Occurrence and Molecular Identification of Azoxystrobin-Resistant *Colletotrichum cereale* Isolates from Golf Course Putting Greens in the Southern United States

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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 and annual bluegrass putting greens throughout the southern United States. Strobilurin (QoI) fungicides such as azoxystrobin are single-site mode-of-action fungicides applied to control *C. cereale*. In vitro bioassays with azoxystrobin at 0.031 and 8 μ g/ml incorporated into agar were performed to evaluate the sensitivity of 175 isolates collected from symptomatic turfgrasses in Alabama, Mississippi, North Carolina, Tennessee, and Virginia. Three sensitivity levels were identified among *C. cereale* isolates. Resistant, intermediate, and sensitive isolates were characterized by percent relative growth based on the controls with means of 81, 23, and 4%, respectively, on media containing azoxystrobin at 8 μ g/ml. The molecular mechanism of resistance was determined by comparing amino acid sequences of the cytochrome b protein. Compared with sensitive isolates, *C. cereale* isolates exhibiting QoI resistance had a G143A substitution, whereas isolates expressing intermediate resistance had a F129L substitution. *C. cereale* isolates displaying azoxystrobin resistance in vitro were not controlled by QoI fungicides in a field evaluation. The dominance of QoI-resistant *C. cereale* isolates identified in this study indicates a shift to resistant populations on highly managed golf course putting greens.

Anthracnose, incited by *Colletotrichum cereale* Manns sensu lato Crouch, Clarke, & Hillman ($\equiv$ *C. graminicola* (Ces.) G.W. Wils.) (11), has become a common and destructive disease of creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.) putting greens in the transition zone and throughout the southern United States (33). Generally, anthracnose occurs on creeping bentgrass putting greens during the summer and early autumn as the turf becomes physiologically weakened from heat stress, predisposing the foliar portion of the plant to infection by *C. cereale*. The symptoms of anthracnose foliar blight on creeping bentgrass putting greens appear as yellow to bronze irregularly shaped areas that may coalesce and are often associated with a loss of turf

density (33). Moreover, symptoms can be exacerbated on heat-stressed creeping bentgrass. Similarly, *C. cereale* infects annual bluegrass putting greens, causing foliar blight or basal rot symptoms further described by Smiley et al. (24).

Fungicides are applied in a preventive manner to creeping bentgrass and annual bluegrass putting greens on a biweekly basis from April to October to manage many turf pathogens, including *C. cereale*. A proactive, preventive fungicide program that includes biochemical (single- or multi-site) and topical (contact or penetrant) mode-of-action (MOA) rotations is an effective approach for anthracnose management. Strobilurin, phosphate, or demethylation inhibitor fungicides applied alone or tank mixed with a contact fungicide have proven to be an effective control (8,30). Environmental conditions may favor disease development, thus requiring curative applications that include single-site MOA fungicides due to their efficacy at low doses and their fungitoxic effect post application (9,21,31).

In the 1970s, research was initiated on a by-product of the fungus *Strobilurus tenacellus* (Pers.) Singer exhibiting fungicidal properties (4,23). The by-product, referred to as strobilurin, targeted the mitochondrial

electron transport chain at the Qo center (Qo inhibitor or QoI) of cytochrome bc₁ (35). The first synthetically produced strobilurin (QoI) fungicide was released in Germany in 1996 under the common name azoxystrobin, a broad-spectrum fungicide labeled for several plant diseases (23).

Shortly after QoI fungicides (QoIs) were introduced to the turf market in the mid-1990s, fungicide resistance was documented (1,2,12,28). Population shifts from QoI-sensitive to QoI-resistant *Pyricularia grisea* (Cooke) Sacc. (gray leaf spot) infecting perennial ryegrass (*Lolium perenne* L.) occurred rapidly under heavy disease pressure. Reduced sensitivity of *P. grisea* to azoxystrobin was initially observed in vitro (28). Subsequent molecular analysis identified an amino acid substitution from glycine (G) to alanine (A) at position 143 (G143A) in the cytochrome b protein of isolates with the greatest resistance to azoxystrobin. Intermediate resistance was also observed in isolates with an amino acid substitution from phenylalanine (F) to leucine (L) at position 129 (F129L) (16). Since these initial findings, QoI resistance has been detected in *C. cereale* isolated from annual bluegrass in two separate studies (2,29). *C. cereale* isolates collected from locations where QoIs were applied were not inhibited when exposed to azoxystrobin at 8 or 10 μ g/ml (2,29). In addition, isolate responses were similar for trifloxystrobin and pyraclostrobin, indicating cross resistance among QoIs (2,29). Molecular techniques confirmed in vitro results with all resistant isolates having the G143A substitution (2,29).

Based on the frequency of QoI fungicide applications for management of anthracnose throughout the transition zone and southern United States, and the increased anecdotal and documented evidence of resistance to this class of fungicides in *C. cereale* populations (2,6,7,10,17–19,25,26,29), studies were established to determine whether *C. cereale* isolates causing anthracnose on creeping bentgrass and annual bluegrass putting greens in the eastern transition zone and southern United States were resistant to azoxystrobin. The objectives of this research were to determine the sensitivity of *C. cereale* isolates to azoxystrobin using in

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vitro bioassays, obtain nucleotide sequences of the *cytochrome b* gene to identify point mutations resulting in amino acid substitutions, and evaluate the efficacy of QoI fungicides in a creeping bentgrass putting green artificially inoculated with *C. cereale* isolates exhibiting azoxystrobin resistance based on in vitro bioassays.

MATERIALS AND METHODS

Isolate collection. *C. cereale* was isolated from creeping bentgrass plants exhibiting symptoms of anthracnose or heat stress during summer 2006 and 2007 in Alabama and Mississippi (Table 1; 32). Individual plants exhibiting signs of *C. cereale* (acervuli and setae) 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 air dried on sterile filter paper in a laminar flow hood (3,15). One hundred plants (shoots and roots) from each location were transferred (five per plate) onto a modified potato dextrose agar (m-PDA; 6 g of Difco 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) with 24-h fluorescent light for 4 to 7 days. Monoconidial isolates of *C. cereale* were placed in -20°C for long-term storage on air-dried, sterilized glass fiber filter paper (Fisher Scientific, Pittsburg, PA; 32). Sen-

sitive *C. cereale* isolates from California and Pennsylvania were obtained from annual bluegrass putting greens for this study from Dr. F. Wong, University of California-Riverside and Dr. G. Olaya, Syngenta Crop Protection, Inc., Vero Beach, FL, respectively (2,29). Isolates never exposed to QoIs were also recovered from turf-type tall fescue (*L. arundinaceum* (Schreb.) Darbysh.) research plots at Mississippi State University (Table 1).

A second set of *C. cereale* isolates were collected from creeping bentgrass and annual bluegrass putting greens exhibiting anthracnose symptoms in North Carolina, Tennessee, and Virginia in 2004 and 2005 (Table 1). Infected plant material was excised and surface disinfested in a 0.6% sodium hypochlorite solution for 5 min. Once dried, sterile plant tissues were plated onto PDA (39 g Difco of PDA per liter) amended with 50 mg each of chloramphenicol, streptomycin, and tetracycline. Isolates were maintained at -80°C on air-dried, sterile filter paper for long-term storage.

In vitro sensitivity evaluations. In vitro bioassay methods were based on those previously described by Wong et al. (29). *C. cereale* isolates were screened at two azoxystrobin concentrations, 0.031 and 8 µg/ml. Azoxystrobin (Heritage 50 WG; Syngenta Professional Products, Greensboro, NC) was dissolved in acetone to obtain stock solutions of azoxystrobin at 31 and 8,000 µg/ml. A stock solution (ml)

was added to 1 liter m-PDA to deliver the desired azoxystrobin concentration. A nonamended control (acetone at 1 ml/liter of m-PDA) was included as a treatment. All treatments were amended with salicyl-hydroxamic acid (SHAM) at 100 µg/ml to inhibit the alternative oxidase pathway (1,2,19,28,29,35).

Infested filter paper was placed on m-PDA to initiate mycelial growth. A seed plate culture was obtained for each isolate by transferring mycelium to fresh m-PDA. A 5-mm-diameter hyphal plug was removed from the margin of a 10-day-old *C. cereale* culture and placed, mycelium-side down, in the center of azoxystrobin-amended or nonamended media. Isolates were incubated at room temperature (approximately 22°C) in the dark for 6 days, after which three equidistant colony growth diameter measurements were recorded.

Data analysis. Percent relative growth was calculated by dividing the average radial colony growth on azoxystrobin-amended media by the average radial colony growth on nonamended media and multiplied by 100. An arbitrarily selected sample of *C. cereale* isolates ($n = 40$), exhibiting variations in percent relative growth and later characterized molecularly, were included in the statistical analysis. Percent relative growth values from the bioassays were arcsine transformed using SAS (version 9.1; SAS Institute, Cary, NC) and analyzed using PROC GLIMMIX. Treatment means were separated by least square means ($P = 0.05$). Following analysis, values were back transformed and reported as percent relative growth. Relative growth of *C. cereale* isolates collected from Alabama and Mississippi (single isolate evaluated per plate, replicated twice) were analyzed separately from isolates from North Carolina, Tennessee, and Virginia (single isolate evaluated per plate, replicated three times) due to the in vitro sensitivity evaluations being conducted in separate laboratories.

The effective concentration required to achieve 50% inhibition of mycelial growth on azoxystrobin-amended media (EC_{50}) was calculated for *C. cereale* isolates ($n = 45$) collected from locations in Alabama and Mississippi and those originating from California and Pennsylvania (2,32). Percent relative growth for an individual isolate at eight azoxystrobin concentrations ranging from 4.9×10^{-4} to 8 µg/ml was subjected to the regression procedure (PROC REG) in SAS to obtain the intercept (b_0) and slope (b_1). The EC_{50} value (µg/ml) for each isolate was calculated using the equation:

$$\text{azoxystrobin}_{50\%} = e[(50 - b_0)/b_1]$$

All bioassays were conducted twice and data were pooled for individual isolates.

Characterization of *cytochrome b* gene sequences. The *C. cereale* isolates in-

Table 1. *Colletotrichum cereale* isolates from various hosts and locations throughout the United States and the frequency of phenotypic responses observed with azoxystrobin at 8 µg/ml

Location	Host	No. of isolates	Phenotypic response		
			Sens. ^y	Int. ^w	Resist. ^x
West Point, MS	Creeping bentgrass	30	0	0	30
Birmingham, AL	Creeping bentgrass	5	0	0	5
Birmingham, AL	Creeping bentgrass	6	0	0	6
Sylacauga, AL	Creeping bentgrass	2	0	0	2
West Point, MS	Creeping bentgrass	6	0	0	6
West Point, MS	Creeping bentgrass	10	0	0	10
Birmingham, AL	Creeping bentgrass	1	0	0	1
Inverness, AL	Creeping bentgrass	6	0	0	6
Olive Branch, MS	Creeping bentgrass	2	0	0	2
Tupelo, MS	Creeping bentgrass	12	0	0	12
Tupelo, MS	Creeping bentgrass	1	0	0	1
Pennsylvania	Annual bluegrass	2 ^y	2	0	0
Temecula, CA	Annual bluegrass	17 ^z	17	0	0
Starkville, MS	Tall fescue	4	4	0	0
Leland, NC	Creeping bentgrass	11	0	0	11
Charlotte, NC	Creeping bentgrass	2	0	0	2
Sanford, NC	Creeping bentgrass	3	0	0	3
Monroe, NC	Creeping bentgrass	7	0	0	7
Virginia Beach, VA	Creeping bentgrass	5	0	0	5
Gatlinburg, TN	Creeping bentgrass	6	0	0	6
Blowing Rock, NC	Annual bluegrass	36	0	9	27
Asheville, NC	Annual bluegrass	1	1	0	0

^y Sensitive: number of isolates from a location exhibiting growth inhibition with azoxystrobin at 8 µg/ml and approximately 50% growth inhibition at 0.031 µg/ml.

^w Intermediate: number of isolates from a location exhibiting minimal growth inhibition with azoxystrobin at 0.031 µg/ml but significant growth inhibition at 8 µg/ml.

^x Resistant: number of isolates from a location exhibiting minimal growth inhibition with azoxystrobin at 8 µg/ml.

^y *C. cereale* isolates courtesy of G. Olaya, Syngenta Crop Protection, Inc., Vero Beach, FL (2).

^z *C. cereale* isolates courtesy of F. Wong, University of California, Riverside (31).

cluded in the statistical analysis from Alabama, Mississippi, and California characterized as resistant ($n = 20$) or sensitive ($n = 5$) based on azoxystrobin in vitro bioassays were sequenced to confirm the molecular mechanisms of azoxystrobin resistance (12). Fresh mycelium was harvested from a 21-day-old culture grown on m-PDA and lyophilized prior to RNA extraction with the RNeasy Mini Kit (Qiagen, Valencia, CA). The AccessQuick RT-PCR System (Promega Corp., Madison, WI) was used to create cDNA from RNA. Extracted RNA (1 μ l) was incorporated with reagents, including primers C1 (5' gga agaggtatgtactacgggtca 3') and C2 (5' acg ctaaacccctcaaac 3') previously described by Wong et al. (29), and incubated in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) at 45°C for 45 min. The *cytochrome b* gene was amplified with the GoTaq PCR Core System (Promega Corp.) and 5 μ l of cDNA (32). Polymerase chain reactions (PCRs) were performed in the thermal cycler with the following program: 95°C for 3 min; 30 cycles of 95°C for 30 s, 51°C for 45 s, and 72°C for 2 min; followed by an extension cycle at 72°C for 5 min and a 10°C holding temperature. The amplicon was visualized using electrophoresis in a 1.5% agarose gel to ensure its desired size of 219 bp. Amplicons corresponding to the size were purified with ExoSAP-IT (USB Corp., Cleveland, OH) according to the manufacturer's instructions. The purified amplicon was cloned according to protocol with the pGEM-T Easy Vector System (Promega Corp.) and plasmids were purified with the Qiaprep Spin Miniprep Kit (Qiagen).

Purified plasmids were prepared for sequencing according to the manufacturer's instructions with the DTCS Quick Start Kit (Beckman and Coulter, Fullerton, CA). Nucleotide sequencing was performed at the Life Sciences and Biotechnology Institute at Mississippi State University using the Beckman CEQ 8000 DNA Analysis System (Beckman and Coulter). DNASTAR Lasergene Software (DNASTAR Inc., Madison, WI) was used to trim, translate, align, and interpret nucleotide sequences.

The *cytochrome b* gene of representative *C. cereale* isolates from North Carolina, Tennessee, and Virginia was sequenced using the following protocol. RNA was extracted from lyophilized mycelium using the protocol for plant tissue from Pure-script (Gentra Systems Inc., Minneapolis, MN). Reverse-transcription (RT)-PCR was performed in same-tube reactions using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA) and the primers previously described (29). The optimal thermal cycling program for reverse transcriptase was performed, and PCR amplification was conducted using the thermal cycling program previously described. Electrophoresis was performed for all amplicons in 1.5% agarose gel to ensure that the product cor-

responded to the expected size of 219 bp. These amplicons were sequenced directly at the Duke University DNA Sequencing Facility in Durham, NC.

The sequence data obtained from all isolates were compared with the original *C. cereale cytochrome b* sequence (GenBank accession number AY285743) to determine

Table 2. Treatments and application rates of strobilurin (QoI) and other fungicides for control of anthracnose on a creeping bentgrass putting green inoculated with azoxystrobin-resistant *Colletotrichum cereale* isolates in North Carolina, 2006^x

Fungicide ^y	Rate (kg or liters of product/ha)
Thiophanate-methyl	12.6 liters
Phosphoric acid	17.6 liters
Chlorothalonil/thiophanate-methyl ^z	17.6 kg
Chlorothalonil/thiophanate-methyl ^z + phosphoric acid	17.6 kg + 17.6 liters
Azoxystrobin	0.61 kg
Fosetyl-Al	12.2 kg
Propiconazole	3.18 liters
Fluoxastrobin	0.57 liter
Fluoxastrobin	1.14 liter
Fluoxastrobin + propiconazole	0.57 liter + 3.18 liters
Fluoxastrobin + chlorothalonil	0.57 liter + 5.5 kg
Fluoxastrobin + polyoxin-D	0.57 liter + 6.1 kg
Fluoxastrobin + propiconazole + potassium phosphate	0.57 liter + 3.18 liters + 12.7 liters
Fluoxastrobin followed by propiconazole	0.57 liter followed by 3.18 liters
Fluoxastrobin followed by polyoxin-D	0.57 liter followed by 6.11 kg
Fosetyl-Al + chlorothalonil	12.2 kg + 9.8 kg
Chlorothalonil	9.8 kg
Phosphoric acid + chlorothalonil	17.6 liters + 9.8 kg
Pyraclostrobin	1.5 kg
Control (water)	...

^x *Colletotrichum cereale* inoculum (9×10^3 conidia/ml) applied as a foliar suspension to the creeping bentgrass the evening of 25 May 2006; CO₂-powered boom sprayer at 276 kPa; 8004 flat fan nozzles, calibrated to deliver water at 814 liters/ha; irrigated with 2.5 mm of water.

^y Treatments applied in water equivalent to 814 liters/ha; 14-day spray interval; application dates 29 June, 13 and 27 July, and 10 and 23 August 2006.

^z Prepackaged fungicide.

Fig. 1. Relative growth of select *Colletotrichum cereale* isolates evaluated in Mississippi with azoxystrobin at 8 μ g/ml. Sensitive isolates evaluated originated from annual bluegrass in California (TCGC 5-63, 5-35, and 5-48) or tall fescue in Mississippi (TF 1C and 1A). Percent relative growth values were arcsine transformed and analyzed in SAS using PROC GLIMMIX. Least-square means were used to separate significantly different relative growth means; bars with the same letter are not significantly different at $P = 0.05$.

percent identity using DNA Lasergene software. All isolates were sequenced twice.

Efficacy of QoI fungicides for anthracnose control in North Carolina. A field evaluation was conducted in 2006 on a creeping bentgrass, putting green cv. Dominant, that was artificially inoculated with *C. cereale* isolates (ABR-1, -3, -5, -7, -46, -48, -50, and -52) exhibiting complete

resistance to azoxystrobin in vitro (*data not shown*) prior to fungicide applications. The putting green had no prior history of anthracnose and symptoms were never observed prior to inoculation with *C. cereale* isolates. Conidia were suspended in 1.6 g of PDA per liter, resulting in a final concentration of 9×10^3 conidia/ml. The inoculum was applied as a foliar suspension to the creeping bentgrass in the eve-

ning of 25 May using a CO₂-powered boom sprayer at 276 kPa with 8004 flat fan nozzles (TeeJet Technologies, Wheaton, IL) and calibrated to deliver water at 814 liters/ha. The study area was immediately irrigated with 2.5 mm of water.

The creeping bentgrass putting green was mowed, fertilized, and watered with a routine management schedule used to maintain golf course putting greens. Fungicide treatments and application rates are shown in Table 2. Fungicide treatments were applied as previously described for the foliar inoculum application, except that irrigation was not applied after the fungicide applications. Anthracnose incidence was determined by estimating the percent plot area exhibiting symptoms using a point-intersect method. Turfgrass quality, based on color, density, and uniformity, was determined visually on a scale of 1 to 9 (9 = best; 6 ≥ acceptable). The plot size was 1.0 m wide by 1.8 m long and treatments were arranged in a randomized complete block with four replications. Data were subjected to analysis of variance, PROC GLM, and means separation by a Waller-Duncan *k*-ratio *t* test (*k* = 100).

RESULTS

In vitro sensitivity evaluations. Three azoxystrobin sensitivity levels were identified among *C. cereale* isolates (Table 1). A resistant group composed of 81% of all isolates had relative growth that ranged from 55 to 119% of the untreated control with a mean of 81% at the discriminatory dose (22) of azoxystrobin at 8 µg/ml. Of the *C. cereale* isolates tested, 5% exhibited intermediate resistance and showed inhibition at the discriminatory dose (8 µg/ml), with relative growth ranging from 14 to 34% with a mean of 23%; however, limited inhibition was observed with azoxystrobin at 0.031 µg/ml, with relative growth ranging from 93 to 109% of the untreated control and a mean of 101%. Sensitive isolates exhibited greater inhibition at both concentrations, with relative growth ranging from 0 to 75% and a mean of 44% for azoxystrobin at 0.031 µg/ml and 0 to 25% with a mean of 4% for azoxystrobin at 8 µg/ml.

Resistant *C. cereale* isolates evaluated in Mississippi had significantly greater mean relative growth with azoxystrobin at 8 µg/ml compared with sensitive isolates (Fig. 1). Relative growth of resistant isolates was 74% greater than that of sensitive isolates. Significant differences in relative growth were observed among resistant isolates; however, all resistant isolates had significantly greater relative growth than the sensitive isolates (Fig. 1).

Of the isolates evaluated in North Carolina, the sensitive isolate, AVGC1, exhibited no growth and was significantly different from all other *C. cereale* isolates with azoxystrobin at 8 µg/ml (Fig. 2). The remaining isolates exhibited significantly

Fig. 2. Relative growth of select *Colletotrichum cereale* isolates evaluated in North Carolina with isolate AVGC1 exhibiting complete inhibition with azoxystrobin at 8 µg/ml. Percent relative growth values were arcsine transformed and analyzed in SAS using PROC GLIMMIX. Least-square means were used to separate significantly different relative growth means; bars with the same letter are not significantly different at *P* = 0.05.

Fig. 3. Effect of azoxystrobin at different concentrations on radial growth of *Colletotrichum cereale*-resistant isolates from creeping bentgrass putting greens in Mississippi and Alabama compared with sensitive isolates from annual bluegrass (TCGC 5-54 and 99-78) or tall fescue (TF 1B).

different relative growth, resulting in isolates classified as resistant or intermediately resistant. The relative growth of intermediately resistant isolates was 65% less than that of resistant isolates (Fig. 2). Differences in relative growth were observed within the resistant group; however, all isolates classified as resistant had significantly greater relative growth than intermediately resistant or sensitive isolates (Fig. 2).

EC₅₀ values for resistant isolates collected in Alabama and Mississippi were considered to be greater than 8 µg/ml azoxystrobin due to the lack of mycelial growth inhibition at the discriminatory dose. Sixteen sensitive isolates had EC₅₀ values for azoxystrobin ranging from 0.003 to 1.079 µg/ml with a mean of 0.130 µg/ml. Relative growth of resistant isolates was slightly inhibited at high concentrations, which was in contrast to sensitive isolates that were progressively more inhibited as azoxystrobin concentrations increased (Fig. 3).

Characterization of cytochrome b gene sequences. Nucleotide sequences of the *cytochrome b* gene were obtained for 40 *C. cereale* isolates exhibiting different levels of sensitivity to azoxystrobin. The *cytochrome b* sequences corresponded to amino acid residues 98 to 170 (2,29), specifically containing residues 129 and 143 that have been previously associated with QoI resistance (1,2,12,16,29). In all, 65% of the *C. cereale* isolates sequenced were classified as resistant in vitro and had a transversion point mutation from guanine to cytosine resulting in the G143A change. The *C. cereale* isolates classified as intermediately resistant had a transversion point mutation from cytosine to guanine resulting in the F129L change, whereas the sensitive isolates retained phenylalanine and glycine at positions 129 and 143, respectively (Table 3). Percent identity for all the *C. cereale* isolates ranged from 94 to 99% compared with the GenBank reference sequence AY285743.

Twenty *C. cereale* isolates from Alabama and Mississippi identified as azoxystrobin-resistant based on in vitro bioassays expressed the G143A substitution. In contrast, the sensitive isolates from California and Mississippi did not exhibit any amino acid substitutions (Table 3). Six resistant *C. cereale* isolates collected from creeping bentgrass in the eastern transition zone had the G143A substitution, whereas the F129L substitution was identified in eight isolates from annual bluegrass in North Carolina expressing intermediate resistance (Table 3). The sensitive isolate from North Carolina had no amino acid substitutions at either location (Table 3).

Efficacy of QoI fungicides for anthracnose control in North Carolina. In July 2006, temperatures ≥32°C coupled with relative humidity ≥85% induced heat stress in the artificially inoculated creeping

bentgrass putting green, predisposing the plants to infection. By early August, symptoms of anthracnose were evident, and microscopic observations confirmed foliar infection by *C. cereale*. Fungicide treatments were applied biweekly beginning 29 June 2006 and treatment differences were observed on 6 August. The three QoI fungicides applied alone were not effective for controlling anthracnose in creeping bentgrass previously inoculated with azoxystrobin-resistant *C. cereale* isolates, except for fluoxastrobin at the low rate (Table 4). Anthracnose incidence in thiophanate-methyl-treated plots was similar to the QoI fungicide treatments and untreated control. The greatest reduction in anthracnose incidence occurred in plots treated with fluoxastrobin tank mixed or alternated with propiconazole; however, anthracnose incidence was similar in plots treated with propiconazole alone. Fosetyl-Al alone or tank mixed with chlorothalonil reduced anthracnose incidence compared with azoxystrobin and the untreated control (Table 4).

Turfgrass quality observed on 6 August was improved in creeping bentgrass plots

treated with fluoxastrobin tank mixed with another non-QoI fungicide (Table 4). Azoxystrobin, pyraclostrobin, or both rates of fluoxastrobin did not improve turfgrass quality compared with the untreated control. Fosetyl-Al tank mixed with chlorothalonil was the only fungicide treatment that provided acceptable turfgrass quality on 6 August. Thiophanate-methyl and chlorothalonil had the lowest turfgrass quality rating of 2.8 (Table 4).

DISCUSSION

Creeping bentgrass and annual bluegrass putting greens in the southern United States and eastern transition zone that were sampled in this study were dominated by azoxystrobin-resistant isolates. Historically, applications of QoIs to manage turfgrass diseases at the locations where *C. cereale* isolates were collected have occurred with great frequency. Based on the use of QoIs, these results were not unexpected considering anecdotal evidence from superintendents and reports of *C. cereale* resistance occurring at other locations (2,6,8,10,17–19,25,26,29). However, this was the first documentation of azox-

Table 3. Molecular characterization of *Colletotrichum cereale* isolates from turfgrasses throughout the southern United States, eastern transition zone, and California

Isolate	Accession no.	Location of analysis	Amino acid position	
			129	143
BO 6 9	FJ799953	Mississippi	Phe	Ala
BO 6 37	FJ799954	Mississippi	Phe	Ala
BO 12 3	FJ799955	Mississippi	Phe	Ala
FL A2-1	FJ799956	Mississippi	Phe	Ala
FL B3-1	FJ799957	Mississippi	Phe	Ala
GS 4 22	FJ799958	Mississippi	Phe	Ala
GS 4 28	FJ799959	Mississippi	Phe	Ala
OO 4 G1-1	FJ799960	Mississippi	Phe	Ala
OO 4 K1-1	FJ799961	Mississippi	Phe	Ala
OO 7 1	FJ799962	Mississippi	Phe	Ala
OO 7 E5-1	FJ799963	Mississippi	Phe	Ala
OO 7 R1-1	FJ799964	Mississippi	Phe	Ala
OW 15 56b	FJ799966	Mississippi	Phe	Ala
OW 15 N5b	FJ799967	Mississippi	Phe	Ala
OW 15 R4-1	FJ799968	Mississippi	Phe	Ala
OW 15 T4-2	FJ799969	Mississippi	Phe	Ala
OW 16 1C	FJ799970	Mississippi	Phe	Ala
OW 16 14B	FJ799971	Mississippi	Phe	Ala
PGC PG 1	FJ799972	Mississippi	Phe	Ala
PGC PG 4	FJ799973	Mississippi	Phe	Ala
TCGC 5-35	FJ799974	Mississippi	Phe	Glu
TCGC 5-48	FJ799975	Mississippi	Phe	Glu
TCGC 5-63	FJ799976	Mississippi	Phe	Glu
TF 1A	FJ799977	Mississippi	Phe	Glu
TF 1C	FJ799978	Mississippi	Phe	Glu
ABR12	GQ255954	North Carolina	Phe	Ala
ABR16	GQ255955	North Carolina	Phe	Ala
ABR47	GQ255960	North Carolina	Phe	Ala
BR7A	GQ255963	North Carolina	Phe	Ala
GBGC2	GQ255967	North Carolina	Phe	Ala
PA5	GQ255968	North Carolina	Phe	Ala
ABR17	GQ255956	North Carolina	Leu	Glu
ABR18	GQ255957	North Carolina	Leu	Glu
ABR23	GQ255958	North Carolina	Leu	Glu
ABR38	GQ255959	North Carolina	Leu	Glu
BR5B	GQ255962	North Carolina	Leu	Glu
BR8A	GQ255964	North Carolina	Leu	Glu
BR13A	GQ255965	North Carolina	Leu	Glu
BR19A	GQ255966	North Carolina	Leu	Glu
AVGC1	GQ255961	North Carolina	Phe	Glu

ystrobin resistance in *C. cereale* isolated from creeping bentgrass in the United States that was confirmed using molecular methods.

Differences in relative growth, based on in vitro bioassays, were evident when comparing sensitive, intermediate, and resistant *C. cereale* isolates at the two azoxystrobin concentrations. Isolates with intermediate resistance exhibited colony growth inhibition with azoxystrobin at 8 µg/ml but were insensitive to azoxystrobin at 0.031 µg/ml. Resistant isolates did not exhibit mycelial growth inhibition with azoxystrobin at 0.031 µg/ml and minimal inhibition was observed at 8 µg/ml, which is consistent with previous reports of *C. cereale* resistance (2,10,29).

C. cereale isolates collected from putting greens previously exposed to QoI fungicides exhibited either intermediate or complete resistance to azoxystrobin. Due to sampling size, these results may not be inclusive of all *C. cereale* isolates within the sampled putting greens. However, these results indicate the propensity of practical resistance within *C. cereale* populations on putting greens in the southern United States and eastern transition zone.

The phenotypic responses of *C. cereale* isolates observed in vitro were supported by sequences of the *cytochrome b* gene. Deduced amino acid sequences revealed two separate substitutions that corresponded to the observed phenotypic responses in vitro. The most unique substitution was the F129L identified in *C. cereale* isolates exhibiting intermediate resistance to azoxystrobin. This is a first report of *C.*

cereale isolates exhibiting this phenotypic response to azoxystrobin and characterized by the F129L substitution. The isolates expressing this substitution responded similarly to azoxystrobin, as did *P. grisea* and *Alternaria solani* Sorauer that expressed the F129L substitution and were classified as intermediately resistant in vitro (16,20).

C. cereale isolates collected from creeping bentgrass and the remainder of the isolates collected from annual bluegrass in North Carolina expressed the G143A substitution. These isolates were characterized as resistant due to minimal in vitro growth inhibition observed with azoxystrobin at 8 µg/ml. These results are consistent with *C. cereale* isolates obtained from annual bluegrass putting greens expressing the G143A substitution in previous reports (2,29).

The fungicide evaluation conducted in North Carolina on a creeping bentgrass putting green with no prior history of anthracnose and subsequently artificially inoculated with azoxystrobin-resistant isolates indicated a phenotypic response similar to that observed by *C. cereale* isolates in azoxystrobin bioassays. The QoI fungicides did not successfully control anthracnose following inoculation with azoxystrobin-resistant isolates. These results are similar to other reports that indicate that QoI fungicides are not effective when azoxystrobin-resistant isolates cause anthracnose in creeping bentgrass putting greens (6–8).

Azoxystrobin, pyraclostrobin, and fluoxastrobin (high rate) applied alone

were not effective for controlling anthracnose. The lower rate of fluoxastrobin applied alone, tank mixed, or alternated with propiconazole significantly reduced anthracnose incidence. The only treatments that resulted in the greatest reduction of anthracnose incidence were fosetyl-Al alone or tank mixed with chlorothalonil. However, this evaluation did illustrate improved efficacy of the QoI, fluoxastrobin, when applied in combination with another fungicide class; in this case, a demethylation inhibitor, to manage *C. cereale* exhibiting practical resistance to QoI fungicides (19). The potential for cross resistance among QoIs was also supported by the results of the fungicide evaluation and is consistent with other studies previously reported (2,7,8,17,26,29,31).

The *C. cereale* isolates evaluated in this study also exhibited multiple resistance (5) due to their lack of sensitivity to thiophanate-methyl determined previously via in vitro analyses and amino acid substitutions identified in the β -tubulin 2 protein (27,34). Multiple resistance is again supported in the fungicide evaluation where thiophanate-methyl did not successfully control anthracnose. The possibility of multiple resistance should raise the awareness of golf course superintendents to implement fungicide resistance management strategies, including reducing the frequency of at-risk fungicide applications, maintaining recommended rates, avoiding curative applications, and using integrated disease management programs (5). Cultural practices and management strategies

Table 4. Anthracnose incidence and turfgrass quality observed on 6 August 2006 resulting from a fungicide evaluation on a creeping bentgrass putting green inoculated with azoxystrobin-resistant *Colletotrichum cereale* isolates in North Carolina^a

Fungicide ^e	Rate (kg or liters of product/ha)	Anthracnose incidence (%) ^w	Turf quality ^x
Thiophanate-methyl	12.6 liters	22 bcdef ^y	2.8 f
Phosphoric acid	17.6 liters	29 ab	3.3 ef
Chlorothalonil/thiophanate-methyl ^z	17.6 kg	16 cdefgh	3.5 def
Chlorothalonil/thiophanate-methyl ^z + phosphoric acid	17.6 kg + 17.6 liters	21 bcdefg	4.5 bcde
Azoxystrobin	0.61 kg	25 bcd	4.0 cdef
Fosetyl-Al	12.2 kg	12 fghij	4.0 cdef
Propiconazole	3.18 liters	13 efghij	3.8 def
Fluoxastrobin	0.57 liter	11 ghij	3.8 def
Fluoxastrobin	1.14 liter	15 defghij	4.0 cdef
Fluoxastrobin + propiconazole	0.57 liter + 3.18 liters	4 ij	5.0 abcd
Fluoxastrobin + chlorothalonil	0.57 liter + 5.5 kg	14 defghij	5.0 abcd
Fluoxastrobin + polyoxin-D	0.57 liter + 6.1 kg	15 defghi	5.0 abcd
Fluoxastrobin + propiconazole + potassium phosphate	0.57 liter + 3.18 liters + 12.7 liters	11 fghij	5.5 abc
Fluoxastrobin followed by propiconazole	0.57 liter followed by 3.18 liters	8 hij	4.3 bcdef
Fluoxastrobin followed by polyoxin-D	0.57 liter followed by 6.1 kg	16 cdefgh	3.8 def
Fosetyl-Al + chlorothalonil	12.2 kg + 9.8 kg	7 hij	6.3 a
Chlorothalonil	9.8 kg	30 ab	2.8 f
Phosphoric acid + chlorothalonil	17.6 liters + 9.8 kg	29 ab	3.8 def
Pyraclostrobin	1.5 kg	15 cdefgh	3.5 def
Control (water)	...	24 bcde	3.3 ef

^a *C. cereale* inoculum (9×10^3 conidia/ml) applied as a foliar suspension to the creeping bentgrass on the evening of 25 May 2006; CO₂-powered boom sprayer at 276 kPa; 8004 flat fan nozzles, calibrated to deliver water at 814 liters/ha; irrigated with 2.5 mm of water.

^v Treatments applied in water equivalent to 814 liters/ha; 14-day spray interval; application dates 29 June, 13 and 27 July, and 10 and 23 August 2006.

^w Anthracnose incidence determined by the percent surface area exhibiting anthracnose symptoms using a point-intersect method.

^x Turf quality based on a visual rating scale of 1 to 9 scale, where 9 = highest quality and 6 = acceptable quality.

^y Values represent means of four replications. Means within columns followed by the same letter are not significantly different according to the Waller-Duncan *k*-ratio *t* test (*k* = 100).

^z Prepackaged fungicide.

such as mowing and rolling frequency, vertical mowing, plant growth regulator applications, and nitrogen fertility have been evaluated on annual bluegrass putting greens in the northeastern United States, resulting in reduced anthracnose severity (13,14). These management strategies should be investigated on creeping bentgrass and annual bluegrass putting greens in the southern United States and eastern transition zone to determine which practices may best manage anthracnose in these regions.

The results of this research illustrate how widespread fungicide resistance has become in *C. cereale* infecting creeping bentgrass and annual bluegrass putting greens in the southern United States and eastern transition zone. These results also document the first F129L substitution in a species of *Colletotrichum* exhibiting intermediate resistance in vitro. The remainder of *C. cereale* isolates evaluated in this study had the G143A substitution and exhibited minimal sensitivity to azoxystrobin in vitro. The dominance of resistant *C. cereale* isolates was not expected at the initiation of this study; however, these results illustrate the shift from predominantly sensitive to resistant isolates on highly managed putting greens. Based on the findings of this research, future work should focus on fungicide resistance through a population and management perspective. If new single-site mode-of-action fungicides are developed to control *C. cereale*, studies should be conducted to determine optimal timing and frequency of applications to minimize selection pressure and maximize the longevity of the new chemistry.

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LITERATURE CITED

- Avila-Adame, C., and Köller, W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. *Curr. Genet.* 42:332-338.
- Avila-Adame, C., Olaya, G., and Köller, W. 2003. Characterization of *Colletotrichum graminicola* isolates resistant to strobilurin-related QoI fungicides. *Plant Dis.* 87:1426-1431.
- Backman, P. A., Landschoot, P. J., and Huff, D. R. 1999. Variation in pathogenicity, morphology, and RAPD marker profiles in *Colletotrichum graminicola* from turfgrasses. *Crop Sci.* 39:1129-1335.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. *Pest Manage. Sci.* 58:649-662.
- Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: How can it be managed? FRAC Monogr. No. 1. Crop Life International, Brussels.
- Burpee, L. L., Stephens, S. L., and Martinez-Espinoza, A. D. 2004. Control of anthracnose caused by a benzimidazole- and QoI-resistant isolate of *Colletotrichum graminicola*, 2003. *Fungic. Nematicide Tests* 59:T016.
- Burpee, L. L., Stephens, S. L., and Martinez-Espinoza, A. D. 2004. Suppression of anthracnose caused by an isolate of *Colletotrichum graminicola* with dual fungicide resistance, 2003. *Fungic. Nematicide Tests* 59:T017.
- Burpee, L. L., Stephens, S. L., and Martinez-Espinoza, A. D. 2005. Control of anthracnose caused by an isolate of *Colletotrichum graminicola* exhibiting dual fungicide resistance, 2004. *Fungic. Nematicide Tests* 60:T044.
- Couch, H. B. 2000. The Turfgrass Disease Handbook, 1st ed. Krieger Publishing Company, Malabar, FL.
- Crouch, J. A., Clarke, B. B., and Hillman, B. I. 2005. Phylogenetic relationships and fungicide sensitivities of *Colletotrichum graminicola* isolates from turfgrass in North America. *Int. Turfgrass Soc. Res.* 10:186-195.
- Crouch, J. A., Clarke, B. B., and Hillman, B. I. 2006. Unraveling evolutionary relationships among the divergent lineages of *Colletotrichum* causing anthracnose disease in turfgrass and corn. *Phytopathology* 96:46-60.
- Fernández-Ortuño, D., Torés, J. A., de Vicente, A., and Pérez-García, A. 2008. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *Int. Microbiol.* 11:1-9.
- Inguagiato, J. C., Murphy, J. A., and Clarke, B. B. 2008. Anthracnose severity on annual bluegrass influenced by nitrogen fertilization, growth regulators, and verticutting. *Crop Sci.* 48:1595-1607.
- Inguagiato, J. C., Murphy, J. A., and Clarke, B. B. 2009. Mowing and rolling strategies to manage anthracnose on annual bluegrass greens. *Golf Course Manage.* No. 5:111-116.
- Khan, A., and Hsiang, T. 2003. The infection process of *Colletotrichum graminicola* and relative aggressiveness on four turfgrass species. *Can. J. Microbiol.* 49:433-442.
- Kim, Y. S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial *cytochrome b* gene. *Phytopathology* 93:891-900.
- McCullough, M. D., and Wong, F. P. 2005. Evaluation of mid-season fungicide applications for the control of anthracnose on a mixed annual bluegrass and creeping bentgrass green in northern California, summer 2004. *Fungic. Nematicide Tests* 60:T056.
- Milus, E. A., Richardson, M. D., Hensler, K. L., and Weight, C. T. 2002. Evaluations of fungicides for control of anthracnose on bentgrass putting greens, 1998. *Fungic. Nematicide Tests* 57:T07.
- Mitkowski, N. A., Madeiras, A. M., Chaves, A., and Wick, A. 2009. Fungicide sensitivity of *Colletotrichum cereale* isolated from turfgrasses in the northeastern United States. *Online. Applied Turf. Sci.* doi:10.1094/ATS-2009-0917-01-RS.
- Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Dis.* 89:269-278.
- Pscheidt, J. W. 2009. Fungicide theory of use and mode of action. *Online. http://plant-disease.ipcc.orst.edu/articles.cfm?article_id=12.*
- Russel, P. E. 2004. Sensitivity baselines in fungicide resistance research and management. FRAC Monogr. No. 3. Crop Life International, Brussels.
- Sauter, H., Steglich, W., and Anke, T. 1999. Strobilurins: evolution of a new class of active substances. *Angew. Chem. Int. Ed.* 38:1328-1349.
- Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 2005. Compendium of Turfgrass Diseases, 3rd ed. American Phytopathological Society, St. Paul, MN.
- Tredway, L. P. 2006. Evaluation of fungicides for prevention of anthracnose foliar blight, 2005. *Fungic. Nematicide Tests* 61:T038.
- Tredway, L. P., and Soika, M. D. 2008. Impact of fungicides applied for brown patch control on anthracnose development in creeping bentgrass, 2007. *Plant Dis. Manage. Rep.* 2:T013.
- Tredway, L. P., Soika, M. D., and Bunting, M. L. 2008. Fungicide sensitivity in North Carolina populations of *Colletotrichum cereale* and molecular characterization of benzimidazole- and QoI-insensitive strains. (Abstr.) *Phytopathology* 98:S159.
- Vincelli, P., and Dixon, E. 2002. Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. *Plant Dis.* 86:235-240.
- Wong, F. P., Midland, S. L., and de la Cerda, K. A. 2007. Occurrence and distribution of QoI-resistant isolates of *Colletotrichum cereale* from annual bluegrass in California. *Plant Dis.* 91:1536-1546.
- Wong, F. P., Rios, J. C., and Ries, S. B. 2007. Evaluation of fungicides for the control of anthracnose in southern California, 2006. *Plant Dis. Manage. Rep.* 1:T063.
- Wong, F. P., Rios, J. C., and Ries, S. B. 2007. Evaluations of fungicides for the curative control of anthracnose in southern California, 2005. *Plant Dis. Manage. Rep.* 1:T064.
- Young, J. R. 2009. Identification of strobilurin and benzimidazole resistance in *Colletotrichum cereale* isolates causing anthracnose on creeping bentgrass putting greens in Mississippi and Alabama. M.Sc. thesis, Mississippi State University, Mississippi State.
- Young, J. R., Tomaso-Peterson, M., and Crouch, J. A. 2008. First report of *Colletotrichum cereale* causing anthracnose foliar blight of creeping bentgrass in Mississippi and Alabama. *Plant Dis.* 92:1475.
- Young, J. R., Tomaso-Peterson, M., de la Cerda, K., and Wong, F. P. 2010. Two mutations in β -tubulin 2 gene associated with thiophanate-methyl resistance in *Colletotrichum cereale* isolates from creeping bentgrass in Mississippi and Alabama. *Plant Dis.* 94:207-212.
- Ziogas, B. N., Baldwin, B. C., and Young, J. E. 1997. Alternative respiration: A biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. *Pestic. Sci.* 50:28-34.