Two Mutations in β -Tubulin 2 Gene Associated with Thiophanate-Methyl Resistance in *Colletotrichum cereale* Isolates from Creeping Bentgrass in Mississippi and Alabama

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ABSTRACT

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Turfgrass anthracnose, caused by *Colletotrichum cereale* ($\equiv$ *C. graminicola*), has become a common disease of creeping bentgrass putting greens during the summer in Mississippi and Alabama over the last 15 years. Thiophanate-methyl is a single-site mode-of-action fungicide applied to control *C. cereale*. In vitro bioassays were performed to evaluate the sensitivity of 103 isolates to thiophanate-methyl concentrations ranging from 0.039 to 10 μ g/ml. Eighty-three isolates were collected from creeping bentgrass in Mississippi and Alabama that had been exposed to thiophanate-methyl. An additional 20 isolates were included from nonexposed turfgrasses. Radial colony growth in amended media was relative to nonamended media for all in vitro bioassays. With thiophanate-methyl at 10 μ g/ml, relative growth of exposed isolates ranged from 77.5 to 130.7% with a mean of 99.3% compared with nonexposed, baseline isolates that ranged from 0.0 to 48.7% with a mean of 20.4%. A representative sample of thiophanate-methyl-exposed and nonexposed isolates was used to determine the mechanism of resistance by comparing amino acid sequences of the β -tubulin 2 protein. All of the thiophanate-methyl-exposed isolates that were sequenced had a point mutation resulting in substitutions from glutamic acid to alanine at position 198 or from phenylalanine to tyrosine at position 200 of the β -tubulin 2 protein. These amino acid substitutions in *C. cereale* isolates from Mississippi and Alabama appear to confer resistance to thiophanate-methyl and differ from those reported previously for this pathogen.

Turfgrass anthracnose, incited by *Colletotrichum cereale* Manns ($\equiv$ *C. graminicola* (Ces.) G.W. Wilson) (10), is a destructive disease of creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.). The disease has been well documented on annual bluegrass golf course putting greens or fairways throughout the United States (2,13,15,33,34); however, there is limited information available describing anthracnose on creeping bentgrass in the southern United States. *C. cereale* infects annual bluegrass year round, causing symptoms of foliar blight or basal rot when conducive environmental conditions occur. Regionally, the pathogen causes foliar blight on creeping bentgrass predominately in the summer

during periods of heat stress (29). The symptoms of anthracnose on creeping bentgrass putting green turf in Mississippi and Alabama include large, irregularly shaped thin areas appearing chlorotic to bronze (37).

Golf course superintendents in Mississippi and Alabama that manage creeping bentgrass putting greens initiate a preventive fungicide program in April to control anthracnose. Fungicides are applied on a biweekly basis through September or October, depending upon the environmental conditions. As a general means of disease prevention, single-site mode-of-action fungicides are applied alone or tank-mixed with multi-site mode-of-action fungicides such as chlorothalonil and mancozeb. Benzimidazoles, strobilurins, dicarboximides, and demethylation inhibitors are groups of single-site mode-of-action fungicides that are labeled for anthracnose control. However, repetitive applications of single-site mode-of-action fungicides to putting greens have resulted in increased selection pressure for fungicide resistance, as has been widely documented for several turf pathogens (2,4,16,32,33).

Benzimidazole fungicides have been applied to highly managed golf turf in the southeastern United States for over 30 years but anthracnose severity on creeping bentgrass putting greens appears to have

been increasing over the last 15 years. Cultural practices such as lower mowing heights, decreased nitrogen fertility, and reduced watering practices may have contributed to increased anthracnose incidence and severity on creeping bentgrass putting greens (8) but reduced fungicide efficacy may also be contributory.

The benzimidazole fungicides inhibit microtubule production by binding to the heterodimeric subunits of the tubulin molecule (26). Benzimidazole-resistant isolates of many fungi have been identified from a variety of crops. These isolates were determined to be resistant due to missense point mutations in DNA that result in amino acid exchanges in the β -tubulin 2 protein (5,7,12,14,17,18,21–23,26,28,35). According to a Fungicide Resistance Action Committee publication (1), there are four amino acid substitutions in the β -tubulin 2 protein that can predispose fungi to develop resistance to benzimidazole chemistries following excessive, repeated applications to a single population. At amino acid position 198, alanine (Ala), glycine (Gly), or lysine (Lys) is exchanged for glutamic acid (Glu); or, at amino acid position 200, tyrosine (Tyr) is exchanged for phenylalanine (Phe). Fungal isolates expressing one of these substitutions will fail to respond to benzimidazole fungicides due to a decreased binding affinity to the specific site within the gene leading to decreased disease control.

Detweiler et al. (13) reported that *C. cereale* isolates collected from annual bluegrass in Michigan as early as 1989 were resistant to benzimidazoles using in vitro and in vivo bioassays. *C. cereale* isolates collected from annual bluegrass in California have also been determined to be resistant to benzimidazoles following repeated exposure to this fungicide class using in vitro bioassays, in vivo studies, and molecular techniques to identify nucleotide missense point mutations associated with the phenotypes (33). All *C. cereale* isolates identified as thiophanate-methyl resistant had Lys substituted for Glu at amino acid position 198 of the β -tubulin 2 protein (33).

Based on the frequency of benzimidazole use for anthracnose control in the southeastern United States and the presence of resistance to this fungicide group in *C. cereale* populations, a study was

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initiated to determine whether *C. cereale* isolates causing anthracnose on creeping bentgrass putting greens in Mississippi and Alabama were resistant to the benzimidazole fungicide thiophanate-methyl. The specific objectives included (i) evaluating *C. cereale* isolates for resistance to thiophanate-methyl using in vitro bioassays and (ii) obtaining nucleotide sequences of the β -tubulin 2 gene to investigate any missense point mutations resulting in amino acid substitutions.

MATERIALS AND METHODS

Isolate collection. *C. cereale* was isolated from creeping bentgrass putting greens in Mississippi and Alabama that exhibited symptoms of anthracnose or heat stress during the summer months of 2006 and 2007 (Table 1) (36). Individual plants exhibiting signs (acervuli and setae) of *C. cereale* were surface disinfested by rinsing in 70% ethanol for 1 min followed by an agitated rinse in 0.6% sodium hypochlorite solution for 90 s, rinsed with sterile distilled water three times, and dried on sterile filter paper in a laminar flow hood (3,20). In all, 100 plants (shoots and roots) from each location were transferred (5 per plate) onto a modified potato dextrose agar (m-PDA) (6 g of PDA and 15 g of agar per liter amended with 100 mg each of chloramphenicol and streptomycin sulfate). The plants were incubated at room temperature (approximately 22°C) on a bench top under 24-h fluorescent light for 4 to 7 days. Fungal colonies producing acervuli, setae, and conidial masses characteristic of *C. cereale* were transferred to fresh m-PDA and incubated as previously described for 21 days. Monoconidial isolates were subsequently obtained through serial dilution. Individual germinating conidia were transferred to m-PDA and incubated as previously described. *C. cereale* isolates were grown on sterilized glass fiber filter paper (Fisher Scientific, Pittsburg) that was overlaid on PDA (39 g/liter), dried, and subsequently stored at

–20°C for long-term storage. Additionally, baseline isolates (never exposed to thiophanate-methyl) were recovered from turf-type tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh) research plots at Mississippi State University and were obtained from Dr. F. Wong, University of California, Riverside (33,34) (Table 1).

In vitro bioassay. Technical-grade thiophanate-methyl (97% active; Cleary Chemical Corporation, Dayton, NJ) was dissolved in acetone to obtain stock solution at 10,000 μ g/ml. Serial fourfold dilutions in acetone were used to obtain stock concentrations of thiophanate-methyl at 2,500, 625, 156, and 39 μ g/ml. In vitro bioassays were performed on m-PDA amended with 1 ml of stock concentrations to obtain final concentrations of thiophanate-methyl at 0 (1 ml of acetone), 0.039, 0.156, 0.625, 2.5, and 10 μ g/ml. The 10 μ g/ml treatment was used as the discriminatory dose at which sensitive or resistant isolates were identified (27,33). A select group of *C. cereale* isolates was also evaluated with thiophanate-methyl-amended medium at 100 μ g/ml.

All isolates were prepared by removing a 5-mm-diameter hyphal plug from the leading edge of a 10-day-old culture and placing it mycelium-side-down in the center of thiophanate-methyl-amended media. Following a 6-day incubation in the dark, three random measurements of colony diameter (mm) were recorded for each isolate to determine percent relative growth. Percent relative growth was calculated by dividing average radial colony growth on thiophanate-methyl-amended media by average radial colony growth on nonamended media multiplied by 100.

The effective concentration required to achieve 50% colony growth inhibition (EC_{50}) on thiophanate-methyl-amended media was calculated for isolates evaluated in in vitro bioassays. Percent relative growth for an individual isolate at all thiophanate-methyl-concentrations was subjected to the regression procedure (PROC REG) of SAS (version 9.1; SAS Institute,

Cary, NC) to obtain the intercept (b_0) and slope (b_1). The EC_{50} value (μ g/ml) for each isolate was calculated using the equation $\text{thiophanate-methyl}_{50\%} = e[(50 - b_0)/b_1]$.

The bioassays were conducted each year, repeated, and data were pooled for each isolate.

Sequence analysis of β -tubulin genes.

The β -tubulin 2 genes from 27 *C. cereale* isolates, including 23 thiophanate-methyl-resistant and 4 thiophanate-methyl-sensitive isolates based on bioassay results, were sequenced to identify point mutations leading to amino acid substitutions that confirm thiophanate-methyl resistance. Fresh mycelium was lyophilized for genomic DNA extraction using the protocol described in the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and stored at –20°C.

Polymerase chain reaction (PCR) was used to amplify two subunits of the β -tubulin gene, β -tubulin 1 (*TUB-1*) (556 bp) and β -tubulin 2 (*TUB-2*) (500 bp), for each isolate. The primers used for PCR were those described by Wong et al. (33). PCR amplification employed in 2006 included 2 μ l of extracted genomic DNA combined with PCR reagent concentrations recommended with the *Taq* DNA Polymerase with Thermopol Buffer (New England Biolabs, Ipswich, MA), resulting in a final volume of 22 μ l. In 2007, PCR amplification was prepared using the GoTaq PCR Core System (Promega Corp., Madison, WI). All PCR reactions were amplified using the following thermal cycling program: denaturation step at 95°C for 5 min; followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 10 min. The amplicon was visualized by performing electrophoresis in a 1.5% agarose gel to ensure a single product. Excess primer or dNTPs in solution were removed using ExoSAP-IT (USB Corporation, Cleveland) according to the manufacturer's instructions.

PCR amplicons were sequenced at the University of California, Riverside Genomics Core Sequencing facility using Big Dye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA) in 2006. In 2007, PCR amplicons were prepared for sequencing using the DTCS Quick Start Kit (Beckman and Coulter, Fullerton, CA) and sequenced at the Life Sciences and Biotechnology Institute at Mississippi State University using the Beckman CEQ 8000 DNA Analysis System (Beckman and Coulter). Resultant nucleotide sequences were translated, aligned, and analyzed using DNASTAR Lasergene Software (DNASTAR Inc., Madison, WI). All isolates were sequenced twice.

RESULTS

In vitro bioassay. Radial colony growth of all 83 *C. cereale* isolates collected from fungicide-treated creeping bentgrass putting greens in Mississippi and Alabama

Table 1. *Colletotrichum cereale* isolates collected in 2006 and 2007 from creeping bentgrass golf course putting greens and turf-type tall fescue in Mississippi and Alabama and baseline isolates from California

Year	City, state	Population code	No. of isolates
2006	West Point, MS	OW 15	30
2006	Birmingham, AL	OO 4	7
2006	Birmingham, AL	OO 7	9
2006	Sylacauga, AL	FL	2
2007	West Point, MS	OW 15	6
2007	West Point, MS	OW 16	7
2007	Birmingham, AL	OO 7	1
2007	Inverness, AL	GS 4	6
2007	Olive Branch, MS	PGC PG	2
2007	Tupelo, MS	BO 6	12
2007	Tupelo, MS	BO 12	1
2007	Starkville, MS	TF ^a	3
2002	Temecula, CA	TCGC 5 ^b	17

^a *Colletotrichum cereale* isolates not exposed to thiophanate-methyl were recovered from tall fescue grown at the Rodney R. Foil Research Center at Mississippi State University.

^b *C. cereale* isolates courtesy of Dr. F. Wong, University of California, Riverside (33,34).

was classified as resistant to thiophanate-methyl at the discriminatory dose of 10 $\mu\text{g/ml}$. Relative growth of these isolates ranged from 77.5 to 130.7% with a mean of 99.3%. In contrast, relative growth of sensitive isolates ranged from 0.0 to 48.7% with a mean of 20.4% with thiophanate-methyl at 10 $\mu\text{g/ml}$.

Relative growth of resistant isolates was similar across all concentrations, whereas sensitive isolates exhibited reduced colony growth with thiophanate-methyl at 0.625 $\mu\text{g/ml}$ and higher (Fig. 1A and B). The EC_{50} values for thiophanate-methyl-resistant *C. cereale* isolates were considered to be greater than 10 $\mu\text{g/ml}$ due to minimal inhibition at this concentration. Similarly, selected isolates exhibited minimal colony inhibition with thiophanate-methyl at 100 $\mu\text{g/ml}$. However, the EC_{50} of the sensitive isolates for thiophanate-methyl ranged from 0.6 to 8.2 $\mu\text{g/ml}$ with a mean of 3.0 $\mu\text{g/ml}$.

Sequence analysis of β -tubulin genes.

Nucleotide sequences of the *Tub-1* gene of nine *C. cereale* isolates were obtained in 2006 corresponding to a representative sample from each creeping bentgrass putting green sampled. The *TUB-1* sequence corresponded to amino acid positions 180 to 329 (25). No differences between deduced amino acid sequences were observed among these isolates. *C. cereale* isolates sensitive or resistant to thiophanate-methyl from Mississippi and Alabama resulted in nucleotide sequences that were similar, ranging from 94.0 to 99.8% identity. The consensus nucleotide sequence of *TUB-1* from these isolates was 98% identical to the *TUB-1* sequences obtained from *C. cereale* isolates from California (33) and 92% identical to the original nucleotide sequence for *C. graminicola*'s *TUB-1* (accession no. M34491). The locations where nucleotide sequences differed between resistant and sensitive *C. cereale* isolates ultimately coded for the same amino acid (data not shown). *TUB-1* was not sequenced for isolates in 2007 due to the similarity of sequences and lack of consistent missense point mutations in resistant isolates compared with sensitive isolates in 2006.

Nucleotide sequences from 27 isolates (23 thiophanate-methyl-resistant and 4 thiophanate-methyl-sensitive) were obtained from purified PCR product of the *TUB-2* gene. The amplified *TUB-2* nucleotide sequence corresponded to amino acid positions 163 to 303 (25), containing the residues at positions 198 and 200 typically associated with benzimidazole resistance. Based on the translation of nucleotide sequences obtained, 52% of the isolates classified as resistant had an amino acid change from Glu to Ala at position 198 (E198A) (Fig. 2). The remaining resistant isolates had an amino acid change from Phe to Tyr at position 200 (F200Y) (Fig. 2). The four sensitive isolates, including

those from tall fescue, retained Glu and Phe at positions 198 and 200, respectively, similar to the original *C. graminicola* *TUB-2* sequence (accession no. M34492) (Fig. 2). There were no other deduced changes in amino acid sequence in the resistant isolates that were consistent with the observed isolate phenotypes or were typical of those conferring resistance to the benzimidazole fungicides. Nucleotide sequences obtained from isolates were identical when the sequencing was repeated.

Benzimidazole-resistant fungi with substitutions at amino acid positions 198 or 200 have been characterized as intermediately or highly resistant based on EC_{50} values (7,21,35); however, selected *C. cereale* isolates with E198A and F200Y substitutions evaluated in this study were not inhibited with thiophanate-methyl at 100 $\mu\text{g/ml}$, expressing highly resistant responses. Relative growth of isolates with

the E198A exchange ranged from 90.0 to 104.7% with a mean of 98.0% for thiophanate-methyl at 100 $\mu\text{g/ml}$, whereas those with the F200Y substitution ranged from 90.9 to 103.4% with a mean of 95.3% at 100 $\mu\text{g/ml}$. Based on these results, EC_{50} values of both groups of isolates were considered to be greater than 100 $\mu\text{g/ml}$.

DISCUSSION

Thiophanate-methyl-resistant isolates of *C. cereale* originating from creeping bentgrass putting greens in Mississippi and Alabama were identified. *C. cereale* isolates were collected from golf course putting greens where thiophanate-methyl was applied on a regular basis for several years to control turfgrass diseases. Based on the history of thiophanate-methyl use at the golf courses, these results were not unexpected, considering similar reports of *C.*

Fig. 1. Representative sample of the relative growth from thiophanate-methyl-sensitive and -resistant *Colletotrichum cereale* isolates cultured on thiophanate-methyl-amended media at 0.039, 0.156, 0.625, 2.5, and 10 $\mu\text{g/ml}$. Radial growth was recorded after incubating at room temperature (approximately 22°C) in the dark for 6 days. Relative growth was calculated by dividing average radial colony growth on thiophanate-methyl-amended media by average radial colony growth on nonamended media and multiplied by 100. Data points are plotted at the concentrations evaluated with the x-axis on a log scale. **A**, Dose response curve, 2006 isolates. **B**, Dose response curve, 2007 isolates.

cereale resistance occurring at other locations in the United States (6,19,24,30,31). However, this is the first identification of thiophanate-methyl resistance in *C. cereale* isolated from creeping bentgrass putting greens.

The phenotypic response of thiophanate-methyl resistance in *C. cereale* isolates in this study was supported at the molecular level based on methods previously reported on benzimidazole-resistant *C. cereale* isolated from annual bluegrass in

California (33). In this study, two separate amino acid substitutions, E198A and F200Y, were observed in the β -tubulin 2 protein of *C. cereale* isolates that were not inhibited at the discriminatory dose of thiophanate-methyl at 10 μ g/ml. This is the

Isolate ID	GenBank Accession #	170	180	190	200
BO 6 9 (res)	FJ476048	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
BO 6 37 (res)	FJ476049	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
BO 12 3 (res)	FJ476050	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
FL A2-1 (res)	FJ476051	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
FL B3-1 (res)	FJ476052	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
GS 4 22 (res)	FJ476053	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
GS 4 28 (res)	FJ476054	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
OO 4 G1-1 (res)	FJ476055	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OO 4 K1-1 (res)	FJ476056	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OO 4 O1-2 (res)	FJ476057	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OO 7 1 (res)	FJ476058	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
OO 7 E5-1 (res)	FJ476059	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OO 7 R1-1 (res)	FJ476060	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OO 7 R2-1 (res)	FJ476061	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OW 15 52B (res)	FJ476062	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
OW 15 56B (res)	FJ476063	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OW 15 I4b-1 (res)	FJ476064	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OW 15 N5b (res)	FJ476065	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OW 15 R4-1 (res)	FJ476066	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	AT FCIDNE
OW 16 1C (res)	FJ476067	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
OW 16 14B (res)	FJ476068	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
PGC PG 1 (res)	FJ476069	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
PGC PG 4 (res)	FJ476070	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	YCIDNE
TCGC 5-35 (sens)	EU116291	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	FCIDNE
TCGC 5-48 (sens)	GQ121136	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	FCIDNE
TF 1A (sens)	FJ476071	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	FCIDNE
TF 1C (sens)	FJ476072	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	FCIDNE
TUB-2 (GenBank)	M34492	ATFSV VPSPKVSDTV	VEPYNATLSV	HQLVENSDET	FCIDNE

Fig. 2. Amino acid sequences spanning residues 165 to 206 of the β -tubulin 2 protein for 27 *Colletotrichum cereale* isolates identified as resistant (res) or sensitive (sens) to thiophanate-methyl based on in vitro data. These sequences were compared with the original β -tubulin 2 protein in GenBank. The key amino acids are located at positions 198 and 200, and changes are highlighted.

first report of the E198A substitution identified in *C. cereale* and is in contrast to thiophanate-methyl-resistant *C. cereale* isolates from annual bluegrass identified with the amino acid substitution E198K (33). The E198A substitution has been previously reported, however, in thiophanate-methyl-resistant *C. gloeosporioides* (Penz.) Penz. & Sacc., a pathogen infecting various crops (7,23,26).

The F200Y substitution was also observed in some of the thiophanate-methyl-resistant isolates collected in this study. To date, no amino acid substitution at position 200 in the β -tubulin 2 gene of *C. cereale* has been reported; however, two isolates of *C. gloeosporioides* exhibiting the F200Y exchange were previously reported (7). Benzimidazole-resistant fungi exhibiting amino acid substitutions at position 200 or 198 have been characterized as intermediately or highly resistant, respectively, based on EC₅₀ values (7,21,35). Various fungal isolates with an amino acid substitution at position 198 were considered highly resistant (EC₅₀ > 100 μ g/ml), whereas isolates with an amino acid substitution at position 200 were considered to have intermediate resistance (7,21,35). *C. cereale* isolates expressing E198A or F200Y substitutions in this study had similar colony growth with thiophanate-methyl at 10 or 100 μ g/ml. EC₅₀ values among these isolates were > 100 μ g/ml, which were in contrast to previous reports (7,21,35). The results in this study indicate that the phenotypic display of colony growth among the Mississippi and Alabama isolates differed from those of other pathogens.

Key differences in amino acid substitutions were observed among *C. cereale* isolates collected from creeping bentgrass putting greens in Mississippi and Alabama compared with previous reports for *C. cereale* infecting annual bluegrass putting greens (33). These differences may be due, in part, to the host or regional location of these isolates. Phylogenetic analysis data were generated by Crouch et al. (11) to separate an extensive group of *Colletotrichum* spp. isolated from numerous warm- and cool-season gramineous species. The study demonstrated the separation of *Colletotrichum* spp., of which three clades primarily contained *C. cereale* isolates from creeping bentgrass or annual bluegrass, making it apparent that the isolates were distinguishable based on host specificity. Geographic regions did not influence the relatedness of *C. cereale* isolates (11). This information led us to hypothesize that the amino acid substitutions observed in *C. cereale* isolates from creeping bentgrass versus amino acid substitutions observed in *C. cereale* isolates from annual bluegrass may be a result of host specificity.

The differences in relative growth based on in vitro bioassays were evident when

comparing sensitive and resistant *C. cereale* isolates at high concentrations of thiophanate-methyl. Sensitive isolates exhibited reduced colony growth at concentrations of thiophanate-methyl ≥ 0.625 μ g/ml. Colony growth inhibition was not observed among isolates considered resistant to thiophanate-methyl at concentrations ≥ 10 μ g/ml. Although the isolates in this study were not tested in planta, they appear to behave similarly to those reported as resistant by Wong et al. (33). The relative growth of these isolates at the discriminatory dose of 10 μ g/ml corresponded to those classified by Wong et al. (33) as resistant. Relative growth values for thiophanate-methyl-sensitive isolates were also consistent with those characterized by Wong et al. (33). The dose response curves (Fig. 1) of selected isolates further illustrate the behavior of all *C. cereale* isolates considered resistant in this study.

Results from this research may not be inclusive of all *C. cereale* isolates within existing populations due to a limited sampling size but thiophanate-methyl-resistant isolates must have been present at high frequencies in the populations sampled. Previously, no data were reported for *C. cereale* isolated from creeping bentgrass putting greens regarding thiophanate-methyl resistance. However, this study identified similar results from in vitro bioassays when compared with what was reported for the pathogen from annual bluegrass (9,13,33). Based on the current research, thorough sampling within these *C. cereale* populations may provide a more accurate measure of the magnitude of resistant versus sensitive isolate occurrence in creeping bentgrass putting greens. The fact that thiophanate-methyl-resistant *C. cereale* isolates are present in creeping bentgrass putting greens in Mississippi and Alabama should raise awareness among golf course superintendents that fungicide resistance management needs to be considered in management programs. Disease management strategies consisting of nitrogen fertility, growth regulators, and vertical mowing that reduced anthracnose on annual bluegrass (15) should be investigated to determine the efficacy of anthracnose control on creeping bentgrass in Mississippi and Alabama.

Based on the documentation of thiophanate-methyl-resistant isolates of *C. cereale* present in creeping bentgrass golf course putting greens in Mississippi and Alabama, fungicide resistance appears to be more pervasive within *C. cereale* populations than previously thought. No sensitive isolates were identified from golf course putting greens with a history of thiophanate-methyl applications. Based on previous reports of a high frequency of fungicide resistance within *C. cereale* populations (33), it is apparent that this trend will continue in highly managed

creeping bentgrass putting greens. Based on the results of this study, it is recommended that proactive disease management strategies be implemented for thiophanate-methyl resistance and resistance to other single-site mode-of-action fungicides.

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