ASSESSMENT OF SUSCEPTIBILITY OF CREEPING BENTGRASS CULTIVARS TO DOLLAR SPOT, SENSITIVITY OF KANSAS ISOLATES OF SCLEROTINIA HOMOEOCARPA TO DEMETHYLATION INHIBITOR FUNGICIDES, AND DETERMINATION OF MUTATIONS IN BETA-TUBULIN GENE ASSOCIATED WITH RESISTANCE

by

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Abstract

Dollar spot disease of turfgrass, caused by the fungus *Sclerotinia homoeocarpa*, is the most economically important disease of intensively managed turfgrass such as creeping bentgrass (*Agrostis stolonifera*) in golf course fairways and putting greens. While several cultural management practices can lessen the severity of the disease, fungicide applications are necessary to manage the disease to acceptable levels. Host resistance is another avenue of improving the quality of turfgrass in response to dollar spot disease, but more information on cultivar susceptibility levels is necessary. Many fungicides are available to control dollar spot, but *S. homoeocarpa* has shown resistance to both demethylation inhibitor (DMI) and benzimidazole class fungicides. The objectives of this thesis were: (1) to evaluate 15 cultivars of creeping bentgrass under putting green and fairway management for both overall quality and disease severity of dollar spot in the presence and absence of fungicide applications; (2) to determine the sensitivity of Kansas *S. homoeocarpa* isolates to the DMI fungicides propiconazole, metconazole, tebuconazole, and triticonazole using *in vitro* mycelia growth assays; (3 to determine a best discriminatory dose concentration for each DMI fungicide that will provide for rapid testing of relative sensitivity, and (4) to sequence the entire β-tubulin gene of several resistant and sensitive isolates to determine mutations associated with resistance. There were differences in turfgrass quality among the 15 cultivars at putting green height but not fairway height. There were no significant differences in disease. In the fungicide resistance assays, the mean EC_{50} values were 0.0163, 0.038, 0.0612, and 0.0994 µg/ml for metconazole, propiconazole, tebuconazole, and triticonazole, respectively. Correlations were significant and positive for all pairwise comparisons of $\log_{10}EC_{50}$ values. Regressions using discriminatory

concentrations tested were significant. The most predictive concentrations were 0.01, 0.05, 0.05, and 0.10 µg/ml a.i, for metconazole, propiconazole, tebuconazole, and triticonazole, respectively. The entire β-tubulin gene was sequenced for four resistant and four sensitive isolates. The resistant isolates all harbored a substitution of alanine for glutamic acid at codon 198 (E198A).

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Chapter 1 - Introduction

Dollar spot, caused by *Sclerotinia homoeocarpa*, F. T. Bennett, is one of the most important diseases of turfgrass (Goodman and Burpee, 1991; Venu et al., 2009; Walsh et al., 1999). Dollar spot is a serious and chronic disease of both warm season (C4) and cool season (C3) grasses in temperate climates. This disease is especially problematic on closely mowed, intensively managed turfgrasses such as golf course fairways and putting greens (Latin, 2006; Smiley et al., 2005).

Creeping bentgrass is the primary species of turfgrass used for putting greens in temperate climates. Symptoms of dollar spot on greens height creeping bentgrass (maintained at a 2.0 to 4.0 mm mowing height) include straw to gray colored sunken patches of necrotic foliage, generally ranging from 2.5 to 5.0 cm in diameter. These sunken patches, or infection centers, increase in size on higher-cut turfgrass stands to a range of 5.0 to 10.0 cm in diameter. On individual plants, small, circular, necrotic lesions develop on individual leaf blades; these lesions will often expand to cover the width of a leaf blade.

Environmental conditions conducive to the disease are periods of high humidity with temperatures ranging from 15-30°C. Though disease severity increases in dry soils and low nitrogen fertility, soil pH and phosphorus fertility are not known to affect disease severity (Golembiewski and Danneberger, 1998; Smiley et al., 2005).

There are a number of cultural practices available to assist in controlling dollar spot. Fertility programs that focus on supplying adequate nitrogen to the turfgrass can reduce disease severity (Smiley et al., 2005). In addition to appropriate fertility, practices to reduce leaf surface moisture can decrease disease pressure. Duration of leaf wetness can be reduced by practices

such as watering during the day when standing water is more likely to evaporate, and watering deeply and infrequently (Landschoot and McNitt, 1997; Smiley et al., 2005; Walsh et al., 1999; Williams et al., 1996). Other studies have found that morning mowing, which displaces dew, can also be utilized to decrease disease pressure (Delvalle et al., 2011). The use of lightweight turf rollers in the afternoon as well as the morning has been shown to reduce disease, indicating that factors other than dew displacement are involved (Giordano et al., 2012). Though cultural practices can often reduce disease severity they are usually not sufficient to reduce disease to acceptable levels by themselves.

Host resistance offers an additional method of disease management. New creeping bentgrass cultivars are being developed with an emphasis on improved resistance to dollar spot in an effort to reduce fungicide inputs and increase the flexibility of disease management approaches (Lee et al., 2006). In a study of three cultivars conducted on a putting green in Manhattan, KS, from 1997 through 1999, Settle et al. (2001) found 'Crenshaw' to be most susceptible to dollar spot while 'Penncross' was moderately susceptible and 'L-93' was most resistant. Crenshaw required three curative, or post infection, applications whereas L-93 required two applications of fungicide to suppress dollar spot to acceptable levels. This suggests that resistant cultivars would require a less stringent spray program earlier in the season to provide season-long acceptable playing quality. At this time, no fully resistant cultivars are available.

There are many classes of fungicides labeled for use to control dollar spot on turfgrass. The contact fungicides chlorothalonil and mancozeb are labeled for dollar spot control. Systemic materials available include the demethylation inhibitors (DMI) such as metconazole and propiconazole; dicarboximides such as iprodione and vinclozolin; stobilurins such as

pyraclostrobin; benzimidazoles such as thiophanate-methyl; and succinate dehydrogenase inhibitors such as boscalid (Vincelli and Munshaw, 2014; Latin, 2011).

Currently, managers of high quality turfgrass rely on multiple fungicide applications to control dollar spot to an acceptable level. Fungicides are applied in anticipation of a disease outbreak many times over the course of a season (Abernathy et al., 2001; Koch et al., 2009). The repeated use of systemic fungicides can lead to fungicide resistance. Cases of resistance to systemic fungicides in *S. homoeocarpa* have been documented (Bishop et al., 2008; Detweiler et al., 1983; Golembiewski et al., 1995; Jo et al., 2008), and it is theorized that these cases are linked towards frequency and number of fungicide applications.

Field resistance of *S. homoeocarpa* to benzimidazoles, a class of systemic fungicides first used to control dollar spot in the 1960's , was first reported in 1973 (Warren et al., 1974; Latin, 2011) Resistance to benzimidazole fungicides has been reported in numerous other plant pathogenic fungi including *Venturia inaequalis, Monilinia fucticola*, *Monilinia laxa, Colletotrichum cereale*, *Cladobotryum dendroides*, and many others (Koenraadt et al., 1992; Ma et al., 2003; Ma et al., 2004; Ma and Michailides, 2005; McKay et al., 1998; Wong et al., 2008; Young et al., 2010; Warren et al., 1977). Benzimidazoles inhibit β-tubulin polymerization in fungi. Resistance to benzimidazoles has been associated with mutations in the β-tubulin gene in many fungal species. These mutations alter the binding site of the fungicide to the β-tubulin protein (Oakley, 2004). The main regions of the gene which have historically been associated with resistance to benzimidazoles have involved the 198th codon, although the specific mutation differs. Mutations observed at the 198th codon include E198A, E198Q, E198G, E198K, and E198V (Ma et al., 2005). In laboratory induced mutants, as well as field isolates of *Aspergillus nidulans* and other species, mutations associated with resistance have occurred at the 6th (H6Y),

50th (Y50C), 167th (F167Y), 200th (F200Y) and 240th (L240F) codons (Koenradt et al., 1992; Jung et al., 1992, Ma et al., 2003; Ma and Michailides, 2005). *S. homoeocarpa* has shown resistance to benzimidazole fungicides in in-vitro laboratory assays and field experiments (Detweiler et al., 1983; Burpee, 1997; Jo et al., 2008; Bishop et al., 2008). There has been only one prior study which examined the sequence of the *S. homoeocarpa* β-tubulin gene, reporting a mutation of glutamic acid to lysine at codon 198 (Koenradt et al., 1992). The primers in that study were designed to amplify codons 167 – 241, omitting other regions of the gene with known sites of mutations associated with benzimidazole resistance in other species of plant pathogenic fungi (Ma and Michailides, 2005).

In addition to benzimidazoles, another class of fungicides implemented in managing dollar spot are the demethylation inhibiting fungicides, or DMI's. DMI's have been used to control dollar spot since the 1970's, and field resistant populations of *S. homoeocarpa* to DMI fungicides have been observed (Detweiler et al., 1983; Bishop et al., 2008; Burpee, 1997; Ok et al., 2011; Putman et al., 2010; Jo et al., 2006; Hsiang et al., 2007; Miller and Stevenson, 2002; Golembiewski et al., 1995). DMI fungicides inhibit the fungal pathogen's growth by interfering with the oxidative sterol 14α-demethylation in the ergosterol biosynthesis pathway (Siegel, 1981). Sterols modulate the movement of phospholipids and ensure the correct cellular membrane fluidity. The disruption of this sterol synthesis pathway interferes with membrane permeability and activity of some enzymes associated with the fungal-cellular membrane (Hollomon et al., 1990). Resistance to DMI's has a quantitative nature, rather than the qualitative nature observed with resistance to thiophanate-methyl (Golembiewski et al., 1995, Jo et al., 2006), and higher application rates of the DMI fungicides can often maintain an adequate level

of control in stands where resistance has been observed. DMI fungicides are still used widely for dollar spot control.

Previous studies have provided conflicting evidence of a correlation between in vitro sensitivity to DMI products. Hsiang et al. (1997) found low to moderate correlations in the sensitivity of *S. homoeocarpa* to propiconazole, tebuconazole, mycobutanil, and fenarimol. In addition, Miller and Stevenson (2002) reported positive correlations of *S. homoeocarpa* sensitivity to propiconazole, fenarimol, and myclobutanil, but not between any of those fungicides and triadimefon. However, Golembiewski et al. (1995) reported that resistance to triadimefon, fenarimol, and propiconazole were all correlated between the 150 isolates tested, and Ok et al. (2011) found strong correlation between five DMI products tested (propiconazole, triadimefon, myclobutanil, metconazole, triticonazole, and tebuconazole) on 58 isolates selected arbitrarily from multiple locations.

Much progress has been made in understanding the biology and management of dollar spot, but knowledge gaps remain. More information is needed on cultivar resistance and overall cultivar performance. The sensitivity of Kansas isolates to DMI fungicides has not previously been examined. Furthermore, more information is needed regarding the relationships between resistance among the different DMI fungicides in order to develop a more efficient screening procedure as well as to properly evaluate fungicide performance. The entire β-tubulin gene of *S. homoeocarpa* has not previously been sequenced. Sequencing the entire gene allows all sites which have been associated with resistance to benzimidazoles in other species of fungi to be examined. Therefore, the objectives of this thesis are to: (i) evaluate the susceptibility of 15 creeping bentgrass cultivars to dollar spot disease; (ii) determine the sensitivity of Kansas *S. homoeocarpa* isolates to propiconazole, metconazole, tebuconazole, and triticonazole using *in*

vitro mycelia growth assays; (iii) to determine a best discriminatory dose concentration for each product that will provide for rapid testing of relative sensitivity; (iv) sequence the entire β-tubulin gene of isolates of *S. homoeocarpa* resistant and sensitive to thiophanate-methyl and identify mutations that may be associated with resistance.

Chapter 2 - Assessment of Susceptibility of Creeping Bentgrass Cultivars to Dollar Spot

Introduction

Dollar spot, caused by *Sclerotinia homoeocarpa*, F. T. Bennett, is one of the most important diseases of turfgrass (Goodman and Burpee, 1991; Venu et al., 2009; Walsh et al., 1999), as it can cause high levels of damage to many species of turfgrass. Dollar spot is particularly serious on highly maintained turfgrass, such as creeping bentgrass (*Agrostis stolonifera* L.) at both fairway and putting green mowing heights. The disease reduces visual quality and also can impact the playability of golf course greens by disrupting surface uniformity (Latin, 2006; Smiley et al., 2005).

There are a number of cultural practices available to assist in controlling dollar spot. Fertility programs that focus on supplying adequate nitrogen to the turfgrass can reduce disease severity (Smiley et al., 2005). In addition to appropriate fertility, practices to reduce leaf surface moisture can decrease disease pressure. Duration of leaf surface moisture can be reduced by practices such as watering during the day when standing water is more likely to evaporate, and watering deeply and infrequently (Landschoot and McNitt, 1997; Smiley et al., 2005; Walsh et al., 1999; Williams et al., 1996). Morning rolling removes the dew and guttation fluid which is thought to be a nutrient source for foliar pathogens. Other studies have found that morning mowing, which displaces dew, can also be utilized to decrease disease pressure (Delvalle et al., 2011). The use of lightweight turf rollers in the afternoon as well as the morning has been shown to reduce disease, indicating that factors other than dew displacement may be involved (Giordano et al., 2012).

Cultural practices alone are often insufficient, however, and managers of high quality turfgrass rely on multiple fungicide applications to control dollar spot to an acceptable level. Fungicides are applied in anticipation of a disease outbreak many times over the course of a season (Abernathy et al., 2001; Koch et al., 2009).

In the absence of resistant cultivars it is necessary to use frequent preventive fungicide applications in order to maintain putting surfaces at an acceptable level of playability (Latin, 2006). The repeated use of systemic fungicides can lead to fungicide resistance. Cases of resistance to systemic fungicides in *S. homoeocarpa* have been documented (Bishop et al., 2008; Detwiler et al., 1983; Golembiewski et al., 1995; Jo et al., 2008), and it is theorized that these cases are linked towards frequency and number of fungicide applications. Therefore, reducing the number of applications through cultural practices or the use of resistant cultivars could lead to longer product efficacy, reduce environmental impacts, and reduce costs of golf course management.

With the limitations and challenges of cultural practices and fungicides, host resistance offers an additional method of disease management. New cultivars are being developed with an emphasis on improved resistance to dollar spot in an effort to reduce fungicide inputs and increase the flexibility of disease management approaches (Lee et al., 2006). In a study of three cultivars conducted on a putting green in Manhattan, KS, from 1997 through 1999, Settle et al. (2001) found 'Crenshaw' to be most susceptible to dollar spot while 'Penncross' was moderately susceptible and 'L-93' was most resistant. Crenshaw required three curative, or post infection, applications whereas L-93 required two applications of fungicide to suppress dollar spot to acceptable levels. This suggests that resistant cultivars would require a less stringent spray program earlier in the season to provide season-long acceptable playing quality.

In a 2008 National Turfgrass Evaluation Program (NTEP) trial, 'Declaration', 'Kingpin' and 'Memorial' were the least susceptible to dollar spot at putting green height. '007', Penncross, 'Mackenzie', and 'LS-44' were rated as being moderately susceptible to dollar spot (NTEP, 2008a). Abernathy et al. (2001) reported finding 'A-4' and Penncross to be moderately resistant and L-93 to be least susceptible. At fairway height, Declaration, Kingpin, L-93, 'Crystal Bluelinks', and LS-44 were all rated as having good resistance to dollar spot (NTEP, 2008b). For the cultivars 'Alpha', 'Bengal', 'Independence', and 'T-1', not much has been previously published concerning their susceptibility to dollar spot.

In order to improve the efficacy and reduce the inputs of turfgrass fungicides, resistant cultivars should be used on golf courses. As such, a thorough evaluation of cultivar susceptibility is needed to determine the resistance of any given cultivar. Therefore, the objective of this study was to compare the susceptibility, to dollar spot, of 15 cultivars of creeping bentgrass. This project is part of a North Central Extension and Research Association (NCERA-192) regional project in collaboration with Iowa State University, Michigan State University, North Dakota State University, Ohio State University, Purdue University, South Dakota State University, Southern Illinois University, the University of Illinois, the University of Minnesota, the University of Missouri, the University of Nebraska and the University of Wisconsin.

Materials and Methods

Research site and plot maintenance

Creeping bentgrass cultivars were evaluated at both fairway and putting green height at the Rocky Ford Turfgrass Research Center in Manhattan, KS. The fairway was established on native soil and the putting green was constructed to United States Golf Association specifications. The cultivars evaluated were 007, A-4, Alpha, Bengal, Crenshaw, Crystal

Bluelinks, Declaration, Independence, Kingpin, L-93, LS-44, Mackenzie, Memorial, Penncross, and T-1. Each cultivar was seeded in September, 2008 at 49 kg·ha^{-1} in three 1.2 x 3.0 m plots. Both the putting green and fairway experiments were each set up in a randomized complete block design. Each complete block was then split, with the cultivar the main plot factor and a fungicide regimen (described below) the strip plot factor.

The fairway and the putting green were mowed with a triplex reel mower at 13 and 3 mm, respectively. The fairway was mowed three days per week and the putting green was mowed six days per week. Urea (46-0-0 NPK) was used to provide nitrogen (N) at 25 kg·ha^{-1} per month to the fairway and at 50 kg·ha^{-1} per month to the green during establishment from September to November of 2008 and in May and June of 2009. After the summer of 2009, a polymer-coated methylene urea N source (Professional Fertilizer, 18-2-20 N-P-K, Spring Valley, Jackson, WI) was applied once monthly from July to November of 2009. In 2010 and 2011, methylene urea was applied from May to November at 25 kg N·ha^{-1} biweekly to the green and once monthly to the fairway.

In 2009 and 2010, aerification and topdressings were implemented as needed (Thompson, 2011). The fairway was irrigated at 75% of reference evapotranspiration (ET) three days per week and the green was irrigated daily at 100% ET. The evapotranspiration was measured using an onsite weather station and the FAO-56 Penman-Monteith equation (Allen et al., 1998).

Fungicide applications

As part of the larger regional study, it was determined that for the putting green and fairway each year, a tank mix of Emerald (boscalid, 3-pyridinecarboxamide,2-chloro-N-(4'-chloro(1,1'-biphenyl)-2-yl), BASF Corporation, Durham, NC) at 0.4 kg·ha^{-1} a.i. and Daconil Ultrex (chlorothalonil, tetrachloroisophthalonitrile, Syngenta Group Company, Greensboro, NC) at 8 kg·ha^{-1} a.i. was applied preventatively to the fungicide-treated subplots at the first

appearance of dollar spot infection centers in all three replicate plots of Crenshaw. Crenshaw was selected as the indicator because of its well-documented susceptibility to the disease (Settle et al., 2001). Preventative fungicide applications were performed on both the fairway and putting green on June 2, 2011.

Subsequent curative treatments were to be made later in the season, as needed, if two of the three replicate subplots of the fungicide-treated Declaration plots had dollar spot affecting an average of 10% of the plot area for that cultivar in the fairway study or an average of 5% of the plot area in the green study. Thresholds were considered met when a value of > 44 dollar spot infection centers $(DSIC)/m^2$ and > 87 $DSIC/m^2$ was reached for the 5% and 10% thresholds, respectively, based on an average DSIC (diameter of 3.8 cm).

Disease assessment and data analysis

Plots were rated biweekly for overall turfgrass quality in 2011 from June 1 to October 13. Visual quality ratings were representative of turfgrass color, texture, density, and uniformity and followed a 1 to 9 scale (1 = poorest quality, 6 = minimum acceptable quality, 9 = optimum quality, including disease damage and weed encroachment) following the guidelines of Morris and Shearman.

Dollar spot severity was rated weekly, when disease was present, by counting the number of dollar spot infection centers (DSIC) in each strip plot. A 30.5 x 30.5 cm square was placed at random three times in each plot, and the number of DSIC were counted, converted to m^2, based on an average diameter of 3.8 cm, and averaged. Though not a specific objective of the study, the severity of brown patch was also rated by visually estimating the percentage of the plot area showing symptoms. Data were subjected to the Shapiro-Wilks test for normality. Quality data for the putting green and fairway were normal with homogenous error. Quality data were analyzed using the PROC MIXED procedure of SAS 9.2 with treatment and cultivar as fixed effects and

block as a random effect. Tukey-adjusted P-values were generated to denote differences (P<0.05).

Area under the disease progress curve (AUDPC) values for dollar spot data were calculated using the formula $\sum_{i=1}^{ni-1}(\frac{[y_i+y_{i+1}]}{2})(t_{i+1} - t_i)$ (Madden et al., 2007), in which n denotes the number of intervals between assessments, y represents the number of DSICs present, and t is the time, in days passed. Dollar spot data for the putting green and the fairway were subjected to the log(x+1) transformation to meet the assumption of normality and homogeneity about the mean. Dollar spot data were analyzed using the PROC MIXED procedure of SAS 9.2 with treatment and cultivar as fixed effects and block as a random effect. Tukey-adjusted P-values were generated to denote differences (P<0.05).

Brown patch data on the green and fairway were subjected to an arcsine transformation to meet assumptions of normality and homogeneity about the mean. Brown patch data were analyzed using the PROC MIXED procedure of SAS 9.2 with treatment and cultivar as fixed effects and block as the random effect. Tukey-adjusted P-values were generated to denote differences (P<0.05).

Results

There were no cultivar x fungicide interactions for any response variable. Therefore, cultivar means were calculated using turf quality and disease severity data from both treated and non-treated strip plots. On the fairway, turfgrass declined severely and uniformly across the plots starting on July 8 due to heat, drought, and thick thatch. As such, subsequent dates are not included in the analysis.

Turfgrass Quality

On the putting green, there were significant differences in turfgrass quality for the overall season average and on several individual rating dates (Table 1). For the season-long average, only 007, L-93, and Crystal Bluelinks maintained an average quality at or above minimum acceptable, though 007 was the only cultivar to have a quality rating significantly higher than any other cultivars (A4, Declaration, Independence, and Penncross). On June 8, 007, L-93, and McKenzie had significantly higher quality than Crenshaw, though only L-93 had an acceptable quality level. On June 15, 007 had the highest rating, above minimum acceptable, and was significantly higher than A-4, Bengal, Crenshaw, Declaration, Independence, Kingpin, LS-44, and Penncross. On June 22, 007 had the highest rating, above minimum acceptable, and was significantly higher than A-4, Bengal, Crenshaw, Declaration, and T-1. On July 20, 007 had the highest rating, above minimum acceptable, and was significantly higher than Declaration, only. On August 31, Penncross and T-1 shared the highest rating of 7.8. Only Penncross, however, was significantly different from another cultivar, Mackenzie. For all other dates that data is shown, there were no significant differences.

On the fairway, there were no significant differences in turfgrass quality on any individual rating dates or for the seasonal average (Table 2). The cultivars 007, Bengal, Declaration, and L-93 had a season-long average quality level which was less than acceptable.

Dollar Spot

On the putting green, dollar spot disease pressure was relatively low in 2011, with only four dates when the number of DSICs averaged across treated and untreated plots for all cultivars exceeded $5/m^2$. No significant differences were observed on those dates (Table 3) or any other dates (data not shown). There were no differences in AUDPC values (Table 3).

Similarly, on the fairway-height study, only the dates in which the average number of DSICs were $>5/m^2$ are shown (Table 4). No significant differences were observed amount cultivars on those or any other dates. In addition, there were no significant differences in the AUDPC values among cultivars (Table 4).

Brown Patch

On the putting green, brown patch severity exceeded 10% on August 13 and 15, and those data are shown in Table 5. On August 13, no significant differences were observed. On August 15th, L-93 had the lowest % of the plot affected, and Bengal had the highest % of the plot affected. L-93 was significantly less affected than all other cultivars except for T-1. Bengal had a higher % affected than all other cultivar aside from 007, Declaration, Independence, LS-44, and Memorial.

On the fairway, brown patch severity exceeded 10% only on July 20. There were no significant differences among cultivars observed on this date (Table 6).

Discussion

The primary purpose of this study was to compare the relative susceptibility of 15 cultivars of creeping bentgrass to dollar spot in putting green and fairway conditions, as part of a multi-year, multi-state project. In this 2011 year of the study, there were no significant differences in dollar spot severity among cultivars. The summer of 2011 had low dollar spot activity due to the longer periods of hot (average daily high 96°F) and dry (total precipitation 11 cm, less than half of average) weather that occurred during July and August. It is hard to come to a definite conclusion based on this year's data alone.

The dollar spot susceptibility of some of these cultivars has been evaluated in other studies. Data from Thompson (2011), in which the same field plots were studied in 2009 and

2010, 007 again had acceptable quality, but was surpassed by Declaration and Memorial. For dollar spot, Thompson (2011) reported that 007 as well as A-4, Alpha, Bengal, Crystal Bluelinks, Declaration, Kingpin, L-93, LS-44, Memorial, and Penncross had significantly lower AUDPCs than Crenshaw, Independence, Mackenzie and T-1 at putting green height. The difference among study years emphasizes the importance of evaluating cultivars over several years in different weather conditions. This data from Kansas will be combined with data from several other states as part of the larger NCERA study.

Though few differences were observed in this study, other research has provided evidence of differences in dollar spot severity among cultivars. Lee (2003) found that L-93 had only 10% of the AUDPC as compared to Crenshaw, and a study by Abernathy (2001) also found evidence that L-93 was less susceptible to dollar spot than Crenshaw, alone or as part of a blend in which Crenshaw and L-93 were the only differences between the polyblend turfgrass stands. Declaration has performed with high quality ratings and low levels of both brown patch and dollar spot in other studies. Liu (2012) found that Declaration had a lower AUDPC value than the (more susceptible) cultivars Penncross and Independence for both untreated plots and in a study where Penncross and Independece were treated with a fungicide (chlorothalonil) twice for each treatment received by Declaration. Using cultivars such as Declaration or L-93as part of an IPM strategy for dollar spot management could lower the environmental impacts that a golf course can have on the ecosystem as well as save money. Further, resistant cultivars can delay the formation of fungicide-resistant isolates of dollar spot disease by lowering the number of seasonal fungicide applications needed to maintain acceptable quality.

Table 1. Turfgrass quality ratings for 15 cultivars of creeping bentgrass maintained under green conditions, 2011.

														Seasonal	
Cultivar	6/1	6/8		6/15		6/22		7/8	7/20		8/3	8/31		9/7	9/21	9/28	10/13	Average	
007	6.7[2]	4.7	A[3]	6.8	a	6.2	a	6.5	6.8	a	7.2	6.3	a,b	7.7	6.0	7.5	6.5	6.6	a
A-4	4.8	4.3	a,b	3.8	b	3.7	b	3.8	5.3	a,b	5.8	6.8	a,b	6.7	6.5	6.5	6.8	5.4	b,c
Alpha	5.7	4.5	a,b	5.0	a,b	4.8	a,b	5.2	6.5	a,b	7.0	7.0	a,b	7.2	5.8	5.8	6.3	5.9	a,b,c
Bengal	4.5	4.2	a,b	3.7	b	3.5	b	3.8	5.3	a,b	6.2	7.3	a,b	7.3	6.5	7.2	7.0	5.5	a,b,c
Crenshaw	4.5	3.0	b	3.5	b	3.5	b	3.3	5.8	a,b	6.7	6.5	a,b	6.3	5.3	5.0	5.0	4.9	a,b,c
Crystal Bluelinks	5.5	4.5	a,b	5.2	a,b	4.7	a,b	4.5	5.5	a,b	6.5	7.7	a,b	7.7	6.3	7.2	6.3	6.0	a,b,c
Declaration	4.7	4.2	a,b	3.7	b	3.7	b	3.0	4.3	b	5.5	6.3	a,b	7.3	6.0	6.5	6.0	5.1	b,c
Independence	5.5	3.8	a,b	4.2	b	4.0	a,b	3.7	5.3	a,b	6.3	6.5	a,b	7.0	5.3	6.0	5.5	5.3	b,c
Kingpin	5.2	4.7	a,b	4.3	b	4.2	a,b	4.3	5.0	a,b	5.8	7.2	a,b	7.3	6.5	6.8	6.5	5.7	a,b,c
L-93	6.0	6.0	a	5.7	a,b	5.5	a,b	4.7	5.7	a,b	6.3	7.0	a,b	7.5	6.2	6.7	6.7	6.2	a,b
LS-44	5.5	4.7	a,b	3.7	b	4.7	a,b	4.5	5.5	a,b	6.7	7.2	a,b	7.8	6.7	6.8	6.8	5.9	a,b,c
Mackenzie	6.0	5.7	a	5.0	a,b	4.7	a,b	4.2	5.2	a,b	5.8	6.2	b	6.3	5.5	6.2	6.0	5.6	a,b,c
Memorial	5.7	4.5	a,b	4.8	a,b	4.7	a,b	4.0	4.8	a,b	6.3	7.7	a,b	7.2	6.5	7.7	6.7	5.9	a,b,c
Pencross	5.0	3.8	a,b	3.8	b	4.3	a,b	3.2	4.5	a,b	5.7	7.8	a	7.2	6.2	6.5	6.3	5.4	b,c
T-1	5.0	4.3	a,b	4.5	a,b	3.7	b	3.0	5.8	a,b	6.7	7.8	a,b	7.3	5.3	6.5	5.3	5.5	a,b,c

Turfgrass Quality[1] by Date

1. Quality was assessed visually using a 0-9 scale, with 0 = lowest quality, 9 = highest quality, 6 = minimally acceptable quality. Quality ratings were based on color, uniformity, texture, and density.
2. Cultivar data are based on six observations: Three fungicide-treated subplots and three non-treated subplots. Data were normal with homogenous errors, therefore no transformations were required. Data were analyzed using the PROC MIXED procedure of SAS, with treatment and cultivar as fixed effects and block as a random effect.
3. Within columns, values followed by the same letter are not significantly different as determined by a Tukey-adjusted P-value of <0.05.

Table 2. Turfgrass quality ratings for 15 cultivars of creeping bentgrass maintained under fairway conditions, 2011.

Cultivar								
	Turfgrass Quality by Date[1]							
	6/1	6/8	6/15	6/22	7/8	7/20	8/3	Seasonal Average
007	5.7[2]	6.2	6.7	7.5	5.2	4.7	2.2	5.4
A-4	7.8	6.3	7.5	7.3	5.8	4.8	3.8	6.2
Alpha	7.5	7.2	7.8	7.8	6.3	5.5	3.7	6.6
Bengal	5.8	6.2	7.5	7.5	5.5	5.0	2.2	5.7
Crenshaw	6.7	7.0	7.3	7.8	5.5	5.5	4.5	6.3
Crystal Bluelinks	6.7	6.8	7.3	7.0	5.8	5.8	3.3	6.1
Declaration	4.2	5.2	7.3	7.2	5.8	5.8	4.7	5.7
Independence	6.8	8.3	8.0	7.7	5.8	6.3	4.5	6.8
Kingpin	7.3	6.7	7.8	7.2	6.3	5.7	4.7	6.5
L-93	5.2	5.0	5.8	6.5	4.8	5.3	3.8	5.2
LS-44	7.2	7.7	7.8	7.7	6.5	6.7	5.8	7.1
Mackenzie	5.7	6.7	6.7	7.3	6.2	5.8	4.0	6.1
Memorial	7.5	7.5	8.0	7.7	6.8	6.5	4.8	7.0
Pencross	7.3	7.5	7.2	7.2	6.5	6.2	4.3	6.6
T-1	5.5	6.8	8.0	7.5	5.7	6.5	4.2	6.3

1. Quality was assessed visually using a 0-9 scale, with 0 = lowest quality, 9 = highest quality, and 6 = minimum level of acceptable quality. Quality ratings were based on color, uniformity, texture, and density.
2. The number of DISCs/m^2 was obtained by randomly tossing a 30.5 cm square into each plot three times, averaging the number of DSICs, and converting to /m^2. The average of the three values is shown.

Table 3. Number of dollar spot infection centers per square meter (DSIC/m^2) on fifteen cultivars of creeping bentgrass.

Cultivar	Dollar spot infection centers^2 per m^2				
	6/15	6/22	6/29	7/08	AUDPC^3
007	19.7[4]	4.8	4.2	8.4	310.1
A-4	35.9	19.1	8.4	22.7	513.9
Alpha	16.7	9.0	7.8	6.0	265.5
Bengal	23.3	10.8	5.4	13.2	392.4
Crenshaw	23.3	4.8	10.8	15.5	984.0
Crystal Bluelinks	29.9	17.3	9.0	10.2	389.9
Declaration	14.9	16.1	1.8	21.5	346.5
Independence	20.9	20.3	20.9	22.7	911.9
Kingpin	12.6	10.8	6.0	1.2	186.3
L-93	13.8	13.2	10.2	7.2	292.6
LS-44	12.6	6.6	9.6	19.1	332.3
Mackenzie	22.7	17.3	20.3	22.1	789.7
Memorial	16.1	9.0	5.4	9.6	229.3
Pencross	13.8	5.4	7.2	0	143.7
T-1	16.1	4.8	5.4	7.2	313.9

[1.] The number of DISCs/m^2 was obtained by randomly tossing a 30.5 cm square into each plot three times, averaging the number of DSICs, and converting to /m^2. The average of the three values is shown.

[2.] Dollarspot disease severity was assessed weekly from 6/15 through 10/13. Dates in which average number of DSICs>5/m^2 are shown.

[3.] The AUDPC was calculated using the equation $\sum_{i=1}^{ni-1}(\frac{[y_i+y_{i+1}]}{2})(t_{i+1} - t_i)$ in which a y_i is the number of DSICs for any given date, and t_i would be the corresponding date. The difference between two dates is the number of days which passed.

[4.] Cultivar data are based on six observations, three fungicide-treated subplots and three non-treated subplots. Data were subjected to the log(x+1) transformation to meet assumptions of normality and homogeneity of variance and analyzed using the PROC MIXED procedure of SAS with treatment and cultivar as fixed effects and block as a random effect. Back-transformed means are shown. Differences were determined by a Tukey-adjusted P-value of less than or equal to 0.05. There were no significant differences.

Table 4. Number of dollar spot infection centers per square meter (DSIC/m^2) on fifteen cultivars of creeping bentgrass.

Cultivar	6/22	6/29	7/08	7/13	7/20	8/03	AUDPC[3]
			Dollar spot infection centers[2] per m^2				
007	6.6[4]	5.4	7.2	5.4	0.0	0.0	148.6
A-4	7.2	6.0	3.6	4.2	0.0	0.0	123.2
Alpha	11.4	10.8	7.8	2.4	0.0	0.0	194.7
Bengal	6.6	3.6	8.4	3.0	0.0	0.2	129.8
Crenshaw	20.9	19.7	31.1	16.7	0.0	0.0	549.3
Crystal Bluelinks	6.6	4.2	8.4	4.8	0.0	3.0	164.8
Declaration	4.2	1.8	1.2	1.8	0.0	0.0	48.1
Independence	8.4	4.8	12.0	13.8	0.0	4.2	263.1
Kingpin	6.0	2.4	5.4	1.2	0.0	4.9	118.9
L-93	0.6	1.8	4.8	5.4	0.0	0.3	84.23
LS-44	11.4	9.0	13.2	9.0	0.0	6.0	299.3
Mackenzie	17.9	6.6	12.0	5.4	0.0	6.0	273.3
Memorial	3.0	3.0	3.0	3.0	0.0	0.0	73.25
Pencross	9.6	7.8	11.4	7.8	0.0	0.0	221.9
T-1	2.4	4.8	7.2	9.0	0.0	0.0	150.7

[1.] The number of DSICs/m^2 was obtained by randomly tossing a 30.5 cm square into each plot three times, averaging the number of DSICs, and converting to /m^2. The average of the three values is shown.

[2.] Dollarspot disease severity was assessed weekly from 6/15 through 10/13. Dates in which average number of DSICs > 5/m^2 are shown.

[3.] The AUDPC was calculated using the equation $\sum_{i=1}^{ni-1}(\frac{[y_i+y_{i+1}]}{2})(t_{i+1} - t_i)$ in which a y_i is the number of DSICs for any given date, and t_i would be the corresponding date. The difference between two dates is the number of days which passed.

[4.] Cultivar data are based on six observations, three fungicide-treated subplots and three non-treated subplots. Data were subjected to the log(x+1) transformation to meet assumptions of normality and homogeneity of variance and analyzed using the PROC MIXED procedure of SAS with treatment and cultivar as fixed effects and block as a random effect. Back-transformed means are shown. Differences were determined by a Tukey-adjusted P-value of less than or equal to 0.05. There were no significant differences.

Table 5. Severity of brown patch in fifteen cultivars of creeping bentgrass maintained under green conditions, 20111.

Cultivar	Brown patch severity[2] (% plot affected)		
	8/13	8/15	
007	28.3[3]	35.9	H[4],i
A-4	29.2	26.8	d,e,f
Alpha	35.8	17.4	b,c
Bengal	24.2	39.8	i
Crenshaw	16.7	22.6	c,d,e
Crystal Bluelinks	26.7	28.7	e,f,g
Declaration	15.0	36.9	h,i
Independence	20.0	35.4	g,h,i
Kingpin	30.8	20.1	b,c,d
L-93	23.3	10.0	a
LS-44	21.7	38.3	h,i
Mackenzie	20.0	32.2	f,g,h
Memorial	28.3	33.8	g,h,i
Pencross	31.7	24.7	d,e
T-1	20.0	14.2	a,b

[1.] August 13th and 15th were the only dates on which the average disease level exceeded 10%. Dates in which average disease was < 10% are not shown.

[2.] Brown patch was assessed biweekly by visually estimating the percentage of each plot displaying brown patch symptoms.

[3.] Cultivar data are based on six observations, three fungicide-treated subplots and three non-treated subplots. Data were subjected to the arcsine transformation to meet assumptions of normality and homogeneity of variance and analyzed using the PROC MIXED procedure of SAS with treatment and cultivar as fixed effects and block as the random effect. Back-transformed means are shown.

[4.] Within columns, values followed by the same letter are not significantly different as determined by a Tukey-adjusted P-value <0.05.

Table 6. Severity of brown patch in fifteen cultivars of creeping bentgrass maintained under fairway conditions, July 20th, 2011[1].

Brown Patch Severity[2] (% plot affected)	
Cultivar	
007	13.3[3]
A-4	15.0
Alpha	8.3
Bengal	14.2
Crenshaw	11.7
Crystal Bluelinks	13.3
Declaration	9.2
Independence	6.7
Kingpin	7.5
L-93	18.3
LS-44	5.8
Mackenzie	5.8
Memorial	2.5
Pencross	5.0
T-1	2.5

[1.] July 10th was the only date on which the average disease level exceeded 10%. Dates in which average disease was < 10% are not shown.

[2.] Brown patch was assessed biweekly by visually estimating the percentage of each plot displaying brown patch symptoms.

[3.] Cultivar data are based on six observations, three fungicide treated subplots and three non-treated subplots. Data were subjected to the arcsine transformation to meet assumptions of normality and homogeneity of variance and analyzed using the PROC MIXED procedure of SAS with treatment and cultivar as fixed effects and block as the random effect.

Chapter 3 - Chapter 3 - Sensitivity of Kansas Isolates of *Sclerotinia homoeocarpa* to Demethylation Inhibitor Fungicides

Introduction

Sclerotinia homoeocarpa is the causal agent of dollar spot, a serious and chronic disease of both warm season (C4) and cool season (C3) turfgrasses. Dollar spot is particularly problematic on closely mowed, intensively managed turfgrass such as golf course fairways and putting greens (Latin, 2006; Smiley et al., 2005). Creeping bentgrass (*Agrostis stolonifera* L.) is the primary species of turfgrass used for putting greens in temperate climates. Symptoms of dollar spot on greens height creeping bentgrass (2.0 to 4.0 mm mowing height) include small, straw to gray colored sunken patches of necrotic foliage, generally ranging from 2.5 to 5.0 cm in diameter, with patch size increasing to 5.0 to 10.0 cm on higher-cut turfgrass stands. On individual plants, small, circular, necrotic lesions develop on individual leaf blades; these lesions will often expand and cover the width of a leaf blade.

While management practices such as appropriate nitrogen fertilization as well as irrigation practices designed to minimize leaf wetness are helpful in decreasing the severity of the disease (Landschoot and McNitt, 1997; Smiley et al., 2005; Walsh et al., 1999; Williams et al., 1996), golf course superintendents typically rely on multiple fungicide applications per year to reduce the disease to an acceptable level. There are many classes of fungicides labeled to control dollar spot on turfgrass. The contact fungicides chlorothalonil and mancozeb are labeled for dollar spot control. Systemic materials available include the demethylation inhibitors (DMI) such as metconazole and propiconazole, dicarboximides such as iprodione and vinclozolin, stobilurins such as pyraclostrobin, benzimidazoles such as thiophanate-methyl, and succinate dehydrogenase inhibitors such as boscalid. These range in their interval of control from 7-14

days (chlorothalonil) up to 28 days (propiconazole and boscalid), with subsequent applications being necessary towards the end of the control window in order to ensure continued disease suppression.

Resistance to several fungicides has been observed in populations of *S. homoeocarpa*, including fungicides in the DMI class of fungicides (Detweiler et al., 1983; Bishop et al., 2008; Burpee, 1997; Ok et al., 2011; Putman et al., 2010; Jo et al., 2006; Hsiang et al., 2007; Miller and Stevenson, 2002; Golembiewski et al., 1995). The sensitivity of isolates of *S. homoeocarpa* collected from locations in Kansas to DMI inhibitor fungicides has not yet been examined.

DMI's first became available for dollar spot control in the 1970's as site-specific penetrant fungicides for turfgrass. DMI fungicides inhibit the fungal pathogen's growth by interfering with the oxidative sterol 14α-demethylation in the ergosterol biosynthesis pathway (Siegel, 1981). Sterols modulate the movement of phospholipids and ensure the correct cellular membrane fluidity. The disruption of this sterol synthesis pathway interferes with membrane permeability and activity of some enzymes associated with the fungal-cellular membrane (Hollomon et al., 1990). Due to the high specificity of the mode of action of DMI fungicides, they are prone to resistance. In the early 1990's, field resistance of *S. homoeocarpa* to DMI's was observed, specifically to the active ingredients triadimefon, fenarimol, and propiconazole (Golembiewski et al., 1995). Resistance to DMI's has a quantitative nature, rather than the qualitative nature observed with resistance to thiophanate-methyl (Golembiewski et al., 1995, Jo et al., 2006), and higher application rates of the DMI fungicides can often maintain an adequate level of control in stands where resistance has been observed. DMI fungicides are still used widely for dollar spot control.

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Previous studies of other fungi have found evidence that DMI resistance occurs from both the active ingredient failing to coming into contact with target proteins due to increased expression of exporter proteins (Köller and Scheinpflug, 1987), as well as an increased production of the 14-α-demethylaze enzyme (Ma et al., 2006). Hulvey et al. (2012) reported evidence that field resistance of *S. homoeocarpa* to propiconazole was linked to over-expression of the *S. homoeocarpa* analog of the *CYP51* gene, designated *ShCYP51B*, which codes for the 14-α-demethylase enzyme, as well as *ShatrD*, an analog to the *Botrytis cinerea atrD* efflux transportor protein known to be active towards DMI fungicides.

Nearly two decades has passed since the first observed case of DMI resistance in *S. homoeocarpa*, and since then several new DMI fungicides have been developed and registered for turfgrass, including metconazole, triticonazole, tebuconazole, and a mixture including difenoconazole. Previous studies have provided conflicting evidence of a correlation between DMI products. Hsiang et al. (1997) found low to moderate correlations in the sensitivity of *S. homoeocarpa* to propiconazole, tebuconazole, mycobutanil, and fenarimol. In addition, Miller and Stevenson (2002) reported positive correlations among propiconazole, fenarimol, and myclobutanil, but not between any of those fungicides and triadimefon. However, Golembiewski et al. (1995) reported that resistance to triadimefon, fenarimol, and propiconazole were all correlated between the 150 isolates tested, and Ok et al. (2011) found strong correlation between five DMI products tested (propiconazole, triadimefon, myclobutanil, metconazole, triticonazole, and tebuconazole) on 58 isolates selected arbitrarily from multiple locations.

Currently, the method employed to determine the sensitivity of an isolate of *S. homoeocarpa* to a DMI fungicide relies on time-consuming mycelial growth assays on media amended with a range of fungicide concentrations to determine the effective concentrations

which reduces fungal growth by 50% relative to an unamended control (EC_{50}). Developing a single discriminatory concentration to predict EC_{50} values will provide a more efficient and rapid testing protocol.

The objectives of this study were to: (i) determine the sensitivity of Kansas *S. homoeocarpa* isolates to propiconazole, metconazole, tebuconazole, and triticonazole and correlations among those fungicides using *in vitro* mycelia growth assays; (ii) to determine a best discriminatory dose concentration for each product that will provide for rapid testing of relative sensitivity.

Materials and Methods

Collection, isolation, and storage of isolates

Plugs of turfgrass, 3-10 cm in diameter and containing active dollar spot infection centers (DSIC) were collected from 12 sites in nine counties in Kansas in 2007, 2008, and 2011 (Table 7). All infection centers taken from the same location were at least 1 m apart. *Sclerotinia homoeocarpa* was isolated from symptomatic leaf blades by surface sterilizing in 0.6% sodium hypochlorite for 30 seconds, rinsing in sterile, distilled water, blotting dry, and placing tissue samples on either ¼ strength potato dextrose agar (¼PDA), which was prepared by combining 6 g potato dextrose broth (Difco PDB, Benton, Dickinson and Company, Sparks, MD), and 15 g agar in 1 L water, and/or water agar (WA). After a few days growth, colonies were examined under a compound microscope to ensure that morphology was consistent with *S. homoeocarpa*. Isolates were hyphal tipped twice to ensure that single isolates were collected.

Isolates were grown on millet for long-term storage. For each isolate, 40 g of millet were placed in a 900 ml glass jar (Ball Corporation) with 50 ml of sterilized, distilled water overnight. The following day the millet was autoclaved, allowed to cool 2 hours, and autoclaved again.

Once the millet had cooled to room temperature, plugs of colonized ¼ PDA were placed in the jar with the prepared millet. The millet cultures were incubated for two weeks; the jar was shaken every few days to promote uniform colonization. Once fully colonized, the millet was removed from the jars and placed in shallow weighing boats in a laminar flow hood and allowed to dry for several days. When fully dry, up to 25 cm^3 of the colonized millet was placed into a 25 ml tube and placed into a freezer at -20°C.

Preliminary in-vitro assays to develop reproducible tests

Six isolates were used in preliminary assays to develop reproducible methods for testing sensitivity to each fungicide. One piece of colonized millet per isolate was placed on an 85 mm diameter plastic Petri dish containing 25 ml of ¼PDA. Colonies were incubated for approximately one week at room temperature. An 8mm diameter section from the margin of each colony was then transferred to an 85 mm plate of WA and incubated at room temperature for 7 days. Plugs from the margins of these colonies were then transferred to fungicide-amended media. Using serial dilutions, WA was amended with propiconazole, metconazole, triticonazole, or tebuconazole at 0.001, 0.01, 0.05, 0.1 or 1.0 µg/ml a.i in addition to a non-amended control.

Each isolate was placed on three replicate plates for each concentration of each fungicide. These plates were incubated at 25° C in the dark. Colony diameters were measured after 48 hours for isolates which had colony diameters > 30 mm at that time on the unamended media, and all other isolates were measured after 72 hours. Two perpendicular diameters of each colony, minus the 8mm plug, were measured, and averaged. For each concentration, the percent relative growth (%RG) for each isolate was calculated by comparing the growth on amended media (GOA) compared to the growth on the non-amended control (GOC) by the equation (GOA/GOC) x 100.

The $\log_{10}EC_{50}$ was then calculated by conducting a linear regression (Microsoft Excel, 2007) of the percent relative growth vs $\log_{10}$ value of the fungicide concentration and using the regression equation to calculate the log value of the fungicide concentration which would inhibit the growth by 50%. The antilog of this value was used to obtain the EC_{50}, representing the fungicides active ingredient (a.i.) concentration in µg/ml.

EC_{50} *values and evaluation of cross-sensitivity*

After confirmation of the reliability and reproducibility of the assays using 6 isolates, additional isolates (60 for metconazole, tebuconazole and triticonazole; 65 for propiconazole) of *S. homoeocarpa* were tested to determine their EC_{50} values. In two separate experiments, each isolate was placed on one plate of WA of each concentration for each fungicide and the $\log_{10}EC_{50}$ and EC_{50} values were calculated. Values were tested for normality using the Shapiro-Wilkes test (SAS 9.2 PROC NORMAL). Correlation analysis was conducted to determine the relationship, if any, of the EC_{50} values among the four fungicides (SAS 9.2 PROC CORR).

Determination of single discriminatory concentrations

For each fungicide, regression of the $\log_{10}EC_{50}$ values against %RG at various concentrations was used to determine potential single discriminatory concentrations. After visually examining scatter plots of %RG against each fungicide concentration, the concentrations that appeared to have the strongest ability to predict EC_{50} values were analyzed further. The concentrations used were 0.01, 0.05, and 0.1 µg/ml a.i for metconazole, tebuconazole, and triticonazole, and 0.01 and 0.05 µg/ml a.i for propiconazole. The coefficient of determination (R^2) was determined using the regression analysis (Minitab 15, Minitab Inc., State College, PA).

Results

Determination of EC$_{50}$ values

For all four fungicides, the log$_{10}$EC$_{50}$ values formed a unimodal curve (Figures 1-4) but were not normally distributed (P<0.001 for all four fungicides). For metconazole, the range of EC$_{50}$ values was 0.00128 to 0.0528 µg/ml a.i, with a mean of 0.0163 µg/ml a.i. For propiconazole, the range of EC$_{50}$ values was 0.0014 to 2.42 µg/ml a.i, with a mean of 0.038 µg/ml a.i. For tebuconazole, the range of EC$_{50}$ values was 0.00268 to 0.415 to µg/ml a.i, with a mean of 0.0612 µg/ml a.i. For triticonazole, the range of EC$_{50}$ values was 0.00477 to 1.51 µg/ml a.i., with a mean of 0.0994 µg/ml a.i.

Cross sensitivity among DMI fungicides

Correlations were significant (P<0.0001) and positive for all pairwise comparisons of log$_{10}$EC$_{50}$ values (figures 5-10). However, there was a high level of variability in the strength of the correlation, with correlation coefficients ranging from 0.46 for propiconazole and metconazole (Figure 5) to 0.76 for triticonazole and tebuconazole (Figure 6). Other correlation coefficients were 0.50 for propiconazole and triticonazole (Figure 7), 0.47 for propiconazole and tebuconazole (Figure 8), 0.67 for metconazole and tebuconazole (Figure 9), and 0.73 for metconazole and triticonazole (Figure 10).

Determination of single discriminatory concentrations

Regressions using all potential discriminatory concentrations tested were significant (P<0.0001, figures 11-14), but had different levels of variability. For metconazole, the concentration of 0.01 µg/ml a.i. had the least variability, with an R^2 of 0.73, compared to 0.62 for 0.05 µg/ml a.i. and 0.46 for 0.1 µg/ml a.i. (Figure 11). For propiconazole, the concentration of 0.05 µg/ml a.i. had the least variability, with an R^2 of 0.70 compared to 0.69 for 0.01 µg/ml a.i.

(Figure 12). For tebuconazole, the concentration of 0.05 µg/ml a.i. had the least variability, with an R^2 of 0.85 as compared to 0.80 for 0.1 µg/ml a.i. and 0.64 for 0.01 µg/ml a.i. (Figure 13). For triticonazole, the concentration of 0.1 µg/ml a.i. had the least variability with an R^2 of 0.82 as compared to 0.79 for 0.05 µg/ml a.i. and 0.57 for 0.01 µg/ml a.i. (Figure 14).

Discussion

The Kansas *S. homoeocarpa* isolates examined in this study exhibited a range of sensitivities to the four DMI fungicides. The range of EC_{50} values was greatest for propiconazole, with the highest isolate's EC_{50} of 2.42 µg/ml a.i. being over 1,700 times higher than the lowest EC_{50} of 0.0014 µg/ml a.i. This is a markedly wider range than that reported by Miller and Stevenson (2002) for EC_{50} values of both previously unexposed (baseline) isolates (0.0006 to 0.0102 µg/ml a.i, a 17-fold difference) and a previously exposed population (0.005 to 0.057 µg/ml a.i, an 11-fold difference). The average EC_{50} value of 0.038 µg/ml a.i from this study is similar, however, to the average value of 0.0283 µg/ml a.i reported by Miller and Stevenson (2002) for a previously-exposed population. In our study, metconazole had the smallest range, with only a 41-fold difference between the lowest EC50 value of 0.00128 and 0.0528 µg/ml a.i.

In this study we found significant predictive power of discriminatory concentrations for each fungicide. These discriminatory concentrations can be used to streamline future studies. Specifically for propiconazole, our results are consistent with those of Jo et al. (2006) who also reported a strong correlation between $logEC_{50}$ values and relative growth on media amended with propiconazole at 0.1 µg/ml a.i. The 0.1 µg/ml a.i concentration has been used in other studies (Koch et al., 2009; Popko et al., 2012) to screen large numbers of isolates. Our results for

triticonazole, tebuconazole, and metconazole indicate that single concentrations have good predictive value for these DMI fungicides.

Moreover, in this study, there were strong, positive correlations among all four fungicides. This is consistent with prior reports of correlation of *S. homoeocarpa* to various DMI fungicides (Golembiewski et al., 1995; Ok et al., 2011) but in contrast to other reports where correlations among DMI fungicides did not occur for all combinations (Hisiang et al., 1997, Miller and Stevenson, 2002). Our results, along with those of Ok et al. (2011) indicate that for streamlining it may be possible to simply test one DMI fungicide at one concentration and gain information about EC_{50} values that is relevant to the several DMI fungicides currently used to control *S. homoeocarpa* in turfgrass.

Miller and Stevenson (2002) reported that isolates with reduced sensitivity to propiconazole exhibited shorter incubation periods and higher disease severity in *in-planta* greenhouse experiments. Jo et al. (2006) also found that disease severity on propiconazole-treated field plots increased as mean EC_{50} values obtained *in-vitro* for propiconazole increased. Popko et al (2012) observed that populations with isolates with relative mycelia growth above 50% when tested at 0.1 µg/ml exhibited practical field resistance. Though exact EC_{50} and relative growth values can vary by assay, we did find that a large number of isolates displayed relative growth values exceeding 50% when grown on media with 0.1 µg/ml a.i propiconazole. Further work with greenhouse or field assays with multiple DMI fungicides may lead to a predictive model that could be used to efficiently screen and predict sensitivity based on a single discriminatory concentration.

The use of a single discriminatory concentration saves resources and speeds up the resistance screening procedure. It is not possible to confidently test future isolates on a

discriminatory dose without first screening a large population in order to best determine an accurate relationship between EC_{50} values and RG. The discriminatory dose will allow for expedited feedback in the event of suspected developing resistance, and will also allow for a more streamlined testing procedure for new isolates.

Table 7. Source and year of collection for Sclerotinia homoeocarpa isolates used in this study.

Site	Turf stand information[a]	Collection year(s)	Number of isolates
AVPB	1 CBG	2007	1
DCCC	2 KBT, 1KBF	2007	3
RFTC	1 CBG	2011	2
AVPV	1 CBG	2008	7
MCC	1 CBG	2007 and 08	8
SRCC	1 CBG	2008	1
CBHCC	1 CBG	2008	10
NCLWN	buffalograss lawn	2008	1
CHCC	Several CBG's	2008	4
OSWT	Several CBG's	2008	21
QRCC	Several CBG's	2008	14
WCGC	1 CBG, 1 KBF	2007	2
TOTAL			**74[b]**

[a]CBG = creeping bentgrass putting green, KBF = Kentucky bluegrass fairway, KBT = Kentucky bluegrass tee

[b]All isolates were not successfully tested on all fungicides

Figure 1. Frequency distribution of the $\log_{10}$ effective concentrations of metconazole that inhibit radial mycelial growth by 50% ($\log_{10}EC_{50}$) for 66 isolates of *Sclerotinia homoeocarpa*. Each isolate was tested at least twice on water agar amended with metconazole at concentrations ranging from 0.001 to 1.0 μg/ml a.i.
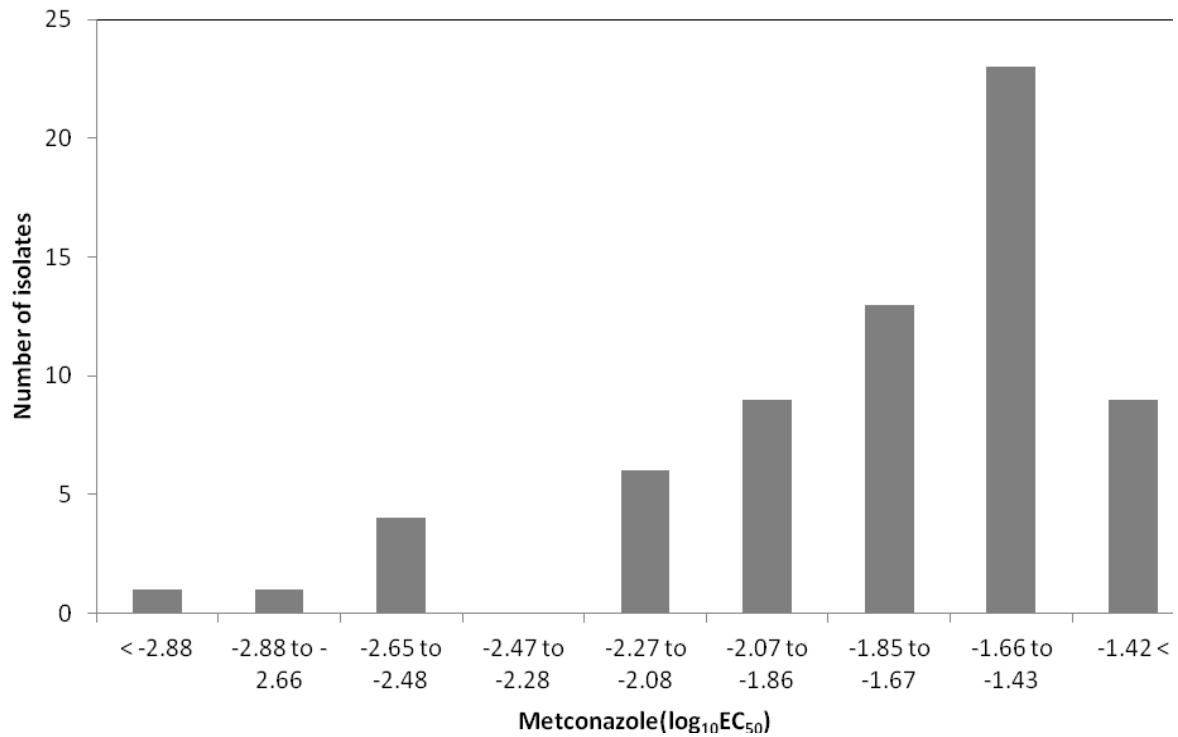
Figure 2. Frequency distribution of the $\log_{10}$ effective concentrations of propiconazole that inhibit radial mycelial growth by 50% ($\log_{10}EC_{50}$) for 71 isolates of *Sclerotinia homoeocarpa*. Each isolate was tested at least twice on water agar amended with propiconazole at concentrations ranging from 0.001 to 1.0 µg/ml a.i.
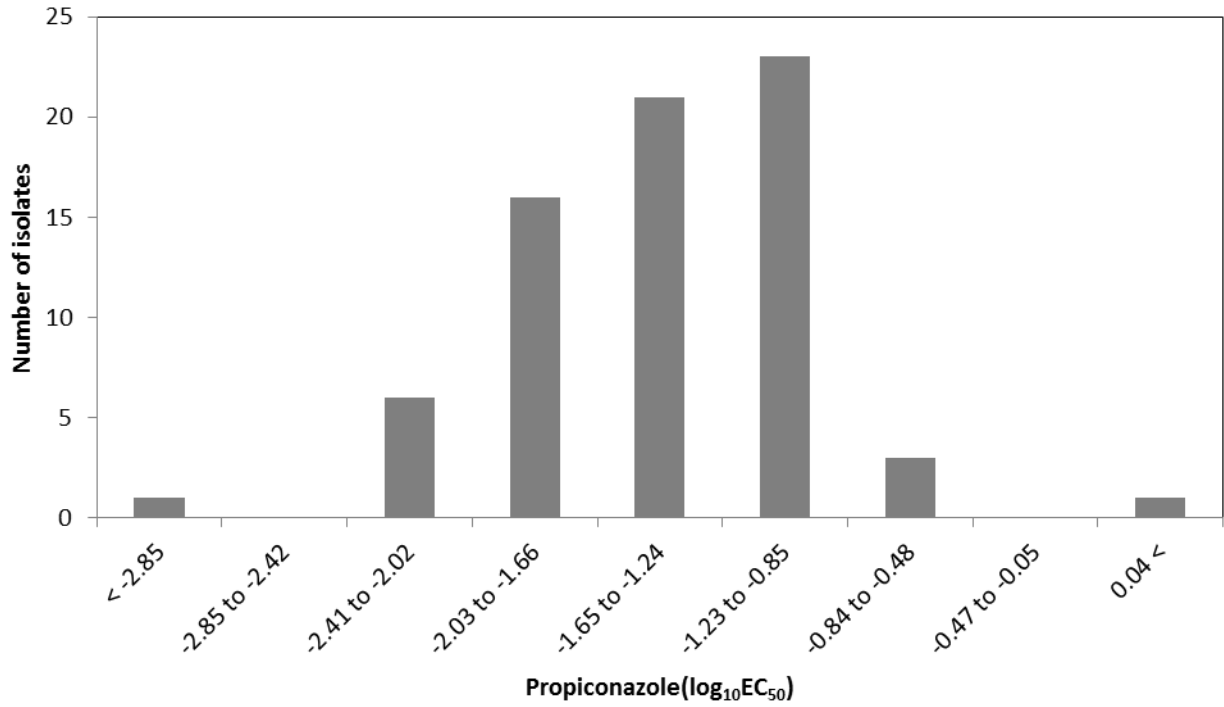
Figure 3. Frequency distribution of the $\log_{10}$ effective concentrations of tebuconazole that inhibit radial mycelial growth by 50% ($\log_{10}EC_{50}$) for 66 isolates of *Sclerotinia homoeocarpa*. Each isolate was tested at least twice on water agar amended with tebuconazole at concentrations ranging from 0.001 to 1.0 μg/ml a.i.
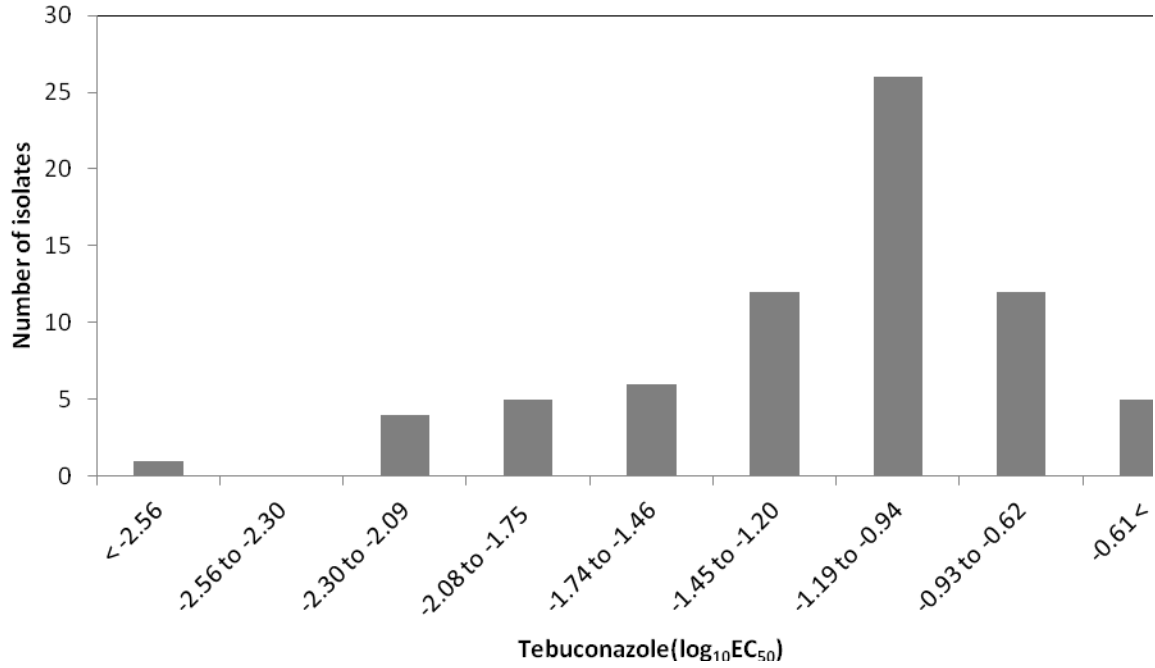
Figure 4. Frequency distribution of the $\log_{10}$ effective concentrations of triticonazole that inhibit radial mycelial growth by 50% ($\log_{10}EC_{50}$) for 66 isolates of *Sclerotinia homoeocarpa*. Each isolate was tested at least twice on water agar amended with triticonazole at concentrations ranging from 0.001 to 1.0 µg/ml a.i.

Figure 5. Relationship between $Log_{10}EC_{50}$ values of 66 isolates of *Sclerotinia homoeocarpa* for metconazole and propiconazole. Each point represents one isolate. Each isolate was tested on the representative fungicides at concentrations of 0.001, 0.01, 0.05, 0.1, and 1 μg/ml and a control of water agar.

Figure 6. Relationship between $Log_{10}EC_{50}$ values of 71 isolates of *Sclerotinia homoeocarpa* for tebuconazole and triticonazole. Each isolate was tested on the representative fungicides at concentrations of 0.001, 0.01, 0.05, 0.1, and 1 µg/ml and a control of water agar.
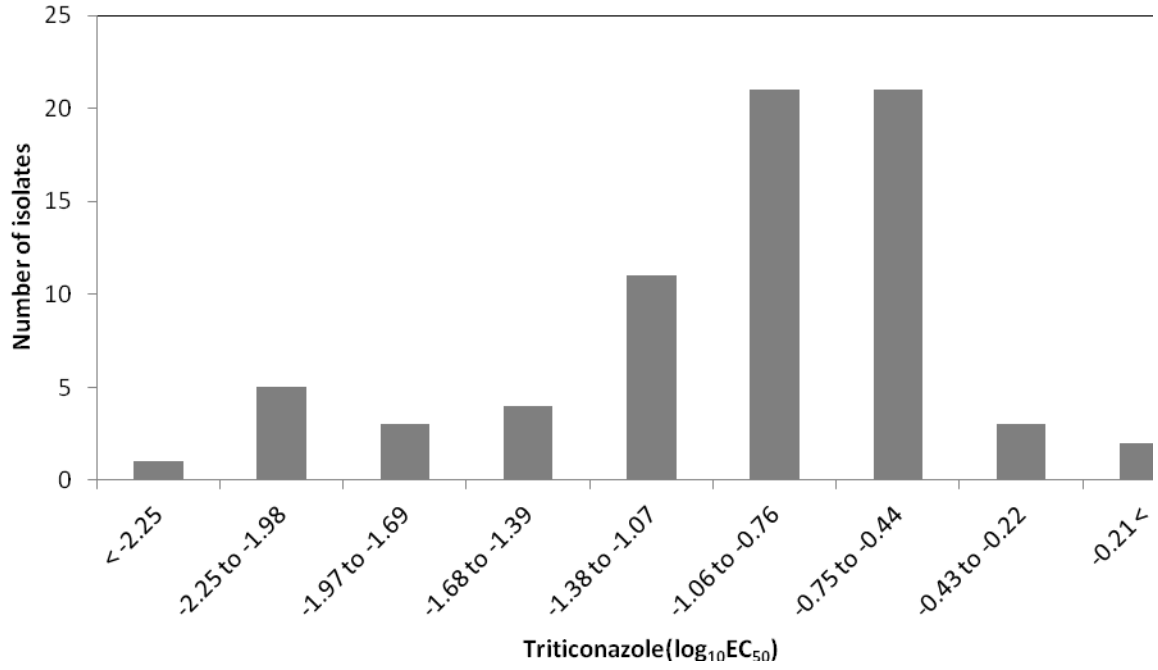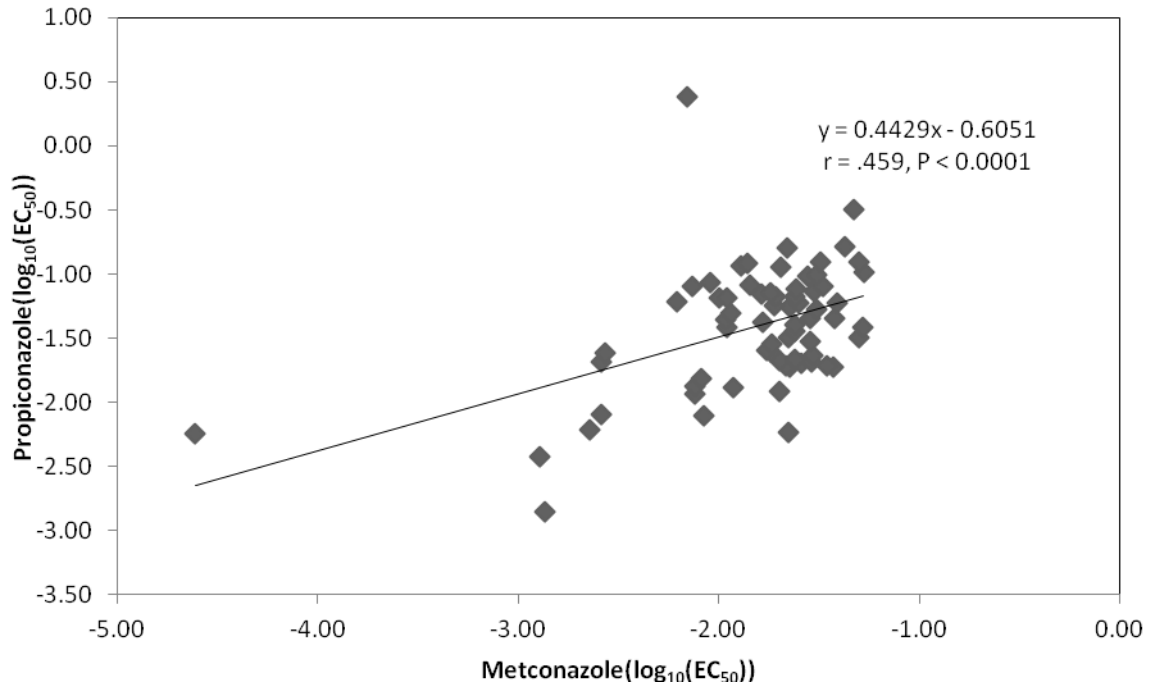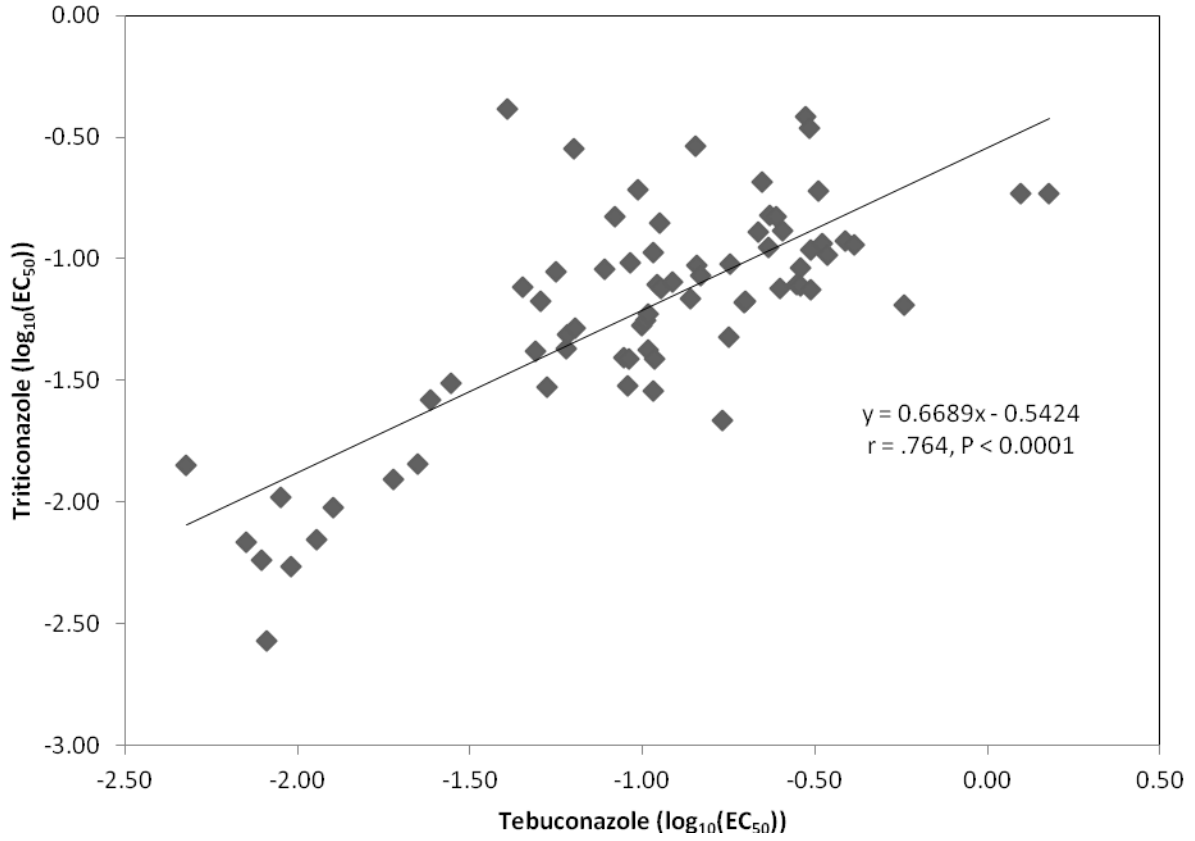
Figure 7. Relationship between $\text{Log}_{10}\text{EC}_{50}$ values of 71 isolates of *Sclerotinia homoeocarpa* for propiconazole and triticonazole. Each isolate was tested on the representative fungicides at concentrations of 0.001, 0.01, 0.05, 0.1, and 1 μg/ml and a control of water agar.

Figure 8. Relationship between $Log_{10}EC_{50}$ values of 71 isolates of *Sclerotinia homoeocarpa* for propiconazole and tebuconazole. Each isolate was tested on the representative fungicides at concentrations of 0.001, 0.01, 0.05, 0.1, and 1 µg/ml and a control of water agar.

The scatter plot shows the relationship with equation:
y = 0.444x - 0.5805
r = .465, P < 0.0001

X-axis: Propiconazole($log_{10}(EC_{50})$)
Y-axis: Tebuconazole($log_{10}(EC_{50})$)

Figure 9. Relationship between $Log_{10}EC_{50}$ values of 66 isolates of *Sclerotinia homoeocarpa* for metconazole and tebuconazole. Each isolate was tested on the representative fungicides at concentrations of 0.001, 0.01, 0.05, 0.1, and 1 µg/ml and a control of water agar.
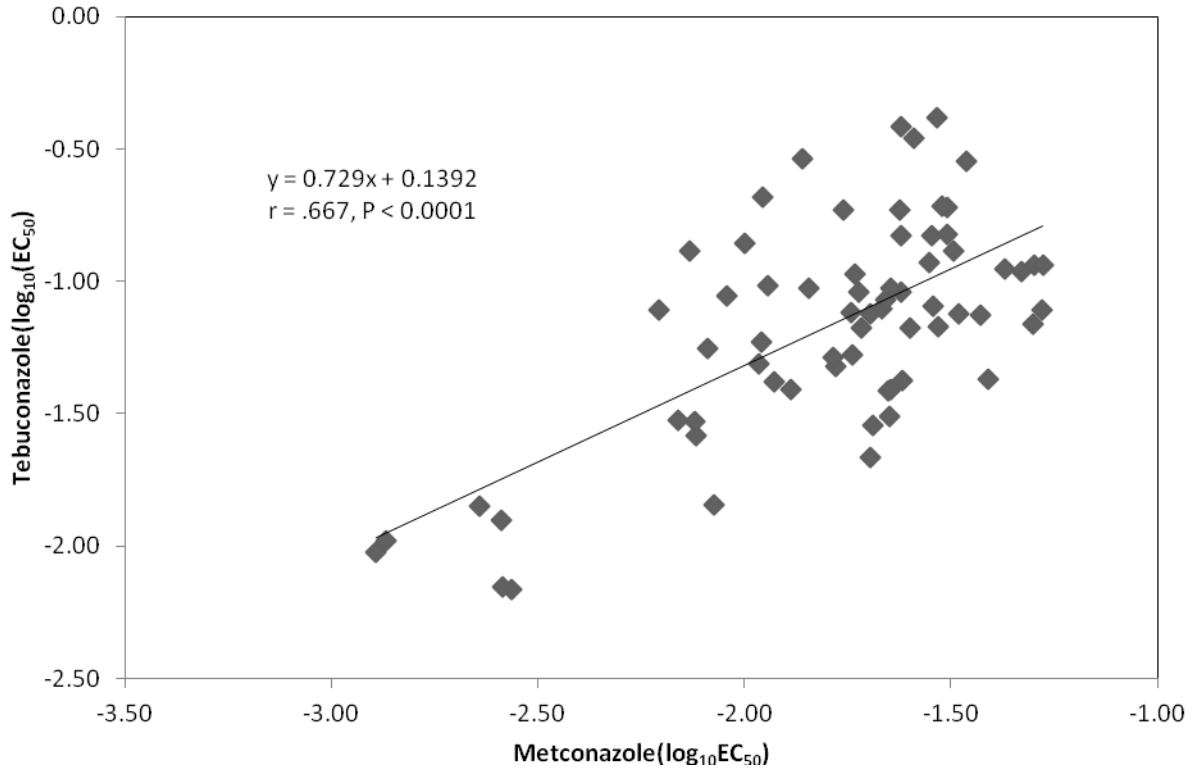
Figure 10. Relationship between $Log_{10}EC_{50}$ values of 66 isolates of *Sclerotinia homoeocarpa* for metconazole and triticonazole. Each isolate was tested on the representative fungicides at concentrations of 0.001, 0.01, 0.05, 0.1, and 1 μg/ml and a control of water agar.

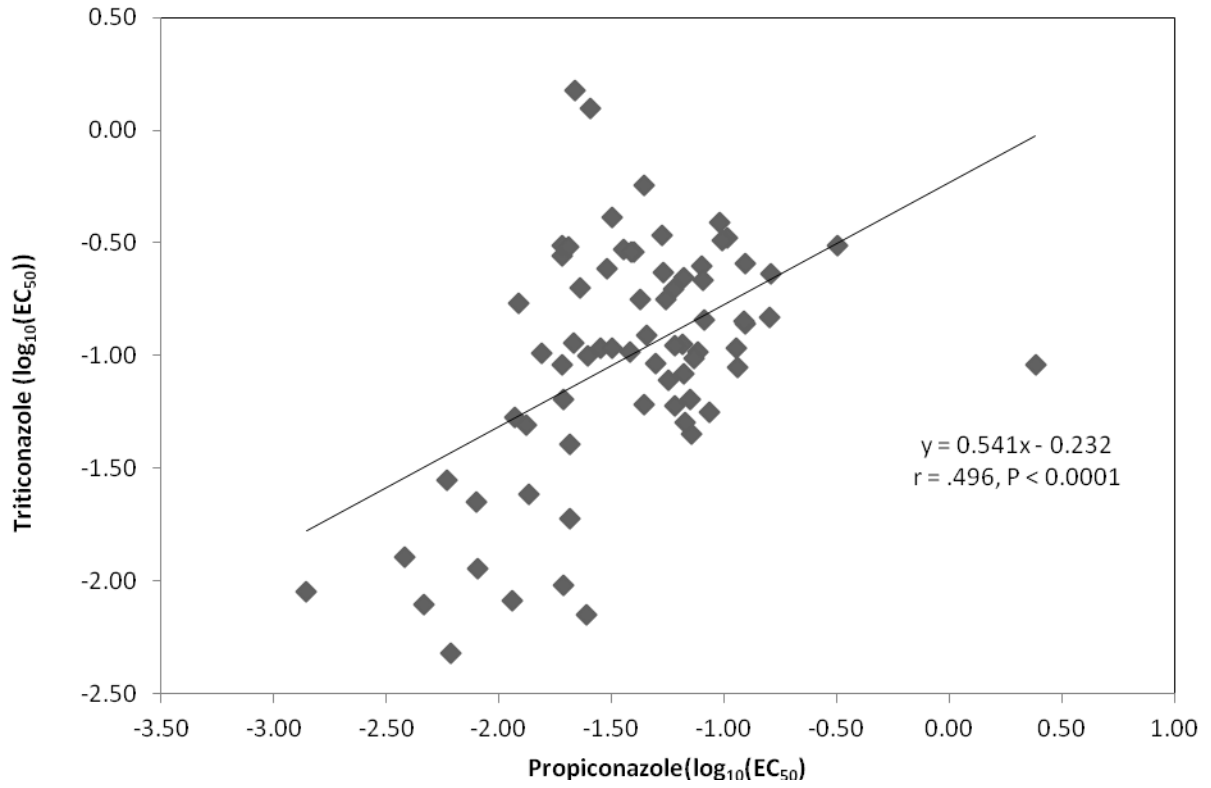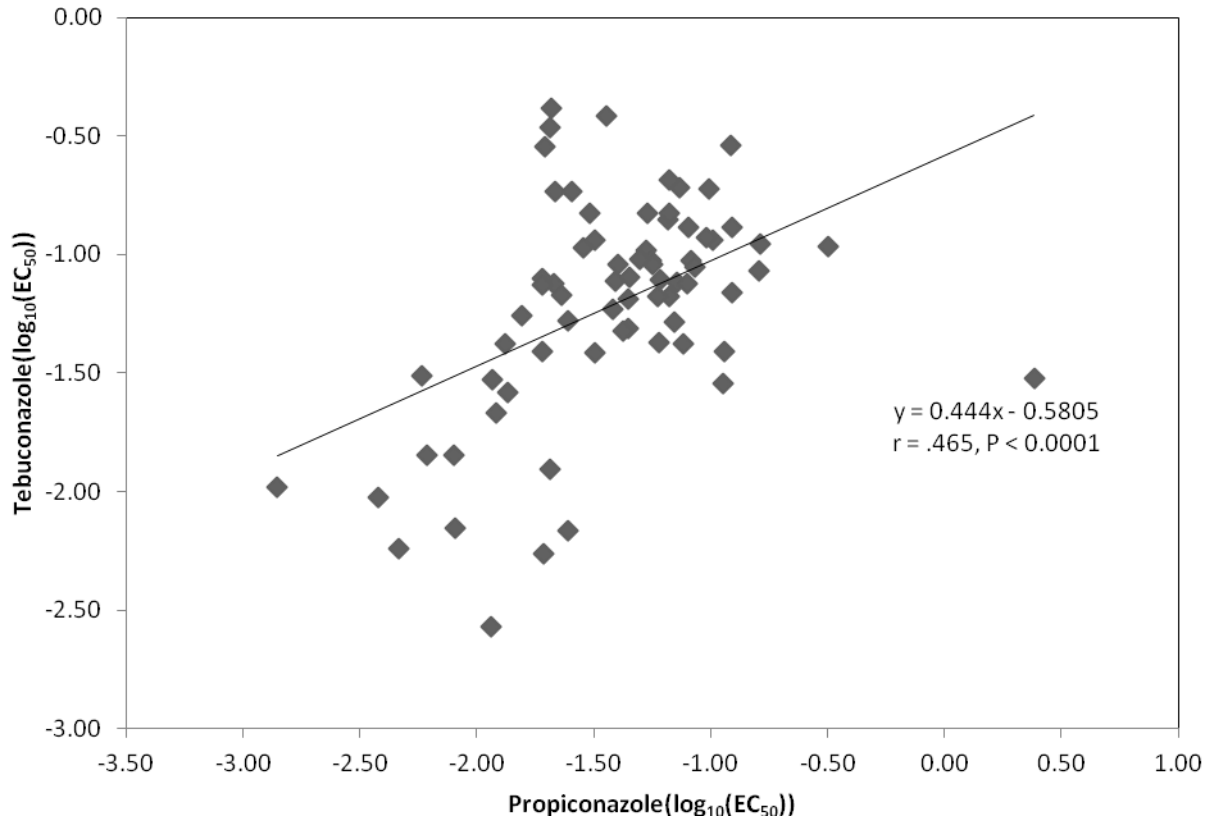**Figure 11.** Relationship between $\log_{10}$ concentrations of metconazole that inhibit relative mycelial growth of 66 isolates of *Sclerotinia homoeocarpa* by 50% as compared to a control ($\mathrm{Log_{10}EC_{50}}$) and growth of those isolates at 0.1, 0.05, and 0.01 μg/ml a.i.

Figure 12. Relationship between $\log_{10}$ concentrations of propiconazole that inhibit relative mycelial growth of 71 isolates of *Sclerotinia homoeocarpa* by 50% as compared to a control ($\text{Log}_{10}\text{EC}_{50}$) and growth of those isolates at 0.1 and 0.05 μg/ml a.i.

Figure 13. Relationship between $\log_{10}$ concentrations of tebuconazole that inhibit relative mycelial growth of 71 isolates of *Sclerotinia homoeocarpa* by 50% as compared to a control ($\text{Log}_{10}\text{EC}_{50}$) and growth of those isolates at 0.1, 0.05, and 0.01 µg/ml a.i.

RG at 0.01
y = 0.0183x - 2.7416
R^2 = 0.6409

RG at 0.05
y = 0.021x - 2.3747
R^2 = 0.8511

RG at 0.10
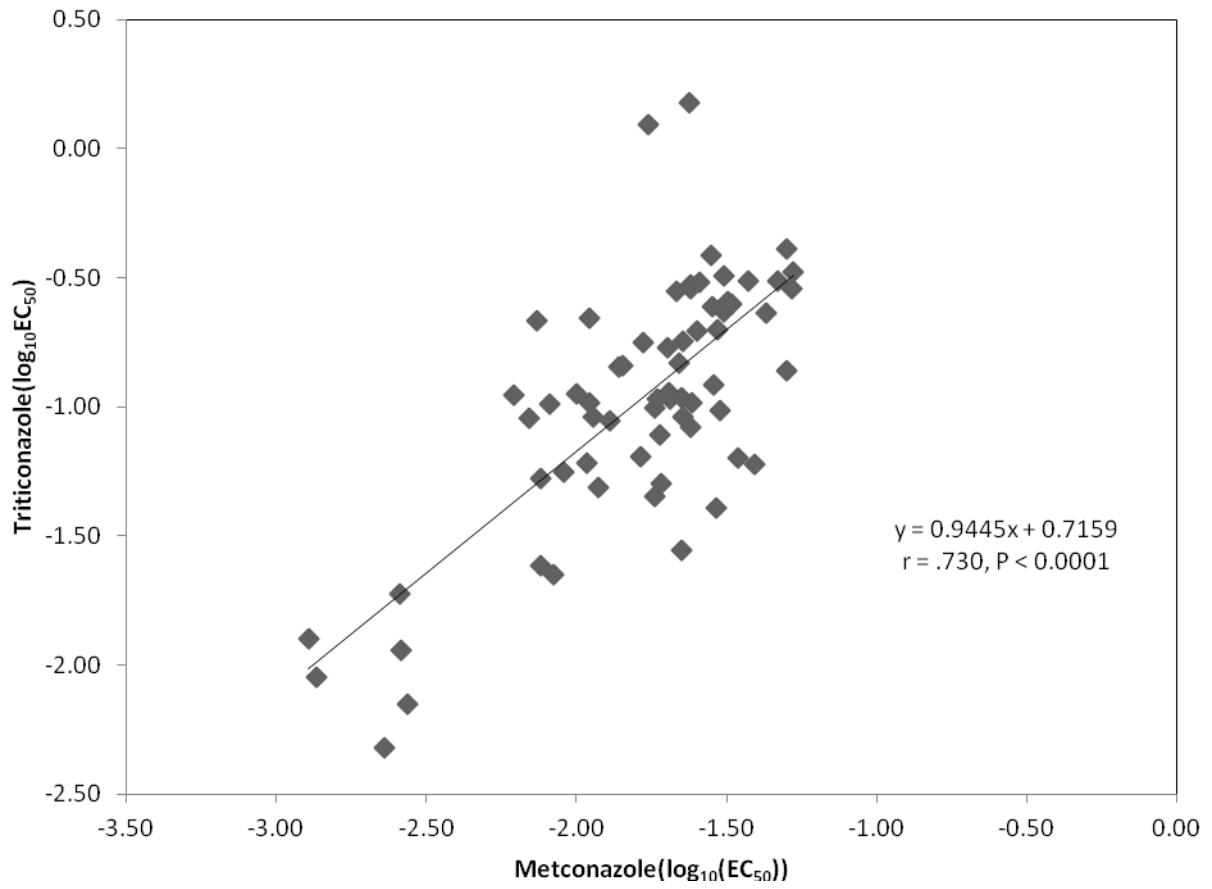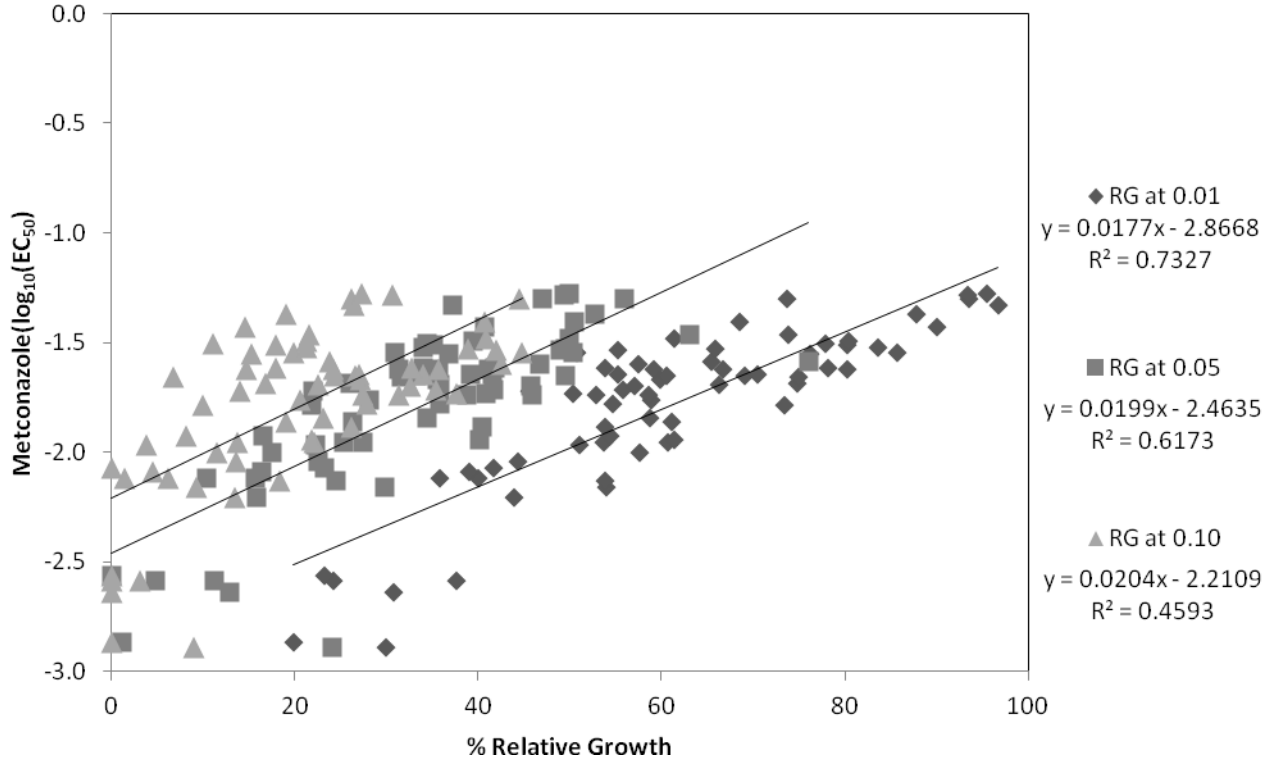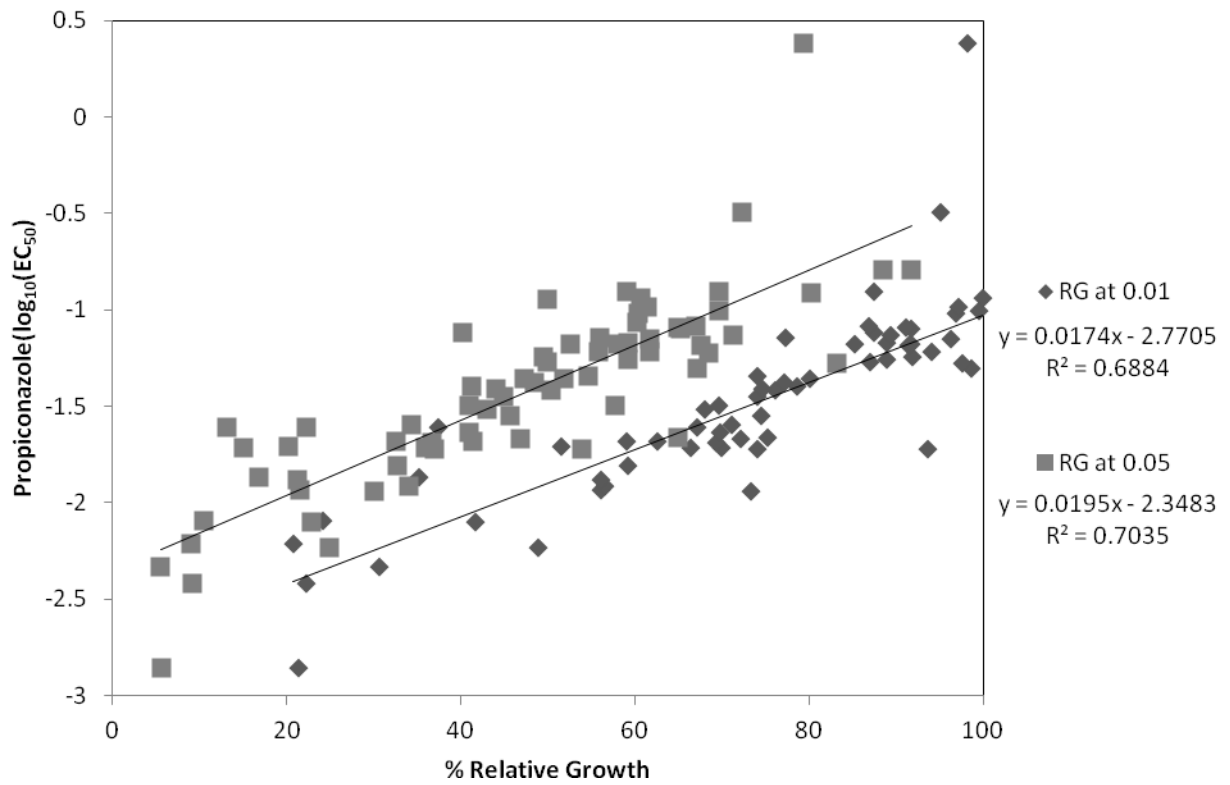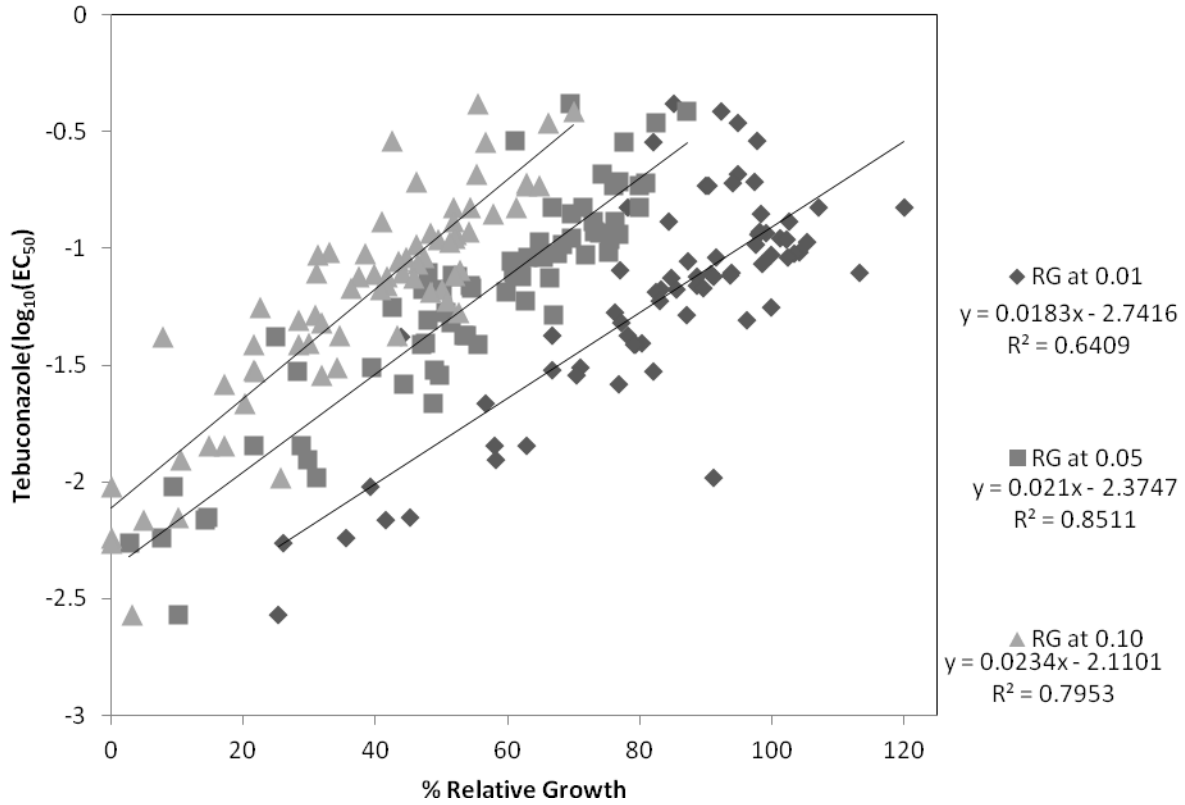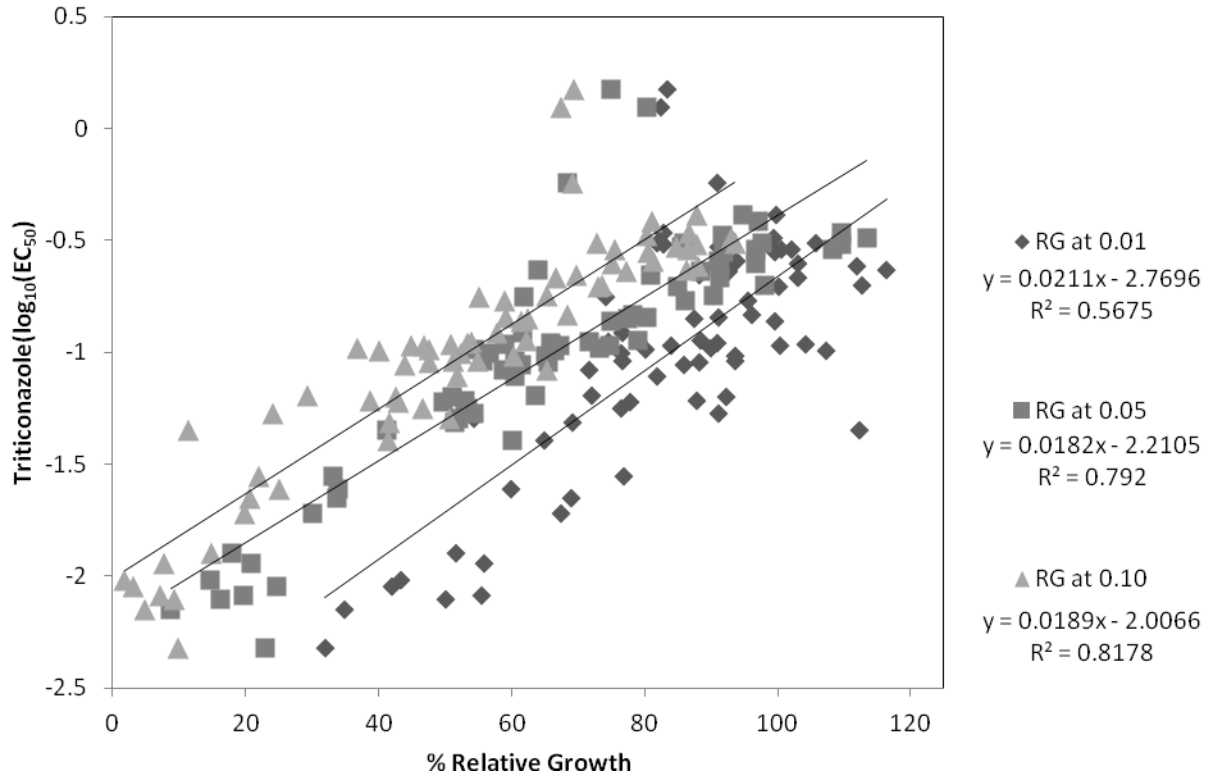y = 0.0234x - 2.1101
R^2 = 0.7953

Figure 14. Relationship between $\log_{10}$ concentrations of triticonazole that inhibit relative mycelial growth of 66 isolates of *Sclerotinia homoeocarpa* by 50% as compared to a control ($\text{Log}_{10}\text{EC}_{50}$) and growth of those isolates at 0.1, 0.05, and 0.01 μg/ml a.i.

Chapter 4 - Determination of Mutations in Beta-Tubulin Gene Associated with Resistance to Thiophanate-Methyl in Kansas Isolates of *Sclerotinia homoeocarpa*

Introduction

Sclerotinia homoeocarpa is the causal agent of dollar spot, a serious and chronic disease of both warm season (C4) and cool season (C3) grasses in temperate climates. While management practices such as appropriate nitrogen fertilization as well as irrigation practices designed to minimize leaf wetness, can reduce the severity of the disease (Landschoot and McNitt, 1997; Smiley et al., 2005; Wal.sh et al., 1999; Williams et al., 1996), golf course superintendents typically rely on multiple fungicide applications per year to reduce the disease to an acceptable level.

There are many classes of fungicides labeled for use to control dollar spot on turfgrass. The contact fungicides chlorothalonil and mancozeb are labeled for dollar spot control. Systemic materials available include the demethylation inhibitors (DMI) such as metconazole and propiconazole; dicarboximides such as iprodione and vinclozolin; stobilurins such as pyraclostrobin; benzimidazoles such as thiophanate-methyl; succinate dehydrogenase inhibitors such as boscalid (Vincelli and Munshaw, 2013; Latin, 2011).

Benzimidazoles primarily inhibit β-tubulin polymerization in fungi, although interactions with other forms of tubulin as well as differential interactions with tubulin in the free and polymerized states have also been reported. Microtubules consist of long chains of β- and α-tubulin dimers, and microtubules are one type of cytoskeletal filament. The structural degradation of this filament results in the interruption of many cellular processes including cell division and the proper movement of organelles within a cell, ultimately resulting in cell death.

Benzimidazole fungicides were first used in the 1960's and the first reported case of resistance was noted in 1970 when a benzimidazole-class compound failed to control powdery mildew on cucurbits (Genet, 2005, FRAC Website). Resistance to benzimidazole fungicides has also been reported in numerous other plant pathogenic fungi. Resistance has been associated with mutations in the beta-tubulin (βt) gene in many fungal species including *Venturia inaequalis, Monilinia fucticola*, *Monilinia laxa, Colletotrichum cereale*, *Cladobotryum dendroides*, and many others (Koenraadt et al., 1992; Ma et al., 2003; Ma et al., 2004; Ma and Michailides, 2005; McKay et al., 1998; Wong et al., 2008; Young et al., 2010; Warren et al., 1977). Benzimidazole resistance most commonly results from mutations that alter the binding site (Oakley, 2004). Most mutations involving these species have involved the 198th codon, although the specific mutation differs. Mutations observed at the 198th codon include E198A, E198Q, E198G, E198K, and E198V (Mal. et al., 2005). In laboratory induced mutants, as well as field isolates of *Aspergillus nidulans* and other species, mutations associated with resistance have occurred at the 6th (H6Y), 50th (Y50C), 167th (F167Y), 200th (F200Y) and 240th (L240F) codons (Koenradt et al., 1992; Jung et al., 1992, Ma et al., 2003; Ma and Michailides, 2005). *S. homoeocarpa* has shown resistance to benzimidazole fungicides in in-vitro laboratory assays and field experiments (Detweiler et al., 1983; Burpee, 1997; Jo et al., 2008; Bishop et al., 2008). In previous work (Koenraadt et al., 1992) a mutation leading to a change of glutamic acid to lysine at codon 198 (E198K) was associated with benomyl resistance in many species of fungi, including one isolate of *S. homoeocarpa*. The primers in that study were designed to amplify codons 167 − 241, omitting other regions of the gene with known sites of mutations associated with benzimidazole resistance in other species of plant pathogenic fungi (Ma and Michailides, 2005). Phylogenetic studies have relied on primers designed to amplify conserved regions within the β-tubulin gene

(Glass and Donal.dson, 1995), and therefore also do not sample all regions of the gene known to be associated with resistance. Another study (DeVries et al., 2008) focused on population structure of *S. homoeocarpa* including isolates sensitive and resistant to thiophanate-methyl. The study utilized the conserved primers Bt2a and Bt2b (Glass and Donaldson, 1985) which amplify codons 12-127 of the beta-tubulin gene. Sequences for resistant and sensitive isolates were identical indicating that the mutation associated with resistance was not found in that region. Therefore, the objective of this study was to sequence the entire β-tubulin gene of isolates of *S. homoeocarpa* resistant and sensitive to thiophanate-methyl and identify mutations that may be associated with resistance.

Materials and Methods

Collection, isolation, and storage of isolates

In previous work (unpublished), 71 isolates of *S. homoeocarpa* were tested for sensitivity to thiophanate-methyl by assessing growth on media amended with the fungicide at 10 μg a.i./ml. Four sensitive and four resistant isolates were chosen for analysis.

The isolates were collected from plugs of turfgrass, 3-10 cm in diameter and contained active dollar spot infection centers (DSIC). These were collected from 12 sites in nine counties in Kansas in 2007 and 2008 (Table 1). All infection centers taken from the same location were at least 1 m apart. *Sclerotinia homoeocarpa* was isolated from symptomatic leaf blades by surface sterilizing in 0.6% sodium hypochlorite for 30 s, rinsing in sterile, distilled water, blotting dry, and placing tissue sample on either ¼ potato dextrose agar (¼PDA), which was prepared by combining 6 g potato dextrose broth (Difco PDB, Benton, Dickinson and Company, Sparks, MD), and 15 g agar in 1 liter water, and/or water agar (WA). After a few days growth, colonies

were inspected under a compound microscope to ensure that morphology was consistent with *S. homoeocarpa*.

For long-term storage, 40 g of millet were placed in a 900 ml glass jar (Ball Corporation) with 50 ml of sterilized, distilled water overnight; the following day the millet was autoclaved, allowed to cool 2 hours, and autoclaved again. Once the millet had cooled to room temperature, 1 cm^2 pieces of the fungal-colonized ¼ PDA were cut from colony and placed in the jar with the prepared millet. The fungus was allowed to grow for two weeks; the jar was shaken every few days to promote uniform colonization. Once fully colonized, the millet was removed from the jars and placed in a sterilized biological. horizontal-flow hood and allowed to dry for several days. When fully dry, up to 25 cm^3 of the colonized millet was placed into a 25 ml tube and placed into a freezer set at -20°C.

Extraction of Genomic DNA, Primer Design, and PCR Analysis

A single piece of colonized millet was placed on a piece of circular cellophane, 80mm in diameter, which was then placed on top of ¼ PDA in an 80 mm Petri dish. The colonies were allowed to grow for 7-14 days, or until the mycelium reached the edge of the dish. The cellophane was removed and placed, with aerial mycelium growth, into a laminar flow hood to dry for 7 days.

Genomic DNA was extracted using a commercial kit (Easy-DNA kit, Life Technologies, Grand Island, NY) with the following minor modifications: room temperature isopropanol was substituted for 100% ethanol at -20°C during the isolation of the DNA and 70% ethanol was substituted for 80% ethanol for the DNA wash. Products were stored at -20°C.

An unpublished genomic sequence contig from *S. homoeocarpa* isolate MB01 (Orshinksi et al., 2012), sensitive to thiophanate-methyl, was used to identify the sequence of the β-tubulin gene. This nucleotide sequence was BLAST searched against The National Center for

Biotechnology Information (NCBI) Microbes sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to locate the β-tubulin gene within the contig (Altshul et al., 1990). Serial Cloner 2.5 (http://serial.basics.free.fr/Home/Home.html) was used to identify start and stop codons and determine the gene structure associated with the *S. homoeocarpa* β-tubulin gene by comparison against known ascomycete β-tubulin sequences *benA* (AN1182) of *Aspergillus nidulans* and the β-tubulin gene (BC1G_00122) of *Botryotinia fuckeliana* (strain B05.10).

PCR reaction and product isolation

Three pairs of PCR primers were designed from the resulting *S. homoeocarpa* β-tubulin sequence to amplify overlapping PCR products for complete sequencing of the gene by the Sanger Sequencing method. Primers used for PCR amplification are as follows: BT1 forward and reverse (BT1F/BT1R); BT2F/R, and BT3F/R. The primer sequence and total nucleotide base pairs (bp) of each amplicon: BT1F (5'-CTCAGGATCTCAACTTCTTGCTTCC-3'), BT1R (5'-GTAATGTCCCTTGGCCCAGTTG-3'),(806 bp); BT2F (5'-CCGTCCATAATACGAGCTGCGG-3'), BT2R (5'-GCCATCATGTTCTTCGGGTCG-3'), (795 bp); BT3F of (5'-GTTTCCCTGGTCAACTCAACTCC-3'), BT3R (5'-CAACAATGCTTGCTGCCAATGAAG-3'), (805 bp).

PCR amplification was carried out using Ex Taq (TaKaRa Bio Company, Mountain View, CA) thermostable proof-reading DNA polymerase and 25 pmol of each primer per reaction. Thermal cycling protocols for PCR amplification consisted of 90 °C for 5 min, followed by 35 cycles of 90 °C for 30 s, a primer pair specific temperature of 57.8°C for primer sets 1 and 2; 59.0°C for primer set 3, and 72°C for 90 s; and ending with a final elongation step of 72°C for 5 min. PCR products were electrophoresed on a 1% agarose gel to verify size,

purified using a Wizard® SV Gel and PCR Clean-up kit (Promega), and all products were directly sequenced at the Kansas State University DNA Sequencing and Genotyping Facility. Sequences were analyzed using Geneious version 5.3.5 created by Biomatters (http://www.geneious.com/). Full sequences were determined for four sensitive isolates (isolates DS4, DS5 and DS6 from site DCC and isolate DS35 from site CBHCC) and four resistant isolates (isolates DS24 and DS31 from site CBHCC, isolate DS58 from site MCC, and isolate DS66 from site QRCC). Partial sequences were obtained for several other isolates.

Results

Sequence searches of the *S. homoeocarpa* contig from the reference isolate MB01 against the NCBI Microbes sequence database revealed a putative β-tubulin gene with sequence similarity to known ascomycete β-tubulin genes. The gene structure was deduced by sequence comparisons with the β-tubulin genes from *A. nidulans* and *B. fuckeliana* (May et al., 1987; Park et al., 1997). The *S. homoeocarpa* β-tubulin gene was 1785 bp long, including seven exons and six introns (Figure 15). Residues 6, 50, 165, 134, 198, 200, and 257, which are known to be involved in benzimidazole resistance in other species, were conserved in the contig from isolate MB01. The sequenced strain did not harbor any mutations in β-tubulin known to confer or be associated with benzimidazole resistance.

We determined the genomic sequence of the β-tubulin gene in 4 sensitive (S) and 4 resistant (R) *S. homoeocarpa* isolates from Kansas. In the genomic sequence, bp number 977 in exon six was an adenosine in sensitive isolates and the reference isolate and a cytosine in resistant isolates, corresponding to a substitution of alanine for glutamic acid at codon 198 (E198A) in the resistant isolates. All other codons implicated in benzimidazole resistance did not harbor mutations compared with the reference sequence.

The BT3F/BT3R amplicon of isolate 32 (S) had several silent mutations that differed from all other isolates, including substitutions of thymine for cytosine at nucleotide 1,633, guanine for thymine at nucleotide 1,642, and thymine for cytosine at nucleotide 1,756. All of these substitutions are silent mutations. Isolate 32 did not have the E198A mutation.

Discussion

Point mutations in the β-tubulin genes of other fungi have been associated with resistance to benzimidazole fungicides. In particular, mutations at amino acid position 198 have been reported for *Penicillium expansum* (Albertini et al., 1999; Baraldi et al., 2003), *Monilinia fructicola* (Koenraadt et al., 1992; Ma et al., 2003), *Tapesia acuformis* (Albertini et al., 1999), *A. nidulans* (June et al., 1992) and *Botrytis cinerea* (Luck and Gillings, 1995). Most mutations found in previous work have been associated with amino acid position 198 and this evidence supports that the mutation of codon 198 in *S. homoeocarpa* is also associated with resistance.

Field isolates of fungi resistant to benzimidazoles exhibit mutations that seem to be restricted to positions 6, 50, 167, 198, 200, and 240 (Ma et al., 2005). There is some evidence that there is a fitness cost associated with these mutations, as (for example) field resistant isolates of *M. fructicola* with a mutation at codon 6 exhibited low temperature sensitivity (Ma et al., 2003).

In other fungi, several point mutations contributing to resistance have been found at other positions in the β-tubulin gene. These include amino acid position 6 in *Monilinia fructicola* (H6Y, Ma et al., 2003), position 50 in *Cladobotryum dendroides* (Y50C, McKay et al., 1998), position 167 in *P. expansum* (F167Y, Baral.di et al., 2003), position 200 in *Venturia inaequal.is* (F200Y, Koenraadt et al., 1992), position 240 in *Monilinia laxa* (L240F, Ma et al., 2005) and residues 6, 50, 134, 165, 200, and 257 in *A. nidulans* (Jung and Oakley, 1990; Jung et al., 1992;

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Jung et al., 1998). Our results indicated that isolate 32 has nucleotide sequence divergence within the β-tubulin gene that does not lead to sequence differences at the protein level.

Different levels of benzimidazole resistance have been observed. These differences have been attributed to the different codons affected by the mutations associated with resistance. For example, in *Monilinia fructicola*, a mutation at codon six led to a low level of resistance to thiophanate-methyl, whereas a mutation at codon 198 led to a high level of resistance (sensitive isolates EC_{50}'s ranged from 0.2644 to 0.7629 μg a.i./ml; a low level of resistance was defined as an isolate with an EC_{50} ranging from 2.3844 to 6.4212 μg a.i./ml, and a high level of resistance was defined as an isolate with an EC_{50} higher than 50.0 μg a.i./ml) (Ma et al., 2003). Other examples of different levels of resistance can be found from studies of species of *Venturia* and *Tapesia* (Ma et al., 2005; Koenraadt et al., 1992; Albertini et al., 1999).

The entire β-tubulin gene of *S. homoeocarpa* has not previously been sequenced in full in a study comparing resistant and sensitive isolates. Many studies have focused only on sequence analysis of codons 167-241 due to the availability of PCR primers that amplify this region. The extreme 5' end of the β-tubulin gene, including codon six which is a known source of benzimidazole resistance, has been difficult to analyze due to the presence of a very short first exon and a relatively large first intron, which lacks conservation of sequence and length. Sequencing the gene in full is necessary to determine all contributing factors within β-tubulin involved with resistance. Even so, other genomic regions could also be investigated, especially if the isolate is multidrug resistant. In *Botrytis cincrea*, resistance to multiple fungicides has been associated with increased efflux protein activity, caused in part by transcription factor mutations (Kretschmer et al., 2009).

In addition to exploring other regions of the β-tubulin gene, site-directed mutagenesis of codon 198 would provide functional evidence that is the sole or major mutation causing resistance to benzimidazole fungicide in *S. homoeocarpa*. If a partial loss in resistance is observed, it may be prudent to investigate other regions of the gene or even perhaps the genome.

Figure 15. Structure of the *S. homoeocarpa* β-tubulin gene showing sequence variation in sensitive and resistant strains at bp 977 in codon 198 on exon 6. Black regions represent exons; gray regions represent introns.
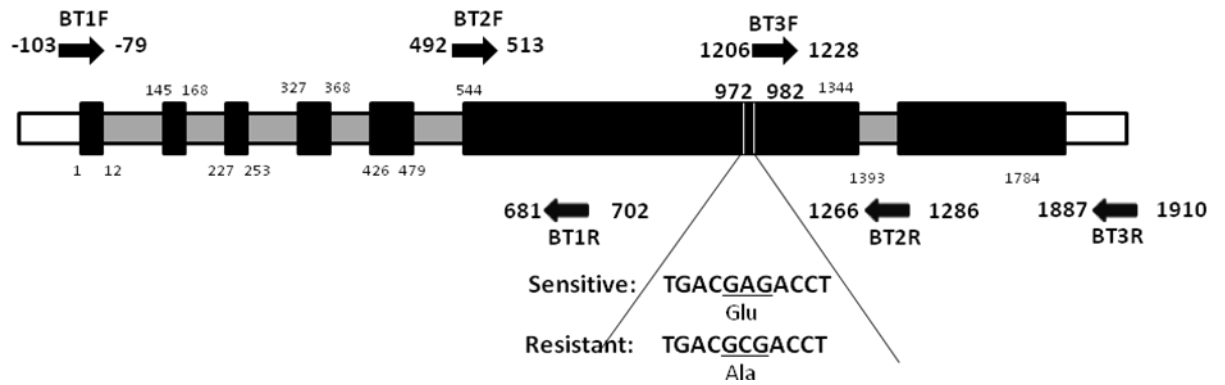
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