

Thiophanate-Methyl and Propiconazole Sensitivity in *Sclerotinia homoeocarpa* Populations from Golf Courses in Wisconsin and Massachusetts

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ABSTRACT

Koch, P. L., Grau, C. R., Jo, Y.-K., and Jung, G. 2009. Thiophanate-methyl and propiconazole sensitivity in *Sclerotinia homoeocarpa* populations from golf courses in Wisconsin and Massachusetts. *Plant Dis.* 93:100-105.

Management of dollar spot, caused by the fungus *Sclerotinia homoeocarpa*, is dependent upon repeated fungicide applications in intensively managed turfgrass such as golf course putting greens and fairways. Repeated fungicide applications could potentially select for fungicide-resistant isolates and result in a reduction of disease control. The objectives of this study were to determine the degree of *S. homoeocarpa* in vitro sensitivity to the fungicides thiophanate-methyl and propiconazole using isolates collected from golf course putting greens, fairways, and roughs; and to determine the relationships of golf course age and fungicide history to the frequency of fungicide-insensitive isolates within the population. More than 1,400 *S. homoeocarpa* isolates were collected from putting greens, fairways, and roughs at six Wisconsin golf courses and one Massachusetts golf course and subjected to in vitro fungicide sensitivity assays with single discriminatory concentrations of thiophanate-methyl and propiconazole. Five of seven pathogen populations from rough areas were not significantly different from one another in propiconazole sensitivity. These populations were collectively the most sensitive to both fungicides and therefore, served as baseline populations for comparison with fungicide-exposed populations from putting greens and fairways. Greater propiconazole insensitivity was observed in populations collected from fairways and putting greens that received more frequent applications of the fungicide than those isolated from the roughs. In nearly all the golf courses, the frequency of thiophanate-methyl insensitivity was higher among isolates of *S. homoeocarpa* collected from fairways than from roughs regardless of the age of the golf course or history of benzimidazole use. Thus, while the development of resistance to propiconazole can be predicted in part by the relative frequency of demethylation inhibitor fungicide applications, the occurrence of populations resistant to thiophanate-methyl appears to be unrelated to recent use of the benzimidazole class of fungicides.

Dollar spot, caused by the fungus *Sclerotinia homoeocarpa* F.T. Bennett, is one of the most common diseases of intensively managed turfgrass in temperate regions of the world (2). *S. homoeocarpa* has a wide host range among turfgrass species, but its primary impact is on creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.) grown on golf course putting greens, tees, and fairways. Symptoms first appear on the leaf blade as small, straw-colored lesions with a reddish brown border. In optimal environmental conditions with temperatures between 18 and 30°C and relative humidity greater than 85%, multiple lesions may coalesce and spread across the entire leaf blade (2). The fungus spreads locally via hyphal contact with surrounding leaf tis-

sue, forming distinct silver dollar-sized bleached patches 3 to 5 cm in diameter on low-cut turfgrass as opposed to larger and more diffuse 15 to 30 cm patches on higher-cut turfgrass (5). Efforts at cultural and biological control of dollar spot have been marginally effective, failing to provide the high degree of control demanded by the golf industry (10,26). Acceptable control in intensively managed turfgrass has been obtained only through multiple fungicide applications made throughout the growing season. As a result, more fungicide applications are made to control dollar spot than any other turfgrass disease in the United States (25).

Frequent fungicide applications to control dollar spot have resulted in well-documented cases of pathogen resistance to fungicides in several chemical classes. In the 1970s, soon after the introduction of benzimidazoles into the market, reduced sensitivity in *S. homoeocarpa* populations to fungicides in this class was reported (27,28). Throughout the past 25 years, reduced sensitivity of *S. homoeocarpa* isolates to several dicarboximide and sterol demethylation-inhibitor (DMI) fungicides

also has been reported (1,3,4,9,12,14,20). The decreasing efficacy of these important chemical classes against dollar spot, coupled with the lack of new compounds in the turfgrass market, has left turfgrass managers with dwindling options.

Past research has shown that development of resistance to the benzimidazoles is conferred by a single mutation in the β -tubulin gene (21,24). The result of this mutation is immediate and complete resistance (qualitative or disruptive selection) to the fungicide at no fitness cost to the pathogen, allowing the rapid build-up of resistant isolates in the presence of fungicides and persistence in the environment in the absence of fungicides (13,15,19). In contrast to the benzimidazoles, development of insensitivity to the DMIs is controlled by several genes that regulate the demethylation of lanosterol to ergosterol in the sterol biosynthesis pathway (16). With each mutation in the pathway, sensitivity to DMIs is reduced (23). The result is a gradual build-up (quantitative or directional selection) of isolates in the population with reduced sensitivities to the DMIs that are less fit than wild-type isolates and do not persist in the environment in the absence of fungicides (6,18,22).

Turfgrass management, especially on golf courses, is unique in agriculture because of the division of the golf course into several distinct sections corresponding with how golf is played. Putting greens, fairways, and rough areas have varying disease thresholds and are in turn subjected to vastly different management practices, affecting the microenvironment of that site and the level of dollar spot disease pressure (8). Putting greens are the most intensively managed and highest value areas of the golf course. They are subjected to daily mowing at heights that range from 2.5 to 4 mm, frequent irrigation, and repeated preventative fungicide applications. Fairways are managed less intensively with mowing taking place two to four times per week at heights of approximately 12 mm. Less frequent and oftentimes curative fungicide applications are made to fairways at most courses because of the cost associated with treating larger areas and the higher tolerance for minor infection. Rough areas are the least intensively managed areas on the golf course, with mowing taking place every 2 to 3 days at heights between 5 and 12 cm and fungicides rarely applied. The

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Accepted for publication 28 August 2008

doi:10.1094/PDIS-93-1-0100
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effect these different environments have on the level of disease pressure at each site may also have an effect on the development of fungicide-insensitive isolates in the dollar spot population. Current strategies for delaying resistance development on golf courses include reducing the overall use of high-risk fungicides, limiting its use to periods of lower disease pressure, and tank mixing or rotating with products that have a different mode of action (14). These strategies do not account for possible location effects within a golf course that may have a significant impact on the development of fungicide resistance.

The objectives of this study were to (i) determine the degree of in vitro sensitivity to the fungicides thiophanate-methyl and propiconazole using *S. homoeocarpa* isolates collected from golf course putting greens, fairways, and roughs and (ii) determine the relationship of golf course age and fungicide history to the frequency of fungicide-insensitive isolates within populations of *S. homoeocarpa*.

MATERIALS AND METHODS

***S. homoeocarpa* isolate collection.** Six golf courses in Wisconsin and one golf course in Massachusetts were selected for sampling and represented a range of course ages and 5-year fungicide histories (Table 1). A 10 × 10-m sampling grid was set up in areas of high dollar spot pressure on one putting green, one fairway, and one area of rough at each golf course. The grid area was kept free of pesticide applications until sampling was completed. Courses OK, PV, and YH were the only courses in which fungicides were withheld from the putting green sampling grids and were the only golf courses in which the putting greens were sampled. Fairways and roughs were sampled at all seven golf courses.

Samples, comprised of symptomatic leaf blades, were collected throughout the summer of 2006 as dollar spot symptoms became present at each site. A total of 300 leaf blades showing lesions symptomatic of dollar spot were collected from each of the OK, PV, and YH golf courses. One hundred leaf blades were collected from one putting green, one fairway, and one rough area at each course. A total of 200 symptomatic leaf blades were collected from each of the BH, UR, HL, and MA golf courses with 100 collected from one fairway and one rough area. Each isolate sampled was at least 1 m apart. More than 1,400 isolates of *S. homoeocarpa* were subjected to in vitro fungicide sensitivity assays.

Isolation of *S. homoeocarpa* was performed within 24 h of collection following the procedures outlined in Jo et al. (14). Symptomatic leaf tissue was surface sterilized for 30 s in a 2.5% sodium hypochlorite solution, rinsed in sterile water, dried on sterile filter paper, and placed on acidified potato dextrose agar (APDA). The

APDA was prepared by adding 1 ml of 85% lactic acid (Fisher Scientific, Fair Lawn, NJ) per 1 liter of PDA (Difco Laboratories, Detroit, MI) after sterilizing in an autoclave for 15 min. Isolates were confirmed as *S. homoeocarpa* on the basis of colony morphology and comparison with known isolates. Hyphal tips of each isolate were acquired from colonies incubated at 25°C for 48 h and transferred to fresh APDA.

In vitro fungicide sensitivity. Fungicide sensitivity assays followed the procedures outlined in Jo et al. (14). Five-mm-diameter agar plugs containing actively growing mycelium of each isolate were transferred to PDA amended with discriminatory concentrations of thiophanate-methyl (Cleary's 3336 50WP; Cleary Chemical Corporation, Dayton, NJ) and propiconazole (Banner MAXX 1.3ME; Syngenta Crop Protection, Greensboro, NC). Discriminatory concentrations established in Jo et al. (14) for thiophanate-methyl and propiconazole were 1,000 and 0.1 µg a.i. ml⁻¹, respectively. All isolates from a single golf course area were tested concurrently on a single batch of thiophanate-methyl- and propiconazole-amended media. Cultures were incubated at 25°C for 48 h before being rated for fungicide sensitivity.

Because of the quantitative nature of fungal insensitivity to propiconazole, each isolate was cultured in replicate plates of nonamended and propiconazole-amended PDA media to generate average mycelial growth measurements. Radial mycelial growth in each culture was measured in two perpendicular directions and averaged separately for each medium. Percent relative mycelial growth (RMG) was then calculated by taking the average radial growth on propiconazole-amended PDA and dividing it by the average radial growth on nonamended PDA and multiplying by 100. To account for variations in propiconazole sensitivity due to experi-

mental error, two reference isolates (1A3 and 1A4) with a known RMG of 35% were included in every test. Each isolate was multiplied by the difference of the reference isolates RMG from 35% to correct for any variation in propiconazole-amended media preparation (14).

Because of the qualitative development of fungal resistance to thiophanate-methyl, each isolate was evaluated for growth on a single plate of thiophanate-methyl-amended PDA. An isolate was rated resistant if mycelial growth was observed or rated sensitive if no mycelial growth was observed.

Data analysis. For isolates grown on propiconazole-amended medium, analysis of variance tables were produced to determine statistical differences in mean RMG among isolates collected from the different golf courses and golf course areas using PROC MIXED (SAS 9.1; SAS Institute, Cary, NC). Pair-wise comparison tests were done for isolates grown on propiconazole-amended medium at each location using PROC MIXED. An analysis was done for the three courses with all three golf course areas sampled and a separate analysis was done for all seven golf courses over two golf course areas. For isolates grown on thiophanate-methyl-amended medium, chi-square analysis was performed to test differences in the ratio of insensitive to sensitive isolates collected from putting green, fairway, and rough populations using PROC FREQ (SAS Institute). The expected number of resistant isolates was determined on the basis of the assumption that the total observed number of resistant isolates collected from a given golf course would be distributed evenly among the sampling areas.

RESULTS

Relationship of golf course area to fungicide sensitivities. Frequency distributions of isolates obtained from three locations at course OK revealed that the

Table 1. Location, age, and fungicide history for golf courses from which isolates of *Sclerotinia homoeocarpa* were collected in 2006

Course	Location	Year established	Five-year fungicide history ^z	
			Benzimidazoles	Demethylation inhibitors
OK	Cottage Grove, WI	2003	None	Moderate on greens Moderate on fairways
PV	Middleton, WI	1958	None	Intensive on greens Sporadic on fairways
YH	Madison, WI	1967	Sporadic on greens None on fairways	Moderate on greens Sporadic on fairways
BH	Madison, WI	1921	Moderate on greens Sporadic on fairways	Intensive on greens Intensive on fairways
UR	Verona, WI	1991	None	Intensive on greens Moderate on fairways
HL	Verona, WI	2001	Sporadic on greens None on fairways	Intensive on greens Moderate on fairways
MA	Osterville, MA	1916	None on greens Sporadic on fairways	Moderate on greens Moderate on fairways

^z Intensive = >15 applications over the past 5 years; moderate = 5 to 15 applications over the past 5 years; and sporadic = <5 applications over the past 5 years.

population collected from the rough area was more sensitive to propiconazole compared with populations collected from both the fairway and putting green (Fig. 1). Populations collected from five of the seven rough areas were not significantly different in propiconazole sensitivity (Table 2). When all five of those statistically

similar mean RMG values were averaged together, an overall RMG of 10% was calculated. Though not a true baseline because these populations have likely been indirectly exposed to DMI fungicides in the past, given the variable age and fungicide histories of the courses included in this study, these populations serve as a

comparison to determine the degree of shift in fungicide sensitivity in directly exposed populations.

Significant differences ($P < 0.0001$) in propiconazole sensitivity were detected among isolate populations derived from golf courses OK, PV, and YH as well as among populations from different areas within each of the three golf courses where all three areas were sampled (Table 3). Isolate populations from rough areas were the most sensitive to propiconazole at all three golf courses, while populations collected from the fairway at OK and putting greens at all three courses saw a significant reduction in sensitivity (Table 4).

Isolates derived from fairways and roughs differed significantly in propiconazole sensitivity within and among all seven golf courses. Populations isolated from fairways in general were significantly ($P < 0.0001$) less sensitive to propiconazole than those from the roughs (Table 5). Fairway isolates from courses PV and YH, however, exhibited the same sensitivity to propiconazole as the isolates from the rough in the same golf course (Table 2).

The percentage of *S. homoeocarpa* isolates resistant to thiophanate-methyl ranged from 0 to 100% of the population. Chi-square analysis was performed on data from golf courses OK, PV, and YH to determine the proportion of thiophanate-methyl-resistant isolates collected from the putting greens, fairways, and roughs. (Table 6). Course OK had a significantly higher number of resistant isolates in the fairway population than expected, while resistant isolates in the putting green population were significantly lower than expected ($P < 0.0001$). A separate chi-square analysis was performed to test whether the proportion of thiophanate-methyl-resistant isolates collected from the fairways and roughs at all seven golf courses were equal (Table 6). All courses, except PV and YH, had statistically higher frequencies of resistant isolates than expected in the fairway than in the rough.

Relationship of course age and fungicide history to fungicide sensitivities.

Course age did not have a direct relationship to the frequency of *S. homoeocarpa* isolates with reduced sensitivity to propiconazole. Course MA was the oldest golf course sampled, and the isolates sampled from the fairway had the lowest mean propiconazole sensitivity (RMG of 65%) of any course area. Course BH was the second oldest course sampled, but the fairway population at this location was more sensitive to propiconazole than the MA fairway population and was similar to those from newer courses. Course OK was established in 2003 and the isolates collected from the fairway had a relatively moderate mean propiconazole sensitivity of 27%. Course HL, established in 2001, yielded isolates with a comparatively high mean sensitivity of 45%.

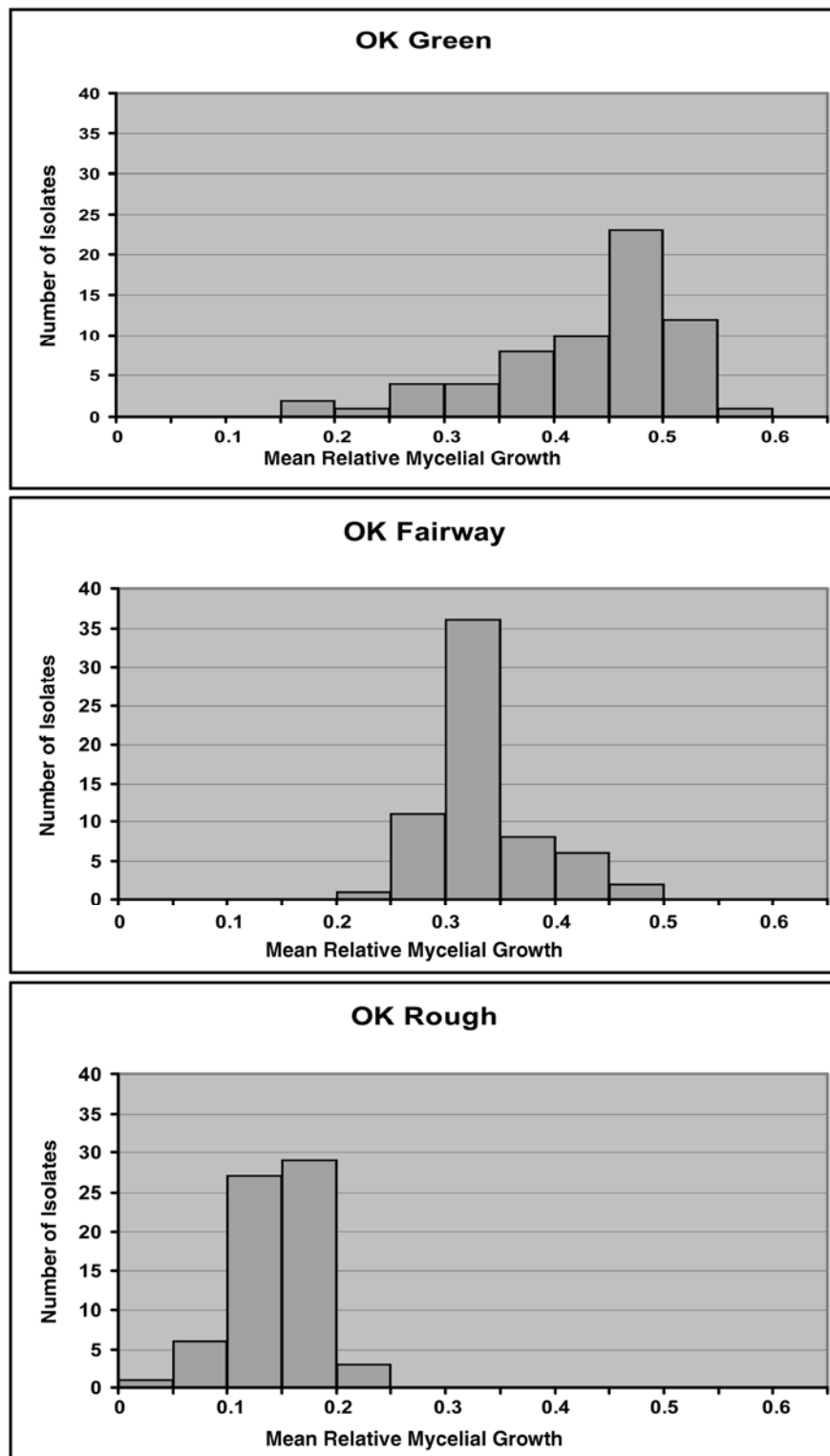


Fig. 1. Frequency distributions of *Sclerotinia homoeocarpa* isolates obtained from a putting green, fairway, and rough area at golf course OK in Wisconsin with in vitro sensitivity to propiconazole measured in mean relative mycelial growth.

Golf courses with greens or fairways that received moderate or intensive applications of DMI fungicides in the past 5 years yielded isolates with lower propiconazole sensitivities. Fairways at PV and YH received sporadic DMI fungicide applications in the past 5 years, and isolates from those areas were similar in propiconazole sensitivity to isolates from the rough areas that had not been directly exposed to fungicide applications.

A relationship of course age or fungicide history to the frequency of thiophanate-methyl-resistant *S. homoeocarpa* isolates was not observed. Even though course BH is 82 years older than course OK, these two fairway populations had the highest frequencies of thiophanate-methyl-resistant isolates. Furthermore, more than 20% of isolates collected from fairways at OK, UR, and HL were thiophanate-methyl-resistant despite those areas having no history of application of benzimidazoles for the past 5 years.

DISCUSSION

Propiconazole sensitivity of *S. homoeocarpa* on propiconazole-amended media was generally lower for isolates derived from golf courses and golf course areas with an intensive 5-year fungicide history. The most sensitive populations to propiconazole were golf course rough populations that had never been directly treated with fungicides. The isolates collected from rough areas served as a baseline to determine the degree of shift in fungicide sensitivities of isolates collected from putting greens and fairways. This baseline was calculated with *S. homoeocarpa* isolates recovered from primarily Kentucky bluegrass, fine fescue, and perennial ryegrass. This approach is valid since only a minor degree of host species specialization has been reported within *S. homoeocarpa* (11). More intensive fungicide programs were used on the fairways compared with the rough areas, likely resulting in the observed shift toward greater fungicide insensitivity. Putting greens at OK and PV received the most intensive fungicide applications and showed a further shift toward insensitivity when compared with fairways at the same courses.

According to the 5-year fungicide records obtained from each course, the fairways at BH received twice the number of fungicide applications as the fairways at MA. However, resistance to propiconazole was significantly higher in *S. homoeocarpa* populations from MA. Both courses employed similar tank mixtures of DMI and non-DMI fungicides, but BH made applications on shorter intervals. These results indicate that the number of fungicide applications within the past 5 years is not a universal predictor of present fungicide sensitivity and that application interval may also impact shifts in population sensitivity.

None of the seven courses studied had previously confirmed cases of practical *S. homoeocarpa* resistance to DMI fungicides. However, on the basis of superintendent observations at MA fairway, HL fairway, PV green, and OK green resistance to DMI fungicides was suspected. Superintendent observations included shorter duration of disease control, increased fungicide rates required for acceptable control and disease breakthrough during times of high disease pressure. These four locations had decreased propiconazole sensitivities compared with other course areas and a higher frequency of isolates with greater than 40% RMG. A 40% RMG may be a threshold for determining when *S. homoeocarpa* resistance detected in vitro begins to generate practical resistance in vivo. Prediction of in vivo resistance in the field on the basis of in vitro resistance in the lab has been difficult because of the number of variables that affect chemical

control of a pathogen as well as the quantitative nature of DMI resistance (7,17,20). Future research detailing the relationship between in vitro fungicide sensitivity and practical resistance in *S. homoeocarpa* is needed.

The documentation of widespread *S. homoeocarpa* resistance to the benzimidazoles in the 1970s resulted in the marked decline in the use of this chemical class (15). Fungicide records obtained from the golf courses we sampled only go back 5 years in accordance with federal pesticide regulations. Research has shown benzimidazole-resistant *S. homoeocarpa* isolates persist in the population indefinitely (13,15), so it was expected that benzimidazole applications made in the past 5 years would not accurately predict the numbers of resistant *S. homoeocarpa* isolates. Course BH and MA both had relatively intensive spray programs on their fairways, and both had high proportions (100% and

Table 2. Mean relative mycelial growth on propiconazole-amended medium of *Sclerotinia homoeocarpa* isolates collected from one fairway and one rough area at golf courses OK, PV, YH, BH, UR, and HL in Wisconsin and MA in Massachusetts

Course	Mean relative mycelial growth on propiconazole-amended medium (%) ^z	
	Fairway	Rough
OK	27 c	8 d
PV	13 d	11 d
YH	9 d	11 d
BH	25 c	11 d
UR	26 c	10 d
HL	45 b	27 c
MA	65 a	25 c

^z Means followed by the same letter do not significantly differ (Tukey–Kramer, $P < 0.05$).

Table 3. Analysis of variance on the mean relative mycelial growth of isolates of *Sclerotinia homoeocarpa* on propiconazole-amended medium collected from putting greens, fairways, and roughs at golf courses OK, PV, and YH in Wisconsin

Source	DF	Type III SS	Mean squares	F value	P value
Course area	2	9.6508	4.8254	475.18	<0.0001
Course	2	3.2541	1.6271	160.23	<0.0001
Course area*course	4	4.7309	1.1827	116.47	<0.0001

Table 4. Mean relative mycelial growth on propiconazole-amended medium of *Sclerotinia homoeocarpa* isolates collected from one putting green, fairway, and rough area at golf courses OK, PV, and YH in Wisconsin

Course	Mean relative mycelium growth on propiconazole-amended medium (%) ^z		
	Putting green	Fairway	Rough
OK	38 b	27 c	8 f
PV	52 a	13 de	11 ef
YH	15 d	9 ef	11 ef

^z Means followed by the same letter do not significantly differ (Tukey–Kramer, $P < 0.05$).

Table 5. Analysis of variance on the mean relative mycelial growth of isolates of *Sclerotinia homoeocarpa* on propiconazole-amended medium collected from fairways and roughs at golf courses OK, PV, YH, BH, HL, and UR in Wisconsin and MA in Massachusetts

Source	DF	Type III SS	Mean square	F value	P value
Course area	1	6.9377	6.9377	576.03	<0.0001
Course	6	16.4551	2.7425	227.71	<0.0001
Course area*course	6	4.2065	0.7011	58.21	<0.0001

75%, respectively) of the *S. homoeocarpa* population resistant to thiophanate-methyl. Conversely, the number of thiophanate-methyl-resistant isolates collected from rough areas was often very low. Sampling error is believed to be the cause for higher frequencies of resistant isolates at courses YH and MA, where many rough isolates were collected within 1 m of the fairway. Populations from infrequently sprayed fairways at PV and YH had a very low frequency of thiophanate-methyl-resistant isolates.

Course area within the golf course also had an effect on thiophanate-methyl resistance. Despite never being exposed to benzimidazole fungicides, course OK had a higher proportion of the fairway population (96%) resistant to thiophanate-methyl than the putting green (21%). Research has shown that hyphae of *S. homoeocarpa* can be transported long distances via mechanical means such as mowing equipment or golfer traffic (2). This may explain the arrival of a previously mutated thiophanate-methyl-resistant isolate of *S. homoeocarpa* at OK, which then proliferated throughout the golf course without the aid of benzimidazole selection. It is unclear why resistant isolates would comprise a greater proportion of the fairway population compared with the putting green. Dollar spot isolates present in the fairway are partially shielded from fungicide applications via the higher mowing height as well as a more significant thatch layer when compared with putting greens. Couple this with the fact that many golf course superintendents allow for minor symptom development to occur on fairways before spraying a fungicide and there is the potential that a large amount of inoculum is receiving a reduced rate of fungicide. Current

resistance management thinking states that applying reduced rates of fungicides to control high inoculum densities will promote rapid resistance development. A lack of other sites with intensive fungicide applications on both putting greens and fairways prevents correlations to other courses from being made. This observation suggests that *S. homoeocarpa* isolates resistant to benzimidazoles may proliferate faster in golf course fairways than putting greens and roughs. If replicated elsewhere, thiophanate-methyl and other high-resistance risk fungicides could be used more frequently and effectively on putting greens where the risk for resistant populations may be lower.

Continued reports of decreased fungicide efficacy from different classes to a number of different pathogens are leaving those who manage turfgrass and other crops with dwindling control options. Resistance to benzimidazoles has long been documented, and we observed at course OK that under similar levels of fungicide selection, higher proportions of thiophanate-methyl-resistant isolates exist in the fairways compared with other parts of the golf course. Only sporadic reports of known *S. homoeocarpa* resistance to DMIs in the field have been observed and recorded in turfgrass (1,9,20). This study shows that DMI resistance in *S. homoeocarpa* is present to some degree in a large number of Wisconsin golf courses and may be more widespread than previously thought. The directional selection that gradually builds up levels of resistance to DMI fungicides means increased reports of practical resistance are likely to increase in the future as DMI fungicides continue to be applied.

In vitro fungicide sensitivity to the fungicides propiconazole and thiophanate-

methyl was significantly different among isolates of *S. homoeocarpa* collected from different golf courses and separate areas within each course. More frequent fungicide applications in the previous 5 years had the largest impact on the sensitivity of *S. homoeocarpa* isolates but the interval at which they were applied also had a significant effect. Management intensity of golf course putting greens, fairways, and roughs were found to significantly affect the occurrence of fungicide resistance. These observations provide further insight into the overall picture of fungicide resistance and may aid in implementing more effective strategies to manage and reduce the significant levels of resistance already present on golf course turf.

ACKNOWLEDGMENTS

Special acknowledgement to Nick Keuler at the University of Wisconsin College of Agriculture and Life Sciences Statistics Consulting Service for his assistance on the statistical analysis of the data presented in this article. Acknowledgments also go to golf course superintendents Trygve Ekern, Aron Hogden, Kevin Hurd, Monroe Miller, Jeff Parks, Neil Radatz, John Tucker, and Mark Williams for allowing their golf courses to be used in this study.

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Table 6. Chi-square analysis of expected and observed numbers of *Sclerotinia homoeocarpa* isolates resistant to thiophanate-methyl collected from putting greens, fairways, and roughs at six Wisconsin golf courses and one Massachusetts golf course

Golf Course	Area	Number of isolates	Number of resistant isolates		χ^2 -test ^z
			Expected ^y	Observed	
OK	Green	66	27	14	***
	Fairway	79	32	76	
	Rough	76	31	0	
PV	Green	77	0.4	0	NS
	Fairway	96	0.3	1	
	Rough	96	0.4	0	
YH	Green	96	6	5	NS
	Fairway	89	5	6	
	Rough	95	6	7	
BH	Fairway	49	17	49	***
	Rough	91	32	0	
UR	Fairway	89	9	18	***
	Rough	87	9	0	
HL	Fairway	87	20	37	***
	Rough	80	18	1	
MA	Fairway	99	57	74	***
	Rough	58	33	16	

^y Expected number of resistant isolates was calculated on the basis of the assumption that the total observed number of resistant isolates would be evenly distributed among the sampling areas at each course.

^z *** = Significant at 0.0001; NS = not significant.

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