Sensitivity Distributions of California Populations of *Colletotrichum cereale* to the DMI Fungicides Propiconazole, Myclobutanil, Tebuconazole, and Triadimefon

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ABSTRACT

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The baseline sensitivity of a California population of *Colletotrichum cereale* (turfgrass anthracnose) to the sterol demethylation inhibitor (DMI) fungicide propiconazole was determined using an in vitro assay with known reproducibility. The 50% effective dose (ED₅₀) values for propiconazole for a nonexposed, baseline population ranged from 0.025 to 0.35 µg/ml with a mean of 0.14 µg/ml. Examination of two DMI-exposed populations indicated an approximate increase of 6.5× in mean ED₅₀ values. In vivo testing of two isolates with ED₅₀ values of propiconazole at 0.15 and 0.90 µg/ml indicated reduced control for the less sensitive isolate by propiconazole at rates ≤38 µg/ml. It was determined that single discriminatory dose testing in vitro with propiconazole at 0.50 µg/ml could differentiate sensitive and resistant isolates. Using this dose, six additional populations were tested and DMI-exposed populations were found to be three to nine times less sensitive compared with the baseline population. Two populations were assayed for sensitivity to myclobutanil, tebuconazole, and triadimefon. Mean ED₅₀ values for a nonexposed population were 0.72, 0.082, and 5.6 µg/ml, respectively; for a DMI-exposed population, mean ED₅₀ values were 3.8, 0.35, and 18 µg/ml, respectively. This work provides information on the development of DMI resistance in populations of *C. cereale* in California and methodologies for future resistance monitoring for this pathogen.

Additional keywords: annual bluegrass, *Poa annua*

Colletotrichum cereale sensu lato Crouch, Clarke, and Hillman (5) is the causal agent of turfgrass anthracnose, a disease that has been increasing in importance for annual bluegrass (*Poa annua*) and creeping bentgrass (*Agrostis stolonifera*) golf course putting greens. Recently, many superintendents in California, as well as other locations in the United States, reported difficulty in controlling this disease with fungicide spray programs (F. P. Wong, *personal observation*). As a result of these reports, we suspected that fungicide resistance development in *C. cereale* may be a factor, and undertook several studies to determine the current status of fungicide resistance in California populations of *C. cereale* from golf course locations.

Systemic, single-site mode of action fungicides labeled for turfgrass anthracnose in the United States include the QoIs, benzimidazoles, sterol demethylation inhibitor (DMI) fungicides, and, most recently, the polyoxins and phenylpyrroles.

Recent studies in California have confirmed that resistance to the QoI fungicides (2) is present in a number of locations that have had difficulty controlling the disease (43). Resistance to benzimidazoles also has been reported (7) and is present in California for this pathogen (42). Although DMI fungicides commonly are used in anthracnose management programs, resistance to these fungicides has not been previously examined.

The DMI fungicides (DMIs) are a large subclass of the sterol-biosynthesis-inhibiting fungicides that function by disruption of the biosynthesis of ergosterol, a key component of cell membrane structure in most true fungi (kingdom mycota), including ascomycetes, basidiomycetes, chytridiomycetes, and zygomycetes (19,21). Specifically, the DMIs inhibit the demethylation of lanosterol by a cytochrome P450 lanosterol 14 α -demethylase, leading to disruption of membrane function by the steric effects of the methylated ergosterol molecules within the cell membrane double-lipid layer (18–21). The DMIs are particularly useful for disease control due to their post-infection activity against fungal plant pathogens (37).

Resistance to DMI fungicides is common for a number of fungal plant pathogens, including *Erysiphe graminis*, *Monilinia fructicola*, *Mycosphaerella fijiensis*, *Rhynchosporium secalis*, *Sphaerotheca*

fuliginea, *Uncinula necator*, and *Venturia inaequalis* (11). Specifically for turf pathogens, DMI resistance has been problematic for *Sclerotinia homoeocarpa* (dollar spot) (12,15,28). Unlike qualitative resistance, typical for benzimidazole or QoI fungicides (2,14,18,21) where resistant isolates are practically immune to the fungicide, resistance to DMIs is manifested as quantitative resistance, where tolerance develops to these fungicides resulting in the need for the high labeled rate of the fungicide or shortened application intervals to achieve adequate control (9,22–24,44,45).

Myclobutanil, propiconazole, and triadimefon are DMIs registered in the United States for the control of anthracnose caused by *C. cereale*. These were registered for use on turf in California in 1995, 1997, and 1980, respectively. Given the historic use of DMIs for the management of this disease, there is an appreciable risk of DMI-resistance development for *C. cereale* which should be investigated. Practically, loss of anthracnose control due to DMI-resistance development in *C. cereale* may not be apparent. Often, DMIs are not used alone in mid- to late-summer when anthracnose is most active due to concerns over detrimental plant growth regulator effects on putting green turf (1,8,16,30). Thus, the use of DMIs early in the season (spring and early summer), limited use in the summer, or use in combination with other fungicides in tank mixes may be hiding practical DMI-resistance development for this pathogen.

At the time the study was initiated, propiconazole was the most recently registered DMI fungicide for turf use in California and commonly is used for anthracnose control. Therefore, it was the primary fungicide focused upon. The overall objective of this work was to develop a method to assay for DMI sensitivity, determine the current level of DMI resistance in several populations of *C. cereale*, and assess the potential impact of DMI resistance on anthracnose management programs. Specifically, we intended to (i) determine the baseline sensitivity distribution of *C. cereale* to propiconazole and assess current levels of propiconazole resistance in several field populations of *C. cereale*; (ii) determine if detected shifts in sensitivity to propiconazole were biologically relevant; (iii) use the developed in vitro testing methodology to determine the sensitivity

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of *C. cereale* populations to the additional DMI fungicides myclobutanil, triadimefon, and tebuconazole, a DMI fungicide currently in late-stage development for use on turf; and (iv) determine levels of cross-resistance among these four DMIs.

MATERIALS AND METHODS

Fungicides. All equipment and chemicals used in this study were obtained from Fisher Scientific (Pittsburgh unless otherwise noted). Technical grade propiconazole (98% active ingredient; Syngenta, Greensboro, NC), myclobutanil (93% active; Dow AgroSciences, Indianapolis, IN), tebuconazole (97% active; Bayer Crop Protection, Kansas City, KS), and triadimefon (95% active; Bayer Crop Protection) were used for this study. The fungicides were dissolved in acetone to provide stock solutions containing each fungicide at 8.0 mg/ml. All stock solutions were stored at -20°C in 15-ml polystyrene centrifuge tubes sealed with Parafilm.

Collection of *C. cereale* populations. In all, 528 single-conidial isolates of *C. cereale* used in a previous study examining the development of QoI resistance (43) were collected in 2002 through 2004 from nine California golf course locations (Table 1). Two populations, TCGC and SCCC, were collected from annual bluegrass in fairways that were reported to never have been sprayed with DMIs or other fungicides. Typically in California, due to economic limitations and the absence of high humidity conditions and the resulting fungal disease pressure, fungicide applications are not made to fairways or roughs, but rather targeted on putting greens and, to a lesser extent, on greens surrounds, approaches, and tees. Although it was possible for some portion of the population to have been exposed to some DMI fungicides, for the purposes of this study, these populations were considered nonexposed, "baseline" populations. The rest were collected from annual bluegrass putting greens where DMI fungicides had been used routinely for over 10 years (more than three applications per year) and considered "DMI-exposed" populations.

Isolates were obtained as described by Wong et al. (43). 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. Sampling was performing using a zigzag sampling strategy with plugs taken at least 10 m apart from each other on each approximate 12,000 m² fairway area to obtain a diverse sample. For the other populations, three to eight 11-cm-diameter plugs were taken from diseased areas on single putting greens (ranging in size from approximately 300 to 600 m²). Distribution of sampling points was variable due to the variable distribution in disease development at the locations; however, 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.

Approximately 12 anthracnose-infected plants with sporulating acervuli were taken as broadly as possible from each plug of collected turf. A single conidia was isolated from each plant by placing the infected plant in 300 µl of sterile distilled water (sdH₂O), streaking the spore suspension on 2% acidified water agar, and picking off single germinating conidia with a pin aided by a dissecting microscope as described by Wong et al. (43). For the duration of the study, isolates were maintained at 28°C on quarter-strength potato dextrose agar (1/4-PDA plates; 9.9 g of potato dextrose agar and 11.3 g of granulated agar per liter of sdH₂O), with regular transfers to new media at 14-day intervals.

Fungicide sensitivity assays. DMI sensitivity assays were based on methods developed by Wong et al. and Miller et al. (28,43,44). Stock solutions for myclobutanil, propiconazole, and tebuconazole were serially diluted in cold acetone to obtain eight working solutions at fourfold concentrations ranging from 0.00098 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 amended with fungicide at 0.0020 to 8.0 µg/ml. For triadimefon, fungicide stocks ranged from

0.0039 to 16 mg/ml which were used as described previously to provide a range of media amended with triadimefon at 0.0078 to 32 µg/ml. The 0 µg/ml (check) treatment was amended with only 1 ml of acetone. Petri dishes were filled with approximately 20 ml of media per dish in a positive-pressure biological safety hood to obtain fungicide-amended 1/4-PDA plates and allowed to solidify overnight. Plates were prepared 24 h prior to use in fungicide assays.

Assays were performed by inoculating fungicide-amended 1/4-PDA plates with 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. Plugs were placed onto the center of the 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, seven fungicide concentrations were used in addition to the nonfungicide-amended check plate. Plates were incubated for 7 days at 28°C without supplemental lighting, and then radial growth was measured by taking the average of three colony diameter measurements, subtracting the size of the original plug used for inoculation, and dividing by two.

Fungicide sensitivity, as measured by the 50% effective dose (ED₅₀), was calculated as described by Wong and Wilcox (44). Briefly, the percent relative growth (RG) was calculated as (radial growth at fungicide concentration/radial growth on the nonamended check plate) × 100. RG values were plotted against the log fungicide concentration and regression performed through the linear portion of the dose response using Microsoft Excel (Redmond, WA). The log ED₅₀ was calculated based upon the x-intercept corresponding to an RG value of 50; ED₅₀ values were calculated by taking the antilog of the calculated log ED₅₀ value.

Assay reproducibility. To determine the reproducibility and variance inherent in a single test, the sensitivity assay was performed using five isolates of *C. cereale* from the baseline population TCGC which were tested five times using new stock solutions each time. Variance, coefficients of variance, and upper and lower 95% confidence intervals were calculated using the normally distributed log ED₅₀ values as described previously (44). Assay reproducibility was examined only for propiconazole.

Distributions of *C. cereale* sensitivities to propiconazole. After establishing test reproducibility, 96 isolates from the baseline population TCGC were tested using the full range of fungicide concentrations. Histograms were constructed using log ED₅₀ classes based upon the least significant difference (LSD; $\alpha = 0.05$) between the five isolates tested five times for assay reproducibility (approximately 0.5 log units). In all, 84 isolates from AHCC and

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

Population	Location	Origin ^y	Exposed to DMIs ^z	Isolates	Collection date
AHCC	San Bernardino, CA	Putting green	Yes	84	2002
ANCC	Pasadena, CA	Putting green	Yes	28	2002
FUGC	Fullerton, CA	Putting green	Yes	65	2002
TCGC	Temecula, CA	Fairway	No	96	2002
CLCC	Canyon Lake, CA	Putting green	Yes	54	2003
EGGC	Corona, CA	Putting green	Yes	60	2003
SCCC	San Jose, CA	Fairway	No	69	2003
SHGC	San Bernardino, CA	Putting green	Yes	40	2003
BHCC	Danville, CA	Putting green	Yes	32	2004
Total	...	...	...	528	...

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

^z Demethylation inhibitor fungicides had been used regularly on putting greens locations. No applications were reported to have been made on the fairways sampled in this study.

65 from FUGC, both DMI-exposed populations, also were tested in the same manner to compare the distribution of sensitivities from a baseline population and exposed population. Population log ED₅₀ means were compared using *t* tests (Microsoft Excel).

In vivo sensitivity of two C. cereale isolates to propiconazole. An isolate from the baseline population (TCGC-5.2, ED₅₀ of propiconazole = 0.15 µg/ml) and one from the exposed population (AHCC-1, ED₅₀ of propiconazole = 0.90 µg/ml) were used to inoculate greenhouse-grown annual bluegrass (*P. annua* 'True-putt'; DLF International, Halsey, OR) treated preventively with propiconazole. Plants were 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 before inoculation. Plants were maintained at ambient greenhouse conditions (approximately 20 to 30°C), clipped at a height of 5 cm once per week, and watered as needed. Plants were inoculated with a spore suspension of conidia from either isolate TCGC-5.2 or AHCC-1 with a CO₂-powered sprayer as described above until run off. Isolates were grown for 14 days on 1/4-PDA at 28°C and conidia harvested by flooding the plates with sdH₂O. The suspension was filtered through a double layer of cheesecloth and adjusted to a final concentration of 1 × 10⁶ conidia/ml. Plants were covered with plastic bags for 48 h 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 and treated with propiconazole (Banner MAXX; Syngenta) at the equivalent of 0, 2.4, 9.6, 38, 150, or 610 µg/ml applied until run-off with a CO₂-powered sprayer using a single TeeJet 8002VS nozzle (R&D Sprayers, Opelousas, LA) at a pressure of 2.0 atm. Three pots of plants were used per experimental treatment. Plants were maintained in the chambers for an additional 12 days with daily misting, and disease severity scored for each pot at this time, rating plants on a linear 0-to-10 scale with 0 equal to no symptoms, 5 = 50% of the plants showing symptoms, and 10 = 100% of the plants showing symptoms. Assays were performed separately for the two isolates. Three pots were used for each fungicide concentration and the test was performed three times. Severity data were normalized using an arcsine (*y*) transformation where *y* = severity rating/10. For each isolate, means of the treatments were analyzed by analysis of variance (ANOVA) and compared using Fisher's Protected LSD with $\alpha = 0.05$.

Single discriminatory dose monitoring of C. cereale populations for sensitivity to propiconazole. Single discriminatory dose testing for fungicide sensitivity can be

utilized to monitor for population shifts in sensitivity and estimate ED₅₀ values as described for pathogens such as *V. inaequalis* and *U. necator* (35,44). Calculated log ED₅₀ values were regressed against RG values for individual isolates from populations AHCC, FUGC, and TCGC. Significance and coefficients of determination (*R*²) were calculated for the relationships at all concentrations (SAS 9.1, Cary, NC). Based on the correlation analysis, it was determined that the single discriminatory dose of propiconazole at 0.50 µg/ml would be most effective. A linear equation describing the relationship was calculated in order to estimate log ED₅₀ values for propiconazole based upon the RG values for isolates assayed with propiconazole at 0.50 µg/ml. The remaining isolates from populations ANCC, BHCC, CLCC, EGGC, SCCC, and SHGC were tested once with propiconazole at 0.50 and 0 µg/ml; RG was measured as described above and used to construct sensitivity distribution histograms in MS-Excel and to estimate isolate log ED₅₀ values for propiconazole.

Sensitivity of C. cereale isolates to myclobutanil, tebuconazole, and triadimefon. ED₅₀ values for a subset of 58 isolates from baseline population TCGC and 60 from DMI-exposed population AHCC were determined for myclobutanil, tebuconazole, and triadimefon using the methodology described above. Isolates were tested against a range of seven fungicide concentrations and a nonamended check treatment. For each population and fungicide, sensitivity distribution histograms were constructed using the log ED₅₀ values. Mean log ED₅₀ values for each population to the three fungicides were analyzed and compared using ANOVA and Fisher's protected LSD (SAS 9.1), whereas mean log ED₅₀ values for the same fungicide between populations AHCC and TCGC were compared using two-tailed *t* tests with $\alpha = 0.05$. Cross-sensitivity among fungicides was performed by regression analysis, comparing log ED₅₀ values for individual isolates for myclobutanil, propiconazole, tebuconazole, and triadimefon (SAS 9.1). The relationship among the individual populations TCGC and AHCC were analyzed separately as well as together to determine the relationships between sensitivity values for all six possible correlations between the four fungicides.

RESULTS

Fungicide assay results. *Assay reproducibility.* Based upon the five isolates assayed independently five times, it appeared that there was high reproducibility in determining log ED₅₀ values for propiconazole using the assay. The average coefficient of variance was 0.22%, with a range of 0.36 to 0.025% (Table 2), reflecting a high amount of reproducibility for this assay.

Distributions of C. cereale sensitivities to propiconazole. For the baseline population TCGC, ED₅₀ values ranged from propiconazole at 0.025 to 0.35 µg/ml with a mean of 0.14 µg/ml (Fig. 1, Table 3). Comparatively, ED₅₀ values for the two DMI-exposed populations ranged from propiconazole at 0.22 to 1.5 µg/ml with means of 0.91 and 0.97 µg/ml for populations AHCC and FUGC, respectively (Fig. 1, Table 3). These results indicated a clear shift in sensitivities between the baseline and DMI-exposed populations, in the range of 6.5- to 6.7-fold with respect to shifts in mean ED₅₀ values.

In vivo sensitivity of two C. cereale isolates to propiconazole. For isolates TCGC-5.2 (ED₅₀ = 0.15 µg/ml) and AHCC-1 (ED₅₀ = 0.90 µg/ml), both isolates were controlled fully at propiconazole concentrations of 150 and 610 µg/ml (Fig. 2). For TCGC-5.2, treatment with propiconazole at 38, 9.8, 2.4, and 0.60 µg/ml resulted in 100, 92, 77, and 19% control, respectively, relative to the 0 µg/ml check treatment. For AHCC-1, the same concentration range provided only 82, 51, 18, and 1.8% control, respectively, relative to the 0 µg/ml check treatment, indicating a differential response between isolates with differing ED₅₀ values in vivo (Fig. 2).

Distributions of C. cereale sensitivities to propiconazole based upon single dis-

Table 2. Assay reproducibility for determining 50% effective dose (ED₅₀) values for propiconazole for *Colletotrichum cereale* using mycelial expansion measurements on fungicide amended quarter-strength potato dextrose agar

Isolate	Mean (µg/ml) ^v	95%LL ^w	95%UL ^x	Variance ^y	CV (%) ^z
TCGC-5.30	0.100	0.046	0.27	0.00036	0.36
TCGC-5.1	0.058	0.026	0.15	0.000067	0.11
TCGC-5.25	0.054	0.024	0.14	0.000032	0.058
TCGC-8.11	0.057	0.028	0.29	0.000014	0.025
TCGC-8.20	0.089	0.041	0.24	0.00048	0.53
Average	...	...	...	0.00019	0.22

^v Mean ED₅₀ value based upon log ED₅₀ values obtained from five repeated assays, isolates grown for 7 days at 28°C on propiconazole-amended media.

^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.

crimutory dose testing. For determining the best correlation between the RG values for growth with propiconazole at 0.0020, 0.0078, 0.031, 0.13, 0.50, 2.0, and 8.0 µg/ml and calculated log ED₅₀ values, using all of the isolates from TCGC, AHCC, and FUGC, the best linear relationship was determined to be for RG with propiconazole at 0.50 µg/ml (Fig. 3). The relationship was described by the function $\log \text{ED}_{50} = 0.015 \times (\text{RG}) - 1.1$, with $R^2 = 0.92$ and $P < 0.001$. Using this relationship, single discriminatory dose testing with propiconazole at 0.50 µg/ml would estimate ED₅₀ values of 0.079 to 2.5 µg/ml based upon RG values of 0 to 100 obtained by this test.

For histograms constructed based on the RG values from the 348 *C. cereale* isolates from populations ANG, BHCC, CLCC, EGGC, SCCC, and SHGC, there was a clear differentiation between the baseline populations (TCGC and SCCC) and the

remaining DMI-exposed populations (Fig. 4). The RG values obtained from DMI-exposed populations generally were outside of the range of RG values obtained for the baseline isolates (Table 4). Based on the estimated log ED₅₀ values for propiconazole, the mean values for the baseline populations TCGC and SCCC were roughly equivalent (0.14 and 0.18 µg/ml, respectively). Estimated mean log ED₅₀ values from the DMI-exposed populations were 4.3 to 8.1 times higher compared with the combined mean of the two baseline populations (Table 4).

Sensitivity distributions of C. cereale isolates to myclobutanil, tebuconazole, and triadimefon. For the 58 isolates from baseline population TCGC, there were significant differences among calculated log ED₅₀ distributions for myclobutanil, tebuconazole, and triadimefon (Fig. 5A; Table 5). Mean log ED₅₀ values were significantly different amongst the four fungi-

cides (ANOVA, $P < 0.001$), with the mean ED₅₀ value for tebuconazole being the lowest (0.082 µg/ml), followed by propiconazole (0.14 µg/ml), myclobutanil (0.72 µg/ml), and triadimefon (5.6 µg/ml).

For population AHCC, mean log ED₅₀ values were significantly different for the four fungicides (ANOVA, $P < 0.001$), with the mean ED₅₀ value for tebuconazole being the lowest (0.35 µg/ml), followed by propiconazole (0.82 µg/ml), myclobutanil (3.8 µg/ml), and triadimefon (18 µg/ml) (Fig. 5B; Table 5). Although ED₅₀ values for exposed population AHCC were higher compared with baseline population TCGC, the relative differences in sensitivity to the four fungicides was similar.

Cross-sensitivity among myclobutanil, propiconazole, tebuconazole, and triadimefon for isolates of C. cereale. For the regression analysis performed for the six interactions between the four fungicides, the relationship between sensitivities for the fungicides within the baseline population TCGC was moderate, and poor for the less sensitive isolates from DMI-exposed population AHCC (Table 6). For population TCGC, the linear regression analysis indicated P values were lowest for the interactions of triadimefon–myclobutanil, tebuconazole–propiconazole, triadimefon–tebuconazole, and myclobutanil–tebuconazole ($P < 0.0034$). R^2 values ranged from 0.21 to 0.34 for these interactions, indicating a low amount of dependence between sensitivities for these fungicides. For the correlation between log ED₅₀ values for propiconazole–myclobutanil and propiconazole–triadimefon, $P = 0.13$ and 0.054, respectively, and R^2 values for these interactions were 0.065 or less. For population AHCC, for all but the interaction of triadimefon–propiconazole, regression analysis indicated nonsignificant P values ($P \geq 0.32$). For the interaction of triadimefon–propiconazole, $P = 0.0092$, but the R^2 value was 0.11.

When the log ED₅₀ values from all of the isolates from both populations were pooled and analyzed together, all interactions were shown to have $P < 0.001$. The correlation between myclobutanil–propiconazole, tebuconazole–propiconazole, and myclobutanil–tebuconazole were strong, as reflected by R^2 values of 0.75, 0.73, and 0.78, respectively (Table 6; Fig. 6). Correlation between the log ED₅₀ values of the remaining fungicide combinations (triadimefon–myclobutanil, triadimefon–propiconazole, and triadimefon–tebuconazole) were lower, with R^2 ranging between 0.48 and 0.58.

DISCUSSION

In this work, we present information on methods for monitoring *C. cereale* sensitivities to propiconazole, evidence for shifts in sensitivity for this fungicide that already have occurred at locations where DMIs have been used regularly, and evi-

Fig. 1. Frequency distributions of sensitivities of three *Colletotrichum cereale* populations to propiconazole based upon bioassays using quarter-strength potato dextrose agar amended with propiconazole at a concentration range of 0.0 to 8.0 µg/ml. Population TCGC ($n = 96$) is a baseline population and AHCC ($n = 84$) and FUGC ($n = 65$) are fungicide-exposed populations. X-axis values represent the midpoint of each histogram class, with histogram classes based upon the calculated Fisher's least significant difference value of 0.5 log units ($\alpha = 0.053$) for calculated 50% effective dose (ED₅₀) values of five isolates based upon five repeated tests.

Table 3. Sensitivity distributions of populations of *Colletotrichum cereale* to propiconazole

Population	<i>n</i>	ED ₅₀ (µg/ml) propiconazole ^z		
		Min	Mean	Max
AHCC (exposed)	84	0.22	0.91 b	1.5
FUGC (exposed)	65	0.64	0.97 b	1.5
TCGC (baseline)	96	0.025	0.14 a	0.35

^z Log 50% effective dose (ED₅₀) calculated by measuring radial growth of isolates on propiconazole-amended quarter-strength potato dextrose agar; relative growth was plotted versus the log concentration of fungicide and regression performed through the linear portion of the dose response to calculate ED₅₀ values. Values here represent the anti-log of the calculated values. Min and Max = minimum and maximum, respectively. Means followed by the same letter are equivalent, based on one-tailed t tests with $\alpha = 0.05$.

dence for cross-sensitivity among four DMI fungicides. This is the first published baseline sensitivity distribution for DMIs for *C. cereale* and the information and methods presented here should allow for future monitoring for DMI resistance for this pathogen and implementation of resistance management strategies for this pathogen.

The method developed here to determine ED₅₀ values for propiconazole had a high amount of reproducibility similar to other systems studied (28,44), based on independently performed replicated tests on five isolates. This method also appeared applicable for the determination of ED₅₀ values for myclobutanil, tebuconazole, and triadimefon. An abbreviated form of the assay using a single discriminatory dose of propiconazole at 0.50 µg/ml was able to differentiate between isolates with ED₅₀ values equivalent to baseline population isolates and those with reduced sensitivity to propiconazole.

With regard to the baseline sensitivity distribution to propiconazole, the 14× range of ED₅₀ values was similar compared with those determined for propiconazole for *Sclerotinia homoeocarpa* (28) and *Cercospora beticola* (17) and unlike the broader range (100× to 950×) observed for propiconazole sensitivity distributions for pathogens such as *Cladosporium caryigenum* (31), *Monilinia fructicola* (33), *M. oxycocci* (27), and *Mycosphaerella fijiensis* (32) or other DMIs for *U. necator* (9,44,45) and *V. inaequalis* (23,24,35). Shifts in propiconazole sensitivity as measured by the ratio of the mean ED₅₀ value of exposed population were similar to what was observed for other pathogens where practical resistance had developed to this fungicide (28,32). Although there is no prior work examining the effects of sensitivity shifts in *Colletotrichum cereale* populations to propiconazole, mean sensitivity shifts of roughly eight times observed for *U. necator* and *V. inaequalis* were associated with the development of practical resistance to myclobutanil (23,44).

In vivo tests with two isolates with a six times difference in propiconazole sensitivity did indicate a differential response to curative applications of propiconazole. Curative treatments were chosen because DMI fungicides typically have no protectant effect on spore germination and initial tissue colonization (37). Differences in disease control between these two isolates were seen only at concentrations of propiconazole at or below 38 µg/ml, approximately 1/16 the labeled rate of the fungicide. This response was similar to what was seen for the control of *V. inaequalis* isolates with differing sensitivities to myclobutanil (24). This implies that isolates that were in this range of decreased sensitivity are less controlled by fungicide applications and could be considered resis-

tant. Practically, the impact of these shifts could be minimal due to the high rate at which fungicides are used on turfgrass. Propiconazole is labeled for anthracnose control on golf course putting greens at rates of 496 to 993 g/ha. Comparatively, propiconazole typically is applied on a

range of agronomic and horticultural crops at rates of approximately 94 to 190 g/ha. As a result, *C. cereale* populations are exposed to a relatively high dose of propiconazole, which is able to provide adequate control of both sensitive and "resistant" populations. The use of such a high-

Fig. 2. Control of two *Colletotrichum cereale* isolates, TCGC-5.2 (baseline population isolate, propiconazole 50% effective dose [ED₅₀] = 0.15 µg/ml) and AHCC-1 (exposed population isolate, propiconazole ED₅₀ = 0.90 µg/ml), with propiconazole in vivo. Six-week-old annual bluegrass grown in 7.5-cm plastic pots were treated with propiconazole at 0, 0.60, 2.4, 9.8, 38, 150, or 610 µg/ml 48 h after inoculation with a conidial suspension of either isolate TCGC-5.2 or AHCC-1. Plants were rated 14 days after inoculation for disease development on a linear 0-to-10 scale, where 0 = no plants showing symptoms, 5 = 50% of the plants showing symptoms, and 10 = 100% of the plants showing symptoms. Points reflect the means of three tests using three replicates per treatment, and error bars reflect the standard error of the mean. For each isolate, means designated with the same letter are statistically equivalent (Fisher's protected least significant difference, $\alpha = 0.05$).

Fig. 3. Correlation between percent relative growth (RG) at the discriminatory dose of propiconazole at 0.50 µg/ml and actual calculated log 50% effective dose (ED₅₀) (µg/ml propiconazole) values for 245 isolates of *Colletotrichum cereale* from three populations. The relationship is useful as an estimate of ED₅₀ values based upon single discriminatory dose testing of isolates.

rate strategy for DMI fungicides has been proposed by Köller and Wilcox. (20,23); however, for most agricultural systems, such rates would be impractical or cost prohibitive. This is not the case for fungi-

cide applications to golf course putting greens.

In examining recent results from university field trials with propiconazole, results are mixed as far as season-long control.

For example, in 2002 Pennsylvania and 2004 Georgia trials, applications of propiconazole at 240 to 993 g/ha provided only 20 to 0% control in season-long programs (4,39,40). In other locations, such as field trials conducted in New Jersey in 2002 (38) and Pennsylvania in 2003 (41), applications provided 91 to 100% control. Furthermore, there were a number of locations in California, Pennsylvania, and Georgia (3,10,13,26) where labeled rates of propiconazole provided control ranging from 65 to 84% in season-long programs. In these cases, the sensitivity of the *C. cereale* populations to propiconazole was not examined, and the role of sensitivity shifts is not understood as far as its contribution to the variable results that were seen in these field trials. There is a possibility that some detrimental plant growth regulator effects caused by propiconazole (30) under warm temperature conditions to the turf could have contributed to the apparent damage reported in some of these trials. This lack of information mandates a further examination to determine what magnitudes of sensitivity shifts actually confer practical resistance (18).

In examining cross-sensitivity among propiconazole, myclobutanil, tebuconazole, and triadimefon, results were similar to those reported for other pathogens such as *S. homoeocarpa* (15,28), *Cercospora beticola* (17), and *U. necator* (9,45). When the baseline population TCGC and exposed population AHCC were analyzed separately, coefficients of correlation gen-

Fig. 4. Frequency distributions of sensitivities of nine *Colletotrichum cereale* populations to propiconazole based upon percent relative growth of isolates tested at the single discriminatory dose of propiconazole at 0.50 µg/ml. Populations TCGC and SCCC are baseline populations, whereas the remainder are demethylation inhibitor fungicide exposed. X-axis values represent the midpoint of each histogram class.

Table 4. Comparison of *Colletotrichum cereale* population sensitivities to propiconazole based on single discriminatory dose testing with propiconazole at 0.50 µg/ml

Population	n	RG with propiconazole at 0.50 µg/ml ^y			Estimated ED ₅₀ (µg/ml) propiconazole ^z		
		Mean	Minimum	Maximum	Mean	Minimum	Maximum
AHCC	84	71	55	100	0.98	0.53	2.6
ANCG	28	77	64	90	1.2	0.73	1.8
BHCC	32	62	51	70	0.68	0.46	0.90
CLCC	54	72	50	79	0.98	0.45	1.2
EGGC	60	78	35	96	1.3	0.27	2.3
FUGC	65	72	64	81	0.97	0.74	1.3
SCCC	69	18	14	20	0.14	0.13	0.16
SHGC	40	63	50	79	0.74	0.45	1.26
TCGC	96	24	13	45	0.18	0.12	0.38

^y RG = relative growth. Percent growth relative to the nonamended check treatment for isolates grown for 7 days on quarter-strength potato dextrose agar amended with propiconazole at 0.50 µg/ml.

^z Calculated based on the formula log 50% effective dose (ED₅₀) (µg/ml) = (0.0153 × RG) – 1.1, as determined by regression analysis of actual calculated log ED₅₀ values versus RG at a dose of propiconazole at 0.50 µg/ml.

Table 5. Sensitivity of two *Colletotrichum cereale* populations to myclobutanil, propiconazole, tebuconazole, and triadimefon

Fungicide	ED ₅₀ (µg/ml) ^y								
	TCGC (baseline, n = 58)				AHCC (exposed, n = 60)				P (t test) ^z
	Mean	Min	Max	Range	Mean	Min	Max	Range	
Myclobutanil	0.72 b	0.19	1.3	7.2	3.8 b	2.0	11	5.4	<0.001
Propiconazole	0.14 c	0.053	0.35	6.6	0.82 c	0.22	1.5	6.9	<0.001
Tebuconazole	0.082 d	0.015	0.57	38	0.35 d	0.19	0.70	3.6	<0.001
Triadimefon	5.6 a	2.2	13	5.9	18 a	5.5	64	12	<0.001

^y Calculations performed using the log 50% effective dose (ED₅₀) values calculated from dose responses on quarter-strength potato dextrose agar amended with fungicides; values reflect the anti-log of the calculated values. Min and Max = minimum and maximum, respectively. Means within columns followed by the same letter are equivalent based on Fisher's protected least significant difference (α = 0.05).

^z P value for a one-tailed t test comparing the means of populations AHCC and TCGC for the same fungicide.

Fig. 5. Frequency distributions of sensitivities of *Colletotrichum cereale* isolates to myclobutanil (MYC), propiconazole (PRO), tebuconazole (TEB), and triadimefon (TRI) for **A**, 58 isolates from baseline population TCGC and **B**, 60 isolates from demethylation inhibitor fungicide-exposed population AHCC. Log 50% effective dose (ED_{50}) values determined by bioassays using fungicide amended quarter-strength potato dextrose agar. X-axis values represent the midpoint of each histogram class.

erally were low. Analysis of both populations resulted in much higher coefficients of correlation, reflecting the possibility that highly resistant isolates likely share some general common mechanisms of resistance. Because the exact genetics and mechanisms of DMI-resistance is unclear, as discussed by Peever and Milgroom (29), the lack of perfect cross-sensitivity between isolates to the four fungicides was not unexpected.

The difference in intrinsic activities between the four DMI fungicides was apparent in both the baseline population TCGC and exposed population AHCC. This has a practical implication discussed by Erickson and Wilcox and Ypema et al. (9,45) in their work with *U. necator* that showed that DMIs with higher intrinsic activities provide better disease control under field conditions. Our work suggests that tebuconazole > propiconazole > myclobutanil > triadimefon for effectiveness against *Colletotrichum cereale* under field conditions. Although not directly tested in this work, these differences in activity are reflected by a field trial conducted in New Jersey in 2002 by Towers et al. (38). Here, seven applications of myclobutanil at 1.2 kg/ha, propiconazole at 0.50 kg/ha, tebuconazole at 1.5 kg/ha, or triadimefon at 1.5 kg/ha were made at 14-day intervals from mid-May to mid-August for anthracnose control on a mixed annual bluegrass and creeping bentgrass putting green. At the end of the trial in September, tebuconazole provided 99% disease control, propiconazole provided 91% disease control, myclobutanil provided 61% disease control, and triadimefon provided 5.8% disease control (control relative to the check treatments, which had an average of 83% area showing symptoms).

Overall, the impact of shifts in DMI sensitivity for *C. cereale* is far from being fully understood. However, this work does lay the groundwork for monitoring shifts in *C. cereale* to DMIs and evaluating the effectiveness of resistance management programs for this pathogen in turfgrass systems. Future studies should address the molecular mechanisms of DMI resistance in *C. cereale*. For example, it is unknown if the mechanism of resistance in this pathogen is mediated by point mutation in or overexpression of the 14 α -demethylase

Table 6. Cross-sensitivity between demethylation inhibitor fungicides for two populations of *Colletotrichum cereale*

Interaction	R^2 (ANOVA P^z)		
	TCGC (baseline) ($n = 58$)	AHCC (exposed) ($n = 60$)	All ($n = 118$)
Triadimefon-myclobutanil	0.24 (<0.001)	0.0113 (0.42)	0.58 (<0.001)
Myclobutanil-propiconazole	0.041 (0.13)	0.0021 (0.71)	0.75 (<0.001)
Tebuconazole-propiconazole	0.21 (0.0034)	0.0022 (0.73)	0.73 (<0.001)
Triadimefon-propiconazole	0.065 (0.054)	0.11 (0.0092)	0.54 (<0.001)
Triadimefon-tebuconazole	0.22 (<0.001)	0.017 (0.32)	0.48 (<0.001)
Myclobutanil-tebuconazole	0.34 (<0.001)	0.014 (0.37)	0.78 (<0.001)

^z ANOVA = analysis of variance. Coefficient of determination (R^2) determined by comparison of log 50% effective dose values for each fungicide from individual isolates.

gene as studied in *U. necator* and *V. inaequalis*, respectively (6,34), or adenosine triphosphate binding cassette transporters such as those studied in *M. graminicola* (36).

The results from this study do indicate that DMI resistance is present in populations of *C. cereale* in California and it is

not unreasonable to assume that resistance development has occurred in other locations where DMIs have been used for anthracnose control. Therefore, DMI resistance should be considered as a factor in anthracnose management programs. The large number of isolates examined in this study would allow for the detection of rare

phenotypes that occurred in 1% or more of the population with 99% confidence (25), suggesting that the sensitivity data would well represent the current DMI-resistance situation for *C. cereale* populations regularly treated with DMI-fungicides. Because the full impact of these shifts is not understood, additional research must be performed to understand which fungicide use patterns and resistance management strategies are most effective. However, it is likely that applications of DMI fungicides for control of *C. cereale* at low-labeled or sub-label rates or at extended application intervals would result in both poor control and resistance development as suggested by Köller and Wilcox (23). Furthermore, DMI fungicides with higher intrinsic activity toward *C. cereale*, such as propiconazole and tebuconazole, should be used preferentially versus myclobutanil or triadimefon. Given the current problems with resistance development to other classes of fungicides (42,43), and limits on multisite fungicide (chlorothalonil) use due to the 1996 U.S. federal Food Quality Protection Act, tactics to manage and delay resistance to DMIs for *C. cereale* must be implemented. Otherwise, it may be highly likely that systemic and curative fungicidal options for this disease will be severely limited.

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Fig. 6. Scatterplots of sensitivity data from two populations of *Colletotrichum cereale* (TCGC and AHCC) illustrating cross-sensitivity relationships between myclobutanil (MYC), propiconazole (PRO), tebuconazole (TEB), and triadimefon (TRI). Correlation was performed by comparing log 50% effective dose (ED_{50}) values for each fungicide from individual isolates. The listed interactions represent fungicides used for the Y-axis $\times$ X-axis values respectively.

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