1981). In stone fruit, they became the mainstay for brown rot control after resistance to the benzimidazoles led to control failures (Jones and Ehret 1976; Michailides et al. 1987; Ogawa et al. 1984; Sonoda and Ogawa 1982) and they are still used extensively for preharvest brown rot control (Horton et al. 2016). The triazoles difenoconazole, propiconazole, tebuconazole, metconazole, flutriafol, and fenbuconazole are currently registered to control peach diseases (Horton et al. 2016) and recent research suggests that some DMI fungicides may be used to manage *Colletotrichum* spp. as well (Gopinath et al. 2006; Shetty et al. 2014; Towers et al. 2003).

The objective of this study was to phylogenetically characterize isolates from the *C. acutatum* species complex collected from peach orchards in South Carolina and Georgia and to determine the sensitivity of all *Colletotrichum* spp. known to cause anthracnose of peach to DMI fungicides.

Materials and Methods

Fungal isolates and culture conditions. *Colletotrichum* spp. were isolated from anthracnose-symptomatic peach fruit from commercial peach orchards, as described previously (Hu et al. 2015b). In all, 12 *Colletotrichum* isolates were collected from Georgia in 2015 and more than 100 were collected from South Carolina in 2004 (Schnabel et al. 2006). Fruit was surface disinfested in 10% bleach for 2 min, then rinsed with distilled water. A cube of necrotic tissue (2 by 2 by 2 mm^3) was cut from the symptomatic area and placed on acidified potato dextrose agar (PDA). Spores were

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obtained by incubating the plates at 25°C with a 12-h cycle of fluorescent light. For single-spore isolation, conidia were scraped off the plate and suspended in 1 ml of sterile distilled water. The conidial suspension (50 µl) was spread on 1.5% (wt/vol) water agar and incubated at 25°C overnight. One germinated conidium was transferred onto PDA and incubated at 25°C with a 12-h cycle of fluorescent light to induce sporulation. Each isolate was stored on filter paper (Whatman Inc., Maidstone, UK) as dried mycelium at -20°C (Zhu et al. 2012).

The *C. gloeosporioides* and *C. truncatum* isolates used in this study were revived from storage (Grabke et al. 2015; Hu et al. 2015b). They originated also from peach and were collected and characterized previously (Grabke et al. 2015; Hu et al. 2015b). Isolates were recovered from stock cultures stored with silica blue gel beans at -20°C on dried filter paper discs (Fisher Scientific, Pittsburgh; Table 1). The isolates from storage were grown for 5 days on PDA before being used for experiments.

Fungicides. Six DMI fungicides were used for sensitivity study: difenoconazole (Inspire EC; Syngenta Crop Protection, Research Triangle Park, Raleigh, NC), propiconazole (PropiMax EC; Dow AgroSciences, Indianapolis, IN), tebuconazole (Elite 45 DF; Bayer CropScience, Morrisville, NC), metconazole (Quash 50 WDG; Valent USA, Walnut Creek, CA), flutriafol (Topguard 11.8%; Cheminova, Wayne, NJ), and fenbuconazole (Indar 75 WSP; Dow AgroSciences). These triazoles were selected because they are currently registered for disease management of peach in the United States.

DNA extractions, polymerase chain reaction amplification, and sequencing. Five isolates of *C. nymphaeae*, seven isolates of *C. fioriniae*-subgroup 2, and three isolates of *C. fioriniae*-subgroup 1 were selected for phylogenetic analysis (Table 1). All of them were grown on PDA at 25°C for 8 days in the dark. Single agar plugs containing actively growing mycelium were transferred to 250-ml flasks containing 40 ml of potato dextrose broth. Flasks were shaken at 120 rpm for 3 days at 25°C. Mycelium was filtered from the broth, rinsed under sterile deionized water, and subjected to DNA extraction using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS) were amplified by primer set ITS-1F

(Gardes and Bruns 1993) + ITS-4 (White et al. 1990). A 240-bp intron of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified by primer set GDF1 + GDR1 (Guerber et al. 2003), and a 540-bp fragment of β -tubulin (*TUB2*) was amplified and sequenced using the primer pair Btub2Fd 5'-GTB CAC CTY CAR ACC GGY CAR TG-3' and Btub4Rd (5'-CCR GAY TGR CCR AAR ACR AAG TTG TC-3') (Woudenberg et al. 2009). Amplifications were performed using a T100 Bio-Rad thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) in a total volume of 25 µl. The polymerase chain reaction (PCR) mixtures contained 1× PCR buffer, 20 ng of template DNA, 0.6 µM each primer, 200 µM each dNTP, and 1 U of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). Conditions for PCR of ITS regions constituted an initial denaturation step of 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C. For the *GAPDH* region, the 35 cycles consisted of 30 s at 94°C, 1 min at 60°C, and 30 s at 72°C; for the *TUB* region, cycles consisted of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C. Sequencing was conducted at the DNA Core Laboratory at Arizona State University (Tempe AZ).

Phylogenetic analysis. Phylogenetic analysis was conducted for the combined data matrix of the three loci (ITS, *GAPDH*, and *TUB2*). Multiple alignments were conducted using DNASTAR (DNASTAR Inc., Nevada City, CA) and CLUSTAL X v. 2.1 (Larkin et al. 2007; Thompson et al. 1997). ITS, *GAPDH*, and *TUB2* nucleotide sequences of *C. acutatum* species complex were obtained from GenBank as reference sequences, including those for *C. nymphaeae*, *C. guajavae*, *C. scovillei*, *C. chrysanthemi*, *C. walleri*, *C. simmondsii*, *C. brisbanense*, *C. paxtonii*, *C. sloanei*, *C. indonesiense*, *C. laticiphillum*, *C. lupini*, *C. melonis*, *C. tamarilloi*, *C. limeticicola*, *C. acutatum*, *C. fioriniae*, *C. salicis*, *C. johnstonii*, *C. pyricola*, *C. godetiae*, *C. australe*, *C. acerbum*, *C. rhombiforme*, *C. kinghornii*, *C. phormli*, and *C. orchidophilum* (Table 2). The maximum-parsimony (MP) tree was constructed by Mega 5.0 software (Tamura et al. 2011) using the MP method (Thompson et al. 1997) and computed with the Subtree-Pruning-Regrafting (level = 1). A branch-swapping method with initial trees was generated by random addition (100 reps). A complete deletion option was used to treat gaps or missing data and the reliability of clusters was evaluated by bootstrapping with 1,000 replicates. For the maximum-likelihood (ML) tree, the following settings were

Table 1. Species, name, origin, and year of isolation of *Colletotrichum* isolates from peach used in this study

Species complex or species	Subspecies	Isolates	Origin (county, state)	Year of isolation
<i>Colletotrichum gloeosporioides</i> ^x	<i>C. fruticola</i>	Cg_RR12_3	Spartanburg, SC	2012
		Cg_RR12_1	Chesterfield, SC	2013
		Cg_SE12_1	Spartanburg, SC	2012
	<i>C. siamense</i>	Cg_Ey12_1	Saluda, SC	2012
		Cg_OD13_2	Saluda, SC	2012
		Cg_Ey12_10	Aiken, SC	2012
<i>C. truncatum</i> ^y		Ct_RR13_1	Saluda, SC	2013
		Ct_RR13_2	Saluda, SC	2013
<i>C. acutatum</i> ^z	<i>C. nymphaeae</i>	CaPH40	Spartanburg, SC	2004
		CaCO4_35	Unknown, SC	2004
		CaPH44	Spartanburg, SC	2004
		CaCO4_42	Unknown, SC	2004
		CaCO4_59	Unknown, SC	2004
	<i>C. fioriniae</i> -subgroup 2	C.2.4.2	Unknown, GA	2015
		C.2.2.2	Unknown, GA	2015
		C.2.3.2	Unknown, GA	2015
		C.3.3	Unknown, GA	2015
		C.5.4	Unknown, GA	2015
		C.2.1.1	Unknown, GA	2015
		C.5.1	Unknown, GA	2015
	<i>C. fioriniae</i> -subgroup 1	CaEY12_2	Spartanburg, SC	2004
		CaEY12_1	Spartanburg, SC	2004
		CaPH4	Spartanburg, SC	2004

^x Refer to Hu et al. (2015b).

^y Refer to Grabke et al. (2015).

^z Identified by internal transcribed spacer (ITS)1-5.8S-ITS2, glyceraldehyde-3-phosphate dehydrogenase, and β -tubulin sequencing in this study.

used (Hu et al. 2015b): the Tamura three-parameter model, γ distributed (five categories), and heuristic method using SPR-extensive. The bootstrap consensus tree, inferred from 1,000 replicates, was constructed.

Fungicide sensitivity assessments in vitro. DMI sensitivity assays were based on methods developed by Miller et al. (2002). Isolates were cultured on PDA plates at 30°C for 5 days (*C. siamense*, *C. fructicola*, and *C. truncatum* isolates) and 25°C for 8 days (*C. nymphaeae* and *C. fiorinae* isolates) in darkness to supply inoculum. Mycelial discs (4 mm in diameter) from the periphery of colonies actively growing on PDA were transferred onto PDA medium containing DMI fungicides with the active ingredient at 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 $\mu\text{g/ml}$. The commercial formulations of difenoconazole, propiconazole, tebuconazole, metconazole, flutriafol, and fenbuconazole were diluted with sterilized distilled water in a series and added to autoclaved PDA medium cooled to 60°C.

Cultures were incubated at 30°C (*C. siamense*, *C. fructicola*, and *C. truncatum*) and 25°C (*C. nymphaeae* and *C. fiorinae*) without

supplemental lighting, and radial growth of colonies was measured when colony diameters of cultures on control dishes reached at least 50 mm (4 days for *C. siamense*, *C. fructicola*, and *C. truncatum* or 8 days for *C. nymphaeae* and *C. fiorinae*). The diameter of each colony was measured in two perpendicular directions and the mean diameter was adjusted by subtracting the diameter of the original plugs used for inoculation.

Fungicide sensitivity, as measured by the 50% effective concentration (EC_{50}) value, was calculated as described by Wong and Wilcox (2002). Briefly, the percent relative growth (RG) was calculated as (radial growth at fungicide concentration/radial growth on the nonamended check plate) $\times$ 100. The EC_{50} value was estimated by linear regression of the probit-transformed relative inhibition (RI) value ($\text{RI} = 1 - \text{RG}$) on $\log_{10}$ transformed-fungicide concentration. The EC_{50} value for each isolate was calculated as the mean of the three replicates.

To measure cross-resistance between pairs of the fungicides tested, the $\log \text{EC}_{50}$ values were correlated and the correlation coefficient

Table 2. GenBank accession numbers for internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -tubulin (*TUB2*) nucleotide sequences from *Colletotrichum* isolates used in this study

Species	Culture ^z	Source	GenBank accession number ^y		
			ITS	<i>GAPDH</i>	<i>TUB2</i>
<i>Colletotrichum nymphaeae</i>	CaPH40	This study	KX066092	KX066095	KX066089
	CaCO4_35	This study	NA	NA	NA
	CaPH44	This study	NA	NA	NA
	CaCO4_42	This study	NA	NA	NA
	CaCO4_59	This study	NA	NA	NA
<i>C. fiorinae</i> -subgroup 2	C.2.4.2	This study	KX066091	KX066094	KX066088
	C.2.2.2	This study	NA	NA	NA
	C.2.3.2	This study	NA	NA	NA
	C.3.3	This study	NA	NA	NA
	C.5.4	This study	NA	NA	NA
	C.2.1.1	This study	NA	NA	NA
<i>C. fiorinae</i> -subgroup 1	C.5.1	This study	NA	NA	NA
	CaEY12_1	This study	KX066093	KX066096	KX066090
	CaEY12_2	This study	NA	NA	NA
	CaPH4	This study	NA	NA	NA
<i>C. nymphaeae</i>	CBS 100064	GenBank	JQ948224	JQ948554	JQ949875
<i>C. guajavae</i>	IMI 350838, CPC 18893	GenBank	JQ948270	JQ948600	JQ949921
<i>C. scovillei</i>	CBS 126529	GenBank	JQ948267	JQ948597	JQ949918
<i>C. chrysanthemi</i>	IMI 36454, CPC 18930	GenBank	JQ948273	JQ948603	JQ949924
<i>C. walleri</i>	CBS 125472	GenBank	JQ948275	JQ948605	JQ949926
<i>C. simmondsii</i>	CBS 294.67	GenBank	JQ948277	JQ948607	JQ949928
<i>C. brisbanense</i>	CBS 292.67	GenBank	JQ948291	JQ948621	JQ949942
<i>C. paxtonii</i>	CBS 502.97	GenBank	JQ948286	JQ948616	JQ949937
<i>C. sloanei</i>	IMI 364297, CPC 18929	GenBank	JQ948287	JQ948617	JQ949938
<i>C. indonesiense</i>	CBS 127551	GenBank	JQ948288	JQ948618	JQ949939
<i>C. laticiphllum</i>	CBS 112989	GenBank	JQ948289	JQ948619	JQ949940
<i>C. lupini</i>	IMI 35126, CPC 18896	GenBank	JQ948177	JQ948507	JQ949828
<i>C. melonis</i>	CBS 159.84	GenBank	JQ948194	JQ948524	JQ949845
<i>C. tamarilloi</i>	CBS 129814	GenBank	JQ948184	JQ948514	JQ949835
<i>C. limetticola</i>	CBS 114.14	GenBank	JQ948193	JQ948523	JQ949844
<i>C. acutatum</i>	IMI 223120, CPC 18870	GenBank	JQ948353	JQ948684	JQ950004
<i>C. fiorinae</i> -subgroup 1	CBS 125396	GenBank	JQ948299	JQ948629	JQ949950
<i>C. fiorinae</i> -subgroup 2	CBS 119292	GenBank	JQ948313	JQ948643	JQ949964
<i>C. salicis</i>	CBS 129972	GenBank	JQ948466	JQ948797	JQ950117
<i>C. johnstonii</i>	IMI 357027	GenBank	JQ948443	JQ948774	JQ950094
<i>C. pyricola</i>	CBS 128531	GenBank	JQ948445	JQ948776	JQ950096
<i>C. godetiae</i>	CBS 796.72	GenBank	JQ948407	JQ948738	JQ950058
<i>C. australe</i>	CBS 116478	GenBank	JQ948455	JQ948786	JQ950106
<i>C. acerbum</i>	CBS 128530	GenBank	JQ948459	JQ948790	JQ950110
<i>C. rhombiforme</i>	CBS 129953	GenBank	JQ948457	JQ948788	JQ950108
<i>C. kinghornii</i>	CBS 198.35	GenBank	JQ948454	JQ948785	JQ950105
<i>C. phormii</i>	CBS 118201	GenBank	JQ948449	JQ948780	JQ950100
<i>C. orchidophilum</i>	CBS 631.80	GenBank	JQ948152	JQ948482	JQ949803

^y NA = not applicable.

^z CBS = culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; and IMI = culture collection of CABI Europe UK Centre, Egham, UK.

strong, with R^2 of 0.8 to 1 ($P < 0.05$). For other fungicide combinations, the correlation coefficients were lower, with R^2 values of 0.1 to 0.6 ($P > 0.05$). For *C. fioriniae*-subgroup 1, the correlation in sensitivities was strong between propiconazole and tebuconazole, metconazole, and difenoconazole; between difenoconazole and tebuconazole, metconazole, flutriafol, and fenbuconazole; and also between flutriafol and metconazole (R^2 values of 0.7 to 1.0, $P < 0.05$). For *C. fioriniae*-subgroup 2, strong relationships were found in all fungicide interactions except for fenbuconazole and propiconazole.

Discussion

Multilocus molecular phylogenetic analysis conducted on the basis of ITS, *ACT*, *TUB2*, chitin synthase (*CHS-1*), *GAPDH*, and histone 3 (*HIS3*) gene sequences delimited 31 species within the *C. acutatum* species complex (Damm et al. 2012). Further analysis determined that all subclades can be recognized with *TUB2* and *GAPDH* sequences alone (Damm et al. 2012). In our study, the combined sequence data of ITS, *TUB2*, and *G3PDH* clearly identified *C. acutatum* isolates to belong to either *C. nymphaeae* or *C. fioriniae*. With the discovery of these additional two *C. acutatum* species, the total number of *Colletotrichum* spp. known to cause anthracnose of peach in the Southeast climbed to five.

Both *C. nymphaeae* and *C. fioriniae* do have a wide host range. *C. nymphaeae* is a pathogen of strawberry fruit (Damm et al. 2012) but can also infect kadsura, gentianaceae, loquat, flowering plants, celery, apple, hoary stock, parsley, plum, peach, mung bean (Sato and Moriawaki 2013), and blueberry (MacKenzie et al. 2009). *C. fioriniae* was isolated from almond, apple, avocado, mango, nectarine (Guerber et al. 2003), and blueberry (MacKenzie et al. 2009).

Within *C. fioriniae*, there is evidence of heterogeneity, because two subclades were apparent in the phylogenetic analysis of this study and one previous study (Damm et al. 2012). All of the isolates collected

in 2015 from Georgia formed into the same clade of *C. fioriniae*-subgroup 2. Isolates of *C. fioriniae* subgroup 1 were collected from South Carolina. Prior to this study, phenotypical differences between the two subgroups were unknown. The sensitivity of subgroup 1 and 2 to difenoconazole, propiconazole, flutriafol, and fenbuconazole were significantly different (Table 3). Isolates from subgroup 2 were more sensitive to polyoxin-D (7 µg/ml average) compared with subgroup 1 (EC₅₀ values > 100 µg/ml) on malt extract agar medium ($P < 0.05$, data not shown). However, neither bootstrap support nor Bayesian probability values were sufficiently high to divide them into different species, and strains from different hosts and countries did not cluster together (Damm et al. 2012).

Species of *Colletotrichum* may exhibit striking differences to fungicides. For example, most wild-type *C. gloeosporioides* isolates are highly sensitive to benomyl and thiophanate-methyl, which is why the latter is still recommended for the control of anthracnose crown rot (Brannen and Smith 2014). However, *C. acutatum* is inherently resistant to benomyl and other MBC fungicides (Adaskaveg and Hartin 1997; Peres et al. 2002, 2004; Talhinhas et al. 2002, 2005). In our study, *C. truncatum* exhibited exceptional resilience to tebuconazole, metconazole, flutriafol, and fenbuconazole. Luckily, this species is an extremely rare pathogen of peach. To the best of our knowledge, the sensitivity of plant-pathogenic *C. truncatum* to DMI fungicides has never been investigated and, thus, we are unable to compare our results with other studies. However, a case of *C. truncatum* species complex fungal keratitis and endophthalmitis has been reported in an immunocompetent human male in whom oral triazole antifungals were contraindicated (Squizzato et al. 2015). Similar to our study, *C. truncatum* showed high minimum inhibitory concentrations and probable resistance to triazoles used in human medicine, including voriconazole, ketoconazole, itraconazole, and miconazole.

Table 3. Mean 50% effective concentration (EC₅₀) values of *Colletotrichum* isolates to demethylation inhibitor fungicides

Species ^z	Mean EC ₅₀ values (µg/ml) ^y					
	Difen	Propi	Tebuco	Metco	Flutri	Fenbu
<i>Colletotrichum fruticola</i>	0.9 ± 0.5 b	0.2 ± 0.1 c	0.8 ± 0.1 b	0.8 ± 0.4 b	13.1 ± 12.0 ab	3.3 ± 1.3 a
<i>C. siamense</i>	0.3 ± 0.3 c	0.2 ± 0.1 c	1.1 ± 0.4 b	0.6 ± 0.2 b	4.1 ± 0.6 b	3.4 ± 2.3 a
<i>C. truncatum</i>	2.7 ± 0.3 a	2.4 ± 0.5 ab	54.7 ± 15.0 a	16.6 ± 0.3 a	>100	>100
<i>C. nymphaeae</i>	1.3 ± 0.3 b	2.5 ± 0.7 a	0.8 ± 0.4 b	1.4 ± 0.9 b	>100	>100
<i>C. fioriniae</i> -subgroup 2	0.5 ± 0.1 b	1.2 ± 0.7 b	0.5 ± 0.1 b	0.5 ± 0.4 b	16.2 ± 8.5 a	5.2 ± 4.2 a
<i>C. fioriniae</i> -subgroup 1	0.2 ± 0.2 c	0.3 ± 0.2 c	0.2 ± 0.1 b	0.03 ± 0.05 b	2.1 ± 1.3 b	0.4 ± 0.3 b

^y Mean values within columns followed by the same letter are equivalent based on Fisher's protected least significant difference ($P = 0.05$). Difen = difenoconazole, Propi = propiconazole, Tebuco = tebuconazole, Metco = metconazole, Flutri = flutriafol, and Fenbu = fenbuconazole.

^z Mean and standard error from the average of three isolates for each species, except *C. truncatum*, for which only two isolates were collected.

Table 4. Cross-sensitivity between demethylation inhibitor fungicides for *Colletotrichum siamense*, *C. fruticola*, *C. nymphaeae*, and *C. fioriniae*

Interaction ^z	R^2 ^y				
	<i>C. siamense</i>	<i>C. fruticola</i>	<i>C. nymphaeae</i>	<i>C. fioriniae</i> -subgroup 1	<i>C. fioriniae</i> -subgroup 2
Tebuco-Propi	0.6	0.5	0.1	1*	1*
Metco-Tebuco	0.7*	0.1	0.6	0.6	1*
Metco-Propi	0.1	0.2	0.2	0.7*	0.9*
Difen-Tebuco	0.2	0.9*	0.1	0.7*	0.9*
Difen-Propi	0.8*	0.9*	1*	0.8*	0.8*
Difen-Metco	0.1	0	0.2	1*	1*
Flutri-Tebuco	0.3	0.3	0.4	0.3	1*
Flutri-Propi	0	0.1	0.8	0.4	0.9*
Flutri-Metco	0.9*	0.9*	0	0.9*	1*
Flutri-Difen	0.2	0	0.8*	0.8*	1*
Fenbu-Tebuco	0	0.3	0.3	0.6	0.6
Fenbu-Propi	0.3	0.9*	0.8*	0.5	0.4
Fenbu-Metco	0.4	0	0	0	0.8*
Fenbu-Difen	0.7*	0.7*	0.8*	0.9*	0.9*
Fenbu-Flutri	0.8*	0.2	1*	0.1	0.7*

^y Coefficient of determination (R^2) determined by comparison of log 50% effective concentration values for each fungicide from the individual *Colletotrichum* spp.; an asterisk (*) indicates correlation coefficient significant at $P = 0.05$.

^z Difen = difenoconazole, Propi = propiconazole, Tebuco = tebuconazole, Metco = metconazole, Flutri = flutriafol, and Fenbu = fenbuconazole.

Isolates of the five *Colletotrichum* spp. included in this study revealed EC₅₀ values of 0.2 to 2.7 µg/ml for difenoconazole and propiconazole, 0.03 to 54.7 µg/ml for tebuconazole and metconazole, and 0.4 to more than 100 µg/ml for flutriafol and fenbuconazole. These values indicate that the sensitivity to difenoconazole and propiconazole of *Colletotrichum* spp. was consistently high. Differences in intrinsic activities between four DMI fungicides were also found in *C. cereale* (Wong and Wilcox 2002). Regardless of previous exposure to DMI fungicides, *C. cereale* isolates were most sensitive to tebuconazole and propiconazole, less sensitive to myclobutanil, and least sensitive to triadimefon. The authors reported that the in vitro intrinsic activity corresponded to the performance of these DMI fungicides for anthracnose control in the field (Towers et al. 2003). Our study showed higher EC₅₀ values to fenbuconazole for almost all the *Colletotrichum* isolates (Table 3), which indicated that fenbuconazole should not be relied on for the control of *Colletotrichum* spp. This was in agreement with a previous study, where fenbuconazole was found to result in higher anthracnose fruit rot incidence caused by *C. gloeosporioides* isolates in cranberry (Wells et al. 2014). Practical implications of differential activity of DMI fungicides to target organisms was discussed previously (Erickson and Wilcox 1997; Ypema et al. 1997). DMI fungicides with lower EC₅₀ values provided better protection from *Uncinula necator* (syn. *Erysiphe necator*) compared with DMI fungicides with higher EC₅₀ values in field trials (Erickson and Wilcox 1997; Ypema et al. 1997). Based on the EC₅₀ values determined in this study, difenoconazole and propiconazole may provide better control efficacy in the field compared with tebuconazole and metconazole against most *Colletotrichum* spp. However, this would need to be verified in field studies. Our data also imply that fenbuconazole and flutriafol may have the lowest control efficacy and, thus, may not provide sufficient protection from peach anthracnose.

Correlation coefficients among difenoconazole, propiconazole, tebuconazole, metconazole, flutriafol, and fenbuconazole varied for different *Colletotrichum* spp. (Table 4). This is consistent with results reported for other pathogens such as *Sclerotinia homoeocarpa* (Hsiang et al. 1997; Miller et al. 2002), *Cercospora beticola* (Karaoglanidis and Thanassouloupoulos 2003), *U. necator* (Erickson and Wilcox 1997; Ypema et al. 1997), *Monilinia fructicola* (Holb and Schnabel 2007), and *Rhynchosporium secalis* (Kendall et al. 1993)

Fig. 2. The 50% effective concentration EC₅₀ values of *Colletotrichum* spp. for difenoconazole (Difen), propiconazole (Propi), tebuconazole (Tebuco), metconazole (Metco), flutriafol (Flutri), and fenbuconazole (Fenbu). *Colletotrichum fructicola* was represented by isolates Cg_RR12_3, Cg_RR12_1, and Cg_SE12_1; *C. siamense* was represented by isolates Cg_Ey12_1, Cg_OD13_2, and Cg_Ey12_10; *C. truncatum* was represented by isolates Ct_RR13_1 and Ct_RR13_2; *C. nymphaeae* was represented by isolates CaPH40, CaCO4_35, and CaPH44; *C. fioriniae*-subgroup 2 was represented by isolates C.2.4.2, C.2.3.2, and C.2.2.2; and *C. fioriniae*-subgroup 1 was represented by isolates CaEY12_2, CaEY12_1, and CaPH4.

to DMI fungicides. Low correlation coefficients in some DMI fungicide interactions may indicate the resistance to be a multigenetic trait or may indicate the resistance to be a single gene trait with different mutations involved, each of which may confer resistance to specific DMI (Fraaije et al. 2007). Some correlation coefficient among the DMI were as low as 0.1 and some as high as 1, indicating that cross-resistance relationships among DMI can vary dramatically, and that no single model of cross-resistance among DMI is appropriate among all *Colletotrichum* spp., which has also been demonstrated in *Pyrenophora teres* (Peever and Milgroom 1993) and *U. necator* (Erickson and Wilcox 1997).

Our study indicates that pathogens causing peach anthracnose display differential sensitivity to DMI fungicides; however, the molecular basis is unknown. One potential mechanism of resistance to DMI fungicides involves point mutations within the substrate recognition site of the P450 demethylation inhibitor (*CYP51*) genes. Examples include the pathogenic fungi *U. necator* (Délye et al. 1997), *Blumeria graminis* (Wyand and Brown 2005), *E. graminis* (Délye et al. 1998), and *Candida albicans* (Favre et al. 1999; Löffler et al. 1997; Marichal et al. 1999; Sanglard et al. 1998), which exhibited alterations in the sequence of *CYP51* genes in resistant isolates. Increased expression of the *CYP51* gene is another potential mechanism of DMI fungicide resistance, which has been reported in *Bulbophyllum jaapii* (Ma et al. 2006), *Penicillium digitatum* (Ghosop et al. 2007; Hamamoto et al. 2000; Sun et al. 2011), *M. fructicola* (Luo and Schnabel 2008), *Venturia inaequalis* (Schnabel and Jones 2001), *Cercospora beticola* (Nikou et al. 2009), and *Mycosphaerella graminicola* (Cools et al. 2012). Expression of ABC transporters was also reported in resistant strains of *Aspergillus nidulas* and *P. digitatum* (Del Sorbo et al. 1997; Nakaune et al. 1998).

This study indicates that at least five *Colletotrichum* spp. occur on peach fruit in South Carolina and Georgia and that *Colletotrichum nymphaeae* and *C. truncatum* are inherently resistant to DMI fungicides. Thus, the importance for practitioners to know the species involved in causing anthracnose disease was underscored by our study. DMI fungicides primarily used for preharvest brown rot control that also have high intrinsic activity to *Colletotrichum* spp. may increase control success for anthracnose disease management if integrated into the summer spray program. However, this would need to be verified in field trials.

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