Occurrence and Distribution of QoI-Resistant Isolates of *Colletotrichum cereale* from Annual Bluegrass in California

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ABSTRACT

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Turfgrass anthracnose, caused by *Colletotrichum cereale* (ex. *Colletotrichum graminicola*), is an important disease of turf used on golf course putting greens. Recent management of the disease has become increasingly difficult, partly due to the possible development of practical resistance to the QoI fungicides. In all, 558 single-conidia isolates of *C. cereale* were collected from 10 California golf courses, 8 of which had been exposed to QoI fungicides and 2 where no fungicides had been used. Isolates were tested using a mycelial expansion assay on azoxystrobin-amended media. For the two nonexposed populations, in vitro 50% effective dose (ED₅₀) values ranged from 0.0060 to 0.089 µg/ml. All isolates from the exposed populations could not be fully inhibited by doses of azoxystrobin as high as 8.0 µg/ml. A subset of these isolates were tested in vitro with the QoI fungicides pyraclostrobin and trifloxystrobin and found to be similar in response, indicating that these isolates were fully cross-resistant to all three fungicides. In greenhouse pot experiments, three isolates nonresponsive to QoI fungicides in vitro were not controlled by label rates of the fungicides. Spore germination assays also were examined; for 10 isolates identified as sensitive by mycelial expansion assays, ED₅₀ values for azoxystrobin ranged from 0.0040 to 0.0047 µg/ml; for 25 isolates identified as QoI-resistant, 93 to 100% of the conidia germinated at azoxystrobin concentrations as high as 8.0 µg/ml relative to the nonamended check treatments. Mitochondrial cytochrome *b* genes from a subset of 15 isolates (12 resistant and 3 sensitive) were partially cloned and sequenced; all resistant isolates had an alanine substitution that corresponded to position 143 of the gene product. These results indicate that QoI resistance is present in California populations of *C. cereale* and is contributing to the difficulty in controlling this disease.

Colletotrichum cereale sensu lato Crouch, Clarke, and Hillman is the causal agent of turfgrass anthracnose, a disease that has been increasing in importance on annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis stolonifera* L.) golf course putting greens. *Colletotrichum cereale* is synonymous with *C. graminicola* (Ces.) G.W. Wilson in previous descriptions of the disease. Recent work has separated this pooid-grass-infecting fungus from the corn-infecting group defined as *C. graminicola* (9).

In recent years, epidemics of anthracnose have been rising in California on annual bluegrass putting greens, as well as in other parts of the United States (9). These epidemics have been difficult to control with fungicide spray programs, which suggested that fungicide resistance could be partially responsible for this lack of control. One class of interest is the QoI

fungicides, which have been very popular for turfgrass disease control. QoI resistance has been reported from and characterized for *C. cereale* isolates collected from annual bluegrass in Delaware and Pennsylvania in 1997 and 1998, respectively (3). Isolates insensitive to azoxystrobin in vitro were reported from California, New Jersey, Pennsylvania (10), and Georgia (5). However, the distribution, frequency, and mechanism of resistance was not documented in these cases.

The QoI class of fungicides includes the strobilurins, azoxystrobin (17), pyraclostrobin (1), and trifloxystrobin (28), and the chemically unrelated imidazolinone and oxazolidinedione fungicides, fenamidone and famoxadone, respectively (19,30). The mode of action of this class of fungicides is the disruption of respiration in mitochondria by binding to and inhibiting the electron transport chain at the Q_o center of the cytochrome bc₁ complex (mitochondrial complex III) (4). The introduction of the strobilurin fungicides from this class were of great interest because they were unique in their broad spectrum of activity toward a large number of basidiomycete, ascomycete, and oomycete plant pathogens. Currently, azoxystrobin, pyraclostrobin, and trifloxystrobin are registered

for use on turfgrass in the United States. For diseases of turf, these fungicides are used frequently due to their aforementioned broad spectrum of activity.

Since their widespread use, QoI resistance has occurred for a number of plant-pathogenic fungi (4,12,27). Resistance is conferred typically by a phenylalanine to lysine amino acid substitution at residue 129 (F129L) or glycine to alanine substitution at residue 143 (G143A) of the mitochondrial cytochrome *b* gene (4). In the United States, resistance has been detected in *Alternaria alternata* on pistachio (26), *A. mali* on apple (25), *A. solani* on potato (34), and *Didymella byroniae* on squash and melon (23,32). Specifically for fungal pathogens of turfgrass, QoI resistance has been characterized for *Pyricularia grisea* (20,43), *Pythium aphanidermatum* (13), and, as mentioned previously, *C. cereale*.

The objectives of this work were to (i) determine the baseline sensitivity distribution for isolates of *C. cereale* from California to azoxystrobin, (ii) determine the frequency of QoI resistance in California populations of *C. cereale*, (iii) confirm the mechanisms of QoI resistance for California isolates of *C. cereale*, and (iv) determine levels of cross-resistance among azoxystrobin, trifloxystrobin, and pyraclostrobin for *C. cereale*. The overall impact of the work is expected to help in determining the necessity to change management strategies for anthracnose and also shed light on the mechanisms of fungicide resistance development for this pathogen from both a population and organismal perspective.

MATERIALS AND METHODS

Fungicides. Technical grade azoxystrobin (98% active ingredient [a.i.]; Syngenta, Greensboro, NC), pyraclostrobin (93% a.i.; BASF Corporation, Research Triangle Park, NC), and trifloxystrobin (96% a.i., Bayer Crop Protection, Kansas City, KS) were used for in vitro assays in this study. The fungicides were dissolved in acetone to provide stock solutions containing each fungicide at 10 mg/ml. All stock solutions were stored at -20°C in 15-ml polystyrene centrifuge tubes sealed with Parafilm. All equipment and chemicals used for the preparation of stock solutions as well as the rest of the experiments was obtained from Fisher Scientific (Pittsburgh) unless otherwise noted. For in vivo studies, the following formulated products

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were used: Heritage 50WG (50% a.i. azoxystrobin; Syngenta), Insignia 20WG (20% a.i. pyraclostrobin; BASF), and Compass 50WG (trifloxystrobin; Bayer).

Collection of *C. cereale* populations.

In all, 558 single-conidial isolates of *C. cereale* were collected in 2002 through 2004 from 10 California golf course locations (Table 1). Isolate populations from two locations, TCGC and SCCC, were collected from annual bluegrass in fairways that had never been sprayed with QoI fungicides. These populations were considered nonexposed, "baseline" populations. The rest were collected from annual bluegrass putting greens where fungicide spray programs had failed to control the disease. Spray records were not available from all of these locations; however, generally, these greens had been sprayed three or more times per year with QoI fungicides starting in 1997 or 1998.

For baseline populations TCGC and SCCC, samples were collected by randomly gathering 10 11-cm-diameter plugs of soil and plants using a cup-cutter (Eagle One Golf Products, Anaheim, CA) from areas affected by anthracnose. For the other populations, three to eight cup-cutter-sized plugs were taken from diseased areas on single putting greens, unless otherwise noted. Distribution of sampling points was variable due to the variable distribution in disease development at the locations, but an attempt was made to take individual samples more than 1 m apart from each other in order to collect a diverse sample from the location.

To obtain individual isolates, typically 12 individual diseased plants with acervuli typical of *C. cereale* were taken from each plug with the aid of a dissecting microscope (MZ 12.5; Leica Microsystems, Nussloch, Germany), the plants being taken from a broad distribution across the sample plug. Each plant was placed in a 1.5-ml microcentrifuge tube with 300 μ l of sterile distilled water (sdH_2O) and was allowed to sit for 5 min to encourage conidial release from the acervuli. Tubes were vortexed gently for 10 s and then 100 μ l of the supernatant was transferred with a micropipette to a 100-mm petri dish containing 2% acidified water agar (20 g of granulated agar plus 250 μ l of lactic acid of per liter of sdH_2O). The liquid was gently streaked across the agar surface using a flame-sterilized wire inoculating loop to spread the conidia across the agar surface. Dishes were incubated overnight at room temperature to allow for conidia to germinate.

Individual germinated conidia were picked from the agar surface using a size 00 insect pin with the aid of a dissecting microscope set at $\times 100$ magnification. Conidia were transferred to petri dishes containing quarter-strength potato dextrose agar (1/4-PDA; 9.9 g of potato dextrose agar and 11.3 g of granulated agar per liter

of sdH_2O), placing five germinated conidia on each dish of agar. Petri dishes were placed at 28°C in an incubator (Model 818; Precision Scientific, Winchester, VA) with 12 h of fluorescent light per day. Three to five days later, dishes were examined for growth, and mycelia from a single colony with typical *C. cereale* morphology was transferred to a 1/4-PDA plate. All isolates were obtained in this manner. All isolates were maintained in the incubator at 28°C for the duration of the study as described above, with periodic transfers to 1/4-PDA plates at 14-day intervals.

Fungicide sensitivity assays. Sensitivity assays were based on methods developed by Avila-Adame et al. (3) with some modifications. Stock solutions of fungicides were serially diluted in cold acetone to obtain eight working solutions with concentrations increasing in fourfold increments from 0.00024 to 4.0 mg/ml. Each solution (1 ml) was added to 500 ml of autoclaved and cooled (40°C) 1/4-PDA to obtain media with fungicide at 0.00049 to 8.0 μ g/ml. The 0 μ g/ml (check) treatment was amended with only 1 ml of acetone. Salicylhydroxamic acid (SHAM; 1 ml) at 50 mg/ml in 1:1 acetone:methanol also was added to all media to obtain a final SHAM concentration of 100 μ g/ml to reduce any effects of alternative oxidase on in vitro growth (2). To obtain fungicide-amended 1/4-PDA plates, petri dishes were filled with approximately 20 ml of media per dish in a positive-pressure biological safety hood and allowed to solidify overnight. Plates were prepared 24 h prior to use in fungicide assays.

Assays were performed by placing a 5-mm plug, removed from the edge of a 7-day-old culture of *C. cereale* grown on 1/4-PDA as described above, onto fungicide-amended 1/4-PDA. Plugs were placed onto the center of the amended agar with the plug inverted so that the top side of the plug was in direct contact with the fungicide-amended media. For each isolate, the eight fungicide concentrations were used in addition to a nonfungicide-amended

check plate. Plates were sealed with Parafilm and incubated for 7 days at 28°C in darkness; light was excluded to minimize any risk of fungicide photolysis in an aqueous environment (1).

Fungicide sensitivity was measured by the 50% effective dose (ED_{50}), calculated as described by Wong and Wilcox (44). Briefly, the radial expansion for each isolate grown at the different fungicide concentrations was measured by taking the average colony diameter (based on three measurements across the colony), subtracting the diameter of the initial 5-mm plug, and dividing by two. Percent relative growth (RG) was calculated as radial expansion at fungicide concentration/radial expansion on nonamended check plate $\times 100$. RG values were plotted against the log fungicide concentration, and linear regression analysis was performed through the points representing the linear portion of the dose response using Microsoft Excel (Redmond, WA). The log ED_{50} was calculated from the interception of the regression line with an RG value of 50. ED_{50} values were calculated by taking the anti-log of the resulting log ED_{50} value.

Assay reproducibility. To determine the reproducibility and variance inherent in a single test as described by Wong and Wilcox (45), the sensitivity assay was performed using five isolates of *C. cereale* from the baseline population. Each isolate was tested five different times using new stock and working solutions and media each time. Variance, coefficients of variance, and upper and lower 95% confidence intervals were calculated using the normally distributed log ED_{50} values as described previously (45). Assay reproducibility was examined only for azoxystrobin.

Distributions of sensitivity to azoxystrobin. After establishing test reproducibility, each of the isolates from the two baseline populations, SCCC and TCGC (60 and 102 isolates, respectively), and three exposed populations, AHCC, ANG, and FUGC (84, 28, and 66 isolates, respectively), were tested using the full range of

Table 1. Origin and number of *Colletotrichum cereale* isolates collected in 2002 to 2004 for fungicide sensitivity testing

Population code	Location	Origin ^y	Exposed to QoIs ^z	Number of isolates	Collection date
AHCC	San Bernardino, CA	Putting green	Yes	84	2002
ANGC	Pasadena, CA	Putting green	Yes	28	2002
FUGC	Fullerton, CA	Putting green	Yes	66	2002
TCGC	Temecula, CA	Fairway	No	102	2002
CLCC	Canyon Lake, CA	Putting green	Yes	65	2003
EGGC	Corona, CA	Putting green	Yes	60	2003
SCCC	San Jose, CA	Fairway	No	60	2003
SHGC	San Bernardino, CA	Putting green	Yes	40	2003
BHCC	Danville, CA	Putting green	Yes	21	2004
DICC	Diablo, CA	Putting green	Yes	32	2004
Total	...	...	...	558	...

^y All isolates were collected from annual bluegrass (*Poa annua*) plants infected with *C. cereale*.

^z QoI fungicides had been in use in exposed locations since 1997, typically with three or more greens applications being made each year for anthracnose control. Fairway turf at the two nonexposed locations did not receive any QoI fungicide applications.

fungicide concentrations. Approximately 25 isolates were tested at a time, making new stock solutions every 5 runs. Histograms were constructed using log ED₅₀ classes based upon the least significant difference ($\alpha = 0.05$) between the five isolates tested five times for assay reproducibility (approximately 0.1 log units). Population means were compared using *t* tests (v. 12.10; Minitab, State College, PA).

During the course of testing, it was found that dose response curves could not be generated for many isolates insensitive to azoxystrobin. Additional sensitivity distributions were constructed based upon RG at azoxystrobin concentrations of 8.0 µg/ml, like those used by Smith et al. (35).

Single discriminatory dose testing of isolates. To facilitate more rapid sensitivity screening, as previously done for *Venturia inaequalis* and *Uncinula necator* (35,45), the utility of single discriminatory dose testing for the determination of fungicide sensitivities was performed. Log ED₅₀ values were regressed against RG values using MS-Excel for population TCGC. Significance and coefficients of variation were calculated for the relationships (Minitab, v. 12.10) with azoxystrobin at 0.031 and 8.0 µg/ml, which appeared to be reasonable discriminatory doses based upon the initial sensitivity data for populations TCGC, SCCC, and AHCC. The remaining isolates from populations ANGC, FUGC, CLCC, SHGC, BHCC, and DICC were tested with azoxystrobin only at 0, 0.031, and 8.0 µg/ml.

Cross-resistance among isolates to azoxystrobin, pyraclostrobin, and trifloxystrobin. To determine levels of cross-resistance among the QoI fungicides, 20 isolates from baseline population TCGC and a selection of 22 isolates from QoI-exposed populations AHCC, ANGC, and FUGC were assayed for sensitivity to pyraclostrobin and trifloxystrobin using the methodology described above. ED₅₀ values for these fungicides were determined for each isolate as described above. Regression analysis was performed using Minitab v.12.10 for comparing ED₅₀ values obtained for the fungicides for all of the isolates. Regression analyses also were performed using RG values for each of the fungicides at 0.031 µg/ml.

In vivo sensitivity of C. cereale isolates to QoI fungicides. To determine whether isolates testing insensitive to QoI fungicides in vitro could be controlled with the fungicides in vivo, three sensitive (TCGC-5.2, -5.3, and -5.4; azoxystrobin ED₅₀ values = 0.074, 0.083, and 0.047 µg/ml, respectively) and three insensitive (AHCC-1, ANGC-1, and FUGC-1; azoxystrobin ED₅₀ values > 8.0 µg/ml) isolates were used in greenhouse experiments. Annual bluegrass (*Poa annua* 'True-putt'; DLF International, Halsey, OR) was seeded at the rate of 19 g/m² in University of California soil-less

mix (UC Mix) in 7.5-cm-diameter plastic pots and grown for 6 weeks. Plants were grown in a greenhouse with ambient light and temperature (approximately 20 to 30°C). Plants were maintained at a height of 5 cm by clipping them once per week, and watered as needed. Plants were treated until run-off with azoxystrobin at 1.5 g/liter, pyraclostrobin at 1.3 g/liter, or trifloxystrobin at 0.94 g/liter (all equivalent to labeled field application rates) using a CO₂-powered sprayer fitted with a single TeeJet 8002VS nozzle (R&D Sprayers, Opelousas, LA) at a pressure of 2.0 atm. A water-only check treatment also was included. Treated plants were inoculated with a suspension of conidia obtained from one of the six isolates. Three pots of plants were used per fungicide-isolate combination.

Conidia were obtained from isolates grown on full-strength PDA in 100-mm petri plates at 28°C for 14 days without supplemental lighting. Conidia were harvested by flooding the plates with sdH₂O and rubbing the plates with a glass rod. The resulting suspension was filtered through a double layer of cheesecloth and adjusted to a final concentration of 1 × 10⁶ conidia/ml. Plants were inoculated by spraying until run-off with the conidial suspension, using approximately 1 to 2 ml per pot.

Plants were covered with plastic bags and incubated at 35°C and 80% relative humidity with 12 h of fluorescent light in a growth chamber (GC15; Environmental Growth Chambers, Chagrin Falls, OH). Bags then were removed 48 h after inoculation and disease severity evaluated 12 days later. Disease severity was evaluated using the Horsfall-Barrat scale (6); severity data was converted to a percentage using the scale midpoints and normalized using an arcsine (*y*) transformation, where *y* = percent severity/100. The experiments were performed a total of three times, evaluating all isolate-fungicide combinations in the repeated experiments. Means of the treatments from each experiment were analyzed by factorial analysis of variance (ANOVA) and compared using Fisher's Protected least significant difference with $\alpha = 0.05$.

Comparison of conidial germination and mycelial growth for sensitivity to azoxystrobin. Other studies have used spore germination in vitro as a measurement of QoI sensitivity (33) because these fungicides are more active against spore germination (4), and in vitro testing using mycelial expansion can be problematic due to the expression of alternative oxidase (2). To determine whether there was any meaningful difference between using spore germination and mycelial growth for evaluating fungicide sensitivity in *C. cereale*, 10 isolates from baseline population TCGC and 25 from exposed population AHCC were assayed using the conidial

germination test. Briefly, conidia were harvested from 7-day-old cultures grown at 28°C on 1/4-PDA plates by flooding them with 10 ml of sdH₂O, rubbing them with glass rod, and filtering the suspension through two layers of cheesecloth. Concentrations were adjusted to 1 × 10⁴ conidia/ml. For each assay, a 100-µl drop of the conidial suspension was placed onto a 1/4-PDA agar plate amended with azoxystrobin at one of the concentrations described above. The drop was streaked across the agar surface and plates were incubated at 28°C in darkness for 24 h, at which time conidia germination was evaluated with the aid of a dissecting microscope. In all, 100 conidia per plate were examined and percent relative germination (RGr) was calculated as percent germinated conidia at fungicide concentration/percent germinated conidia on non-amended check plate × 100. Log ED₅₀ and ED₅₀ values were determined as described above. Regression analysis was performed for log ED₅₀ values for conidial germination versus log ED₅₀ values for mycelial expansion (Minitab v.12.10).

Characterization of cytochrome *b* gene sequences from isolates of *C. cereale*. Three isolates of *C. cereale* were chosen from each of five populations representing one baseline (TCGC) and four exposed sites (AHCC, CLGC, EGGC, and SHGC). The methodology was based upon previous work (3) with some modifications. Isolates were grown for 5 days in 100 ml of potato dextrose broth (24 g per liter of sdH₂O) in closed Erlenmeyer flasks in a rotary shaker set at 120 rpm and 28°C. Mycelia were harvested by vacuum filtration through sterile filter paper (47-mm-diameter, 0.8-µm pore size), air dried, and ground with a mortar and pestle using liquid nitrogen.

RNA was extracted from the ground mycelia using Purescript (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's instructions for plant tissue. RNA yields were approximately 300 µg per sample at a concentration of 600 µg/ml. Primers for polymerase chain reaction (PCR) were designed based upon a known *C. cereale* cytochrome *b* gene sequence (GenBank accession no. AY285743) to amplify a 219-bp fragment encompassing a region containing the codons corresponding to amino acid residues 98 to 171. The primers were as follows: C1, 5' ggaagaggtatgtactacggtca 3' and C2, 5' acgctaaacccctcaaac 3'. Reverse-transcription (RT)-PCR was performed on the extracted RNA from each isolate to create cDNA from the expressed mitochondrial RNA using the AccessQuick RT-PCR System (Promega Corp., Madison, WI) as per the manufacturer's instructions.

PCR amplification was performed on the cDNA using PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Pis-

cataway, NJ). A single bead was added to 5 µl of cDNA, 3 µl each of primers C1 and C2 (10 pmol/µl), and 14 µl of nuclease-free water in a 0.2-ml microcentrifuge tube. PCR then was performed using a PTC-200 DNA Engine (MJ Research, Waltham, MA) with the following program: 94°C for 3 min; 35 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 1 min; followed by 72°C for 5 min and a 10°C holding temperature. PCR products were resolved on 1.5% agarose-Tris-borate-EDTA gels. A single band corresponding to the desired 219-bp product was purified using the Qiaquick gel Extraction System (Qiagen Inc, Valencia, CA).

Purified products were cloned into a vector using pGEM-T Easy Vector System (Promega Corp.) following the manufacturer's instructions and sequenced at the Genomics Core Sequencing facility the University of California, Riverside using Big Dye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA). Nucleotide sequences were translated to a

polypeptide using BioTools (UMass Medical School, Worcester, MA). Both nucleotide and amino acid sequences were aligned and compared with the aforementioned *C. cereale* reference sequence (3) and to each other using ClustalW (18). Percent sequence similarity and differences in both nucleotide and amino acid sequence were calculated and noted.

RESULTS

Fungicide sensitivity assay reproducibility. Based upon the testing of five isolates each five times, it appeared that the assay was very precise in determining log ED₅₀ values. The average coefficient of variance was 0.13%, with a range of 0.0081 to 0.40% (Table 2).

Distributions of sensitivity to azoxystrobin of *C. cereale* isolates from baseline and exposed locations. For the two populations from baseline locations TCGC and SCCC which had not been treated previously with QoI fungicides, the sensi-

tivity distributions were similar (Fig. 1). For TCGC, the mean ED₅₀ value was 0.037 µg/ml, with a range of 0.0060 to 0.089 µg/ml; for SCCC, the mean ED₅₀ was 0.033 µg/ml, with a range of 0.0063 to 0.064 µg/ml (Table 3).

To determine a practical discriminatory dose for future sensitivity testing, 0.031 µg/ml was determined to give the best correlation between RG values and log ED₅₀ values for (Fig. 2). The relationship was described by the function $\log \text{ED}_{50} = 0.015 \times \text{RG} - 2.2$, with $R^2 = 0.80$. Theoretically, by using this single discriminatory dose of azoxystrobin at 0.31 µg/ml in the in vitro tests, isolates with ED₅₀ values of 0.0062 to 0.19 µg/ml could be distinguished based solely on their relative growth compared with a nonamended check treatment.

The 84 isolates from population AHCC (e.g., AHCC10-1 and AHCC10-10; Fig. 3) were nonresponsive to the azoxystrobin concentration range of 0 to 8.0 µg/ml compared with those collected from baseline sites. Because ED₅₀ values could not be generated from assays conducted for these isolates, it was reasoned that RG at 8.0 µg/ml would be a suitable discriminatory dose for examining isolates with a high degree of insensitivity to azoxystrobin.

Histograms were constructed based upon the RG values for isolates tested at 0.031 and 8.0 µg/ml, and there was a clear difference between the baseline populations TCGC and SCCC and the population collected from the QoI-exposed location AHCC (Fig. 4). The average RG of isolates from populations TCGC and SCCC with azoxystrobin at 0.031 µg/ml were 47 and 35%, respectively, whereas that from AHCC was 100% (Table 3). The average

Table 2. Reproducibility of radial expansion assay on fungicide amended quarter-strength potato dextrose agar for determining 50% effective dose (ED₅₀) values for azoxystrobin in *Colletotrichum cereale*

Isolate	Mean (µg/ml) ^y	95%LL ^w	95%UL ^x	Variance ^y	CV (%) ^z
TCGC-5.30	0.064	0.030	0.17	0.00011	0.17
TCGC-5.1	0.067	0.031	0.18	0.00027	0.40
TCGC-5.25	0.058	0.026	0.14	0.0000043	0.0081
TCGC-8.11	0.014	0.0065	0.036	0.0000024	0.016
TCGC-8.20	0.011	0.0050	0.029	0.0000029	0.027
Average	...	...	...	0.000078	0.13

^y Mean ED₅₀ value based upon log ED₅₀ values obtained from five repeated assays; isolates grown for 7 days at 28°C.

^w Lower 95% confidence interval based upon log ED₅₀ values.

^x Upper 95% confidence interval based upon log ED₅₀ values.

^y Variance based upon log ED₅₀ values.

^z Coefficient of variance based upon log ED₅₀ values.

Fig. 1. Sensitivity distributions of two baseline populations of *Colletotrichum cereale* to azoxystrobin. Sites where populations TCGC ($n = 102$) and SCCC ($n = 60$) were collected had not been treated with QoI fungicides. Log 50% effective dose (ED₅₀) values are used to reflect the log-normal distribution of the data and indicated values represent the midpoint of each sensitivity category.

Table 3. Sensitivity of *Colletotrichum cereale* populations to azoxystrobin using relative growth (RG) measurements on quarter-strength potato dextrose agar amended with azoxystrobin at 0.031 and 8.0 µg/ml, plus salicylhydroxamic acid at 100 µg/ml

Population	No. tested ^z	Mean RG (%) ^y	
		0.031 µg/ml	8.0 µg/ml
AHCC	84	100 a	79 b
ANGC	21	98 a	79 ab
BHCC	32	99 a	78 bc
CLGC	54	99 a	71 d
DICC	32	99 a	79 b
EGGC	60	99 a	79 b
FUGC	24	98 a	76 c
SHGC	40	100 a	82 a
SCCC	69	35 c	0 f
TCGC	105	48 b	2.9 e

^y RG = percent radial growth of isolate in media amended with fungicide/radial growth in non-amended media with isolates evaluated after 7 days of growth at 28°C. Means in a column followed by the same letter are statistically equivalent based upon Fisher's protected least significant difference at $\alpha = 0.05$.

^z Number of isolates tested.

RG of isolates from populations TCGC and SCCC with azoxystrobin at 8.0 µg/ml were 2.9 and 0%, respectively, whereas that from AHCC was 79%. Individual *t* tests between populations indicated that RG values for isolates tested at 0.031 and 8.0 µg/ml were different between each of the baseline populations and the exposed population AHCC ($P < 0.001$).

Isolates from the remaining seven populations from QoI-exposed locations (ANGC, BHCC, CLGC, DICC, EGGC, FUGC, and SHGC) were insensitive and reduced in sensitivity to azoxystrobin

doses of 0.031 and 8.0 µg/ml, respectively (Fig. 5). Sensitivity distributions for these populations were similar to the results obtained for population AHCC. Mean RG values for these populations ranged from 98 to 100% growth when tested on azoxystrobin at 0.031 µg/ml (Table 3). Mean RG values for these populations tested on azoxystrobin at 8.0 µg/ml ranged from 71 to 82%. Although there was some variation in mean RG values for the QoI-exposed populations, there was a clear difference between these populations and the two baseline populations TCGC and SCCC.

Fig. 2. Estimation of log 50% effective dose (ED_{50}) values using single discriminatory dose testing at an azoxystrobin concentration of 0.031 µg/ml, plus salicylhydroxamic acid at 100 µg/ml, for isolates from baseline population TCGC. Relative growth values represent the ratio of growth on the azoxystrobin at 0.031 µg/ml treatment to growth on the nonamended check treatment after 7 days at 28°C.

Fig. 3. Dose response curves of two isolates of *Colletotrichum cereale* from baseline population TCGC and two isolates from population AHCC on quarter-strength potato dextrose agar amended with azoxystrobin at 0 to 8.0 µg/ml plus salicylhydroxamic acid at 100 µg/ml.

There appeared to be no isolates that showed an intermediate response to azoxystrobin (i.e., ED_{50} value between the upper range of the baseline populations and 8.0 µg/ml).

Cross-resistance among isolates to azoxystrobin, pyraclostrobin, and trifloxystrobin. For 20 isolates from the baseline population TCGC, mean ED_{50} values for sensitivity to azoxystrobin, pyraclostrobin, and trifloxystrobin were 0.031, 0.0032, 0.014 µg/ml, respectively (Table 4). For the 22 isolates from locations exposed to QoI fungicides, the mean ED_{50} values for pyraclostrobin and trifloxystrobin were 0.66 and 3.3 µg/ml, respectively. Mean ED_{50} values for the exposed population were significantly higher than those of the nonexposed population for both pyraclostrobin and trifloxystrobin (one-tailed *t* test, $P < 0.001$). The decrease in sensitivity to pyraclostrobin and trifloxystrobin for the exposed populations was more than a factor of 200 compared with the nonexposed populations. A comparable statistical comparison could not be made for azoxystrobin because the ED_{50} values for the exposed populations to azoxystrobin exceeded the limit of the assay system. Nonetheless, the mean ED_{50} for this population was greater than 8.0 µg/ml compared with the nonexposed population (mean ED_{50} of 0.031 µg/ml), reflecting more than a 258-fold decrease in sensitivity.

Due to the inability to obtain ED_{50} values to azoxystrobin for all of the isolates, RG values determined at a dose of fungicide at 0.031 µg/ml were used for regression analysis (Fig. 6). The results clearly illustrated cross-sensitivity among the isolates. For the regression of RG in azoxystrobin at 0.031 µg/ml and pyraclostrobin at 0.031 µg/ml, the coefficient of determination (R^2) was 0.97, with $P < 0.001$ (Fig. 6A). R^2 for the regression of azoxystrobin versus trifloxystrobin was 0.91, with $P < 0.001$, and was 0.92 for trifloxystrobin versus pyraclostrobin, with $P < 0.001$ (Fig. 6B and C).

Isolates that did not exhibit a dose response to azoxystrobin at 8.0 µg/ml also had low sensitivity to pyraclostrobin and trifloxystrobin. Their sensitivity also clearly was outside of the distribution for the baseline population. There was a clear difference between these two phenotypes; thus, those not responsive to azoxystrobin at 8.0 µg/ml and those within the azoxystrobin baseline sensitivity distribution subsequently were referred to as QoI resistant or QoI sensitive, respectively.

In vivo performance of fungicides against QoI-resistant and -sensitive isolates. When six isolates of *C. cereale* were inoculated onto plants treated with azoxystrobin, pyraclostrobin, or trifloxystrobin, there was a clear difference in response to the fungicides between those considered QoI sensitive and those considered resis-

tant. In the factorial ANOVA, there were significant ($P < 0.001$) effects of isolate, fungicide, and isolate–fungicide interaction on disease severity. Control of the QoI-resistant isolates AHCC-1, ANG-1, and FUGC-1 with any of the fungicides (Table 5) was equivalent to the water-only check ($P > 0.54$). Conversely, all fungicides provided 97 to 99% control of the QoI-sensitive isolates TCGC-5.2, -5.3, and -5.4 relative to the water-only treatment ($P < 0.001$ for all). These results did indicate that isolates considered QoI resistant in vitro also were insensitive to the QoI fungicides under in vivo conditions.

Comparison of spore germination versus mycelial growth assays for resistance determination. ED_{50} values for spore germination for the 10 isolates from population TCGC with azoxystrobin ranged between 0.0040 and 0.0047 $\mu\text{g/ml}$, with a mean of 0.0044 $\mu\text{g/ml}$ (data not shown). RGr values for the 10 QoI-sensitive isolates ranged from 3 to 21% at 0.0078 $\mu\text{g/ml}$, and no germination was observed at azoxystrobin concentrations of 0.031 $\mu\text{g/ml}$ or higher. RGr values ranged from 93 to 100% for the 25 isolates from population AHCC tested at azoxystrobin concentrations as high as 8.0 $\mu\text{g/ml}$.

There was no correlation between log ED_{50} values obtained by spore germination and mycelial growth assays ($P = 0.86$; data not shown). Qualitatively, it does appear that QoI-resistant isolates do not respond to the fungicide in either spore germination or mycelial growth assays.

Characterization of cytochrome *b* gene sequences in *C. cereale* isolates. In the 15 isolates for which partial sequences of cytochrome *b* corresponding to residues 98 to 171 were obtained, nucleotide sequence similarity ranged from 97 to 99% to the reference sequence AY285743. The deduced amino sequence for the amplified regions showed a glycine-to-alanine substitution at residue 143 in all of the isolates resistant to azoxystrobin in vitro (Fig. 7). The only other amino acid substitution detected was a tryptophan-to-cysteine substitution at residue 142 for isolate EGGC-23.

DISCUSSION

In this article, we provide results that impact the management of turfgrass anthracnose. First, we describe a detailed baseline sensitivity distribution for isolates of *C. cereale* to azoxystrobin with a methodology that allows for reproducible determination of azoxystrobin sensitivities. Second, we have identified azoxystrobin-resistant isolates in California populations of *C. cereale* that were found to have the G143A mutation in cytochrome *b*. Third, isolates resistant to azoxystrobin were found to be completely cross-resistant to pyraclostrobin and trifloxystrobin. Fourth, our resistance monitoring data from several locations in California indicates a high frequency of QoI resistance that has re-

sulted in difficulties managing this disease at those locations. Finally, we show that single discriminatory dose monitoring can be performed for this pathogen using either mycelial growth or spore germination assays for future determinations of resistance development.

Resistance to the QoI fungicides has been documented for a wide range of pathogens since their first commercial use in 1997 (4,12,27), and resistance in *C. cereale* was documented in 1999 (3). This is the first study to present a detailed baseline sensitivity distribution for this pathogen to azoxystrobin as well as limited but functional sensitivity distributions for pyraclostrobin and trifloxystrobin. Based on the limited number of isolates characterized, the mechanism of resistance was

identical to that previously described by Avila-Adame et al. (3) for *C. cereale* and for a number of other pathogens. The large number of isolates used in the study should have detected rare isolates occurring at a frequency of 0.6% with 95% confidence (24). With this relatively large sample size, no isolates with intermediate insensitivity phenotypes were detected, including those typical for F129L mutants as found in *Pyricularia grisea* (20), *A. solani* (34), or *V. inaequalis* (13). No quantitative shifts in population sensitivity, like those documented for *U. necator* (45) were seen, either. Based upon the results, it appears that qualitative resistance, or complete immunity, to QoI fungicides is the most common phenotype for QoI-resistant isolates of *C. cereale*.

Fig. 4. Sensitivity distributions of *Colletotrichum cereale* to azoxystrobin at 0.031 and 8.0 $\mu\text{g/ml}$. All isolates were tested in the presence of salicylhydroxamic acid at 100 $\mu\text{g/ml}$. Numbers for relative growth represent the midpoint of the histogram category.

Fig. 5. Sensitivity distributions of seven populations of *Colletotrichum cereale* from fungicide-exposed locations and a single baseline population (TCGC) to azoxystrobin at 0.31 and 8.0 µg/ml. All isolates were tested on quarter-strength potato dextrose agar amended with fungicide and salicylhydroxamic acid at 100 µg/ml. Numbers for relative growth represent the midpoint of the histogram category.

Table 4. Cross-resistance to QoI-fungicides in isolates of *Colletotrichum cereale*

Factor	No. tested ^v	ED ₅₀ values (µg/ml) ^u								
		Azoxystrobin			Pyraclostrobin			Trifloxystrobin		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Nonexposed ^w	20	0.031	0.011	0.052	0.0032	0.0016	0.0052	0.014	0.0017	0.039
Exposed ^x	22	>8.0 ^y	7.1	>8.0 ^y	0.66	0.22	1.1	3.3	1.8	8.0
Resistance factor ^z	...	>258 ^y	...	...	206	...	...	235	...	...

^u ED₅₀ = 50% effective dose values as determined by in vitro testing of single-spore isolates on quarter-strength potato dextrose agar amended with fungicides and salicylhydroxamic acid at 100 µg/ml. Min and Max = minimum and maximum, respectively.

^v Number of isolates tested.

^w Isolates from baseline population TCGC.

^x A selection of isolates from QoI-exposed populations AHCC, ANG, and FUGC.

^y Values are estimates because actual values exceeded those that could be determined by in vitro testing.

^z Ratio of the mean ED₅₀ for exposed isolates to the mean ED₅₀ for the nonexposed (baseline) isolates.

Among the 15 isolates for which partial cytochrome *b* sequences were obtained, the G143A mutation was found in QoI-resistant isolates, like that previously documented by Avila-Adame et al. (3). The amplified nucleotide sequence corresponding to amino acid residues 98 to 171 would have covered six amino acid substitutions in the 127 to 147 cd loop in cytochrome *b* known to confer high levels of QoI resistance in fungi (13) as well as that reported by Kraiczy at residue 153 (22). Mutations in residues 254, 254, 256, 261, 274, and 275 described by Kraiczy et al. (22) and di Rago et al. (11) in a number of basidiomycetes and *Saccharomyces cerevisiae* were not examined in this case. However, these have not been implicated in the development of field resistance for any plant-pathogenic fungi (12,27).

It would be interesting to note that, in this work, we used a primer set different from that used by Avila-Adame et al. (3). Initial experiments using this published methodology and primer set were unsuccessful; thus, we used our own primers to amplify a smaller region of the cytochrome *b* gene, only to discover a mistake in the published sequence of primer P2. Although published as 5' catagtaacagcttctgc 3', the correct sequence should have been 5' catagtaacagcttctg 3' (C. Avila-Adame, *personal communication*). If the correct primer sequence for P2 had been used, it is likely that it would have been acceptable for our work.

With respect to the observed cross-resistance observed for *C. cereale* isolates, in vitro ED₅₀ values for *C. cereale* isolates to pyraclostrobin and trifloxystrobin were lower than for azoxystrobin; however, the shift in sensitivity for resistant versus sensitive isolates was within the same range (>200×). Based on the glasshouse assays, all of the resistant isolates tested could not be controlled with the highest labeled rate of all three fungicides, indicating that these shifts were adequate for the loss of control with field rates of the fungicides. Because resistance in isolates of QoI-resistant *C. cereale* appears similar, future monitoring with azoxystrobin based on the method-

ology presented herein could be applicable for future studies on QoI resistance.

The locations from which QoI-resistant isolates were found had been using QoI fungicides for disease management since 1998. At a minimum, three sprays per year had been used at each location, with some using up to six applications per year specifically for the control of anthracnose and summer patch, caused by *Magnaporthe poae*, diseases which often occur at the same time (8). These locations had been treated anywhere from 9 to 15 times before fungicide effectiveness became questionable. It was somewhat surprising that QoI resistance was detected at high frequencies from these locations. In general, California golf courses do not have as much disease pressure as those in more humid environments, such as the in the eastern United States, and the reliance on fungicides is less in comparison. However, there is a precedent for resistance development after limited exposure because practical resistance to QoI fungicides has been reported rather soon (1 to 2 years) after their use in a number of cases. These include wheat powdery mildew in northern Europe, cucurbit powdery and downy mildew in Japan, black Sigatoka of banana in Costa Rica and Panama, and vine downy mildew in Italy (16).

Two factors also may have contributed to the rapid development of QoI resistance in populations of *C. cereale* in California. Due to the lack of low winter temperatures and winter dormancy, *C. cereale* in many parts of California can stay active year round as a saprophyte. Anthracnose basal rot is known to occur in the state during these periods under wet conditions. Thus, populations of the fungus inadvertently were exposed continually to QoI fungicides used for other fall and winter diseases caused by *Rhizoctonia* spp., *Microdochium nivale*, and other fungi. These permissive conditions also would allow for the development of large saprophytic populations which would be subjected to fungicide applications; selection against larger populations often results in a higher incidence of resistance development (21). Finally, when anthracnose was seen developing at these locations, many of these applications were made in a curative manner, which would result in incomplete disease control and the survival of larger populations to be selected against (44,45). Thus, three conditions that tend to drive the development of practical resistance existed: (i) repeated exposed to one fungicide class, (ii) exposure of large populations to selection pressure, and (iii) numerous curative applications.

Although the sampling technique used in this study was not ideal, with samples tending to be clustered from diseased portions of greens instead of randomly distributed, the high frequency of QoI resistance in these samples does suggest

uniformity of resistant genotypes in areas affected by anthracnose. The spread of resistant genotypes quickly throughout a putting green is not unexpected. Chen et al. (7) had seen that isolates of *C. cereale* from an *Agrostis palustris* putting green were fairly clonal and that individual isolates were capable of spreading 20 m or

more based on intensive cultural practices. Thus, under strong selection pressure by fungicide applications on greens, a QoI-resistant genotype could spread quickly, given that putting greens are typically 200 to 500 m² in size. The sampling procedure employed in this study likely underestimates the proportion of

Fig. 6. Cross-resistance to QoI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin in 32 isolates of *Colletotrichum cereale*: linear regression performed on relative growth (RG) values determined at fungicide concentrations of 0.031 µg/ml plus salicylhydroxamic acid at 100 µg/ml.

Table 5. Effectiveness of QoI fungicides applied preventively to *Poa annua* in controlling isolates of *Colletotrichum cereale*

Isolate	Azoxystrobin ED ₅₀ (µg/ml) ^z	Disease (%) ^y			
		Water only	Azoxystrobin (1.5 g/liter)	Pyraclostrobin (1.3 g/liter)	Trifloxystrobin (0.94 g/liter)
AHCC-1	>8.0	68 a	63 a	65 a	64 a
ANGC-1	>8.0	61 ab	63 a	54 ab	62 ab
FUGC-1	>8.0	46 b	46 b	50 ab	51 ab
TCGC-5.2	0.074	49 ab	0.33 c	1.3 c	0.76 c
TCGC-5.3	0.083	51 ab	1.3 c	0.33 c	1.3 c
TCGC-5.4	0.047	54 ab	0.50 c	1.0 c	0.50 c

^y Six-week old *P. annua* plants in 7.5-cm-diameter pots were sprayed with water or fungicide solutions and inoculated 24 h later with a spore suspension of 1×10^6 conidia/ml; disease severity was evaluated 14 days later based on the Horsfall-Barrat (0-to-11) scale. Means reflect three experiments using three pots per experiment. Values are based upon converted Horsfall-Barrat midpoint values; statistics were performed on arcsin-transformed data. Means followed by the same letter are equivalent according to Fisher's protected least significant difference ($\alpha = 0.05$).

^z In vitro 50% effective dose (ED₅₀) of azoxystrobin.

isolates still sensitive to the QoI fungicides. For all of the locations where QoI-resistant populations were found, all of these locations already had reported fungicide failures. It was likely that the detection of a high frequency of resistance at these locations reflects sampling after the development of practical resistance. At a field experiment location in northern California where six applications of azoxystrobin (306 g/ha) and pyraclostrobin (306 g/ha) at 14-day intervals provided 34 and 44% control, respectively (29), 40 isolates were collected from the untreated portion of the site and 40 from each of the azoxystrobin- or pyraclostrobin-treated plots. All were tested at a dose of azoxystrobin at 8 µg/ml plus SHAM at

Isolate	Gen Bank			100 ^b	110	120
	Accession	% ^a				
TCCG-5.31 (sen)	EF636928	98.8	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
TCCG-5.33 (sen)	EF636929	98.8	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
TCGC-5.34 (sen)	EF636930	98.8	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
CLGC-11 (res)	EF636931	97.5	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
CLGC-12 (res)	EF636932	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
CLGC-13 (res)	EF636933	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
EGGC-21 (res)	EF636934	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
EGGC-22 (res)	EF636935	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
EGGC-23 (res)	EF636936	97.5	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
SHGC-26 (res)	EF636937	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
SHGC-27 (res)	EF636938	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
SHGC-28 (res)	EF636939	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
AHCC-10 (res)	EF636940	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
AHCC-11 (res)	EF636941	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
AHCC-13 (res)	EF636942	98.2	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF
Cyt b (sen)	AY285743	100	GR	GMYGYSYRAP	RTLWVVMGTM	MTVAMMGMGF

Isolate	130	140	150	160	170
TCCG-5.31 (sen)	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ	DIVESVWGGF	SV
TCCG-5.33 (sen)	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ	DIVESVWGGF	SV
TCGC-5.34 (sen)	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ	DIVESVWGGF	SV
CLGC-11 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
CLGC-12 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
CLGC-13 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
EGGC-21 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
EGGC-22 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
EGGC-23 (res)	LGYVLPYGQM	SLCAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
SHGC-26 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
SHGC-27 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
SHGC-28 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
AHCC-10 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
AHCC-11 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
AHCC-13 (res)	LGYVLPYGQM	SLWAATVITN	TMSAIPWIGQ	DIVEFVWGGF	SV
Cyt b (sen)	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ	DIVESVWGGF	SV

^a % nucleotide sequence similarity to previously characterized cytochrome b sequence

AY285743

^b amino acid residue position

Fig. 7. Amino acid sequences of cytochrome *b* expressed by isolates of *Colletotrichum cereale*. Isolates with 50% effective dose (ED₅₀) values for azoxystrobin >8.0 µg/ml were considered resistant (res), while those with ED₅₀ values within the baseline distribution were considered sensitive (sen). Deviations from known amino acid residues from a previously-characterized, sensitive isolate AY285743 (3) are highlighted.

100 µg/ml using the methodology described above. Of the tested population from the untreated areas, 68% was resistant, whereas 100% taken from the treated areas was resistant. This indicates that sampling of survivor populations from areas where fungicides already have been applied greatly would result in the identification of only resistant isolates and suggests that the amount of control obtained by the application of fungicides is proportional to the frequency of QoI resistance at the location. Subsequent studies on the frequency of QoI-resistant isolates needed for practical resistance to occur and on the distribution of QoI-resistant and -sensitive genotypes within, among, and outside of putting greens should be conducted to better understand how and at what rate these genotypes spread.

Under field conditions, the G143A mutation in cytochrome *b* and the QoI-resistant phenotype appears to be the major trait being selected for by repeated fungicide applications. Studies by Crouch et al. (10) have indicated that azoxystrobin-resistant isolates were associated with a wide range of genotypes based on internal transcribed spacer sequence information. According to that work, QoI resistance is developing independently among isolates without any observable pattern. The presence of high frequencies of QoI-resistant isolates of *C. cereale* from tested locations, the resistance being found in individuals of diverse genotypes, and rather rapid development of suspected field resistance in multiple locations indicate that this pathogen is at high risk for future QoI-resistance development.

Practically, the results of this study have tangible impacts on the management of this disease, especially on annual bluegrass putting greens where anthracnose has been a more severe problem compared with the disease on creeping bentgrass. Although resistance was not confirmed by in vitro or molecular testing, it appears that QoI-resistant *C. cereale* may be fairly widespread, as reflected by poor control in University field studies on either annual bluegrass or creeping bentgrass test sites. Locations where poor or no control was reported include Arkansas (31), California (14,15,29), Pennsylvania (37–42), and New Jersey (36). Because QoI-resistance development appears to have occurred at levels to cause control failures at multiple locations, judicious use of these fungicides at locations where QoI fungicides still have good performance is mandated. Applications of QoI fungicides in mixtures with other fungicide classes, especially multisite materials, would not necessarily delay resistance development, but might help to avoid catastrophic failure in the presence of QoI resistance. Resistance monitoring, using the methods developed in this study, would be useful for helping to determine the relative status of sensitiv-

ity of populations on greens and to determine whether QoI fungicide use should be discontinued. Regardless of the continuing development of QoI resistance in populations of *C. cereale*, the QoI fungicides still have continued utility for the control of other turfgrass diseases. Other than for *Pyricularia grisea* (20,43), no other turfgrass pathogens have appeared to develop practical resistance to QoIs under field conditions. Thus, QoI fungicides are still quite valuable for turfgrass disease control, but their use should be carefully monitored and managed.

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