CHARACTERIZING HORMESIS AND THE IN VITRO EFFECTS OF SUB-LETHAL FUNGICIDE EXPOSURE IN *SCLEROTINIA HOMOEOCARPA*

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by

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DEDICATION

This thesis is dedicated to my parents, Greg and Teresa. Thank you for giving me your love and encouragement in everything I do. It means more than I could ever express.

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ABSTRACT

Dollar spot, caused by *Sclerotinia homoeocarpa* F.T. Bennett, is the most economically important disease of turfgrass. Dollar spot reduces aesthetics and playability of turfgrass, and when left uncontrolled can result in plant death and weed encroachment. Sterol demethylase inhibiting (DMI) fungicides are valuable for dollar spot control due to their broad spectrum of activity with single applications. Previous studies have shown sub-lethal concentrations of fungicides can cause plant pathogens to exhibit hormesis. The first goal of this research was to evaluate the effects of sub-lethal doses of DMI fungicides on the mycelial growth of a range of *S. homoeocarpa* isolates via an *in vitro* fungicide assay. The second goal was to examine the impact of sub-lethal doses of DMI fungicides on the production of oxalic acid in *S. homoeocarpa*. The third goal was to determine the effects of low doses of DMI fungicides on the disease severity of resistant and sensitive populations of *S. homoeocarpa* on creeping bentgrass. Sub-lethal concentrations of DMI fungicides did not cause significant increases of mycelial growth *in vitro* when compared to mycelial growth of untreated isolates. A method to detect oxalic acid via high performance liquid chromatography was developed in this research. Oxalic acid concentrations were greatest when isolates were treated with a high rate of DMI fungicides. In the greenhouse, area under the disease progress curve (AUDPC) was consistently increased by a sub-lethal rate of DMI fungicides when compared to untreated inoculum. A sublethal rate of DMI fungicides consistently caused increased mean disease severity as early as 21 days after treatment. Results of this study suggest sub-lethal concentrations of fungicides may induce hormesis in *S. homoeocarpa*, resulting in increased dollar spot pressure in creeping bentgrass.

Chapter 1

Introduction and Literature Review

Pathogen Biology & Taxonomy

Sclerotinia homoeocarpa F.T. Bennett is the causal agent of dollar spot on turfgrass. The fungus is in the Sclerotiniaceae family and, like many other fungal plant pathogens, is currently placed within the order Helotiales (Whetzel, 1945). This pathogen was initially defined by its microsclerotia and classified as a *Sclerotinia* species due to the broad definition of the species at the time (Walsh et al., 1999). However, the nomenclature and taxonomy of *S. homoeocarpa* is currently under debate.

Although the dollar spot pathogen is cited throughout literature as *Sclerotinia homoeocarpa*, the genus name is erroneous. Taxonomic monographs on *Sclerotinia* and related fungi exclude this pathogen due to its failure to produce tuber-like sclerotia and differing apothecial morphology from other *Sclerotinia* species (Allen et al., 2005). Instead of producing tuber-like sclerotia, *S. homoeocarpa* produces a flat stroma (Smiley et al., 2007). Adding to the difficulty of reclassification, conidial or teleomorphic forms of *S. homoeocarpa* isolates have not been observed in the field or produced in the lab since the original pathogen description (Chang et al., 2014). The only report of *S. homoeocarpa* isolates producing conidia or fertile apothecia comes from initial observations of the disease on fescues in the United Kingdom (Bennett, 1937). Recent molecular evidence suggests the fungus belongs in a new genus, with the proposed name being *Clarireedia* spp. (proposed by Beirn, Clarke, and Crouch). Production and examination of the sexual reproductive stage of this pathogen would help clarify the taxonomic placement (Liberti et al., 2012). As a result, the present name of the dollar spot pathogen remains *S. homoeocarpa.*

Dollar Spot: Disease Cycle & Pathosystem

Sclerotinia homoeocarpa has a wide range of hosts, with most classified under the Poaceae family. In North America, all cultivated and highly maintained turfgrass species are thought to be susceptible to *Sclerotinia homoeocarpa* (Walsh et al., 1999). The disease is most significant on creeping bentgrass (*Agrostis palustris* Huds.) and Kentucky bluegrass (*Poa pratensis*) (Fry and Huang, 2004). Dollar spot is found throughout the world, but severe outbreaks are most often observed in North America, Japan, and Australia where high amenity turfgrasses are maintained (Smiley et al., 2007).

Dollar spot symptoms occur readily within temperatures of 15 – 32°C along with prolonged levels of high humidity (Smiley et al., 2007, Vargas, 2005). Cool nights causing heavy dews are favorable environmental conditions for this disease (Allen et al., 2005). Areas with poor drainage and poor air circulation are particularly conducive for disease development; however, dry soils or soils with low fertility may also promote severe infections (Fry and Huang, 2004).

Dollar spot symptoms vary with turf height. Turf maintained at 1.27 cm or less exhibit small bleached spots rarely exceeding 5 cm in diameter. On higher cut turf, bleached spots are commonly 5 to 15 cm in diameter. On turf at low and high mowing heights, spots may coalesce into large irregular dead areas during severe disease epidemics (Fry and Huang, 2004, Smiley et al., 2007). Individual leaf blades develop single or many lesions that begin by turning chlorotic, with a water soaked appearance, that eventually develop into a white bleached lesion with a tan or reddish brown margin (Smiley et al., 2007). These lesions and patches reduce aesthetics and playability, and when left uncontrolled result in plant death and weed encroachment.

Sclerotinia homoeocarpa primarily overwinters as darkly pigmented stroma on margins of dollar spot lesions from previous outbreaks (Rioux et al., 2014, Smiley et al., 2007). Pathogen dissemination was thought to rely primarily on physical displacement from humans, maintenance equipment, and grass clippings (Smiley et al., 2007, Walsh et al., 1999). Recent research, however, detected the dollar spot pathogen in commercial seed, implicating seed as a potential primary inoculum source (Rioux et al., 2014). When conditions are conducive, local distribution can occur by mycelium that grows from one plant to another (Allen et al., 2005).

The infection process begins by direct penetration of *S. homoeocarpa* hyphae through the leaf cuticle, by entering through natural openings (i.e. stomata, hydathodes, or lenticels), or by entering through cut leaf tips. Once infection occurs, the production of pectolytic enzymes, cellulase, hemicellulase, phosphatidase, and oxalic acid aid in pathogenesis of *Sclerotinia* species (Lumsden, 1970, Riou et al., 1991, Zhou and Boland, 1999). The combined effects of these enzymes result in the degradation of host tissue in *Sclerotinia* species (Bateman and Beer, 1965). Host tissue degradation occurs by lowering pH with oxalate ions, which promotes optimal activity of cell-wall degrading enzymes and exposes host tissues to catabolic enzymes (Bateman and Beer, 1965, Riou et al., 1991). These metabolites are believed to work in similar ways in the infection process of *S. homoeocarpa*.

Oxalic Acid

Oxalic acid has many roles in fungal growth and colonization of substrates, such as in pathogenesis, competition, and control of environmental nutrients (Dutton and Evans, 1996). This organic acid aids in fungal pathogenicity by acidifying host tissues,

increasing polygalacturonase activity, and linking with calcium in the cell wall leading to the degradation of pectic substances and necrosis of host tissue (Venu et al., 2009). Oxalic acid production can also aid pathogens by inhibiting growth of other fungi, even to the point of protecting seedlings against other pathogens, such as *Fusarium oxysporum* E. F. Sm. & Swingle (Dutton and Evans, 1996). The secretion of oxalic acid also increases the rate of soil weathering, leading to enhanced availability of nutrients in the soil within the vicinity of hyphae (Cromack et al., 1978). Fungal produced oxalic acid has been shown to be associated with essential pathogenicity factors of *Sclerotinia* diseases, including *S. homoeocarpa*. (Dutton and Evans, 1996, Maxwell and Lumsden, 1970, Williams et al., 2011, Zhou and Boland, 1999).

Sclerotinia sclerotiorum (Lib.) de Bary produces oxalic acid as a key factor in pathogenesis, as demonstrated by the lack of infection by knockout mutants unable to make oxalic acid (Godoy et al., 1990). Also, a positive correlation between disease severity and accumulation of oxalic acid in infected hypocotyls of bean has been reported (Maxwell and Lumsden, 1970). Oxalic acid is also produced by *S. homoeocarpa* and is believed to have a similar importance in fungal development and pathogenicity for *S. homoeocarpa* as it does for other *Sclerotinia* species (Liberti et al., 2012, Rioux, 2014, Venu et al., 2009). Rioux et al. (2014) indicated that oxalic acid production by *S. homoeocarpa* increased in response to host tissue and xylan monomers. Additionally, a positive correlation was found between endogenous oxalate levels of the host and dollar spot symptom severity, suggesting the interaction of host and pathogen oxalate production may influence symptom development.

Cultural Practices for Dollar Spot Control

Cultural practices alone may limit severity, but are usually insufficient in controlling dollar spot in turfgrass stands with low disease thresholds. As leaf wetness duration increases, the severity of most foliar diseases, including dollar spot, increases (Walsh, 2000). Minimizing the duration of leaf wetness by removing early morning dew can result in a decrease in dollar spot occurrence (Ellram et al., 2007). Maintaining appropriate soil fertility and moisture are also important factors in reducing dollar spot severity, since stressed turfgrass is more susceptible to dollar spot infection (Walsh et al., 1999).

Low nitrogen fertility can make the plant vulnerable to infection and slows recovery after infection (Allen et al., 2005, Vargas, 2005). Also, senescent leaf tissue is more likely to develop in nitrogen stressed plants when compared to plants with adequate nitrogen. The senesced plant tissue provides the pathogen with a viable food source and can increase spread to healthy plant tissue (Walsh et al., 1999).

In greenhouse studies, moisture stress in turfgrass was shown to promote dollar spot occurrence (Couch and Bloom, 1960). Moisture stress in turfgrass can be compounded in field conditions because of heavy thatch layers that contribute to lower soil moisture. As a result, cultural practices to control thatch such as aerification or verticutting are helpful in reducing dollar spot severity. Plant vigor, and therefore disease severity of dollar spot, can be limited by cultural practices that limit leaf wetness, keep soil moisture near field capacity, promote proper drainage, and help maintain adequate fertility (Couch, 1962).

Dollar Spot Control with Fungicides

Fungicide use is the main tool for controlling dollar spot in high amenity turfgrass. Repeated fungicide applications are often necessary due to the low or non-existent threshold for disease tolerance and the wide window of environmental conditions conducive for dollar spot occurrence. Products in multiple fungicide classes are available for dollar spot control, including the nitriles, dicarboximides, benzimidazoles, succinate dehydrogenase inhibitors (SDHI), and sterol demethylase inhibitors (DMI). Fungicides are applied preventively to high amenity turfgrasses due to the seasonal nature of dollar spot occurrence and lack of host resistance in commonly used species or cultivars. (Settle et al., 2001). With this application strategy, fungicides may need to be applied on 7 – 21 day intervals during environmental conditions conducive for disease development (Settle et al., 2001, Thompson, 1998). Due to the necessity for frequent applications, a broad set of conducive environmental conditions, and low thresholds of disease tolerance more money is spent on dollar spot control than any other turfgrass disease (Goodman and Burpee, 1991).

Sterol demethylase inhibitor (DMI) fungicides are a class of acropetal penetrant fungicides used widely on turfgrass. The DMI group is varied in chemistry, including triazole, imidazole, pyrimidine, pyridine, and triforine chemical compounds. This class acts on fungal cells by inhibiting a demethylation step in the biosynthesis of fungal sterols, which are important for fungal growth (Hendrix, 1970). Sterol inhibition by this fungicide group is induced by binding to cytochrome P-450, a multi-functioning hemoprotein (Hutson and Miyamoto, 1998). The inhibition of sterols, particularly

ergosterol in the cytoplasm, causes improper regulation of compounds that enter and exit fungal cells, causing leakage and stopping growth (Latin, 2011).

Fungicide Degradation

Many factors may contribute to fungicide degradation in a turfgrass system. Soils composed of higher organic matter have been shown to increase fungicide half-life (Hockemeyer and Latin, 2015), while microbial breakdown, photodecomposition, and volatilization can lead to dissipation of a fungicide in the form of mineralization or transformation (Sigler et al., 1999). Fungicide activity is compounded in turfgrass due to the high organic matter of the thatch layer and the large leaf-surface area of a turfgrass sward.

The leaves and thatch within a turf system are strong sorbents of organic compounds and have a significant impact on the fate of applied fungicides (Lickfeldt and Branham, 1994). Once applied, a fungicide does not remain on the foliage of turfgrass long. Horst et al. (1994) used gas chromatography to show the fungicide metalaxyl could be reduced by up to 82% in 7 to 10 days in Kentucky bluegrass leaf tissue. Another study used high-pressure liquid chromatography to demonstrate a 5 to 6 day half-life of mefenoxam in creeping bentgrass foliage (Gardner and Branham, 2001). The thatch layer (partially decomposed plant material found between the plants and the soil surface) is possibly the largest sink of fungicide residue. In many studies, the amount of pesticide residue found on the soil surface or roots of turfgrass was miniscule compared to that found and retained within the thatch (Hockemeyer and Latin, 2015, Horst et al., 1994, Schumann et al., 1999). Liu et al. (1996) used a bioassay to determine 19.5% to 93.2% of applied benomyl bound to thatch, while a different study found the fungicide metalaxyl to

be seven times more abundant in the thatch of a turf system when compared to the foliage (Horst et al., 1994). Although the thatch layer may represent a main site of fungicide residue accumulation, it has been shown to have low dissipation half-life (DHL) values. Hockemeyer and Latin used chromatography-tandem mass spectrometry to show the DHL of propiconazole in thatch is only 6 to 10 days, while the DHL in roots ranged from 14 to 24 days. However, within the same study they found propiconazole values within the thatch did not approach zero until 21 days after application. Results for azoxystrobin were similar, with DHL values of 4 to 6 days in the thatch compared to 10 to 13 days in the roots (Hockemeyer and Latin, 2015). Both the thatch and roots of a turf system may represent sites of fungicide accumulation, possibly increasing persistence of sub-lethal fungicide levels over a period of time.

Fungicide Resistance

The risk of fungicide resistance development depends on characteristics of both the pathogen population and the fungicide. Fungicide resistance occurs when the fungicide is unable to provide acceptable levels of disease control due to the majority of individuals in the pathogen population becoming insensitive to the active ingredient (Latin, 2011). The site(s) of action and the particular mechanisms of action of fungicides can allow for different levels of risks of resistance or sensitivity (Latin, 2011). Observations of fungicide resistance were rare until site-specific fungicides were introduced. Site-specific fungicides are active against only one point in a metabolic pathway, a single critical protein, or a single critical enzyme within a pathogen (McGrath, 2004). These fungicides have high activity and usually act systemically throughout the plant, giving opportunities for curative or preventive treatments.

Multi-site compounds attack multiple vital processes within fungal cells. A common site targeted by these fungicides is the sulfhydryl functional group that is associated with numerous cellular proteins (Latin, 2011). Although insensitivity occurs in these fungicides, resistance is more unlikely than single site fungicides because the pathogen must simultaneously develop all necessary mutations needed for fungicide resistance (Scholl and Beckerman, 2009).

Along with characteristics of the fungicide, characteristics of the pathogen life cycle can determine the risk for fungicide resistance development. Resistance begins with a genetic mutation of a cell that results in an altered capacity of the fungicide to interrupt the growth and development of the pathogen (Latin, 2011). Individuals of pathogen populations acquire resistance in one step, due to a major gene mutation, or multiple steps where several mutant genes each have a small effect. Qualitative resistance, also known as "major gene" resistance or "monogenic" resistance, is development of resistance associated with a single gene at the target site (Brent and Hollomon, 2007). Individuals expressing this resistance can quickly take over a population due to the fungicide selecting for their complete resistance. Quantitative resistance, also known as "continuous" or "polygenic" resistance, is characterized by single mutations with small effects (Brent and Hollomon, 2007). Individuals of a population expressing quantitative resistance take longer to dominate a population and reduce fungicide efficacy compared to those with qualitative resistance. Resistance risk associated with a fungicide is not only related to mutation type. Disease epidemiology factors such as isolation, frequency, and the reproductive cycle of the pathogen population are also important.

Fungicide Resistance in Turfgrass Pathogens

Depending on geographic location and weather conditions, a golf course putting green may be subject to up to 20 fungicide applications per year due to disease susceptibility and constant fungicide removal from mowing (Sigler et al., 1999). This intensive use can result in rapid development of fungicide resistant pathogen populations from constant selection pressure for fungicide insensitive individuals.

Alternating the use of fungicides with different modes-of-action can deter the development of pathogen resistance to one individual fungicide. Valued for its use on dollar spot, the contact, multisite fungicide cholorothalonil has importance in fungicide resistance management programs (McDonald et al., 2006). DMI fungicides are often used in resistance management of dollar spot as well, due to their broad spectrum of activity with single applications (Koller and Scheinpflug, 1987).

Fungicide resistance has developed in several turfgrass pathogen populations, causing concern among turfgrass managers. *Pythium aphanidermatum* (Edson) Fitzp, the causal agent of Pythium blight, has developed resistance to the site-specific fungicide mefenoxam, likely due to its wide use over many years (Vargas, 2005). Reduced sensitivity to quinone outside inhibiting fungicides (QoIs) has developed in the anthracnose pathogen, *Colletotrichum cereale* Manns, and the grey leaf spot pathogen, *Pyricularia grisea* Sacc. (Avila-Adame et al., 2003, Kim et al., 2003). Lastly, *Sclerotinia homoeocarpa* has developed resistance to several fungicides, largely due to the high disease pressure of dollar spot and prolonged use of particular fungicides.

Fungicide Resistance in *Sclerotinia homoeocarpa*

Fungicide resistance in dollar spot pathogen populations have been confirmed for several fungicide classes including metal-based compounds like cadmium (Warren et al., 1977), the dicarboximides (Detweiler et al., 1983), the benzimidazoles (Warren et al., 1974), and the DMI fungicides (Hsiang et al., 1997, Miller et al., 2002). Through a single gene mutation, *S. homoeocarpa* developed resistance to the benzimidazole fungicide benomyl and resistant populations quickly established (Warren et al., 1974). The gene mutation resulting in benomyl resistance conferred resistance to all other fungicides in the benzimidazole class; a phenomenon known as cross resistance. A single dollar spot isolate or population may also be resistant to more than one fungicide class, which is termed multiple resistance. Vargas et. al. (1992) observed that some isolates of *S. homoeocarpa* found to have the highest resistance to DMI fungicides are also resistant to benzimidazoles.

The manifestation of fungicide resistance in the field for dollar spot varies with resistance type and population dynamics. Resistance to benzimidazole fungicides results in monogenic resistance, characterized by a complete, abrupt loss of fungicide control, while DMI resistance occurs gradually. This gradual resistance, or polygenic resistance, results in shorter application intervals and the need for higher application rates. Jo et al. (2008) examined a single wild-type population of *S. homoeocarpa* and determined resistance was selected to benzimidazole fungicides quicker than to DMI fungicides. The variation in resistance of this population is due to the resulting resistance type exhibited after the respective fungicide applications. A qualitatively resistant pathogen population was able to arise by the abrupt mechanism of resistance to benzimidazole fungicides.

This is compared to the gradual resistance shift of quantitatively resistant pathogen populations caused by the mechanism(s) of resistance to DMI fungicides. Within the same study, population dynamics were examined between two phenotypically different *S. homoeocarpa* populations within the same area. The population structure varied significantly on turfgrass sites managed differently, leading to significant variation in sensitivity to DMI fungicides within these two populations. This reiterates how not only resistance type, but population dynamics of the pathogen are dependent on determining the risk for fungicide sensitivity or resistance (Jo et al., 2008).

History of Hormesis as a Toxicological Concept

Hormesis is defined as a biphasic dose response, identifiable by inhibition following a high-dose exposure of a stressor and a stimulated response following a low-dose exposure of the same stressor (Calabrese and Baldwin, 2000). The features of this biphasic dose-response relationship have been proven to be widespread across biological and toxicological sciences, indicating the incidence of basic biological regulatory processes (Calabrese, 2003, Calabrese and Baldwin, 2002). Plants, viruses, bacteria, fungi, insects, rodents, primates, and other organisms have demonstrated this response (Calabrese and Blain, 2005). External stimuli including chemicals (Belz and Duke, 2014), radiation (Calabrese et al., 2013), antibiotics (Stebbing, 1998), heat, and stress (Rattan, 2008) may provoke hormesis. Hormesis has not received much standing as a toxicological concept until recently. The lack of credibility likely derives from its initial inconsistencies within the scientific community, difficulties in replicating results due to poor study designs, and greater interests in high-dose responses (Calabrese, 2002).

Hugo Schulz initially described a biphasic dose response in the 1880s when he exposed yeast cultures to a range of doses of bactericidal agents such as phenol and mercury. He observed a stimulation of yeast metabolism when the culture was exposed to low doses of these agents and an inhibition of metabolism with exposure to higher concentrations. However, due to his prior beliefs, Schulz was quick to elaborate his findings within yeast dose response as a toxicological explanation for homeopathy (Calabrese, 2002). Although Schulz's beliefs in homeopathy were a barrier to the ideas of this biphasic dose response within the scientific community, his studies were reproduced numerously throughout the turn of the century and, eventually, disassociated with homeopathy. Major research within the biphasic dose response became quite prominent; however, the idea of hormesis became marginalized due to false claims of its impacts with commercialized products, the focus of testing high-dose effects instead of modest low dose responses, and eventual attacks from the scientific community concerning the difficulty of reproducing results (Calabrese, 2002).

In 1943, Southam and Ehlrich released a study in which they described an event of increased fungal growth in the wood decaying pathogen *Fomitopsis officinalis* (Vill.) Bondartsev and Singer, when treated with low doses of a natural antibiotic from western-red cedar (*Thuja plicata)* leaf extract. Cedar extract was a well-known toxin to the fungus at high doses, so upon the discovery of stimulation in low doses, Southam and Ehrich coined the term 'hormesis' from the Greek word meaning 'to excite.' Although hormesis had been defined and observed, it wasn't generally accepted due to lack of complete understanding. Little to no research was published from the time hormesis was defined

until the 1980s (Calabrese et al., 1987, Garzón and Flores, 2013, Luckey, 1983, Stebbing, 1982).

From the late 1980s through early 1990s, publications on radiation hormesis were prominent (Liu et al., 1987, Miller and Miller, 1987, Parsons, 1990). In 1985, the first ever conference on radiation hormesis was held, and the proceedings were published a few years later in the journal *Health Physics*. These acknowledgements caused the resurgence of hormesis as a toxicological concept within the scientific community. In 1990, a meeting was assembled at the University of Massachusetts, Amherst to determine a plan for a balanced leadership for assessing how biological systems respond to low levels of chemical and physical stressors. The Biological Effects of Low Level Exposures (BELLE) organization was created and began to map out the history and significance of hormesis by providing a database with clear and consistent examples of the concept (Calabrese, 2003). The collection of hormetic responses collected by the BELLE organization allowed for insights into quantitatively evaluating hormesis.

Proving Hormesis

In order to prove the concept of hormesis, Calabrese and Baldwin (1997) suggest an experimental design fulfilling the requirements of determining a no observed adverse effect level (NOAEL), testing five evenly spaced doses below the NOAEL for growth stimulation, and separating those doses by values smaller than one order of magnitude. When evaluating results from an adequately designed experiment for hormesis, Crump (2001) suggested it is critical to have strong evidence, sound data, consistency, and biological plausibility. Model-based approaches can be used to fit the hormetic response and evaluate these critical principles. Since these criteria were defined, the Brain-

Cousens model has been the most widely fitted statistical model for hormesis detection and characterization (Garzón and Flores, 2013).

Chemical Hormesis in Plant Pathogens

Detection of hormetic responses in plant pathogens is potentially crucial for disease management but has often been overlooked in past studies determining fungicide resistance (Flores and Garzón, 2013). Although Southam and Ehlrich (1943) described the first labeled hormetic response of fungal growth in *F. officinalis*, little research has been done to determine other hormetic responses in plant pathogens. Garzón et al. (2011) examined hormetic effects from four *Pythium cryptoirregulare* Garzón, Yánez, & G.W. Moorman isolates and two *P. aphanidermatum* isolates when treated with sub-lethal rates of mefenoxam *in vitro*. Seedling assays using one resistant *P. aphanidermatum* isolate revealed disease severity of damping-off of geranium seedlings increased when treated with sub-lethal doses of mefenoxam. Other reports of hormesis in plant pathogens come from *in vitro* studies by Flores and Garzón (2013). Their findings include other accounts of hormetic responses from the same resistant isolate of *P. aphanidermatum,* this time to sub-lethal doses of propamocarb, cyazofamid, and ethanol (Flores and Garzón, 2013, Garzón et al., 2011). Flores and Garzón (2013) also found one isolate of *Rhizoctonia zeae* Voorhees expressed a hormetic response to ethanol, but not the DMI fungicide propiconazole (Flores and Garzón, 2013).

For detection of hormesis in fungal plant pathogens provoked by fungicide exposure, Flores and Garzón (2013) outlined an appropriate model-based approach. The effective concentration at which half the maximum response is observed (EC_{50}) and the no observable effect level (NOAEL) at which there is no significant difference in the

amount of growth compared to the non-amended control, are useful for evaluating disease management. Therefore, a modified version of the Brain-Cousens model by Schabenberger et al. (1999) was used. With these parameters, Flores and Garzón were able to yield appropriate dose-response curves that represented the biphasic dose response observed for *P. aphanidermatum* and *R. zeae*. These initial findings provide evidence that hormesis is present in plant pathogens and can be quantified. The extent of the hormetic response in other turfgrass pathogens, such as dollar spot, is currently unknown, as is the relevance of this biological response in practical field control with fungicides.

Research Objectives

The incidence of exposure of important fungal and oomycete pathogens to sub-lethal doses of fungicides may occur readily in turfgrass systems, due to the multiple applications in a season utilized for disease control and subsequent fungicide degradation towards the end of an application interval. Pathogen isolates expressing hormesis have the potential to exhibit a rebound effect, and cause increased disease severity when fungicides are routinely applied. Additionally, hormetic isolates may predominate over those that don't display hormesis when fungicides are routinely applied, resulting in a population shift towards increased aggressiveness. Evidence for the expression of hormesis in *S. homoeocarpa* isolates has been observed in previous *in vitro* fungicide resistance assays but not quantified, or reported. Additionally, no information exists on a potential link between fungicide resistance and hormesis occurrence in *S. homoeocarpa* isolates. Oxalic acid production by *S. homoeocarpa* may also attribute to pathogen

aggressiveness, and a hormetic increase by sub-lethal fungicide doses may contribute to increased disease severity.

This research was initiated with the following objectives.

1. Evaluate the effects of low doses of sterol demethylase inhibiting (DMI) fungicides on a range of *Sclerotinia homoeocarpa* isolates with an *in vitro* fungicide assay.

Thirty *S. homoeocarpa* isolates will be utilized in this *in vitro* fungicide assay based upon their previously determined DMI sensitivity by Ma and Tredway (2013) and Burpee (1997). This set of isolates includes a range of sensitivities to DMI fungicides that includes unexposed or baseline isolates, moderately resistant isolates, and resistant isolates. The four DMI fungicides to be used in this assay are difenoconazole, propiconazole, triadimefon, and triticonazole. This *in vitro* study will give evidence to whether low-doses of DMI fungicides stimulate growth of these varying isolates and provide a range of concentrations where this stimulation may occur. Although some have been exposed to DMI fungicides, none of these isolates have yet been exposed to the newer DMI fungicide difenoconazole. If hormesis is occurring, cross-hormesis between DMI fungicides will be assessed by comparing isolate responses from difenoconazole treatments to isolate responses from the other three DMI fungicides.

2. Examine the impact of sub-lethal doses of DMI fungicides on the production of oxalic acid in *S. homoeocarpa*.

High performance liquid chromatography (HPLC) will be used to assess the production of oxalic acid by isolates exposed to low concentrations of the DMI fungicides difenoconazole, propiconazole, triadimefon, and triticonazole. If more oxalic acid is

produced at low concentrations, and is in fact a virulence factor, isolates may be more aggressive and cause more severe disease symptoms after fungicide degradation towards the end of field application intervals.

3. Determine the effects of low doses of DMI fungicides on exposed and baseline populations of *Sclerotinia homoeocarpa in vivo.*

A subset of isolates ranging in sensitivity to DMI fungicides will be evaluated in a greenhouse study. Creeping bentgrass (*Agrostis palustris* Huds.) will be established in Cone-Tainers™ and inoculated with select isolates of *S. homoeocarpa.* Inoculated cone-Tainers will be exposed to sub-lethal doses of DMI fungicides, exposed to label rates of DMI fungicides, or untreated. Disease severity will be assessed to determine if sub-lethal fungicide doses result in increased disease severity from this subset of isolates.

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Chapter 2

Characterizing Hormesis in *Sclerotinia homoeocarpa* Incited by Sub-Lethal Doses of Fungicides

Abstract

Thirty *Sclerotinia homoeocarpa* isolates ranging in sensitivities to demethylation inhibiting (DMI) fungicides were evaluated for the ability to exhibit hormesis as a response to sub-lethal exposure to technical grade difenoconazole, propiconazole, triadimefon, and triticonazole via a mycelial plate assay. Mean EC_{50} values of the thirty isolates to DMI fungicides ranged from 0.011 to 0.399 µg ml^{-1}. Isolates were categorized into three sensitivities based on mean EC_{50} values to the four DMI fungicides – sensitive, moderately resistant, or resistant. Mean no observable adverse effect levels (NOAEL) of isolates to the four DMI fungicides ranged from 9.98 x 10^{-9} to 5.98 x 10^{-3} µg ml^{-1} with a mean of 3.59 x 10^{-4} µg ml^{-1}. Isolates categorized as resistant to DMI fungicides displayed higher NOAELs compared to moderately resistant isolates ($P = 0.022$) and sensitive isolates ($P = 0.018$). Ten sub-lethal fungicide concentrations ranging from 2 x 10^{-5} to 2 x 10^{-9} µg ml^{-1} were selected to determine whether these isolates have the ability to exhibit hormesis. Sub-lethal fungicide concentrations did not induce greater mycelial growth of individual isolates or of any sensitivity category in this study ($P > 0.05$). Although our statistical analysis indicated no significant increase, greater relative growth values were commonly exhibited by resistant isolates grown on media amended with sub-lethal fungicide concentrations when compared to mycelial growth on unamended media. The maximum average relative growth value observed within the specific sub-lethal fungicide concentrations for DMI fungicides was by resistant isolate D3 when treated with propiconazole (14% greater than mycelial growth on unamended media). This increase was observed with propiconazole at a rate of 2 x 10^{-5} µg ml^{-1}.

Introduction

Dollar spot of turfgrass, caused by the fungal pathogen *Sclerotinia homoeocarpa*, is the most economically important disease of high amenity turfgrasses (Goodman and Burpee 1991). Dollar spot outbreaks are most often observed in North America, Japan, and Australia between temperatures of 15-32°C (Smiley, Dernoeden and Clarke 2007). All cultivated and highly maintained turfgrass species in North America are susceptible to *S. homoeocarpa* (Walsh, Ikeda and Boland 1999), however, the disease is most significant on creeping bentgrass (*Agrostis palustris* Huds.) and Kentucky bluegrass (*Poa pratensis*) (Fry and Huang 2004). Dollar spot has a large economic impact on bentgrass due to its common use for golf course putting greens. Cultural practices limit severity of the disease but are often insufficient in controlling dollar spot in turfgrass stands with low disease thresholds (Detweiler, Vargas and Danneberger 1983). Fungicides are typically applied on turfgrass on 7-21 day intervals (Settle, Fry and Tisserat 2001, Thompson 1998). Rigorous fungicide use can select for resistant strains within pathogen populations, ultimately decreasing fungicide efficacy (Detweiler et al. 1983). A golf course can have a wide range of insensitivities to a fungicide in dollar spot infected areas due to the nature of the fungal population dynamics, cultural practices, and history of fungicide applications (Jo et al. 2008).

Sterol demethylase inhibiting (DMI) fungicides are valuable for dollar spot control. Fungicides within the DMI class act on fungal cells by inhibiting a demethylation step in the biosynthesis of fungal sterols, which are important for fungal growth (Hendrix 1970). These systemic fungicides can provide control with a broad spectrum of activity for long intervals at low application rates (Koller and Scheinpflug 1987). Moreover, *S.*

homoeocarpa strains resistant to dicarboximide and benzimidazole fungicides can be controlled with the DMI class (Detweiler et al. 1983, Warren et al. 1974). However, insensitivity to DMI fungicides began to emerge in the early 1990s (Vargas et al. 1992). Insensitivity of a *S. homoeocarpa* population to DMI fungicides results in a gradual shift of reduced efficacy known as quantitative, or polygenic, resistance. Benzimidazole and dicarboximide fungicides, on the other hand, result in a qualitative, or monogenic, type of resistance in *S. homoeocarpa*. Polygenic resistance, like that of DMI fungicides, results in initial disease control from higher application rates but a reduction in control duration.

Hormesis describes a biphasic dose response with an increased biological response occurring with low amounts of a stressor compared to the response without the stressor (Calabrese and Baldwin 2000). The response is widespread across biological and toxicological sciences, demonstrating the incidence of basic biological regulatory processes (Calabrese and Baldwin 2002). This dose response has been recorded in many organisms including plants, fungi, viruses, bacteria, and insects (Calabrese and Blain 2005). Examples of external stimuli that may provoke hormesis include, but are not limited to, chemicals (Belz and Duke 2014), antibiotics (Stebbing 1998), and heat (Rattan 2008). Experimental designs to prove hormesis include the determination of a no observed adverse effect level (NOAEL) and testing five evenly spaced doses below the NOAEL for growth stimulation, with those doses separated by values smaller than one order of magnitude (Calabrese and Baldwin 1997). In order to detect and characterize hormesis under these criteria, the Brain-Cousens statistical model is often used to fit data (Garzón and Flores 2013, Brain and Cousens 1988).

In vitro mycelial growth assays have commonly been used to detect fungicide resistance in *S. homoeocarpa* (Vargas et al. 1992, Miller et al. 2002). More recently, *in vitro* mycelial growth assays have been used to determine the ability of fungal and fungal-like plant pathogens to express hormesis. Pathogens assessed include *Pythium aphanidermatum*, *Rhizocotonia solani, R. zeae,* and *Sclerotinia sclerotiorum* (Flores and Garzón 2013, Zhou et al. 2014). The reports of chemical hormesis *in vitro* indicate the need for further evaluation of the effect of sub-lethal fungicide rates on an array of plant pathogens.

The effects of sub-lethal fungicide exposure on *S. homoeocarpa* has not been evaluated in previous fungicide resistance studies. The research reported in this study was initiated to investigate the effects of sub-lethal fungicide exposure on *S. homoeocarpa*. The objective of the research was to evaluate the effects of sub-lethal concentrations of four DMI fungicides on thirty *S. homoeocarpa* isolates with an *in vitro* fungicide assay.

Materials and Methods

Fungal cultures and *in vitro* DMI fungicide assays. Sensitivity to four DMI fungicides was evaluated for thirty *Sclerotinia homoeocarpa* isolates via an *in vitro* mycelial growth assay. Isolates were selected based on their sensitivities to DMI fungicides, as determined previously (Ma and Tredway, 2013, Jo et al. 2008, Putman et al. 2010, Tredway 2005, Miller et al. 2002, Burpee 1997). This collection included sensitive isolates with no previous exposure to DMI fungicides, sensitive isolates with a history of DMI exposure, moderately sensitive isolates with reduced DMI sensitivity compared to unexposed isolates, and resistant isolates that display a low sensitivity to DMI fungicides.

Cultures of *S. homoeocarpa* were prepared by placing sections of infested filter paper on the surface of potato dextrose agar (Difco Potato Dextrose Agar, Becton, Dickinson and Company, Franklin Lakes, NJ) media (PDA) containing chloramphenicol, streptomycin, and tetracycline at 0.05 g/L each. Isolate cultures were placed on a laboratory bench for three days at room temperature (25°C). Sections of agar were transferred from the actively growing colony margins to petri dishes containing water agar (WA) media. Isolate cultures were then stored at room temperature for 3 days prior to the experiment. Agar plugs (8 mm) were taken from the emerging hyphal tip region of the WA cultures and directly plated on fungicide amended PDA. Cultures were placed on a laboratory bench at room temperature and radial growth of mycelia was measured 3 to 4 days after plating. The diameter of a colony was measured in two perpendicular directions with calipers (6" Fractional Digital Caliper, Carrera Precision, La Verne, CA). The two measurements were adjusted by subtracting the 8 mm diameter of the plug before averaging.

No observable adverse effect levels (NOAEL) and EC_{50} values were determined by an initial mycelial growth assay. Fungicide concentrations were prepared with technical grade propiconazole (95% a.i., Syngenta Crop Protection, Greensboro, NC), difenoconazole (95% a.i., Syngenta Crop Protection, Greensboro, NC), triadimefon (99.5% a.i., Ultra-Scientific Analytical Solutions, North Kingstown, Rhode Island), and triticonazole (92.5% a.i, BASF, Research Triangle Park, NC) dissolved in acetone. Fungicide concentrations were added to autoclaved PDA after being cooled to 55°C. Media was amended with the fungicides to a concentration of 0, 0.0002, 0.0006, 0.002, 0.006, 0.02, 0.06, 0.2, 0.6, 2, 6, or 20 $\mu g\ ml^{-1}$. Final solutions of acetone in media were

0.1% (vol/vol) in all treatments, including the non-amended control. Growth of *S. homoeocarpa* is not inhibited by acetone at this concentration (Hsiang, Yang and Barton 1997).

A second mycelial growth assay was utilized to determine if thirty *S. homoeocarpa* isolates have the ability to exhibit hormesis. Ten sub-lethal fungicide concentrations were utilized based on the mean NOAEL of the four DMI fungicides. Fungicide concentrations of propiconazole, difenoconazole, triadimefon, and triticonazole were prepared as described previously. Potato dextrose agar media was amended with the fungicides to a concentration of 0, 2×10^{-9}, 6×10^{-9}, 2×10^{-8}, 6×10^{-8}, 2×10^{-7}, 6×10^{-7}, 2×10^{-6}, 6×10^{-6}, 2×10^{-5}, 6×10^{-5}, and 0.02 $\mu g\ ml^{-1}$.

Experimental design and data analysis. Treatments included twelve fungicide concentrations, four DMI fungicides, and thirty *S. homoeocarpa* isolates. The experiment was completely randomized with each isolate/fungicide combination replicated twice. Relative growth [RG = (the mean adjusted colony diameter on fungicide-amended medium divided by the mean adjusted colony diameter on non-amended medium) X 100%] was determined for each isolate and sub-lethal fungicide concentration combination. Relative growth values for each isolate and fungicide combination were based on the average of two perpendicular measurements.

The EC_{50} value for each isolate and fungicide combination was assessed by linear regression of the probit-transformed relative inhibition value (RI = 1 – RG) on the $\log_{10}$ – transformed fungicide concentration (Hsiang et al. 1997, Miller et al. 2002). The no observable adverse effect level (NOAEL) was determined for each isolate and fungicide combination by utilizing the Brain-Cousens model (Schabenberger et al. 1999). The

model was generated using the non-linear procedure PROC-NLIN (SAS 9.4, SAS Institute, Cary, NC). Mean NOAELs for each isolate, fungicide, and isolate/fungicide combination were based on the mean relative inhibition values of the two experimental runs. The mean NOAELs for each DMI fungicide were combined for use as a representative NOAEL for each isolate.

Least square means for the mycelial growth of each isolate/fungicide combination were subjected to analysis of variance in PROC GLIMMIX (SAS 9.4; SAS corporation, Cary, NC) and means were separated using Fisher's protected least significant difference ($\alpha = 0.05$). Increased mycelial growth at sub-lethal DMI fungicide concentrations was detected by comparison of the mean mycelial growth of individual isolates on non-amended control media to the mean mycelial growth of individual isolates on media amended with sub-inhibitory fungicide concentrations.

Results

Estimating EC_{50} and the no observable adverse effect level. Estimated EC_{50} values for thirty individual *S. homoeocarpa* isolates were determined with a mycelial growth assay (Table 2.1). Mean EC_{50} values of isolates to the four DMI fungicides used in this study ranged from 0.011 to 0.399 µg ml^{-1} with a mean of 0.121 µg ml^{-1}. Isolates previously exposed to DMI fungicides displayed significantly larger EC_{50} values when compared to unexposed isolates ($P = 0.007$). The range of mean EC_{50} values for isolates previously exposed to DMI fungicides was larger (0.013 to 0.398 µg ml^{-1}) than the range for isolates previously unexposed to DMI fungicides (0.011 to 0.032 µg ml^{-1}).

A correlation ($r = 0.552$, $P < 0.0001$) was observed among EC_{50} values for propiconazole estimated in this study when compared to those reported by Ma and

Tredway (2013) (Figure 2.1). Phenotypic levels of resistance and *in vitro* sensitivities to DMI fungicides were previously evaluated for isolates S088 and S084. Estimated EC_{50} values for propiconazole in this study were 0.005 µg ml^{-1} and 0.044 µg ml^{-1} for S084 and S088 respectively, which is lower than the values reported by Burpee (1997) (0.03 and 0.31 µg ml^{-1}), and Miller et al. (2002) (0.0056 and 0.0753 µg ml^{-1}) (Table 2.2).

Correlation analysis of EC_{50} values from the thirty isolates in this study indicated a positive correlation between all four DMI fungicides. The strongest correlation among fungicides was displayed between EC_{50} values for propiconazole and triadimefon (r = 0.701, $P < 0.0001$) (Figure 2.2E) . A moderate correlation was observed between the EC_{50} values of propiconazole and difenoconazole (r = 0.569, $P < 0.0001$) (Figure 2.2A), as well as triadimefon and difenoconazole (r = 0.468, $P < 0.0001$) (Figure 2.2B). The weakest correlations were observed when EC_{50} values of individual fungicides were compared to EC_{50} values of triticonazole (Figure 2.2C; Figure 2.2D; Figure 2.2F).

Isolates were designated into three categories based on mean EC_{50} values to the four DMI fungicides utilized in this study. Categories included sensitive (EC_{50} of 0.011 to 0.033 µg ml^{-1}), moderately resistant (EC_{50} of 0.049 to 0.122 µg ml^{-1}), or resistant (EC_{50} of 0.125 to 0.399 µg ml^{-1}). Among the thirty isolates, 11 were designated as sensitive, 9 were designated as moderately resistant, and 10 were designated as resistant to DMI fungicides (Figure 2.3). A significant effect was observed when isolate EC_{50} values were subject to ANOVA after being designated into sensitivity categories ($P < 0.0001$). Resistant isolates displayed the largest EC_{50} values compared to all other sensitivity categories ($P < 0.0001$), while moderately resistant isolates displayed larger EC_{50} values than sensitive isolates (P = 0.039) (Figure 2.4).

The mean no observable adverse effect level (NOAEL) to DMI fungicides was estimated for each isolate (Table 2.1). Mean NOAELs of isolates to the four DMI fungicides utilized in this study ranged from 9.98×10^{-9} to 5.98×10^{-3} µg ml^{-1} with a mean of 3.59×10^{-4} µg ml^{-1}. When mean NOAELs to DMI fungicides were evaluated by isolate sensitivity, resistant isolates displayed higher NOAELs compared to moderately resistant isolates ($P = 0.022$) and sensitive isolates ($P = 0.018$) (Figure 2.5). No effect was observed among mean NOAELs when compared by fungicide type.

***In vitro* assay evaluation of chemical hormesis to DMI fungicides.** Mean mycelial growth of *S. homoeocarpa* isolates in the second experimental run was greater than the first experimental run ($P < 0.0001$). Isolate sensitivity had an effect on mycelial growth at sub-lethal fungicide concentrations in both experimental runs ($P < 0.0001$). In the first experimental run, sensitive isolates produced more mycelial growth than moderately resistant isolates ($P < 0.0001$) and resistant isolates ($P < 0.0001$). No difference was observed between the amount of mycelium produced by moderately resistant and resistant isolates ($P = 0.637$) (Figure 2.6A). In the second experimental run, moderately resistant isolates produced less mycelial growth than sensitive isolates ($P < 0.0001$) and resistant isolates ($P < 0.0001$). No difference was observed between the mycelial growth of sensitive isolates and resistant isolates ($P = 0.586$) (Figure 2.6B). Fungicide type had an effect on mycelial growth at sub-lethal fungicide concentrations in both experimental runs ($P < 0.0001$). Isolates grown on media amended with sub-lethal concentrations of difenoconazole displayed the least mycelial growth in each experimental run (Figure 2.7A; Figure 2.7B)

Sub-lethal fungicide concentrations did not cause increased mycelial growth of individual isolates when compared to unamended media in either experimental run ($P > 0.05$). Additionally, sub-lethal fungicide concentrations did not cause increased mycelial growth of any sensitivity category when compared to unamended media in either experimental run ($P > 0.05$). The combination of sub-lethal fungicide concentrations and fungicide type did not cause increased mycelial growth of individual isolates when compared to unamended media in either experimental run ($P > 0.05$). Additionally, the combination of sub-lethal fungicide concentrations and fungicide type did not cause increased mycelial growth of any sensitivity category when compared to unamended media in either experimental run ($P > 0.05$).

Although no statistical significance was observed, exposure to sub-lethal fungicide rates caused resistant isolates A4 and D3 to frequently display growth values greater than the control throughout experimental runs (Figure 2.9A; Figure 2.9B). The maximum average relative growth value of isolate A4 between two experimental runs was greatest with propiconazole (17.5% greater than mycelial growth on unamended media). This increase in mycelial growth was observed with propiconazole at a concentration of 2×10^{-3}. This concentration was utilized in the initial fungicide assay, but was not included as a sub-lethal fungicide concentration in the second fungicide assay. The maximum average relative growth value of isolate D3 between two experimental runs was also greatest with propiconazole (14% greater than mycelial growth on unamended media). This maximum increase in mycelial growth by isolate D3 was observed with propiconazole at a concentration of 2×10^{-5}. This concentration was utilized in the second fungicide assay as a sub-lethal fungicide concentration.

Discussion

Thirty *S. homoeocarpa* isolates were selected based on previous reports indicating the various sensitivities to DMI fungicides (Ma and Tredway 2013). The range of sensitivities for these isolates to four DMI fungicides was confirmed and isolates were designated into three sensitivity categories based on the mean EC_{50} values of all four fungicides. Specific fungicide history was unavailable for a majority of the isolates used in this study, however, the active ingredient difenoconazole was not approved for use on turfgrass within the collection dates of these thirty isolates. Positive correlations were observed among the mean EC_{50} values to each DMI fungicide, including difenoconazole, implying resistance to all four fungicides may be linked. The observation of DMI cross resistance in this study corresponds with previous reports of *S. homoeocarpa* exhibiting cross resistance within the DMI fungicide class (Miller et al. 2002).

The no observable adverse effect level (NOAEL) was determined for each isolate/fungicide combination to estimate fungicide concentrations that cause stimulatory effects. Resistant isolates displayed the greatest mean NOAELs within the initial fungicide assay, indicating sub-lethal fungicide concentrations that cause increased mycelial growth in resistant isolates may be greater than sub-lethal fungicide concentrations that cause increased mycelial growth in moderately resistant or sensitive isolates. There was no difference in mean NOAELs for moderately resistant and sensitive isolates.

Hormesis is defined as a highly generalizable and reproducible biological phenomenon, with maximum stimulatory values often approximated at 30 to 60% when compared to the control (Calabrese 2015, Calabrese, et al. 1999). Quantification and

assessment of chemical hormesis in plant pathogens requires an array of standardizations for adequate results (Garzón and Flores 2013) followed by stringent key factors that are needed for evaluation (Crump 2001). Coinciding with the protocol presented by Flores and Garzón (2013), the factors of determining the NOAEL and dose separation smaller than one order of magnitude are fulfilled by the current study. However, less replications and experimental runs were utilized in the current study than studies by Flores and Garzón.

Typically, the presence of hormesis is detected via a Brain-Cousens model, which is generated using non-linear procedures in statistical software. Significant hormetic responses are evaluated by the 95% confidence interval of the parameter γ, as γ represents the rate of increase at small doses within the Brain-Cousens model. If hormesis is significant, only positive values will be reported in the 95% confidence interval for γ. Although statistical curve modeling was executed for the determination of NOAELs for each isolate, difficulties in hormesis curve modeling occurred within this study and modeling was not achieved. Stimulatory responses can also be determined by comparing mycelial growth on sub-lethal fungicide amended media to mycelial growth on non-amended media via ANOVA (Di et al., 2016). ANOVA has limitations in detecting stimulation, such as the inability to distinguish all results that fit hormetic criteria. However, due to the difficulties of utilizing curve modeling to prove hormesis, statistical analysis of stimulation was executed with ANOVA.

No significant increase at sub-lethal fungicide concentrations was recorded by any of the thirty isolates with the ANOVA method. Although insignificant, isolates grown on sub-lethal fungicide amended media frequently displayed greater relative mycelial

growth values when compared to mycelial growth on unamended media. Resistant isolates D3 and A4 displayed the most frequent relative growth values above 1 when exposed to sub-lethal concentrations of DMI fungicides. Overall, isolate D3 displayed the largest relative mycelial growth value at a sub-lethal fungicide concentration, indicating a 14% increase in growth when compared to the non-amended control. Isolates A4 and D3 displayed the largest EC_{50} values to DMI fungicides when compared to the other 28 isolates in this study. Additionally, Ma and Tredway (2013) reported isolates A4 and D3 had a larger resistance factor to DMI fungicides than 22 of the other isolates used in this study. Garzon et al. (2011) reported a stimulatory effect of mean radial mycelial growth by a mefenoxam resistant isolate of *Pythium aphanidermatum* up to 10% when exposed to low doses of mefenoxam, while Flores and Garzon (2013) recorded stimulation up to 6% when the same isolate of *P. aphanidermatum* was exposed to low doses of other phenylamides. Although no significant stimulation was observed in the current study, our report coincides with previous findings that fungicide resistant isolates of plant pathogens may display greater mycelial growth while exposed to low doses of a resisted fungicide class when compared to mycelial growth with no fungicide exposure. The percent increases observed in this study are similar to increases reported in previous studies of hormesis in plant pathogens, yet no significance was detected among increases in mycelial growth. The analytical method used to prove hormesis in this study may not be suitable to clearly indicate hormetic responses. Future studies attempting to prove hormesis should consider multiple approaches, including multiple statistical models.

These *in vitro* results provide insight into the effects DMI fungicides have at low doses on *S. homoeocarpa* isolates. Although significant stimulation was not observed,

this study describes the ability of isolates to grow adequately in the presence of sub-lethal DMI fungicides. More importantly, this study provides evidence that isolates of *S. homoeocarpa* expressing high resistance to DMI fungicides may have the capability to display more mycelial growth when treated with sub-lethal rates of DMI fungicides compared to untreated isolates. Due to this ability, sub-lethal DMI fungicide exposure to *S. homoeocarpa*, particularly in populations exhibiting resistance to DMI fungicides, could lead to more severe dollar spot occurrences in field conditions. Future attempts to prove hormesis in *S. homoeocarpa* should focus on the *in vitro* effects of sub-lethal fungicide concentrations on *S. homoeocarpa* isolates expressing fungicide resistance.

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Table 2.1. History, EC_{50} value, and sensitivity category of 30 *Sclerotinia homoeocarpa* isolates

Isolate	Origin	Year Collected	[a] History of DMI Exposure	[b] EC_{50} Value	[c] NOAEL ($\mu g\ ml^{-1}$)	[d] Resistance to DMI Fungicides
LWC10	Raleigh, NC	2003	Baseline	0.011	1.00E-08	Sensitive
VGC5	Edgartown, MA	2007	Baseline	0.013	6.35E-05	Sensitive
RE18G8	Shallotte, NC	2003	Exposed	0.013	8.07E-04	Sensitive
LWC5	Raleigh, NC	2003	Baseline	0.017	2.76E-06	Sensitive
RE18G26	Shallotte, NC	2003	Exposed	0.018	9.28E-07	Sensitive
PST4	Rolesville, NC	2003	Baseline	0.018	1.83E-03	Sensitive
RE18G4	Shallotte, NC	2003	Exposed	0.020	5.98E-03	Sensitive
S084	State College, PA	1980	Baseline	0.021	2.76E-05	Sensitive
CHCC10	North Attleboro, MA	2007	Exposed	0.022	7.63E-05	Sensitive
RE18G45	Shallotte, NC	2003	Exposed	0.031	8.95E-05	Sensitive
LWC27	Raleigh, NC	2003	Baseline	0.033	9.00E-09	Sensitive
RE18G38	Shallotte, NC	2003	Exposed	0.049	9.45E-04	Moderately Resistant
451ShCT76	Madison, CT	2007	Exposed	0.063	1.06E-04	Moderately Resistant
725ShME	South Portland, ME	2007	Exposed	0.095	1.11E-04	Moderately Resistant
PhPG4	Pinehurst, NC	2003	Exposed	0.097	2.93E-05	Moderately Resistant
RE18G35	Shallotte, NC	2003	Exposed	0.098	1.50E-04	Moderately Resistant
RE18G16	Shallotte, NC	2003	Exposed	0.102	1.45E-04	Moderately Resistant
RCC18G15	Raleigh, NC	2003	Exposed	0.113	1.90E-05	Moderately Resistant
PhPG9	Pinehurst, NC	2003	Exposed	0.118	3.18E-05	Moderately Resistant
557ShCT173	Woodbridge, CT	2007	Exposed	0.122	2.40E-05	Moderately Resistant
SO88	Chicago, IL	1993	Exposed	0.125	1.15E-05	Resistant
G62	Waynesfield, OH	2004	Exposed	0.148	2.85E-05	Resistant
500ShCT123	Coventry, CT	2007	Exposed	0.190	1.00E-08	Resistant
H127	Springfield, OH	2005	Exposed	0.197	1.60E-05	Resistant
363ShCT18	East Falmouth, CT	2006	Exposed	0.207	5.27E-05	Resistant
G5	Canal Winchester, OH	2003	Exposed	0.272	1.08E-05	Resistant
PhPG22	Pinehurst, NC	2003	Exposed	0.323	4.18E-05	Resistant
A22	Powell, OH	2001	Exposed	0.338	1.18E-05	Resistant
A4	Gainesville, VA	2001	Exposed	0.367	9.35E-05	Resistant
D3	Grove City, OH	2002	Exposed	0.399	7.88E-05	Resistant

[a] History of DMI exposure is based upon field conditions at the time of collection for the individual isolate.
[b] EC_{50} value is based upon the average EC_{50} value of four DMI fungicides determined via a mycelial growth assay.
[c] No observable adverse effect level based upon the average value of four DMI fungicides
[d] Designated resistance to DMI fungicides based on EC_{50} values to four DMI fungicides

Table 2.2. Characteristics of *Sclerotinia homoeocarpa* isolates used as reference based on previous reports of sensitivities to propiconazole. Values are based on mycelial growth on media amended with propiconazole. EC_{50} values are reported in µg ml^{-1}.

Isolate	Location	Year Collected	EC_{50} Value (Burpee)	EC_{50} Value (Miller et al.)	EC_{50} Value (Current Study)	Phenotype to DMIs
S084	State College, PA	1980	0.03	0.0056	0.0053	Sensitive
S088	Chicago, IL	1993	0.31	0.0753	0.0443	Resistant

Table 2.3. Effects of isolate sensitivity to DMI fungicides, sub-lethal fungicide concentration, and DMI fungicide type on radial mycelial growth on agar media throughout the first and second experimental run. ANOVA table was produced using PROC GLIMMIX in SAS.

Disease Severity		**Run 1**	**Run 2**
Effect	**DF**	**Pr > F**	**Pr > F**
Run	1	<.0001	
Sensitivity	2	<.0001	<.0001
Sub-lethal Concentration	10	<.0001	<.0001
Fungicide	3	<.0001	<.0001
Fungicide*Sensitivity	6	<.0001	<.0001
Sub-lethal Concentration*Sensitivity	20	<.0001	<.0001
Fungicide*Concentration	30	<.0001	<.0001

Figure 2.1. Correlation of EC_{50} values for 29 *Sclerotinia homoeocarpa* isolates between the current study and those reported by Ma and Tredway (2013). Values are based on isolate mycelial growth on media amended with propiconazole. ◈ represents the reference isolate S088, which was utilized in both studies.

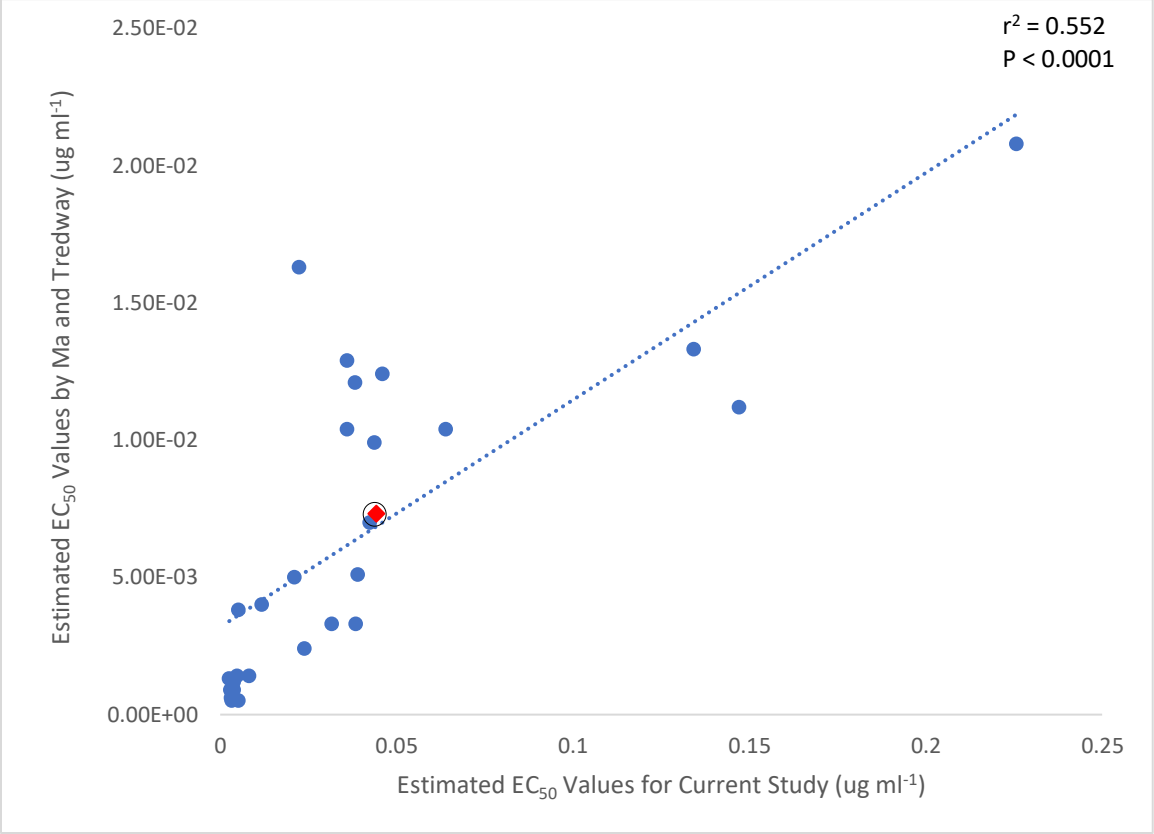
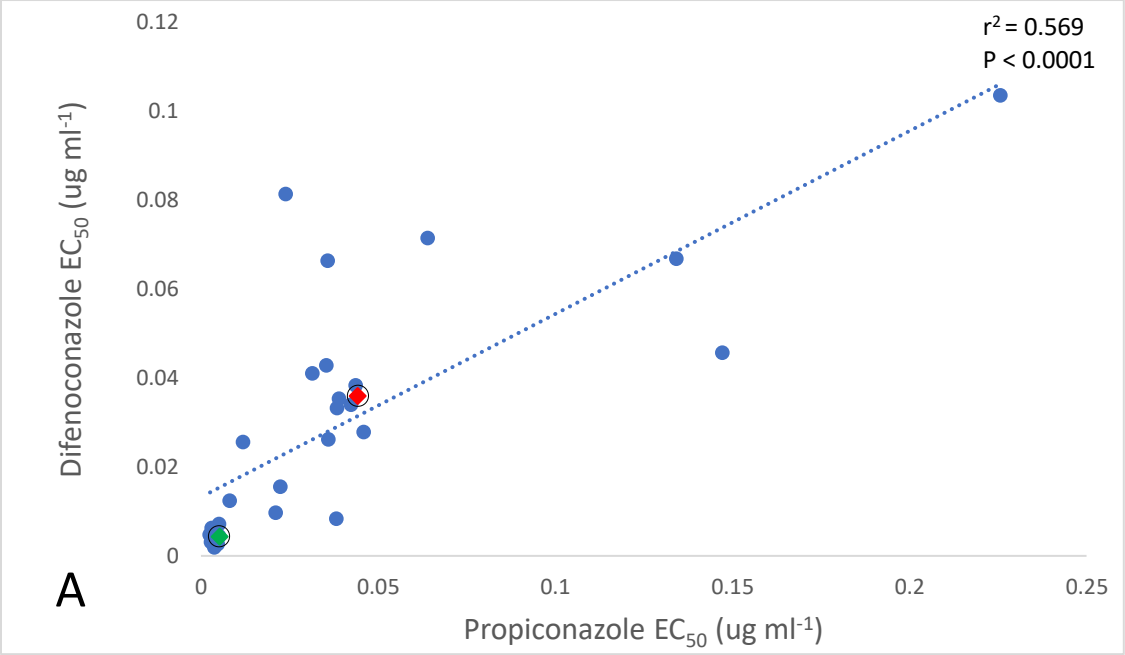
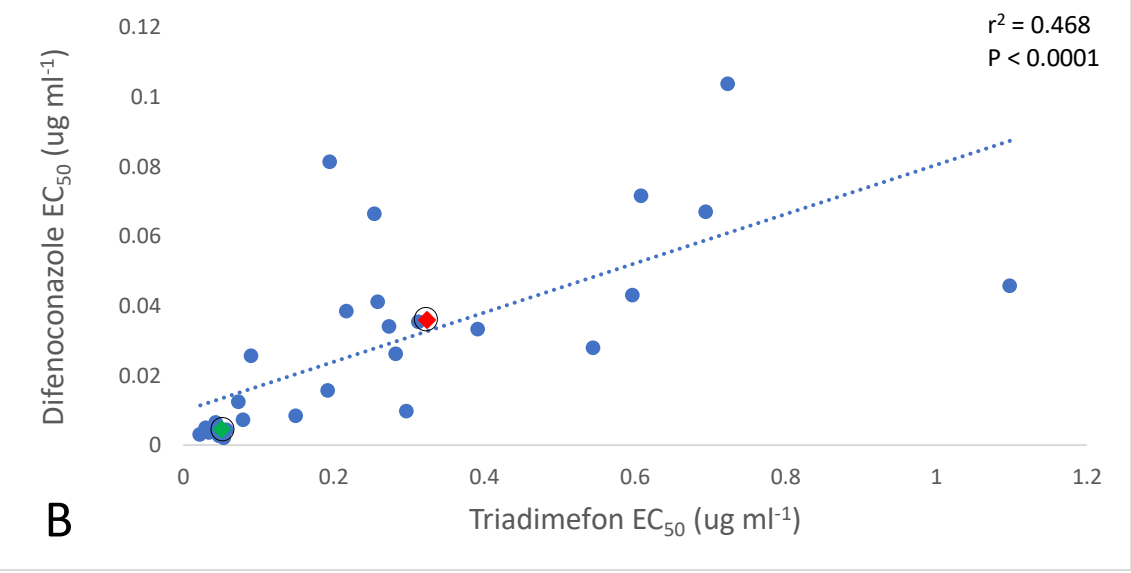
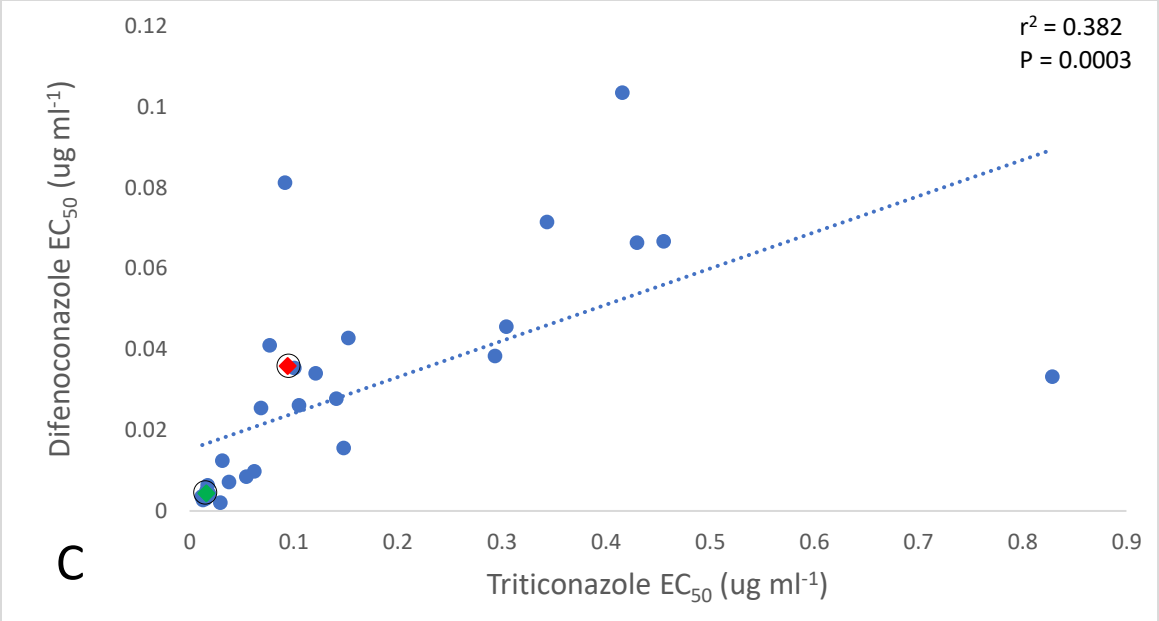


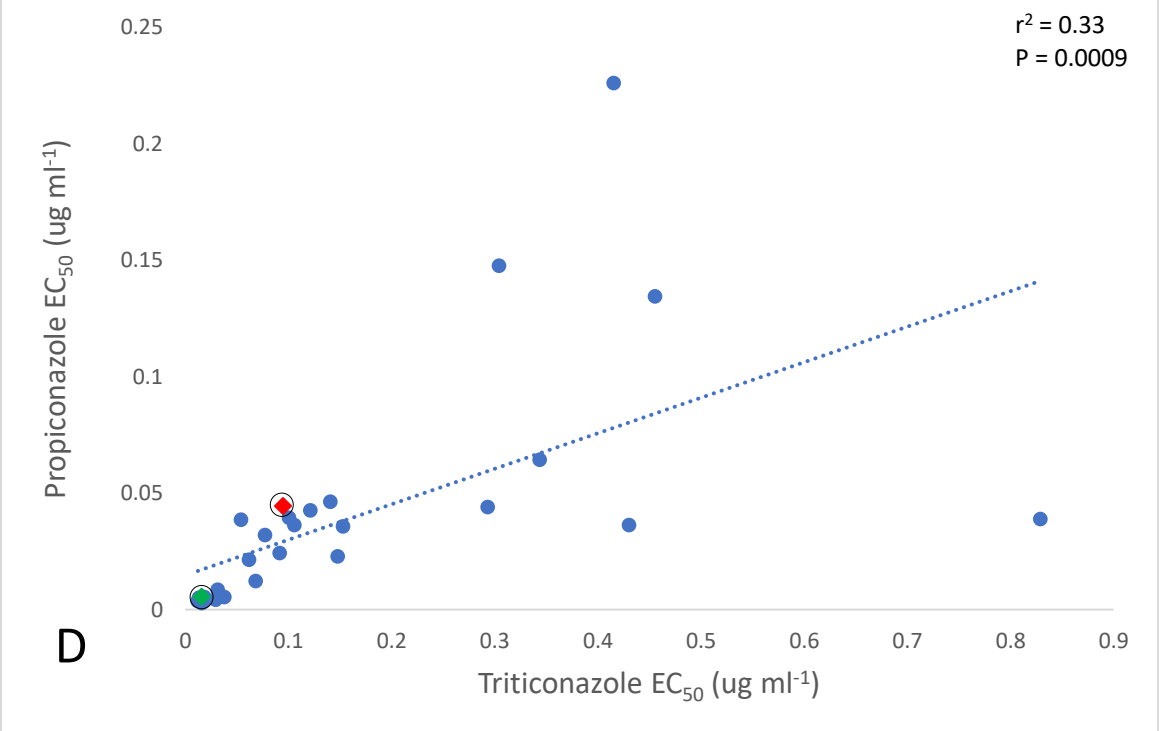
Figure 2.2. Correlation between sensitivities to DMI fungicides in thirty *Sclerotinia homoeocarpa* isolates. Estimated EC_{50} values were determined by mycelial growth on media amended with DMI fungicides. Red and green ◈ markers represent reference isolates S088 and S084 respectively.

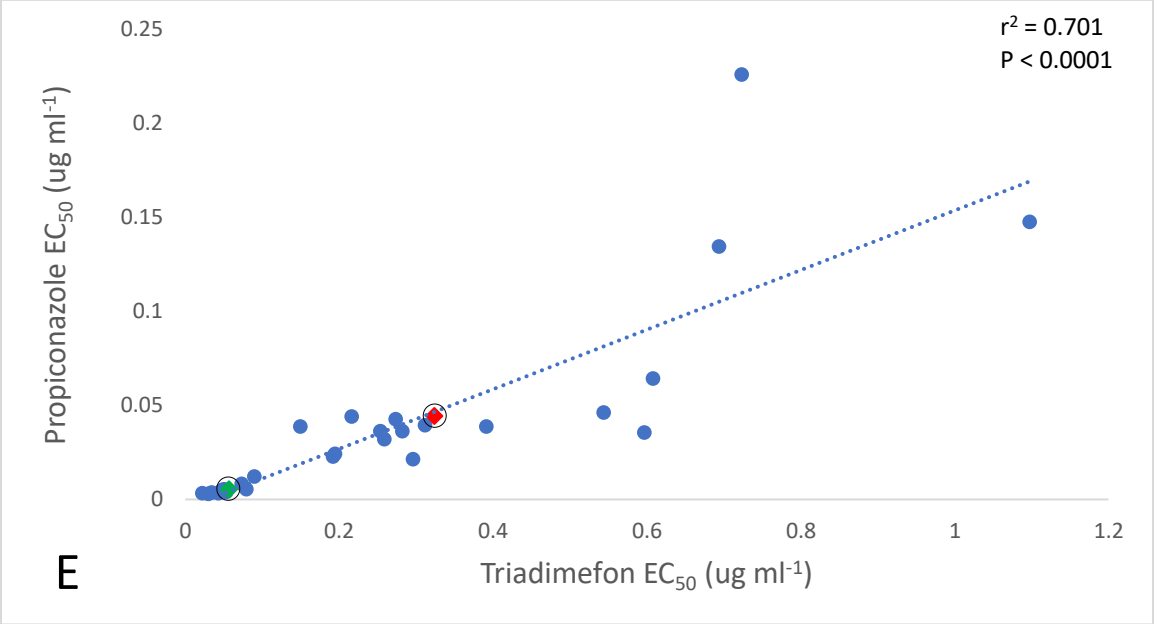
A - difenoconazole vs. propiconazole; B - difenoconazole vs. triadimefon; C - difenoconazole vs. triticonazole; D - propiconazole vs. triticonazole; E - propiconazole vs triadimefon; F - triticonazole vs. triadimefon






r² = 0.382
P = 0.0003
Difenoconazole EC50 (ug ml-1)
Triticonazole EC50 (ug ml-1)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
C


r² = 0.33
P = 0.0009
Propiconazole EC50 (ug ml-1)
Triticonazole EC50 (ug ml-1)
0.25
0.2
0.15
0.1
0.05
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
D


r² = 0.701
P < 0.0001
Propiconazole EC50 (ug ml-1)
Triadimefon EC50 (ug ml-1)
0.25
0.2
0.15
0.1
0.05
0
0
0.2
0.4
0.6
0.8
1
1.2
E

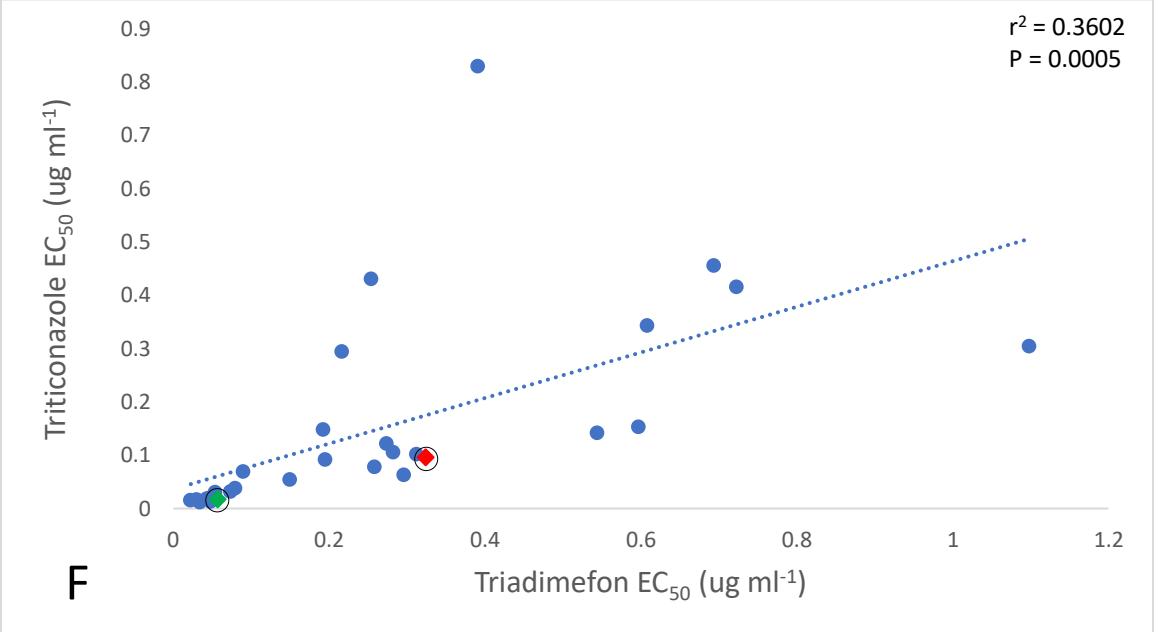

r² = 0.3602
P = 0.0005
Triticonazole EC50 (ug ml-1)
Triadimefon EC50 (ug ml-1)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1.2
F

Figure 2.3. Categorized sensitivity of *Sclerotinia homoeocarpa* isolates based upon mean EC_{50} values of four DMI fungicides. Values were determined by mycelial growth on media amended with propiconazole, difenoconazole, triticonazole, or triadimefon. EC_{50} values were determined by two replications and two experimental runs. ^ indicate baseline isolates that have not been previously exposed to DMI fungicides.

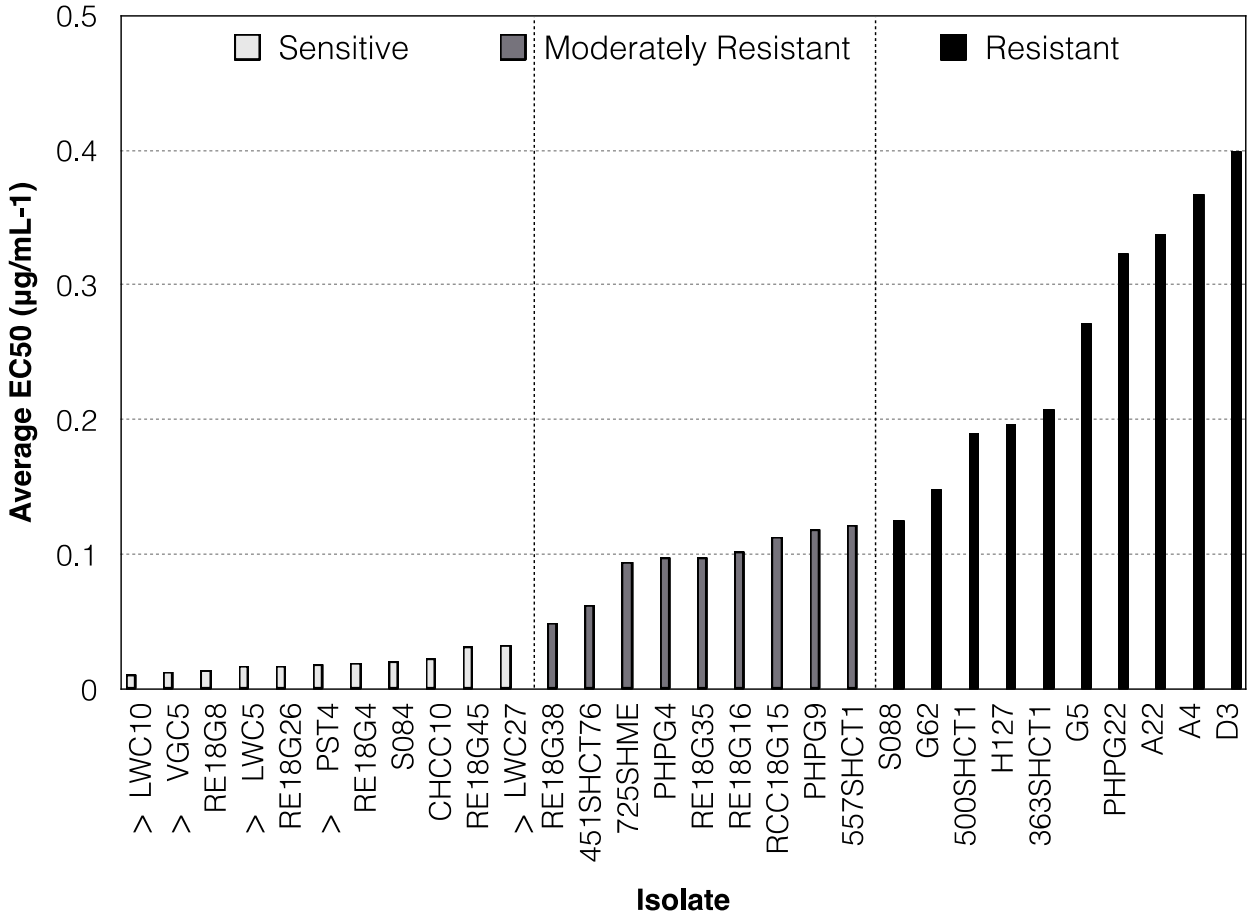


Figure 2.4. Mean EC_{50} values of an initial fungicide plate assay consisting of fungicide concentrations ranging from 0.0002 μg ml^{-1} to 20 μg ml^{-1}. Mean EC_{50} values are based on the EC_{50} of each isolate to each of the four DMI fungicides in two experimental runs. Sensitivity categories are based on EC_{50} values to DMI fungicides determined in this study. Error bars represent the standard error of the mean (0.0242). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).

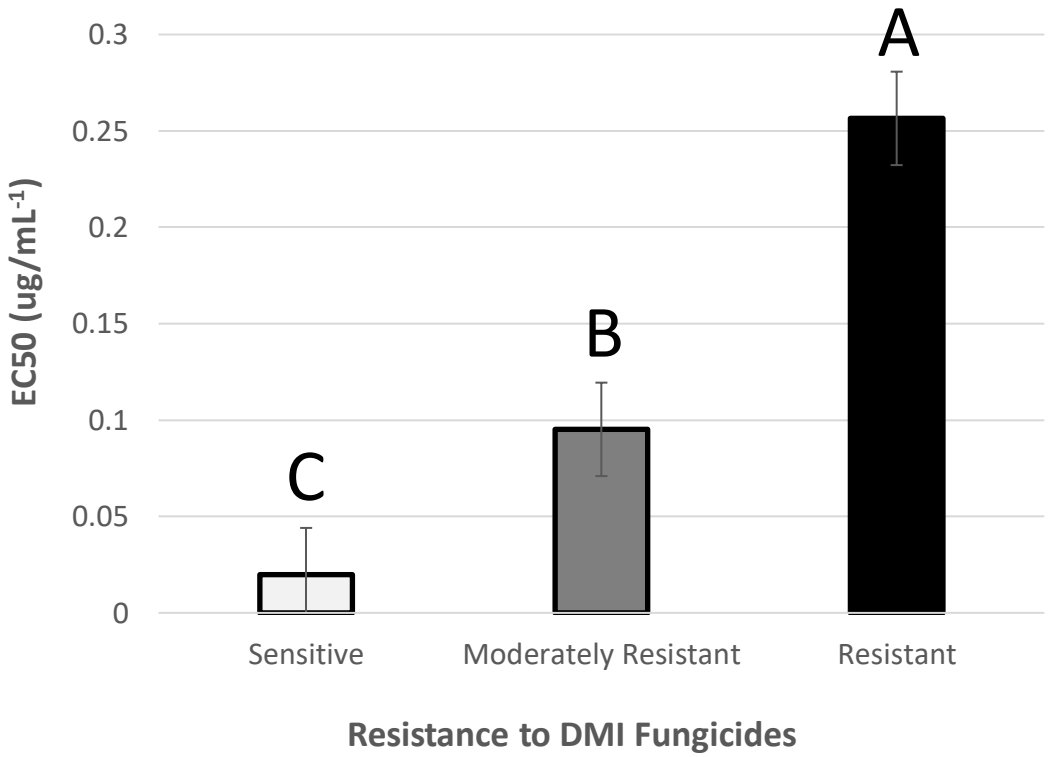


Figure 2.5. Mean no observable adverse effect levels (NOAEL) of an initial fungicide plate assay consisting of fungicide concentrations ranging from 0.0002 μg ml^{-1} to 20 μg ml^{-1}. Mean NOAELs are based on the NOAEL of each isolate to each of the four DMI fungicides in two experimental runs. Sensitivity categories are based on EC_{50} values to DMI fungicides determined in this study. Error bars represent the standard error of the mean (0.000274). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).

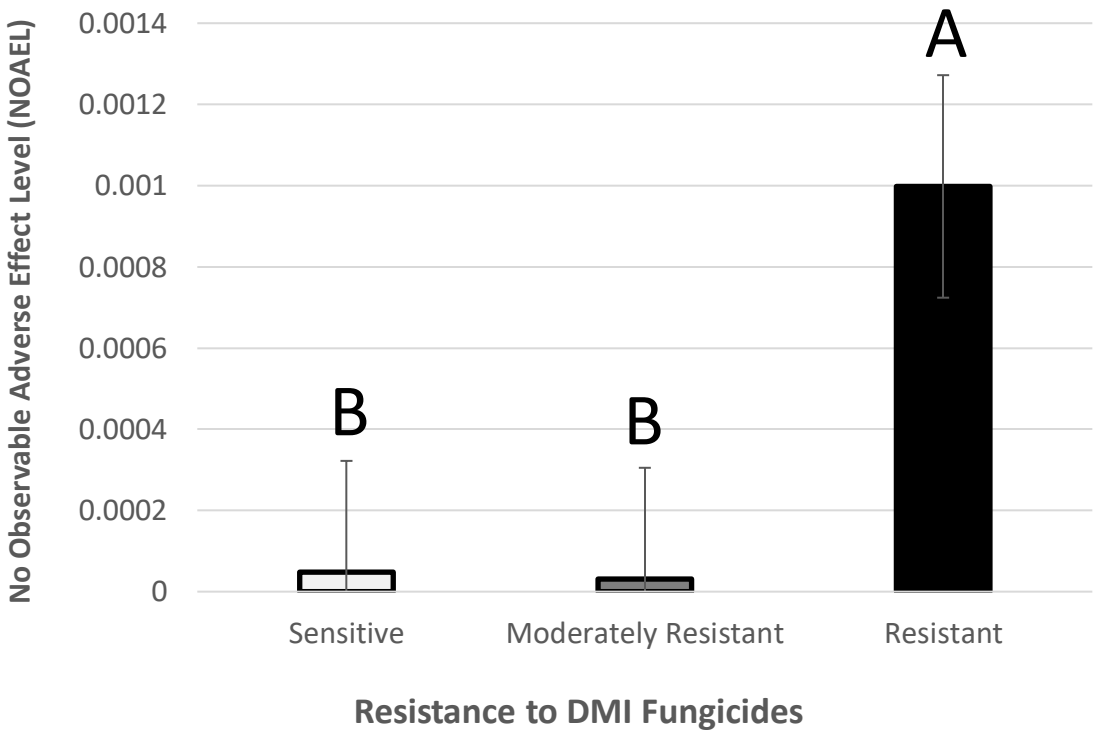
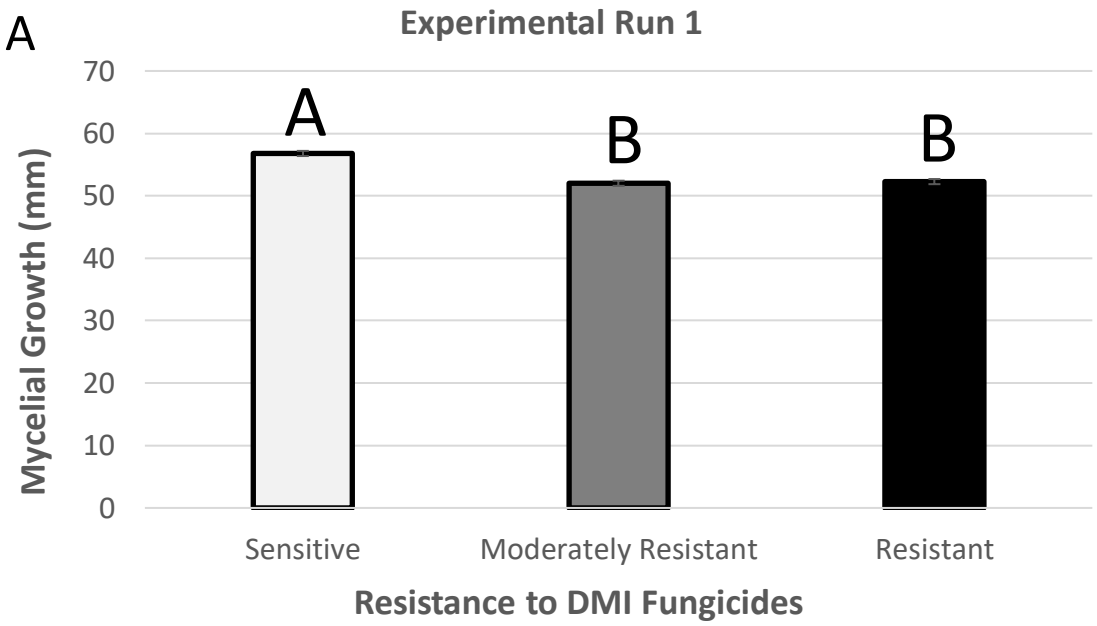


Figure 2.6. Mean radial mycelial growth on agar plates amended with sub-lethal rates of DMI fungicides in experimental run 1 (A) and experimental run 2 (B) by sensitivity. Mean mycelial growth is based on the radial growth of isolates on media amended with one of four fungicides in two replications. Sensitivity categories are based on EC_{50} values to DMI fungicides determined in this study. Error bars represent the standard error of the mean (Run 1 – 0.411; Run 2 – 0.412). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).



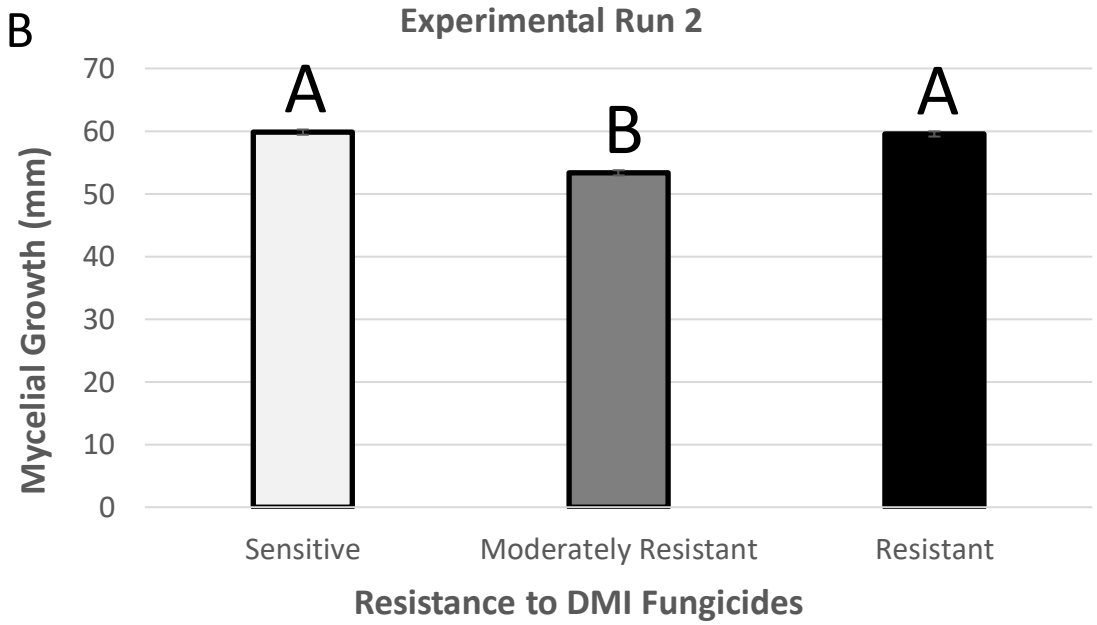
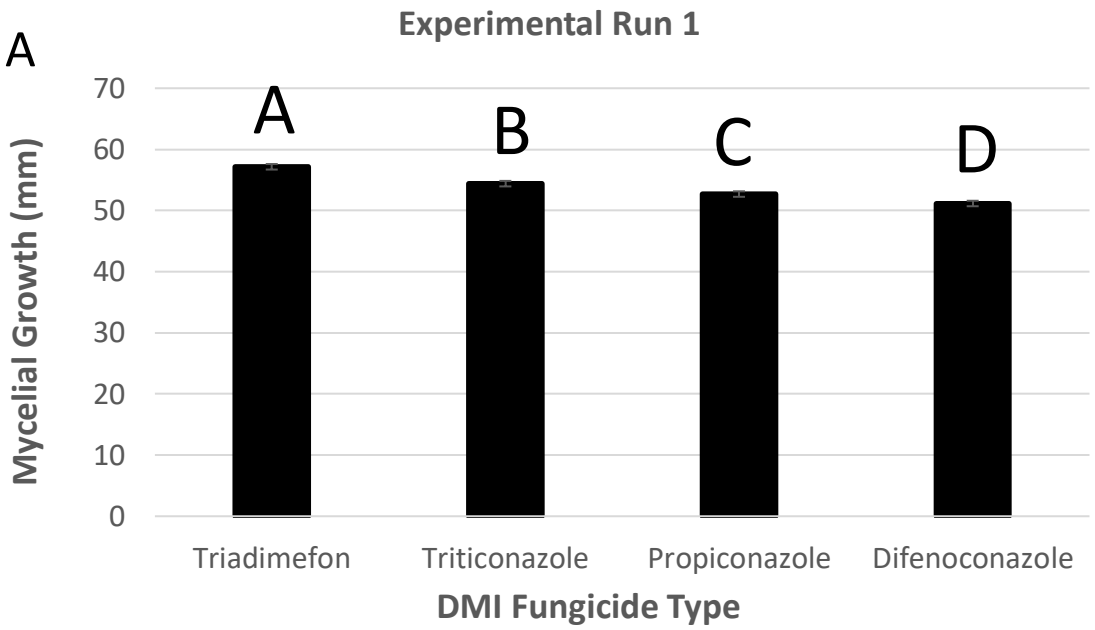


Figure 2.7. Mean radial mycelial growth on agar plates amended with sub-lethal rates of DMI fungicides in experimental run 1 (A) and experimental run 2 (B) by fungicide type. Mean mycelial growth is based on the radial growth of isolates in two replications. Error bars represent the standard error of the mean (Run 1 – 0.450; Run 2 – 0.459). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).



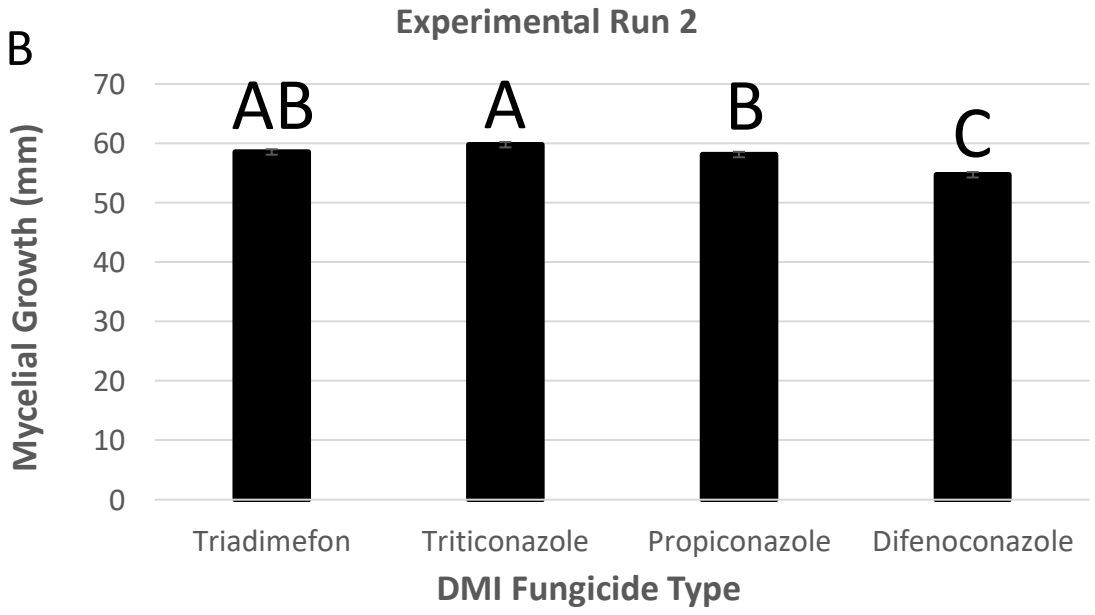
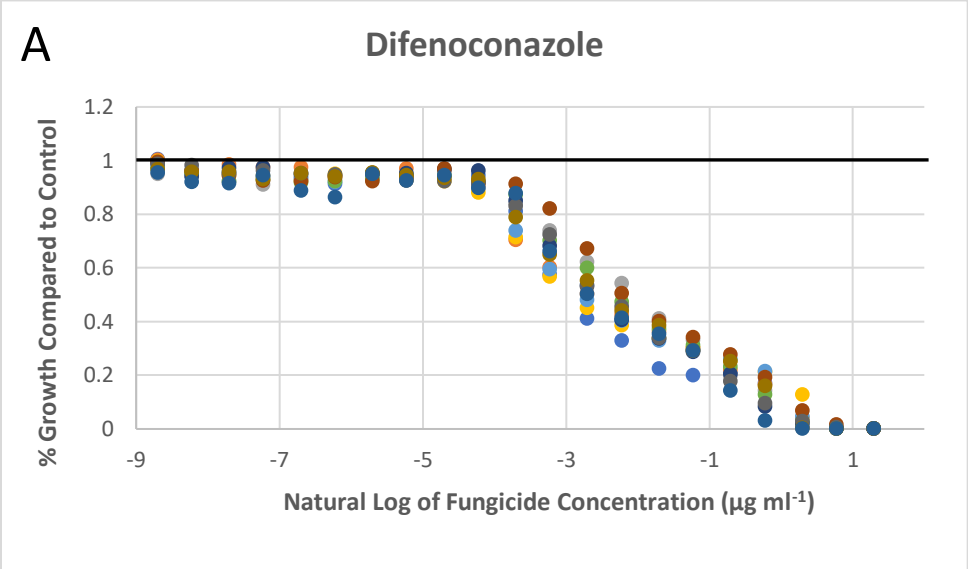
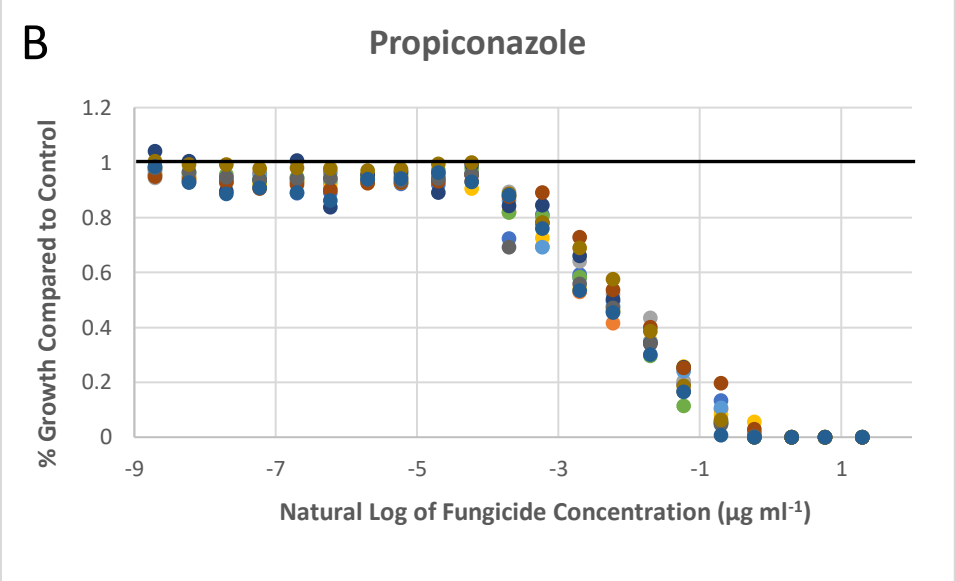
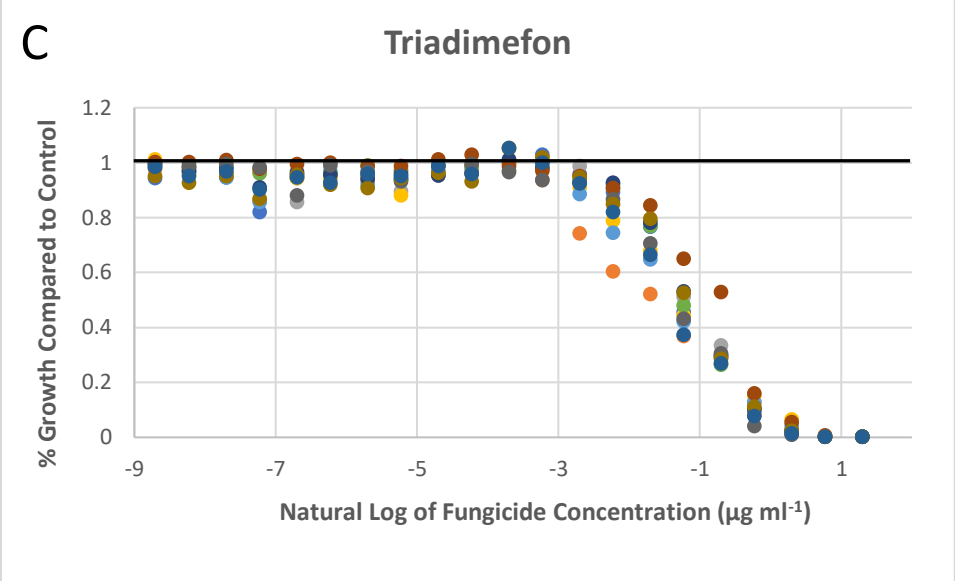


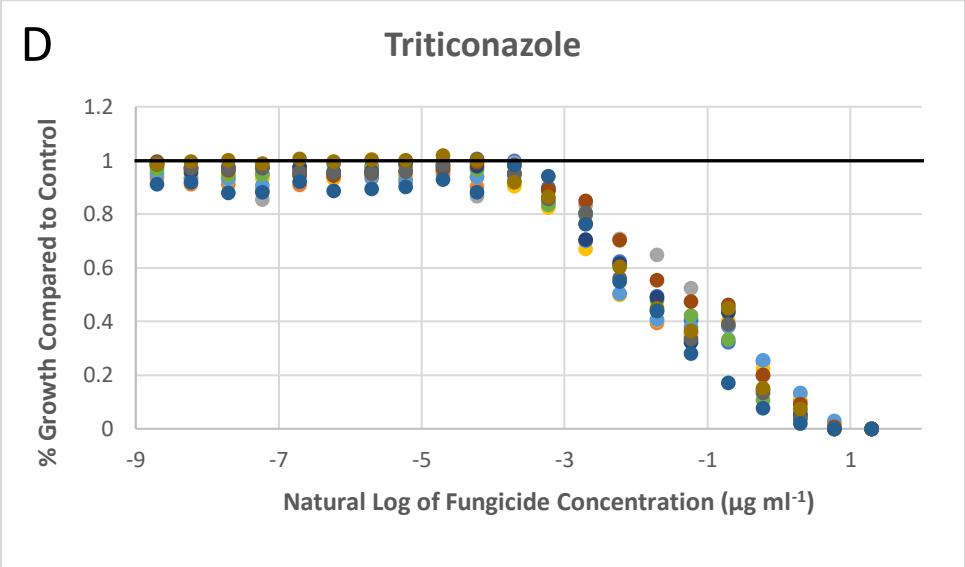
Figure 2.8. Mean relative mycelial growth of *Sclerotinia homoeocarpa* isolates ranging in sensitivities to DMI fungicides. Values are based on relative growth of mycelium on media amended with a DMI fungicide compared to growth of mycelium on non-amended media. Means are averaged across 2 replications and 2 experimental runs.

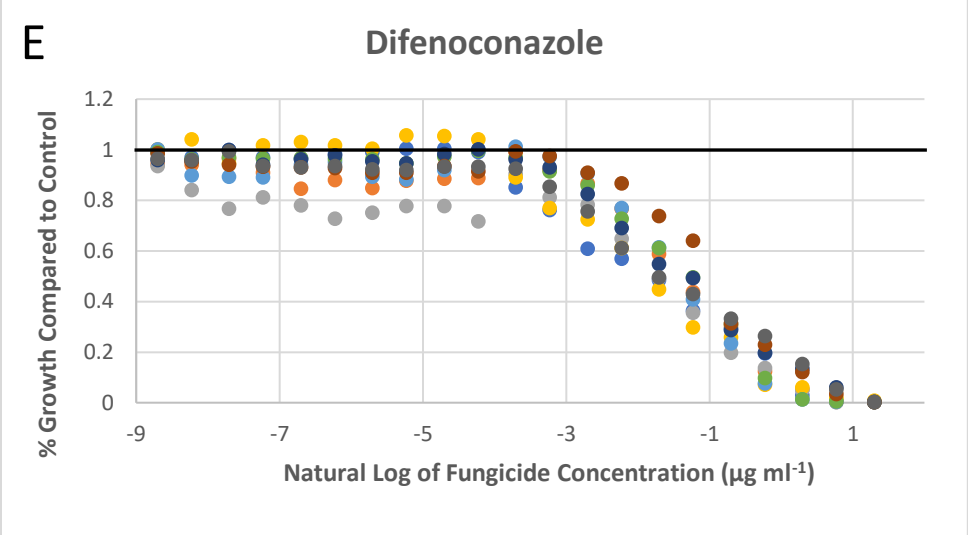
Graphs A-D: 11 isolates sensitive to DMI fungicides
Graphs E-H: 9 isolates moderately resistant to DMI fungicides
Graphs I-L: 10 isolates resistant to DMI fungicides

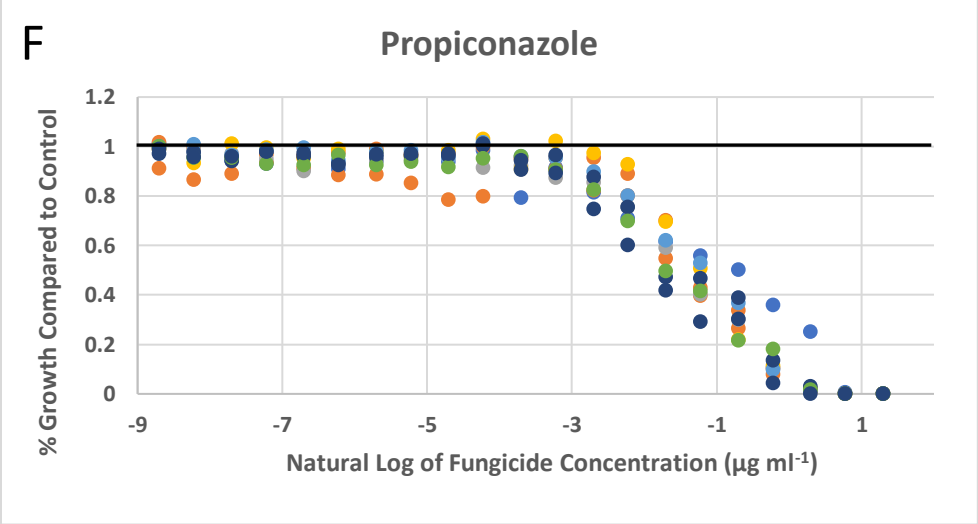


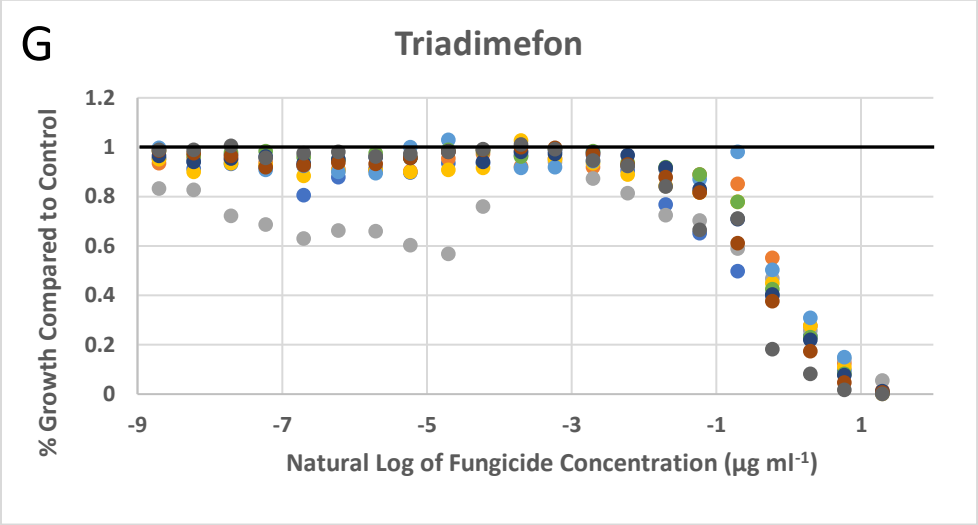


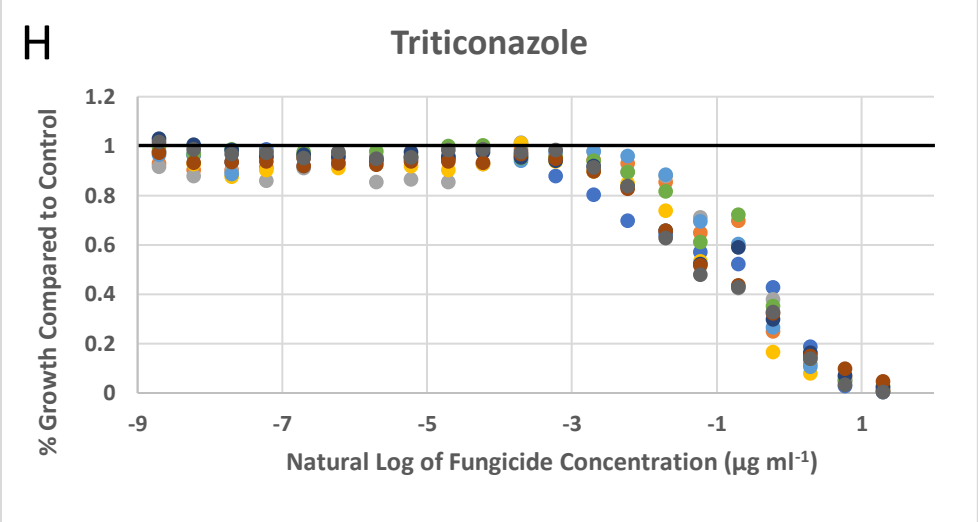

C
Triadimefon
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)


D
Triticonazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

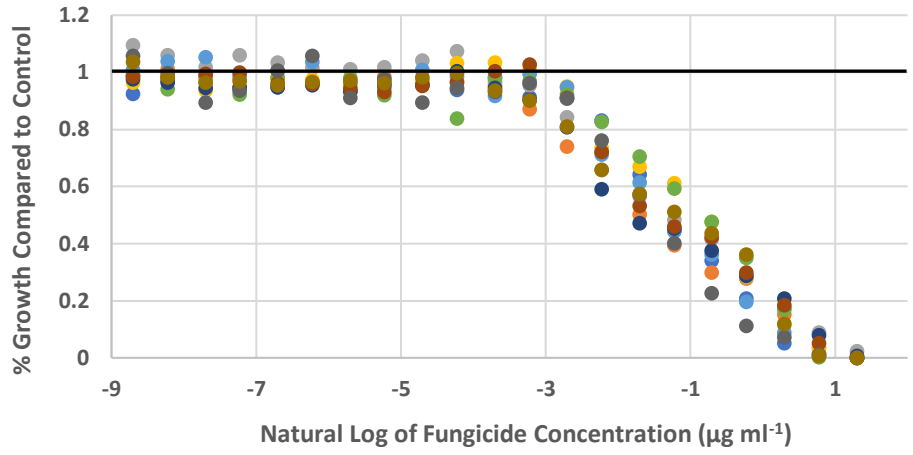

E
Difenoconazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)


F
Propiconazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

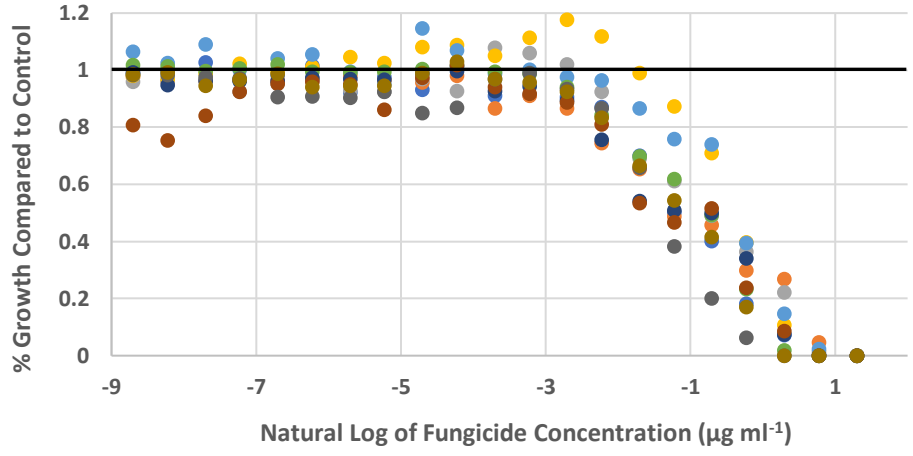

G
Triadimefon
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)


H
Triticonazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

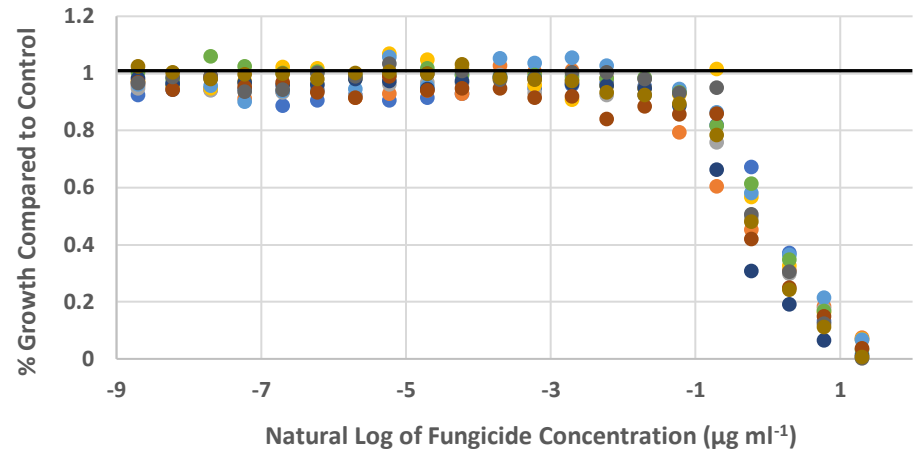
I


Difenoconazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

J


Propiconazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

K


Triadimefon
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

L

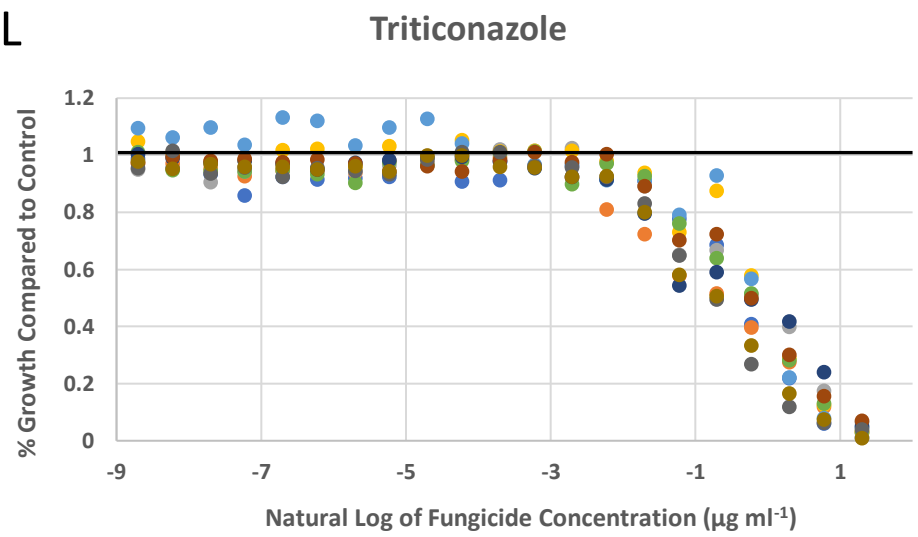
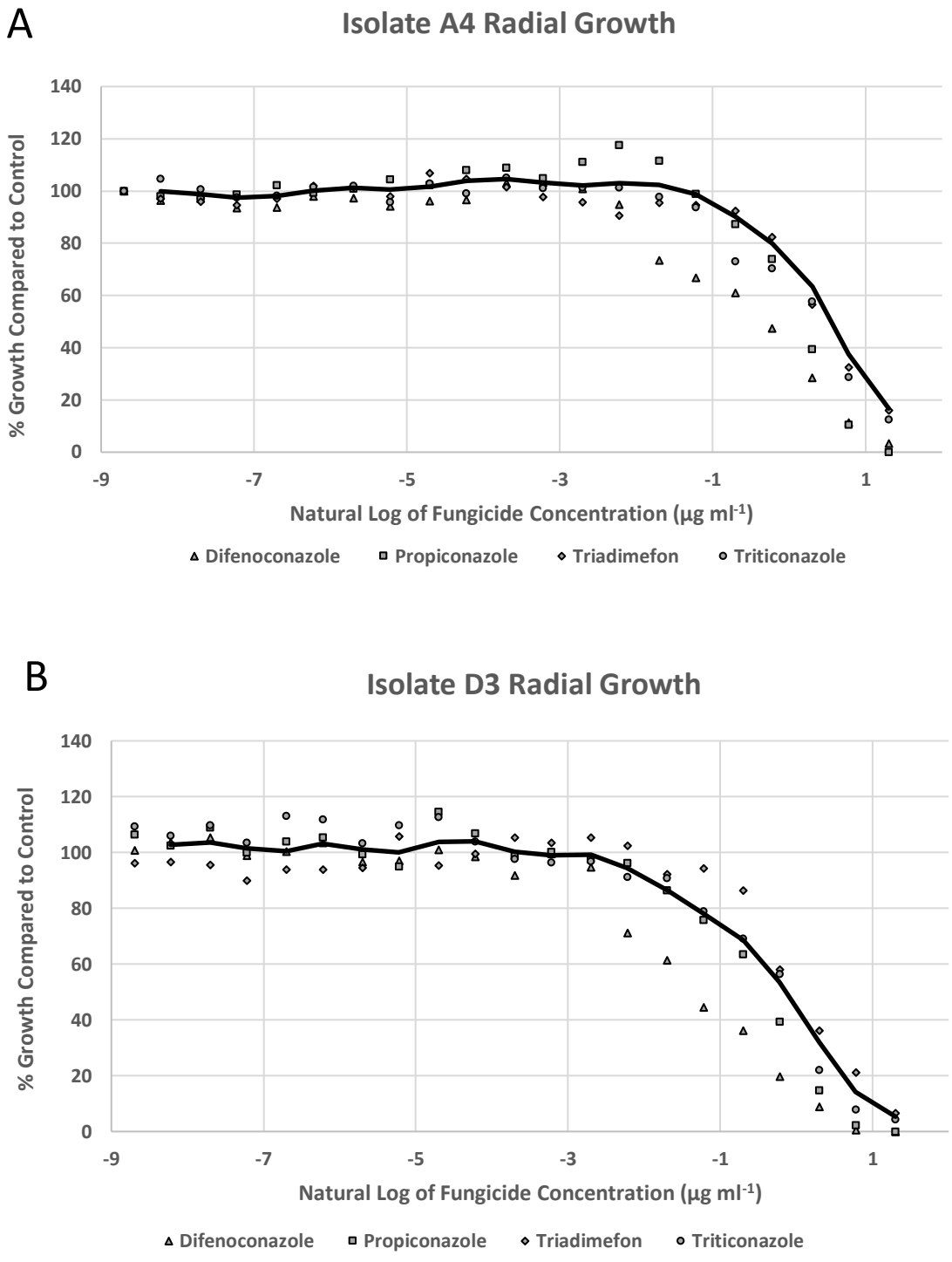

Triticonazole
% Growth Compared to Control
1.2
1
0.8
0.6
0.4
0.2
0
-9
-7
-5
-3
-1
1
Natural Log of Fungicide Concentration (µg ml-1)

Figure 2.9. Observed values and modeled curve of the relative mycelial growth of isolates A4 (A) and D3 (B) in response to concentrations of DMI fungicides. Values were averaged across two replications and two experimental runs. Modeled curves represent the average relative growth value of the isolate to all four DMI fungicides.



Chapter 3:

Effect of Sub-Lethal Doses of Fungicide on Oxalic Acid Production by *Sclerotinia homoeocarpa*

Abstract

Oxalic acid is produced by *Sclerotinia homoeocarpa* to link to calcium in the plant cell wall and acidify host tissue, thereby increasing enzyme activity and causing necrosis. Oxalic acid production of thirty *S. homoeocarpa* isolates ranging in sensitivities to demethylation inhibiting (DMI) fungicides was evaluated via high performance liquid chromatography (HPLC) for increases in production when grown in the presence of a single sub-lethal DMI fungicide dose. An agar plug of mycelium from each isolate was grown in 10 ml of HPLC grade water amended with 0, 0.0002, or 20 µg ml^{-1} of technical grade difenoconazole, propiconazole, triadimefon, or triticonazole for 10 days. Oxalic acid production was detected with HPLC in 17.6% of fungal plugs throughout the study. All 30 isolates produced oxalic acid in at least one experimental run, with only 18 of the isolates exhibiting oxalic acid production in both experimental runs. Final concentrations of oxalic acid ranged from 0 to 0.383 mM, with an average of 0.004 mM. Isolates in 20 µg ml^{-1} of fungicide produced greater oxalic acid concentrations than those grown in 0.0002 µg ml^{-1} ($P = 0.001$) and no fungicide ($P = 0.0002$). The interaction of fungicide type and isolate sensitivity impacted oxalic acid production ($P = 0.0002$), revealing sensitive isolates that were treated with propiconazole produced more oxalic acid when compared to all other sensitivity and fungicide type combinations. Cell leakage is caused by the mechanistic activity of DMI fungicides and may be the cause of greater oxalic acid concentrations observed in sensitive isolates and in isolates exposed to fungicide concentrations of 20 µg ml^{-1}. Results of this study indicate sub-lethal concentrations of DMI fungicides may not induce hormetic increases of oxalic acid production in *S. homoeocarpa*.

Introduction

Sclerotinia homoeocarpa F.T. Bennett is the causal agent of dollar spot, a disease which severely impacts turfgrass quality, utility, and aesthetics. In North America, all cultivated and highly maintained turfgrass species are susceptible to *S. homoeocarpa*, however, the disease is most significant on creeping bentgrass (*Agrostis palustris* Huds.) and Kentucky bluegrass (*Poa pratensis*) (Fry and Huang, 2004). The infection process begins by direct penetration of hyphae through the leaf cuticle or by entering through natural openings such as stomata (Smiley et al., 2007). Infection causes tissue necrosis, which leads to the characteristic dollar spot symptoms of brown to tan circular spots, typically 1-5 cm in diameter. Long term survival of *S. homoeocarpa* is facilitated through the formation of a flat stroma in plant tissue, thatch, and/or clippings (Smiley et al., 2007).

Cultural practices alone may limit disease severity but are typically insufficient in controlling dollar spot in high amenity turfgrasses. Fungicide use is commonly needed to control dollar spot, particularly in areas with low aesthetic thresholds for disease such as golf course putting greens. Sterol demethylase inhibitor (DMI) fungicides are acropetal penetrants commonly used to control dollar spot via the inhibition of a demethylation step in the biosynthesis of fungal sterols within the cell (Hendrix, 1970). This inhibition of sterols, particularly ergosterol in the cytoplasm, results in improper regulation of compounds that enter and exit the fungal cells and ultimately stops growth (Latin, 2011).

The fungal genus *Sclerotinia* contains many plant pathogens that infect a range of important crops. The production of oxalic acid has been confirmed for many *Sclerotinia* species, such as *S. minor*, *S. rolfsii*, *S. sclerotiorum*, *S. trifoliorum*, and *S. homoeocarpa*

(Bateman and Beer, 1965, Livingstone et al., 2005, Maxwell and Lumsden, 1970, Pierson and Rhodes, 1992, Venu et al., 2009). Oxalic acid production, along with the production of pectolytic cell wall-degrading enzymes, is well documented within this genus as a key pathogenicity factor. Host tissue degradation occurs by lowering pH with oxalate ions, which promotes optimal activity of cell-wall degrading enzymes, such as cellulase (Bateman and Beer, 1965, Riou et al., 1991). Oxalic acid production has been confirmed in *S. homoeocarpa* (Beaulieu, 2008, Liberti et al., 2012, Venu et al., 2009), and is believed to have a similar importance in fungal development and pathogenicity for *S. homoeocarpa* as it does for other *Sclerotinia* species (Rioux et al., 2014, Venu et al., 2009).

The term hormesis describes a dose response by an organism to a particular stressor. This phenomenon is characterized by stimulation of a particular biological endpoint upon low-dose exposure to the stressor and an inhibitory effect of the biological endpoint upon high-dose exposure to the same stressor (Calabrese et al., 1987). This response is thought to be a generalizable biological phenomenon independent of biological endpoint and stressors (Calabrese et al., 1999). Previous *in vitro* studies have characterized hormesis in fungal plant pathogens and oomycetes, with stimulatory effects described by mycelial growth (Flores and Garzón, 2013, Garzón et al., 2011).

The effect of sub-lethal DMI fungicide exposure on metabolite production in *S. homoeocarpa* isolates is not understood. Moreover, it is unknown how hormetic responses are impacted by an isolate's sensitivity to DMI fungicides. Increased metabolite production by sub-lethal DMI fungicide rates has been observed in previous studies (Audenaert et al., 2010). A hormetic increase in oxalic acid production due to

sublethal exposure to the DMI fungicides may result in an increase in aggressiveness of *S. homoeocarpa* and increased dollar spot severity. The effects of low and high DMI fungicide exposure on the production of oxalic acid must be evaluated in *S. homoeocarpa* to better understand how fungicides affect metabolite production and the overall infection process of plant pathogens. The objective of this study was to examine the impacts of sub-lethal doses of DMI fungicides on the production of oxalic acid in *S. homoeocarpa*.

Materials and Methods

Fungal Isolates and Modified Melin-Norkrans Media. Thirty *Sclerotinia homoeocarpa* isolates were selected based on their sensitivities to DMI fungicides, as determined previously (Burpee, 1997, Jo et al., 2008, Miller et al., 2002, Putman et al., 2010, Tredway, 2005) (Table 3.1). Isolates ranged from sensitive, unexposed isolates to resistant isolates with a low sensitivity to DMI fungicides. For a majority of these isolates, specific history of fungicide use was unavailable. Individual isolates were stored on colonized filter paper at -80°C until use.

Modified Melin-Norkrans (MMN) media (Marx, 1969) is a defined nutrient medium to promote fungal growth and was selected for use in this study based on the quality of *S. homoeocarpa* mycelial growth. Additionally, this media was chosen for the absence of ingredients that may contain endogenous oxalic acid. Media was prepared according to the Talbot Lab Protocol: MMN (Talbot, 2014).

Cultures of *S. homoeocarpa* were prepared for each experiment by placing a piece of colonized filter paper onto a petri dish of potato dextrose agar (PDA) media containing chloramphenicol, streptomycin, and tetracycline at 0.05 g/L each. Petri dishes were then stored at room temperature (25°C) for three days to allow for colony growth. Sections

from the actively growing fungal colonies on PDA were then transferred to petri dishes containing 25 ml of MMN. Petri dishes were stored on a lab bench at room temperature for three days prior to the experiment.

Fungicide Concentrations and HPLC Treatment Preparation. Fungicide concentrations were prepared with technical grade propiconazole (95% a.i., Syngenta Crop Protection, Greensboro, NC), difenoconazole (95% a.i., Syngenta Crop Protection, Greensboro, NC), triadimefon (99.5% a.i., Ultra-Scientific Analytical Solutions, North Kingstown, Rhode Island), and triticonazole (92.5%, BASF, Research Triangle Park, NC) dissolved in acetone. Polypropylene conical centrifuge tubes (Falcon 50 ml 30x115 mm style, Corning Science, Tamaulipas, Mexico) were filled with 10 ml of HPLC grade water (submicron filtered, Fisher Chemical, Fair Lawn, NJ), and amended with the fungicides to a concentration of 0, 0.0002, or 20 μg ml^{-1}. Final solutions of acetone concentrations were 0.1% (vol/vol) in all treatments, including the non-amended control. Mycelial plugs (10 mm) were taken from the actively growing region of three-day old colonies growing on petri dishes containing MMN media and placed into each solution. Tubes were then placed on an orbital shaker (DS-500 Orbital Shaker, VWR International, Radnor, PA) set at 165 rotations per minute for a period of 10 days.

Assessment of Oxalic Acid by High Performance Liquid Chromatography. Conical centrifuge tubes were removed from the orbital shaker after 10 days to assess the solution for accumulated oxalic acid. The liquid solution was separated from the fungal plug by vacuum filtration (Gast Manufacturing, Inc., Benton Harbor, MI) through a 55-mm circular filter paper (Whatman #1, GE Healthcare, Little Chalfont, Buckinghamshire, UK), and collected in a 250 ml Pyrex® filter flask. The liquid filtrate was transferred into

a suitable vial (12 x 32 mm, Thermo Scientific, Rockwood, TN) for assessment via high performance liquid chromatography (HPLC). The filter flask was triple rinsed with warm tap water, followed by a triple rinse of DI water between the filtration of each culture.

The liquid filtrate of each culture was individually assessed for oxalic acid accumulation using a Dionex Ultimate 3000 UHPLC machine (Thermo Fisher Scientific, Waltham, MA) equipped with a pump, autosampler, column compartment, and a rapid separation variable wavelength detector. The pump was fitted with an Accucore C18 LC Column (150 x 3 mm, 2.6 μm particle size, Thermo Fisher Scientific, Waltham, MA). Oxalic acid was detected with a mobile phase solution of 2% potassium phosphate (KH_2PO_4) in HPLC grade water. Hydrochloric acid was used to adjust the final pH of the mobile phase to 2.3. A fresh solution of mobile phase was created prior to each use of the machine. Standard solutions of synthetic oxalic acid (99+%, Aldrich Chemical Company, Milwaukee, WI) were prepared to confirm and quantify oxalic acid produced by fungal plugs via a standard curve (Figure 3.5). Solutions of synthetic oxalic acid for the standard curve were prepared prior to each use of the machine at concentrations of 0.0375 mM, 0.05 mM, and 0.25 mM. Chromatographic conditions for detection and quantification of oxalic acid were as follows: flow rate of 0.5 ml/min; injection volume of 20 μl/minute; total run time of 3 minutes for each injection. Detection of oxalic acid was performed at a wavelength of 209 nm. Each liquid filtrate was injected twice. Final oxalic acid concentrations were determined by utilizing Chromeleon Chromatography Data System Software (Version 7.2, Thermo Fisher Scientific, Waltham, MA).

Experimental Design and Statistical Analysis. Thirty *S. homoeocarpa* isolates were evaluated for oxalic acid production when exposed DMI fungicides. The thirty isolates

were designated into three classes of sensitivities based on EC_{50} values to DMI fungicides. EC_{50} values were previously determined by an *in vitro* fungicide assay (Table 3.1). Categories of sensitivities included sensitive, moderately resistant, or resistant to DMI fungicides. Four DMI fungicides were utilized in this study. Fungicides included difenoconazole, propiconazole, triadimefon, and triticonazole. The thirty isolates were treated with no fungicide, 0.0002 μg ml^{-1}, or 20 μg ml^{-1} of each individual fungicide. The experimental design was completely randomized with each isolate/treatment combination replicated 3 times. The entire experiment was repeated twice.

Oxalic acid concentrations of the two injections per each liquid filtrate were averaged for analysis. Single, non-averaged values were utilized for liquid filtrates with only one positive oxalic acid injection. Least square means (LSMEANS) were subjected to analysis of variance with the PROC GLIMMIX procedure in SAS (SAS 9.4, SAS Institute, Cary, NC). No difference was observed among experimental runs; therefore, the experimental runs were averaged for this study. Oxalic acid concentrations were analyzed by means separation for single effects and interactions of isolate sensitivity, fungicide type, and fungicide rate. Means were separated using Fisher's protected least significant difference ($\alpha = 0.05$).

Results

Detection and Quantification of Oxalic Acid. Oxalic acid production by thirty *S. homoeocarpa* isolates was evaluated via high performance liquid chromatography (Table 3.1). Methodology for detection and quantification of oxalic acid production by *S. homoeocarpa* in an HPLC system was developed within this study to allow for oxalic acid detection in the presence of fungicide concentrations. The performance of the

method used to quantify oxalic acid was evaluated in terms of interday precision based on two injections of 5 mM standard solutions of oxalic acid (Table 3.2). Analysis of oxalic acid via HPLC was conducted within a seven-month period. Data were collected by the HPLC machine in seven separate instances in the first experimental run, and five separate instances in the second experimental run. Each HPLC analysis representing collected data for this study was included in the evaluation of the developed method. Recovery values of individual 5 mM standard solutions ranged from 77.91 to 158.06% for the entire experiment. The average quantity of standard 5 mM oxalic acid solutions recovered within this method was 5.096 mM.

Production of Oxalic Acid in the Presence of DMI Fungicides. No difference was observed in oxalic acid concentrations among experimental runs ($P = 0.287$). Oxalic acid was detected in each of the two injections for 11.6% of liquid filtrates. Oxalic acid was not detected in five *S. homoeocarpa* isolates in the first experimental run. Oxalic acid was not detected in seven separate isolates in the second experimental run. Eighteen of the thirty isolates in this study produced oxalic acid in both experimental runs (Table 3.1).

Fungicide rate impacted the oxalic acid production of *S. homoeocarpa* isolates ($P < .0008$) (Table 3.3). Isolates treated with fungicide concentrations of 20 μg ml^{-1} produced more oxalic acid than isolates treated with fungicide concentrations of 0.0002 μg ml^{-1} ($P = 0.0009$) and isolates with no fungicide treatment ($P = 0.0002$) (Figure 3.2). There was an interaction between fungicide type and isolate sensitivity on oxalic acid production ($P < 0.0001$) (Table 3.3). Sensitive isolates that were treated with propiconazole produced more oxalic acid when compared to all other combinations of sensitivity and fungicide type (Figure 3.4).

Discussion

Oxalic acid production has previously been confirmed in *Sclerotinia homoeocarpa* (Rioux, 2014, Venu et al., 2009). Although production of oxalic acid in *S. homoeocarpa* has been evaluated, quantification of oxalic acid production by *S. homoeocarpa* isolates ranging in sensitivities to demethylation inhibiting (DMI) fungicides has not been reported. Additionally, no study has evaluated the production of oxalic acid by *S. homoeocarpa* in the presence of low or high concentrations of DMI fungicides. Within this study, a HPLC method to detect oxalic acid production by fungal isolates in the presence of fungicide concentrations was developed. With this method, the ability of *S. homoeocarpa* isolates to produce oxalic acid in the presence of DMI fungicides was confirmed and concentrations of oxalic acid were quantified.

Preliminary experiments within this study showed oxalic acid was present in potato dextrose broth and potato dextrose agar (data not shown). Previous methods of detecting oxalic acid production by S. *homoeocarpa* typically utilized media which included potato dextrose in the form of broth or agar (Andrew et al., 2012, Venu et al., 2009). Although the concentration and consistency of endogenous oxalic acid within laboratory grade potato dextrose has not been confirmed, endogenous oxalic acid within potatoes has been confirmed via high performance liquid chromatography (Bushway et al., 1984). Detection and quantification of oxalic acid production could be obstructed if endogenous oxalic acid is present in media. Additionally, endogenous oxalic acid of hosts has been shown to influence oxalic acid production in *S. homoeocarpa* (Rioux, 2014). To deter the interference of endogenous oxalic acid within our media, modified Melin-

Norkrans media (Marx, 1969) was utilized for the determination of oxalic acid production by *S. homoeocarpa.*

Commonly, oxalic acid detection is executed by use of oxalate quantification kits that utilize bromophenol blue (Andrew et al., 2012, Rioux, 2014, Venu et al., 2009), however, previous studies have confirmed oxalic acid production via high performance liquid chromatography (Rioux, 2014, Venu et al., 2009). Additionally, quantification of whole plant oxalate content of *S. homoeocarpa* inoculated bentgrass leaves has been performed by using a UV/Vis spectrophotometer (Rioux, 2014). Methods for HPLC detection and quantification of oxalic acid in this study were modified from previous studies of oxalic acid determination in *S. homoeocarpa* (Venu et al., 2009) and from methods of oxalic acid determination in fruits and vegetables (Carriedo-Nisperos et al., 1992, Libert, 1981). Evaluation of the method used in the current study indicated recovery values of 5 mM standard solutions ranged from 77.91 to 158.06%, however, the mean concentration of oxalic acid quantified in these standards was 5.096 mM. The wide range of detection among concentrations of standard solutions could partly be due to the result of error in preparation of standard solutions for each use of the HPLC machine. Additionally, wide ranges of quantified standard solutions could be caused by the deterioration of the HPLC column over time due to the high amount of injections analyzed and the high acidity of oxalic acid.

Oxalic acid production by the thirty *S. homoeocarpa* isolates within this study was sporadic. The metabolite was detected in 256 out of the 1,080 liquid filtrates analyzed by HPLC in the first experimental run and 124 out of the 1,080 liquid filtrates analyzed by HPLC in the second experimental run. Oxalic acid was not detected in

filtrates with isolates S084, 451SHCT76, 725SHME, G62, and A22 in the first experimental run, and isolates H127, RE18G8, RE18G38, VGC5, S088, RCC18G15, and G5 in the second experimental run. Eighteen of the thirty *S. homoeocarpa* isolates exhibited oxalic acid production in at least one fungal plug within both experimental runs. Previous studies have shown evidence that host material, such as endogenous oxalic acid and xylan monomers, can induce the production of oxalic acid by the pathogen (Rioux, 2014). The inconsistency of oxalic acid detection within isolates of *S. homoeocarpa* may be attributed to the effect of growing these isolates in an environment without host interaction. Additionally, oxalic acid production could have been affected by the ability of the isolate to grow in an aquatic environment, as isolates were grown in 10 ml of HPLC grade water for a period of 10 days before HPLC analysis.

In the current study, production of oxalic acid by *S. homoeocarpa* was confirmed (Figure 3.2). Additionally, oxalic acid production was observed by *S. homoeocarpa* in the presence of DMI fungicides. The mechanistic action of DMI fungicides inhibits a demethylation step in the biosynthesis of fungal sterols, which are important for overall fungal growth (Hendrix, 1970). Inhibition of sterols within fungal cells, particularly in the cytoplasm, causes leakage of compounds entering and exiting the cell (Latin, 2011). Dahmen et al. (1988) reported significant cell wall leakage of *Puccinia graminis* f. sp. *tritici* and *Monilinia fructicola* at high concentrations (25 μg ml^{-1} and above) of propiconazole when compared to nontreated cultures (Dahmen et al., 1988). In the current study, the interaction of fungicide type and isolate sensitivity impacted oxalic acid concentrations, indicating sensitive isolates treated with propiconazole produced more oxalic acid than all other combinations of sensitivity and fungicide type. This result

may reveal the ability of DMI fungicides to inhibit the production of fungal sterols in sensitive *S. homoeocarpa* isolates, ultimately causing fungal cell leakage and subsequent oxalic acid accumulation within solutions containing fungicides. Fungicide concentration also impacted oxalic acid production. Isolates that were grown in the presence of the high rate of fungicides (20 $\mu g\ ml^{-1}$) produced the largest oxalic acid concentrations. This observation corresponds to the previous result that oxalic acid concentrations examined in this study could be due to leakage from fungal cells caused by the mechanistic activity of DMI fungicides. Additionally, this result suggests higher fungicide rates can promote more fungal cell wall leakage.

Based on previous experiments conducted as a part of this study, 0.0002 ug/ml^{-1} was below the average NOAEL for propiconazole and triticonazole, however, increased oxalic acid production was not observed by *S. homoeocarpa* when exposed to any DMI fungicide at this concentration. Similarly, Zhou et al. (2014) did not observe an increase in oxalic acid production of *Sclerotinia sclerotiorum* at low doses of dimethachlon *in vitro.* However, Audenaert et al. (2010) reported a stimulatory effect on the production of the fungal metabolite deoxynivalenol in *Fusarium graminearum in vitro* and *in vivo*. Although the stimulation of deoxynivalenol was initiated by a DMI fungicide, it was reported that the stimulatory effect was ultimately caused by host produced hydrogen peroxide (Audenaert et al., 2010). In the current study, fungal isolates were grown in a liquid solution without host interaction, potentially affecting stimulatory production of oxalic acid. Additionally, a single concentration of sub-lethal fungicides was utilized in this study. Further research on stimulation of oxalic acid production in phytopathogenic

fungal species should focus on host pathogen interaction and include more low-dose fungicide concentrations.

Previous studies have found conflicting results regarding the relationship between oxalic acid production and mycelial growth. Batemann and Beer (1965) reported that oxalic acid concentrations were not directly related to the total amount of mycelium for *Sclerotinia sclerotiorum*. Venu et al. (2009) reported a correlation between conditions for optimal mycelial growth of *S. homoeocarpa* and greater oxalic acid production. Mycelial growth of *S. homoeocarpa* isolates was not directly quantified prior to oxalic acid detection in the current study, however, no visual difference was observed among mycelial growth of *S. homoeocarpa* isolates treated with no fungicide when compared to mycelial growth of isolates treated with 0.0002 µg ml^{-1} (Figure 3.6A, 3.6B). Isolates grown in the presence of 20 µg ml^{-1} of fungicide typically had little mycelial growth around the agar plug. Instead, melanized mycelium resembling flattened stroma, or survival structures of the pathogen, encompassed the fungal plug (Figure 3.6C). Significant increases in oxalic acid concentrations were observed when isolates were grown in the presence of 20 µg ml^{-1} of fungicide, although mycelial growth was visually inhibited. This may suggest that mycelial growth is not directly related to oxalic acid production. Future experiments attempting to determine the effect of mycelial growth on oxalic acid production should be directed at evaluating mycelial dry weight in combination with metabolite quantification via HPLC. Additionally, studies focusing on the relationship between the production of oxalic acid and the formation of survival structures could provide insight into the function of oxalic acid within fungal plant pathogens.

In the future, studies should focus on elucidating the effect of DMI fungicides on the fungal production of oxalic acid in plant models. Previous studies have examined *S. homoeocarpa* oxalate activity, focusing on oxaloacetate acetylhydrolase (oah) gene activity within *Arabidopsis thaliana* (Andrew et al., 2012). However, Rioux (2014) concluded that *A. thaliana* was a poor model system for *S. homoeocarpa.* Although oxalic acid was detected and quantified *in vitro* in the current study, further studies are needed to determine the effect of DMI fungicides on oxalic acid production by *S. homoeocarpa* in appropriate model systems, such as creeping bentgrass.

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Table 3.1. History, EC_{50} value, and sensitivity category of 30 *Sclerotinia homoeocarpa* isolates.

[a]Isolate	Origin	Year Collected	[b]History of DMI Exposure	[c]EC_{50} Value	[d]Resistance to DMI Fungicides
LWC10	Raleigh, NC	2003	Baseline	0.011	Sensitive
*VGC5	Edgartown, MA	2007	Baseline	0.013	Sensitive
*RE18G8	Shallotte, NC	2003	Exposed	0.013	Sensitive
LWC5	Raleigh, NC	2003	Baseline	0.017	Sensitive
RE18G26	Shallotte, NC	2003	Exposed	0.018	Sensitive
PST4	Rolesville, NC	2003	Baseline	0.018	Sensitive
RE18G4	Shallotte, NC	2003	Exposed	0.020	Sensitive
*S084	State College, PA	1980	Baseline	0.021	Sensitive
CHCC10	North Attleboro, MA	2007	Exposed	0.022	Sensitive
RE18G45	Shallotte, NC	2003	Exposed	0.031	Sensitive
LWC27	Raleigh, NC	2003	Baseline	0.033	Sensitive
RE18G38	Shallotte, NC	2003	Exposed	0.049	Moderately Resistant
*451ShCT76	Madison, CT	2007	Exposed	0.063	Moderately Resistant
*725ShME	South Portland, ME	2007	Exposed	0.095	Moderately Resistant
PhPG4	Pinehurst, NC	2003	Exposed	0.097	Moderately Resistant
RE18G35	Shallotte, NC	2003	Exposed	0.098	Moderately Resistant
RE18G16	Shallotte, NC	2003	Exposed	0.102	Moderately Resistant
*RCC18G15	Raleigh, NC	2003	Exposed	0.113	Moderately Resistant
PhPG9	Pinehurst, NC	2003	Exposed	0.118	Moderately Resistant
557ShCT173	Woodbridge, CT	2007	Exposed	0.122	Moderately Resistant
*SO88	Chicago, IL	1993	Exposed	0.125	Resistant
*G62	Waynesfield, OH	2004	Exposed	0.148	Resistant
500ShCT123	Coventry, CT	2007	Exposed	0.190	Resistant
*H127	Springfield, OH	2005	Exposed	0.197	Resistant
363ShCT18	East Falmouth, CT	2006	Exposed	0.207	Resistant
*G5	Canal Winchester, OH	2003	Exposed	0.272	Resistant
PhPG22	Pinehurst, NC	2003	Exposed	0.323	Resistant
*A22	Powell, OH	2001	Exposed	0.338	Resistant
A4	Gainesville, VA	2001	Exposed	0.367	Resistant
D3	Grove City, OH	2002	Exposed	0.399	Resistant

[a] * indicate isolates that produced OA in only one of the two experimental runs.
[b] History of DMI exposure is based upon field conditions at the time of collection for the individual isolate.
[c] EC_{50} value is based upon the average EC_{50} value of four DMI fungicides determined previously via a mycelial assay.
[d] Resistance to DMI fungicides based on EC_{50} values.

Table 3.2. Evaluation of the developed HPLC method for detection of oxalic acid produced by *Sclerotinia homoecarpa.* Standard concentrations of 5 mM oxalic acid were quantified with each HPLC run utilized to collect data for this experiment. All standard solutions were injected twice.

		Quantified Amt (mM)				
Experimental Run	**Standard Concentration (mM)**	**Inj 1**	**Inj 2**	**Avg**	**[a]Standard Deviation**	**[b]Recovery %**
1	5	4.67	4.84	4.76	0.12	95.2
1	5	4.97	4.94	4.95	0.02	99.1
1	5	4.78	4.89	4.84	0.08	96.8
1	5	5.55	6.10	5.82	0.39	116
1	5	4.60	4.13	4.37	0.33	87.3
1	5	3.64	4.15	3.90	0.36	77.9
1	5	5.02	4.77	4.89	0.18	97.9
2	5	4.29	4.17	4.23	0.09	84.6
2	5	4.03	5.29	4.66	0.89	93.3
2	5	5.23	5.45	5.34	0.15	107
2	5	8.78	7.02	7.90	1.24	158
2	5	4.90	6.10	5.50	0.84	110

[a]Standard deviation values are calculated based upon two injections for the standard solutions.
[b]Recovery percentages calculated by the average quantified amount of the 5 mM standard concentration divided by 5.

Table 3.3. ANOVA table describing the effects of replication, experimental run, isolate sensitivity to DMI fungicides, DMI fungicide concentration, and DMI fungicide type on detected oxalic acid concentrations. ANOVA table was produced using PROC GLIMMIX in SAS.

Oxalic Acid Concentration		
Source	**DF**	**Pr > F**
Replication	2	<.0001
Run	1	0.4622
Sensitivity	2	0.0046
Concentration	2	0.0008
Fungicide	3	0.0005
Fungicide*Sensitivity	6	<.0001
Concentration*Sensitivity	2	0.9595
Fungicide*Rate	3	0.3759
Fungicide*Rate*Sensitivity	6	0.8892

Table 3.4. Mean oxalic acid (OA) production by *Sclerotinia homoeocarpa* isolates when untreated or treated with concentrations of propiconazole, difenoconazole, triadimefon, and triticonazole.

		Control	Propiconazole		Difenoconazole		Triadimefon		Triticonazole	
[a]Isolate	[b]Resistance to DMI Fungicides	[c]Mean OA (mM)	Mean OA (mM) 0.0002	Mean OA (mM) 20	Mean OA (mM) 0.0002	Mean OA (mM) 20	Mean OA (mM) 0.0002	Mean OA (mM) 20	Mean OA (mM) 0.0002	Mean OA (mM) 20
LWC10	S	0.000	0.000	0.139	0.000	0.012	0.000	0.012	0.000	0.013
VGC5	S	0.003	0.003	0.002	0.001	0.000	0.003	0.000	0.003	0.005
RE18G8	S	0.000	0.000	0.014	0.000	0.000	0.009	0.000	0.004	0.009
LWC5	S	0.009	0.009	0.008	0.012	0.011	0.009	0.015	0.006	0.009
RE18G26	S	0.001	0.000	0.042	0.003	0.000	0.000	0.000	0.000	0.021
PST4	S	0.004	0.000	0.139	0.022	0.000	0.000	0.011	0.000	0.000
RE18G4	S	0.004	0.087	0.014	0.018	0.012	0.000	0.007	0.000	0.009
S084	S	0.001	0.002	0.004	0.000	0.000	0.000	0.000	0.000	0.004
CHCC10	S	0.006	0.006	0.013	0.012	0.004	0.001	0.006	0.003	0.011
RE18G45	S	0.000	0.000	0.000	0.000	0.004	0.001	0.000	0.000	0.000
LWC27	S	0.015	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
RE18G38	MR	0.000	0.000	0.014	0.000	0.006	0.000	0.000	0.000	0.010
451ShCT76	MR	0.002	0.000	0.004	0.007	0.000	0.000	0.000	0.000	0.012
725ShME	MR	0.002	0.000	0.000	0.000	0.000	0.002	0.050	0.004	0.004
PhPG4	MR	0.001	0.004	0.005	0.001	0.002	0.000	0.000	0.007	0.005
RE18G35	MR	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
RE18G16	MR	0.000	0.008	0.028	0.000	0.006	0.000	0.000	0.000	0.006
RCC18G15	MR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
PhPG9	MR	0.000	0.000	0.000	0.000	0.000	0.006	0.036	0.005	0.000
557ShCT173	MR	0.000	0.000	0.003	0.000	0.002	0.000	0.000	0.000	0.005
SO88	R	0.000	0.000	0.003	0.000	0.001	0.000	0.000	0.000	0.001
G62	R	0.001	0.003	0.015	0.000	0.002	0.004	0.002	0.003	0.002
500ShCT123	R	0.000	0.000	0.002	0.000	0.005	0.003	0.001	0.000	0.002
H127	R	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.003
363ShCT18	R	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.022
G5	R	0.007	0.015	0.004	0.006	0.004	0.012	0.008	0.013	0.011
PhPG22	R	0.000	0.002	0.003	0.002	0.001	0.000	0.001	0.003	0.003
A22	R	0.000	0.002	0.000	0.001	0.000	0.000	0.000	0.001	0.001
A4	R	0.000	0.001	0.003	0.001	0.010	0.000	0.000	0.000	0.001
D3	R	0.004	0.004	0.008	0.004	0.006	0.002	0.003	0.006	0.016

[a] * indicate isolates that produced OA in only one of the two experimental runs.
[b] Resistance to DMI fungicides is based on EC_{50} values determined previously via a mycelial growth assay.
[c] Mean oxalic acid production (mM) is based on the total production by isolates treated with each DMI fungicide throughout the study. Fungicide concentrations are in $\mu g\ ml^{-1}$.

Figure 3.1. Mean oxalic acid production by *Sclerotinia homoeocarpa* isolates based on sensitivity to DMI fungicides. Sensitivities were determined previously and are based on EC_{50} values to four DMI fungicides. Mean oxalic acid production is based on the total oxalic acid production by each isolate throughout the study. Error bars represent the standard error of the mean (0.001). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).

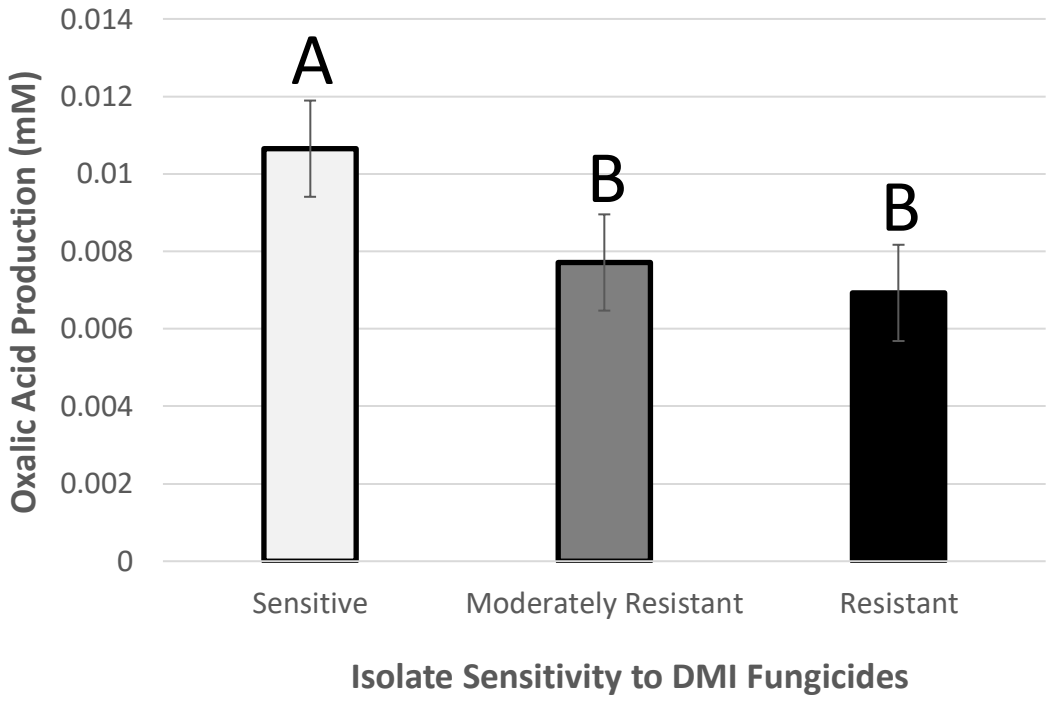


Figure 3.2. Mean oxalic acid production by *Sclerotinia homoeocarpa* isolates based on concentration of DMI fungicide. Mean oxalic acid production is based on the total oxalic acid production by each isolate throughout the study. Error bars represent the standard error of the mean (0.001). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).

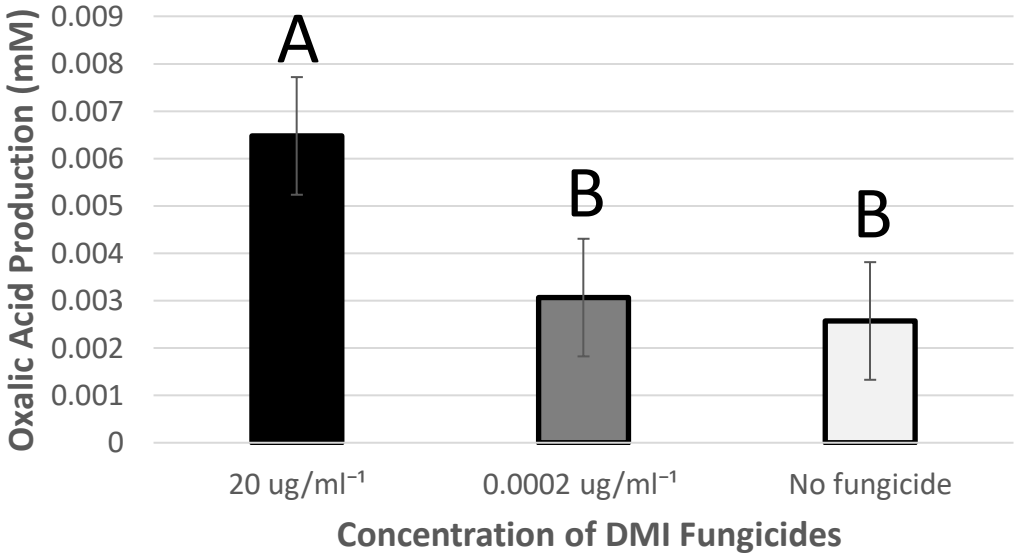


Figure 3.3. Mean oxalic acid production by *Sclerotinia homoeocarpa* isolates based on DMI fungicide type. Mean oxalic acid production is based on the total oxalic acid production by each isolate throughout the study. Error bars represent the standard error of the mean (0.001). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).

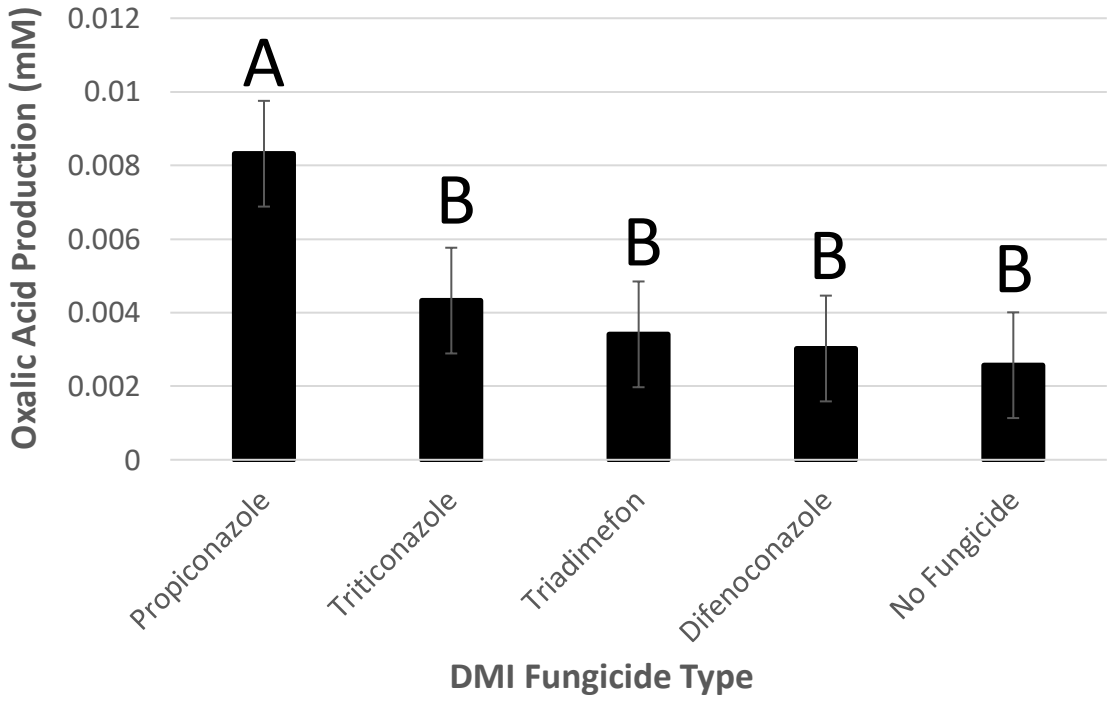


Figure 3.4. Mean oxalic acid production by sensitivity categories of *Sclerotinia homoeocarpa* isolates based on DMI fungicide type. Mean oxalic acid production is based on the total oxalic acid production by each isolate throughout the study. Error bars represent the standard error of the mean (0.002). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).

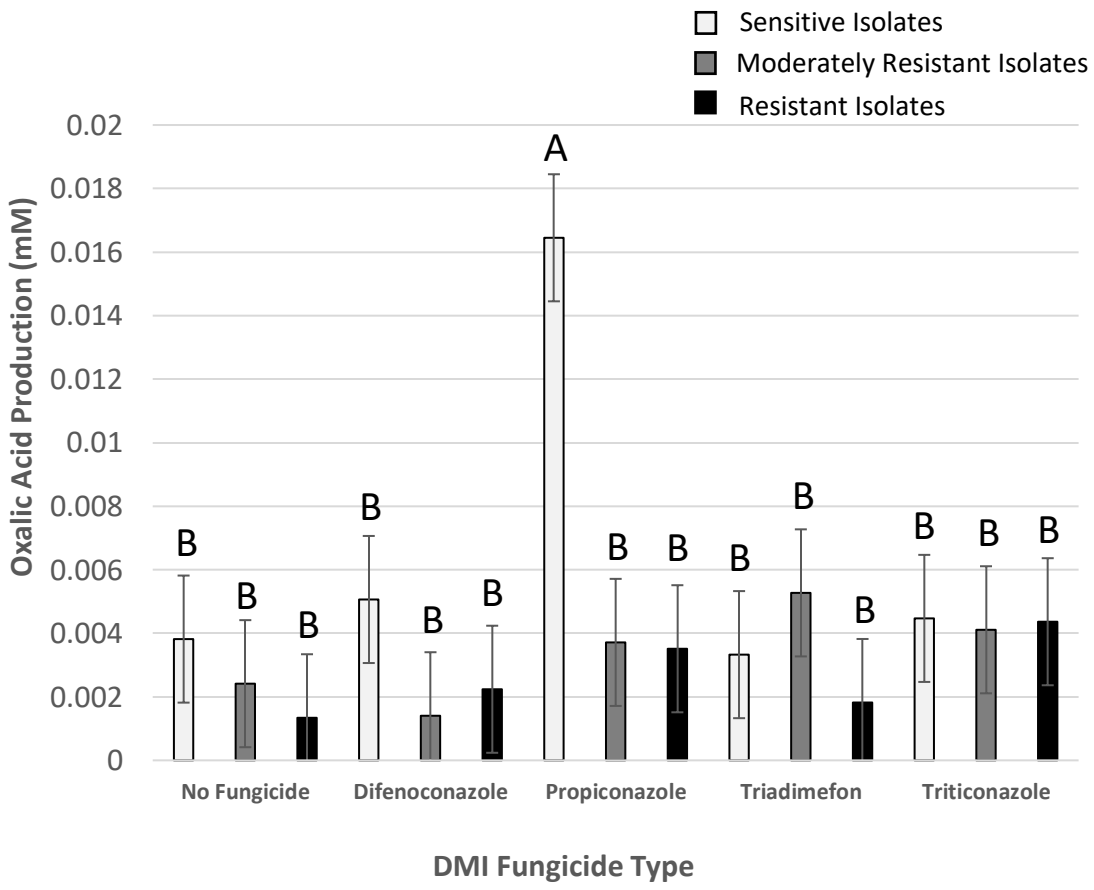


Figure 3.5. Chromatographs produced via Chromeleon Software following HPLC analysis with the described method. (A) Represents an oxalic acid standard concentration used for confirmation of oxalic acid detection in isolates of *Sclerotinia homoeocarpa*. (B) Represents oxalic acid production by an isolate of *S. homoeocarpa*.

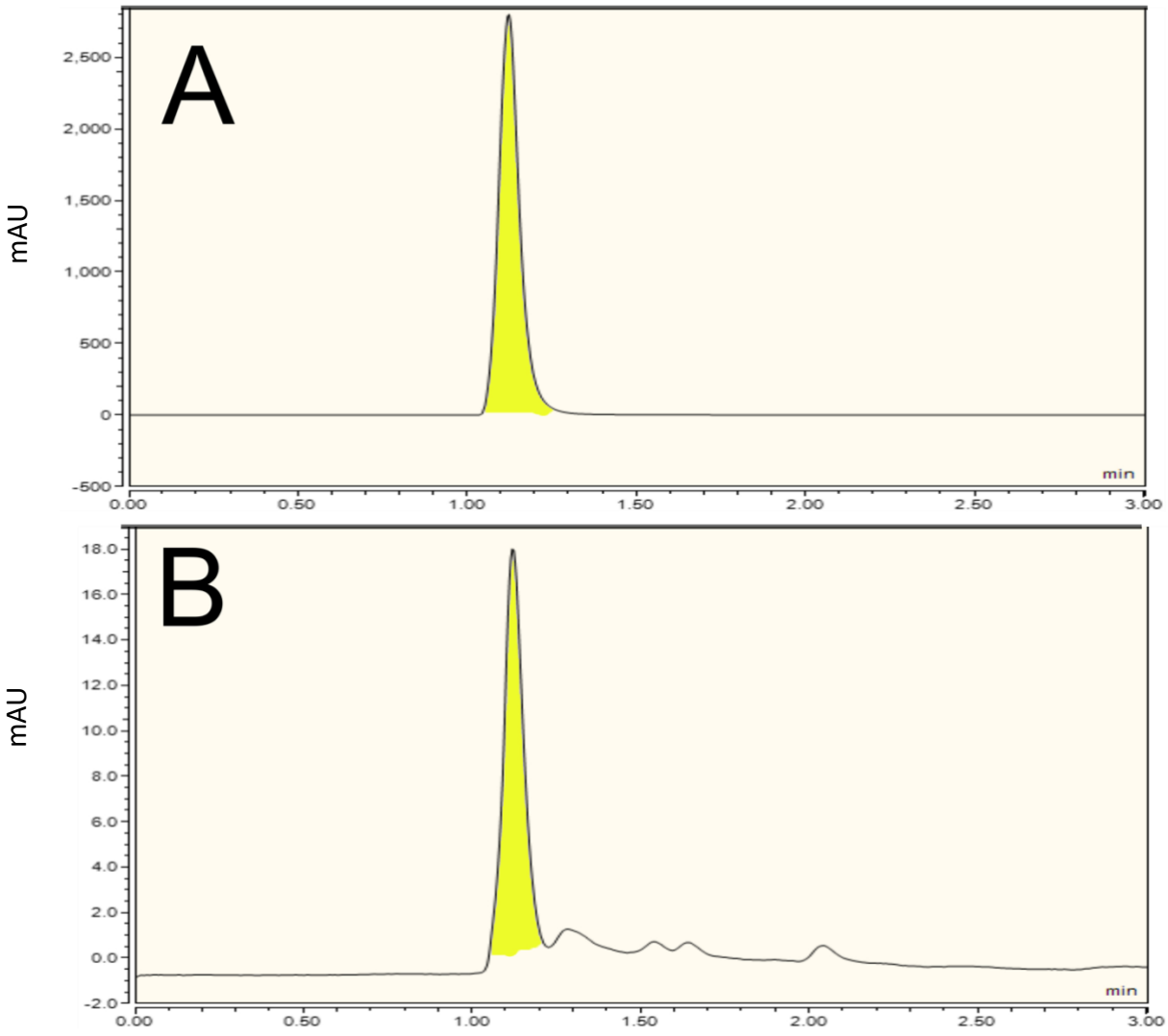
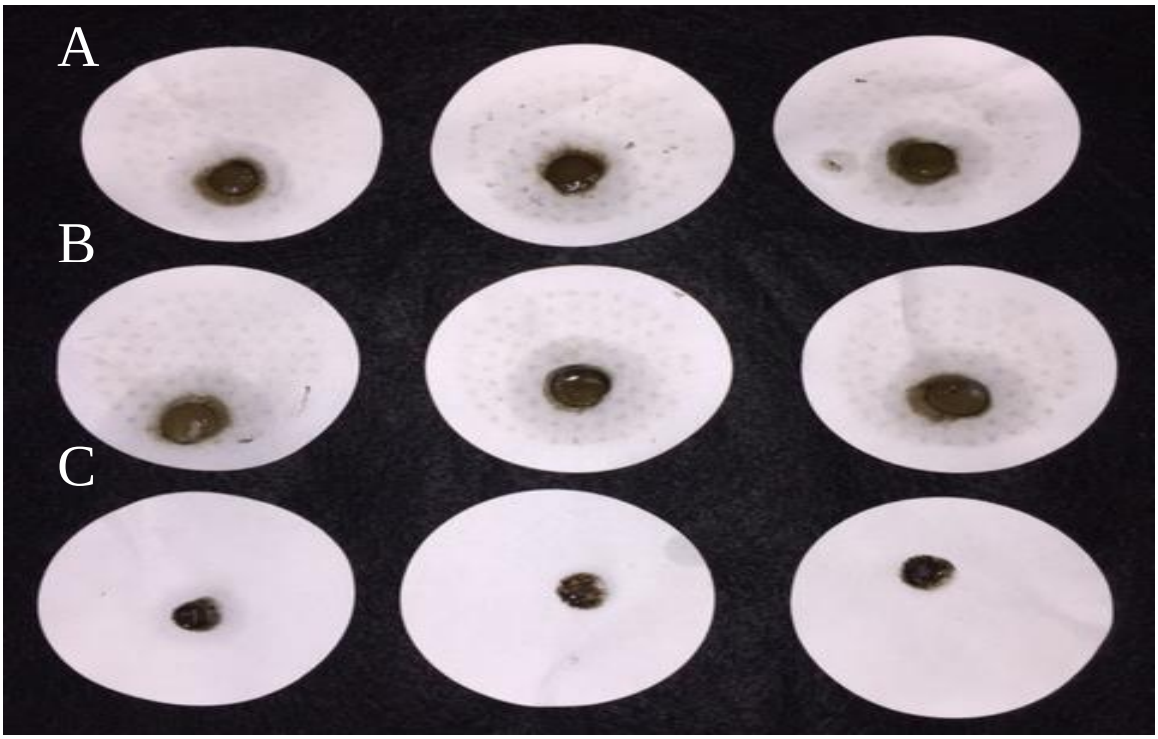


Figure 3.6. Three replications representing mycelial growth of a *Sclerotinia homoeocarpa* isolate on plugs of modified Melin-Norkrans media. Isolates were grown for 10 days in centrifuge tubes on an orbital shaker. Treatments included HPLC grade water with three fungicide rates represented by rows. (A) No fungicide, (B) 0.0002 µg ml^{-1}, (C) 20 µg ml^{-1}.



Chapter 4:

Effect of Sub-Lethal Fungicide Exposure on Control of Dollar Spot

Abstract

Exposure to sub-lethal fungicide concentrations can result in stimulated pathogenicity of particular plant pathogens, leading to increased disease pressure. The objective of this study was to determine the *in vivo* effects of sub-lethal doses of DMI fungicides on disease development from DMI resistant isolates and DMI sensitive isolates of *S. homoeocarpa*. Creeping bentgrass cv. 'Penncross' was inoculated with a subset of six *S. homoeocarpa* isolates ranging in sensitivity to DMI fungicides. Inoculated bentgrass was treated with two sub-lethal rates and one label rate of difenoconazole, propiconazole, triadimefon, or triticonazole. Sub-lethal fungicide rates were calculated based on the high label rate x 10^{-5} (HSLR) and the high label rate x 10^{-10} (LSLR). Dollar spot infections were evaluated for disease severity and area under the disease progress curve (AUDPC) throughout a 36-day period. Disease severity was increased by sub-lethal fungicide applications when compared to untreated inoculum in both experimental runs ($P < 0.05$) at 21 and 36 days after treatment. Total disease severity, represented by AUPDC, was increased by the HSLR of fungicides in both experimental runs. The interaction of fungicide rate and fungicide type had an effect on total disease severity. In the first experimental run, total disease severity of inoculum was increased when treated with both sub-lethal rates of triadimefon (HSLR – $P = 0.0004$; LSLR – $P = 0.048$). In the second experimental run, total disease severity of inoculum was increased when treated with the HSLR of difenoconazole + azoxystrobin ($P = 0.0005$) and the HSLR of triticonazole ($P = 0.047$). Results of this study suggest sub-lethal concentrations of fungicides can induce hormesis in *S. homoeocarpa* inoculum in the form of increased disease pressure *in vivo*.

Introduction

Dollar spot disease, caused by the fungal pathogen *Sclerotinia homoeocarpa,* is the most economically important disease of high amenity turfgrasses (Goodman and Burpee 1991). The disease is most aggressive in temperatures between 21°C and 27°C with high humidity, but can be initiated in temperatures ranging from 15°C to 32°C (Couch 1962, Smiley et al. 2007). Infection by *S. homoeocarpa* results in 1-5 cm straw-colored spots that can cause pitting in the turf canopy. Spots can eventually coalesce to damage large areas of turf (Smiley et al. 2007).

When disease thresholds are low, cultural practices alone can limit severity of dollar spot but are typically insufficient for control (Detweiler et al. 1983). Fungicides are often applied preventively multiple times per annum on 7-21 day intervals when conditions are most favorable for dollar spot occurrence (Fry and Huang 2004, Settle et al. 2001). Fungicide use to this magnitude can lead to the selection of resistant strains of pathogen populations (Brent and Hollomon 2007) and increase the chance of exposure to sub-lethal doses of fungicides (Garzón and Flores 2013).

The development of resistant strains of pathogen populations along with increased chances of sub-lethal fungicide exposure may result in an increased chance of the pathogen population to express hormesis. Hormesis is defined as a biphasic dose–response to an agent, identified by a low dose stimulatory effect and a high dose inhibitory or toxic effect (Mattson 2008). The hormetic response is a generalizable and reproducible biological phenomenon, with evidence suggesting that it may be independent of biological endpoint and stressors (Calabrese et al. 1999). Hormesis has been characterized in fungal and fungal like plant pathogens, with emphasis on the

biological endpoints of mycelial growth *in vitro* and disease severity *in vivo* (Garzón et al. 2011, Flores and Garzón 2013).

The sterol demethylation inhibiting (DMI) fungicide class provides both protectant and curative activity and is commonly used for dollar spot control (Koller and Scheinpflug 1987). The use of this fungicide class becomes more valuable when controlling benzimidazole and dicarboximide resistant strains of fungal pathogens, including resistant strains of *S. homoeocarpa (*Vargas et al. 1992). Disease control failures of DMI fungicides have been reported and tested, confirming the presence of DMI resistant populations of *S. homoeocarpa* (Vargas et al. 1992, Miller et al. 2002). Little research has been conducted on the stimulatory effects of sub-lethal fungicide exposure on *S. homoeocarpa*, particularly in artificial infection studies *in vivo*. In addition, little to no research has been done to compare the difference of potential stimulatory effects within isolates expressing quantitative resistance, which is characterized by a range of expressed sensitivities to a particular fungicide. If sub-lethal concentrations of DMI fungicides induce hormesis in *S. homoeocarpa* in the form of increased dollar spot severity, this phenomenon could be a contributing factor to control failures.

Chemical hormesis caused by sub-lethal doses of DMI fungicides must be evaluated in dollar spot infections caused by isolates varying in sensitivities to the DMI fungicide class. This study will allow for a better understanding of the resistance expression of *S. homoeocarpa* and the results of *in vitro* DMI fungicide assays. Additionally, this study will allow for the assessment of dollar spot severity and disease progression within the presence of low doses of DMI fungicides. Therefore, the objective

of this study was to determine the *in vivo* effects of low doses of DMI fungicides on sensitive and resistant isolates of *S. homoeocarpa*.

Materials and Methods

Fungal Isolates and Inoculum Preparation. Sensitivities to four DMI fungicides were previously confirmed and hormetic doses were assessed *in vitro* for thirty isolates of *Sclerotinia homoeocarpa*. Isolates were designated into sensitivity categories based on EC_{50} values to DMI fungicides. Three sensitive isolates (LWC10, S084, and LWC27) with EC_{50} values ranging from 0.011 to 0.033, and three resistant isolates (S088, A4, and D3) with EC_{50} values ranging from 0.125 to 0.399 were selected for the *in vivo* study (Ma and Tredway, 2013). Isolates S088 and S084 were included based on previous studies which have determined the resistance and sensitivity of these isolates to DMI fungicides (Burpee, 1997).

Inoculum for each isolate was prepared by placing infested filter paper onto petri dishes of potato dextrose agar (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ) media (PDA) containing chloramphenicol, streptomycin, and tetracycline at 0.05 g/L each. Isolates were placed on a laboratory bench for three days at room temperature (25°C). A 10 mm plug was removed from the margin of an actively growing colony and placed on petri dishes containing non-amended PDA. After 3 days of growth on non-amended PDA, two mycelial plugs (10 mm) were removed from the actively growing fungal colony and placed in a culture tube (18x150 mm, Fisher Scientific) containing 6 g of sterilized rye grains, 10 ml of deionized water, and approximately 300 mg of calcium carbonate. Grain cultures were incubated for 4 weeks at 20°C to allow for colonization of the rye grains.

Bentgrass Establishment. Bentgrass cv. Penncross was seeded at a rate of 4.9 g/m^2 in Cone-Tainers™ (Steuwe & Sons, Inc., Corvallis, OR) measuring 3.8 cm in diameter and 21 cm long, with sand as the growth medium. Racks containing 98 Cone-Tainers each were placed on a greenhouse bench and allowed to mature for 7 weeks before inoculation. Pots were fertilized with a total of 36.6 g/m^2 of starter fertilizer (20-20-20 General Purpose Water-Soluble Fertilizer, Jack's Professional, J.R. Peters, Inc., Allentown, PA) throughout the 7 weeks. Final fertility applications were applied two weeks prior to inoculation. Bentgrass was trimmed twice a week with scissors to a height of approximately 1.27 cm to promote full growth of the turf stand in each Cone-Tainer.

Inoculation and Fungicide Application. The center of each Cone-Tainer™ was inoculated with two infested rye grains placed on a matte pin (3.3 cm long). Rye grains were adjusted to make contact with leaf blades within the grass canopy. The head of the matte pin was measured at a height of 1.27 cm above the surface of the Cone-Tainer. One Cone-Tainer per replication was not inoculated with rye grain inoculum to serve as a non-treated control.

Immediately after inoculation, Cone-Tainers™ were treated with difenoconazole + azoxystrobin (Briskway, Syngenta Crop Protection, Greensboro, NC), propiconazole (Banner Maxx II, Syngenta Crop Protection, Greensboro, NC), triadimefon (Bayleton Flo, Bayer Environmental Science, Research Triangle Park, NC), and triticonazole (Trinity, BASF Corporation, Research Triangle Park, NC). Treatments of each fungicide were formulated based on the respective recommended high label rate. Application rates defined in kg a.i. x ha^{-1} were transformed to ppm a.i. based on a water carrier of 0.082 L/m^2. Final fungicide concentrations were applied at a high label rate (HLR), a high sub-

lethal rate (HSLR), and a low sub-lethal rate (LSLR) (Table 4.1). The high sub-lethal rates and low sub-lethal rates were calculated by the high label rate x 10^{-5} and the high label rate x 10^{-10} respectively. Non-inoculated control and inoculated, no-fungicide treatments were separated from the fungicide application area. Fungicide treatments were applied with a CO_2-powered sprayer calibrated to 193 kPa using 8008 Teejet XR nozzles (Teejet Technologies, Glendale Heights, IL). Bentgrass treated with fungicide was allowed to dry for two hours in a separate space from bentgrass with control treatments before randomizing.

After fungicide treatments were applied, Cone-Tainers were placed in a growth chamber maintained at a temperature of 26/20°C day/night with a 12-h photoperiod. Turf leaf wetness was controlled by allowing an atomizing humidifier (Trion, Sanford, NC) to run for 6 h throughout the night period, followed by briefly drying the turf with a hair dryer (Remington, Spectrum Brands Inc) at the beginning of each day period. The turf was ultimately allowed 10-12 h of leaf wetness per day. Bentgrass was maintained at a height of 1.27 cm throughout the study by cutting with scissors twice a week.

Assessment of Disease Pressure. The experiment was designed as a split plot. Main plots consisted of the fungicide, non-inoculated control, and inoculated no-fungicide treatments, while subplots consisted of 4 replications of each isolate arranged as randomized blocks within each main plot. The entire experiment was repeated twice. Disease severity (Y), indicated as the proportion of diseased turf/area in each Cone-Tainer, was measured in two perpendicular directions with calipers (6" Fractional Digital Caliper, Carrera Precision, Maxtool, La Verne, CA) and averaged for analysis. Disease severity was recorded daily for 14 consecutive days after treatment, excluding day 10.

After day 14, disease severity was recorded at 21, 28, and 36 days after treatment. Area under the disease progress curve (AUDPC) was calculated for each isolate and fungicide treatment combination based on the following equation: AUDPC = $\sum_{i=1}^{N_i} \frac{(y_i+y_{i+1})}{2} (t_{i+1} - t_i)$ where y_i = disease symptom percentage, and t_i = time of the *i*th rating. Least square means for AUDPC and disease severity were subjected to analysis of variance in PROC GLIMMIX (SAS 9.4; SAS corporation, Cary, NC) and means were separated using Fisher's protected least significant difference ($\alpha = 0.05$).

Results

AUDPC Values of Inoculum Exposed to Sub-lethal Rates of Fungicides. Mean AUDPC values in the second experimental run were greater than the first experimental run ($P < 0.0001$). Bentgrass inoculated with resistant isolates displayed greater AUDPC values than bentgrass inoculated with sensitive isolates in both experimental runs ($P < 0.0001$). Moreover, inoculum of resistant isolates treated with the HLR of fungicides displayed greater AUDPC values than inoculum of sensitive isolates treated with the HLR of fungicides in both experimental runs ($P < 0.0001$). Inoculated bentgrass treated with the HLR of fungicides displayed the lowest mean AUDPC values within both experimental runs (Figure 4.4A; Figure 4.4B). Inoculated bentgrass treated with the HSLR of fungicides displayed greater AUDPC values in both experimental runs when compared to inoculated bentgrass with no fungicide treatment (Run 1 – $P = 0.027$; Run 2 – $P = 0.003$) (Figure 4.4A; Figure 4.4B).

The interaction of fungicide rate and fungicide type affected AUDPC values in both experimental runs (Table 4.2). In the first experimental run, AUDPC values of inoculum were increased when treated with both sub-lethal rates of triadimefon (HSLR –

P = 0.0004; LSLR – *P* = 0.048) (Figure 4.5A). In the second experimental run, AUDPC values of inoculum were increased when treated with the HSLR of difenoconazole + azoxystrobin (*P* = 0.0005) and the HSLR of triticonazole (*P* = 0.047) (Figure 4.5B). The interaction of isolate inoculum and fungicide rate affected AUDPC values in the first experimental run (Table 4.2), however, AUDPC values of inoculum treated with sub-lethal fungicide rates were not increased when compared to AUDPC values of inoculum with no fungicide treatment. The interaction of isolate inoculum and fungicide rate did not impact AUDPC values in the second experimental run.

Disease Severity of Inoculum Exposed to Sub-lethal Rates of Fungicides. Disease severity in the second experimental run was greater than disease severity in the first experimental run ($P < 0.05$) at each rating day. Inoculum treated with the HLR of fungicides displayed significantly lower disease severity than inoculum with no fungicide treatment ($P < 0.05$) 11 to 36 days after treatment in the first experimental run and 4 to 36 days after treatment in the second experimental run.

The interaction of isolate sensitivity, fungicide rate, and day after treatment had an effect on disease severity in both experimental runs (Table 4.3), though no increases in disease severity due to sub-lethal fungicide rates were observed (Figure 4.2; Figure 4.3). However, the interaction of fungicide rate and day after treatment did reveal instances of increased disease severity due to sub-lethal fungicide rates in both experimental runs. Disease severity of inoculum exposed to sub-lethal fungicide rates was greater than disease severity of inoculum with no fungicide application ($P < 0.05$) at day 21 and day 36 (the final rating day) in both experimental runs. In the first experimental run the increase at day 21 was observed with the HSLR of fungicides, while the increase at day

36 was observed with the HLSR and LSLR of fungicides. In the second experimental run the increase at day 21 and day 36 was only observed with the HSLR of fungicides. Additionally, disease severity of inoculum exposed to sub-lethal fungicide rates was greater than disease severity of inoculum with no fungicide application at 9 and 14 days after treatment (Figure 4.1A) in the first experimental run. Increased disease severity at day 9 was caused by LSLRs and HSLRs of fungicides. Increased disease severity at day 14 was only observed with HSLRs of fungicides. Also, disease severity of inoculum exposed to sub-lethal fungicide rates was greater than disease severity of inoculum with no fungicide application ($P < 0.05$) at 7, 12, and 28 days after treatment in the second experimental run (Figure 4.1B). Each instance of increased disease severity in the second experimental run was caused by HSLRs of fungicides. No increase was caused by LSLRs of fungicides in the second experimental run.

Discussion

A subset of six *Sclerotinia homoeocarpa* isolates were selected to analyze the effects of sub-lethal DMI fungicide exposure on dollar spot infected bentgrass. Isolates S088, A4, and D3 were selected to represent isolates expressing resistance to DMI fungicides and isolates S084, LWC10, and LWC27 were selected to represent isolates sensitive to DMI fungicides. Two sub-lethal fungicide rates were selected for evaluation based on NOAEL values determined *in vitro*. Previous studies focusing on the effects of sub-lethal fungicide exposure on plant pathogens have described differences between *in vitro* and *in vivo* studies (Zhou et al. 2014, Di et al. 2016). Moreover, it has been reported previously that *in vitro* fungicide dose responses by plant pathogens may not be comparable to *in vivo* fungicide dose responses (Bruin and Edgington 1981). Therefore,

an *in vivo* study was conducted to determine the effects of sub-lethal concentrations of DMI fungicides on disease severity and AUDPC from isolates ranging in sensitivities to DMI fungicides.

Mean AUDPC values of bentgrass inoculated with resistant isolates were greater than values of bentgrass inoculated with sensitive isolates in both experimental runs. Although the interaction of isolate sensitivity and fungicide rate was not significant for either experimental run, the HLR of fungicides provided more control of sensitive isolates than resistant isolates in each experimental run, supporting EC_{50} values determined previously *in vitro*.

Incidence of increased AUDPC values caused by sub-lethal fungicide rates of individual DMI fungicide types were inconsistent. Increased AUDPC values in the first experimental run were caused by both sub-lethal rates of triadimefon, while increased AUDPC values in the second experimental run were caused by the HSLR of difenoconazole + azoxystrobin and the HSLR of triticonazole. The hormetic growth curve typically displays a zone of no observable effects prior to the zone of hormetic growth (Lushchak, 2014). The consistent indications of increases in AUDPC values due to the HSLR of DMI fungicides suggests the concentration of the fungicide label rate x 10^{-5} may be within the hormetic range of DMI fungicides for some *S. homoeocarpa* isolates, while the LSLR of DMI fungicides may represent a concentration within the zone of no observable effects. No instances of increased AUPDC values were observed by the interaction of individual isolates and sub-lethal fungicide rates.

Inoculum treated with sub-lethal fungicide concentrations displayed instances of greater disease severity when compared to non-fungicide treated inoculum at particular

days throughout the study. Increased disease severity due to sub-lethal fungicide exposure across all isolates in the first experimental run was observed at 9, 14, 21, and 36 days after treatment. Increased mean disease severity due to sub-lethal fungicide exposure across all isolates in the second experimental run was observed at 7, 12, 21, 28, and 36 days after treatment. Increased disease severity due to sub-lethal rates of fungicides occurred as early as 7 days after treatment, however, increased severity due to sub-lethal fungicide rates were more frequent later in the study (from 12 to 36 days after treatment). Fungicide degradation occurs in a turfgrass system due to microbial breakdown, photodecomposition, and volatilization. Lower fungicide rates were likely present 36 days after treatment due to degradation, therefore, lower rates of fungicide may be responsible for the observations of increased mean disease severity near the end of the study. The HSLR of fungicides caused increased mean disease severity at the final rating day in both experimental runs, suggesting an increase in disease severity may be more frequently initiated by a range of DMI fungicide doses lower than the HSLRs used in this study. More sub-lethal DMI fungicide concentrations near the HSLRs utilized in this study should be tested to determine the full hormetic range for *S. homoeocarpa*.

Hormesis is a reproducible biological phenomenon characterized by inhibition at a high dose of a stressor and stimulation at a low dose of the same stressor (Calabrese et al. 1999). The parameters of hormesis are often described as consistent throughout biological organisms and replications (Calabrese 2015), however, the fundamental mechanism of hormesis has not been fully elucidated. Quantification and assessment of chemical hormesis in fungal plant pathogens requires stringent experimental factors and is typically executed in laboratory conditions. In the current study, assessment of

fungicide induced hormesis was tested in growth chamber conditions with standardized fungicide concentrations, fungal inoculum, and host species. An increase in disease severity by *S. homoeocarpa* isolates due to applications of sub-lethal doses of DMI fungicides was observed in this study.

Greenhouse conditions in this experiment were maintained to resemble field conditions. However, there are numerous differences in environmental conditions and cultural practices between greenhouse and field settings. Stimulatory effects of dollar spot severity by sub-lethal doses of DMI fungicides in controlled *in vivo* conditions may differ from effects in field conditions. Future studies should focus on determining the effects of multiple sub-lethal DMI fungicide concentrations on the disease severity of various dollar spot isolates in field conditions.

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Table 4.1. Fungicide concentrations of DMI fungicides applied to inoculated bentgrass in Cone-Tainers™. Concentrations are reported in ppm (μg ml^{-1}) of active ingredient (a.i.). Treatments of each fungicide were formulated based on the respective recommended high label rate. Application rates defined in kg a.i. x ha^{-1} were transformed to ppm a.i. based on a water carrier of 0.082 L/m^2.

Fungicide	High Label Rate	High Sub-Lethal Rate	Low Sub-Lethal Rate
Difenoconazole + Azoxystrobin	356.4	3.56x10-3	3.56x10-8
Propiconazole	1217.05	1.21x10-2	1.21x10-7
Triadimefon	1942.1	1.94x10-2	1.94x10-7
Triticonazole	1582.1	1.58x10-2	1.58x10-7

Table 4.2. Effects of isolate sensitivity, DMI fungicide type, and fungicide rate on mean AUDPC values of dollar spot infected bentgrass throughout the first and second experimental run. AUDPC values represent the total disease severity calculated over the 36 day period. ANOVA table was produced using PROC GLIMMIX in SAS.

AUDPC		**Run 1**	**Run 2**
Effect	**DF**	**Pr > F**	**Pr > F**
Run	1	<.0001	
Sensitivity	1	<.0001	<.0001
Rate	3	<.0001	<.0001
Fungicide	3	0.2	0.2058
Fungicide*Rate	9	0.0002	0.0172
Sensitivity*Rate	3	0.0568	0.4016
Sensitivity*Rate*Fungicide	9	0.4061	0.7436

Table 4.3. Effects of day after treatment (DAT), isolate inoculum, incoulum sensitivity to DMI fungicides, DMI fungicide type, and fungicide rate on disease severity of dollar spot infected bentgrass throughout the first and second experimental run. ANOVA table was produced using PROC GLIMMIX in SAS.

Disease Severity		**Run 1**	**Run 2**
Effect	**DF**	**Pr > F**	**Pr > F**
Run	1	<.0001	
Sensitivity	1	<.0001	<.0001
Rate	3	<.0001	<.0001
Fungicide	3	<.0001	<.0001
DAT	13	<.0001	<.0001
DAT*Sensitivity	13	<.0001	<.0001
DAT*Rate	39	<.0001	<.0001
Sensitivity*DAT*Rate	39	<.0001	<.0001

Table 4.4. Total disease severity of bentgrass inoculated with isolates of *Sclerotinia homoeocarpa*. EC_{50} values are based upon the average EC_{50} value to four DMI fungicides determined previously via a mycelial growth assay. AUDPC represents the total disease severity calculated over 36 days. Values are based upon the mean AUDPC values of four replications of the isolate treated with the respective rate of four DMI fungicides. Rates include - No fungicide; High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); High label rate (HLR).

AUDPC									
		No Fungicide		HSLR		LSLR		HLR	
		Exp Run		Exp Run		Exp Run		Exp Run	
Isolate	**EC50**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
LWC10	0.011	10.6	17.3	13.1	16.3	10.7	14.5	1.22	3.40
S084	0.021	12.6	13.7	11.6	13.7	13.4	13.5	2.11	3.37
LWC27	0.033	10.8	12.7	9.72	13.5	8.77	11.2	0.65	3.19
S088	0.125	11.2	15.2	14.9	20.1	13.0	18.1	5.63	8.12
A4	0.367	12.5	21.1	12.9	21.2	13.5	17.8	6.58	9.47
D3	0.399	16.2	18.3	13.5	19.3	14.3	16.1	5.71	9.10

Table 4.5. Disease severity at 7 days after treatment (DAT) of bentgrass inoculated with isolates of *Sclerotinia homoeocarpa.* EC_{50} values are based upon the average EC_{50} value to four DMI fungicides determined previously via a mycelial growth assay. Disease severity is indicated as the proportion of diseased turf/area in each Cone-Tainer™. Values are based upon the mean disease severity at 7 days after treatment of four replications of the isolate treated with the respective rate of four DMI fungicides. Rates include - No fungicide; High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); High label rate (HLR).

Disease Severity – 7 DAT									
		No Fungicide		HSLR		LSLR		HLR	
		Exp Run		Exp Run		Exp Run		Exp Run	
Isolate	**EC50**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
LWC10	0.011	0.00	28.7	12.7	34.7	6.27	26.1	0.00	0.00
S084	0.021	0.00	31.9	5.89	20.1	10.4	19.0	0.00	0.00
LWC27	0.033	0.00	10.5	1.82	29.2	1.54	16.1	0.00	0.00
S088	0.125	5.80	31.7	17.5	55.2	14.4	47.5	0.00	7.28
A4	0.367	7.84	57.2	10.4	57.9	9.13	49.8	0.00	9.58
D3	0.399	8.05	48.0	8.07	52.1	14.0	30.2	2.37	9.25

Table 4.6. Disease severity at 14 days after treatment (DAT) of bentgrass inoculated with isolates of *Sclerotinia homoeocarpa*. EC_{50} values are based upon the average EC_{50} value to four DMI fungicides determined previously via a mycelial growth assay. Disease severity is indicated as the proportion of diseased turf/area in each Cone-Tainer™. Values are based upon the mean disease severity at 14 days after treatment of four replications of the isolate treated with the respective rate of four DMI fungicides. Rates include - No fungicide; High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); High label rate (HLR).

Disease Severity – 14 DAT									
		No Fungicide		HSLR		LSLR		HLR	
		Exp Run		Exp Run		Exp Run		Exp Run	
Isolate	**EC50**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
LWC10	0.011	19.0	50.7	24.8	45.1	23.4	37.0	0.00	0.00
S084	0.021	33.9	38.5	22.8	33.7	31.8	37.0	1.81	1.51
LWC27	0.033	22.6	37.9	18.6	31.7	15.4	27.6	0.00	0.00
S088	0.125	17.1	45.2	36.0	59.8	29.0	57.0	4.93	16.5
A4	0.367	9.90	62.8	30.9	66.2	24.5	53.4	0.00	21.6
D3	0.399	33.1	50.1	24.8	56.1	29.2	47.3	5.76	17.0

Table 4.7. Disease severity at 21 days after treatment (DAT) of bentgrass inoculated with isolates of *Sclerotinia homoeocarpa*. EC_{50} values are based upon the average EC_{50} value to four DMI fungicides determined previously via a mycelial growth assay. Disease severity is indicated as the proportion of diseased turf/area in each Cone-Tainer™. Values are based upon the mean disease severity at 21 days after treatment of four replications of the isolate treated with the respective rate of four DMI fungicides. Rates include - No fungicide; High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); High label rate (HLR).

Disease Severity – 21 DAT									
		No Fungicide		HSLR		LSLR		HLR	
		Exp Run		Exp Run		Exp Run		Exp Run	
Isolate	**EC50**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
LWC10	0.011	39.3	56.1	48.3	57.2	41.3	50.5	5.90	0.00
S084	0.021	54.0	43.0	48.0	48.3	49.5	43.9	8.40	0.00
LWC27	0.033	33.5	40.1	40.4	49.8	33.9	39.6	0.90	0.00
S088	0.125	43.3	48.9	54.0	64.0	48.1	58.1	26.0	21.6
A4	0.367	52.6	63.2	45.8	67.2	48.3	56.9	25.2	29.5
D3	0.399	59.6	51.7	47.3	61.4	50.1	52.9	16.3	27.2

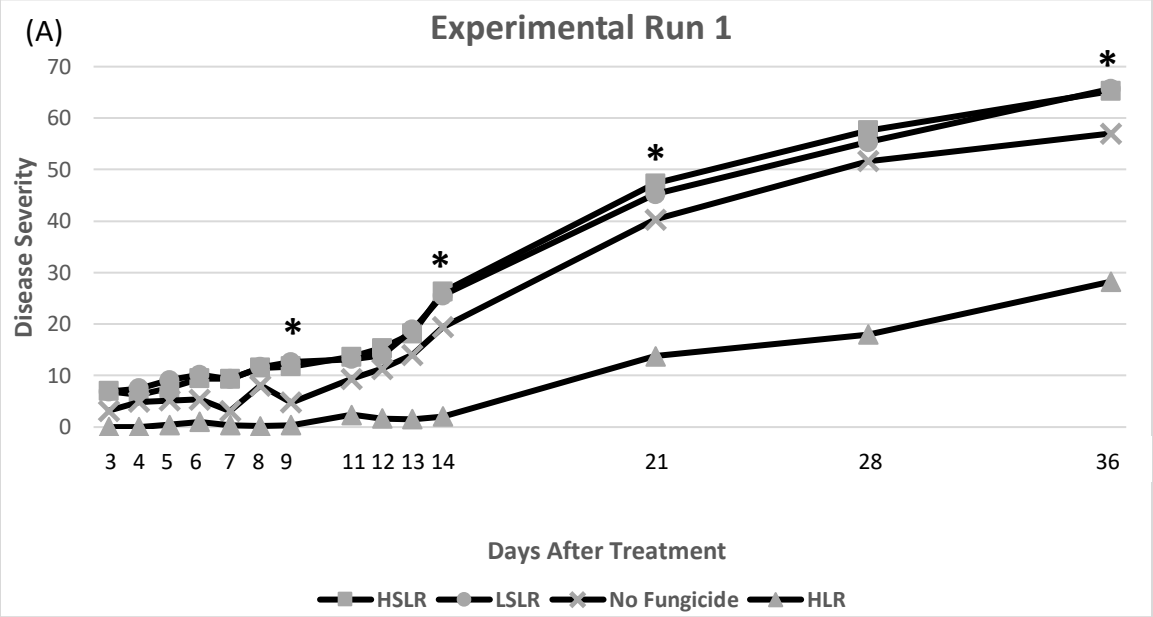
Table 4.8. Disease severity at 28 days after treatment (DAT) of bentgrass inoculated with isolates of *Sclerotinia homoeocarpa*. EC_{50} values are based upon the average EC_{50} value to four DMI fungicides determined previously via a mycelial growth assay. Disease severity is indicated as the proportion of diseased turf/area in each Cone-Tainer™. Values are based upon the mean disease severity at 28 days after treatment of four replications of the isolate treated with the respective rate of four DMI fungicides. Rates include - No fungicide; High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); High label rate (HLR).

Disease Severity – 28 DAT									
		No Fungicide		HSLR		LSLR		HLR	
		Exp Run		Exp Run		Exp Run		Exp Run	
Isolate	**EC50**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
LWC10	0.011	55.2	63.3	60.2	58.4	50.4	56.1	5.20	22.6
S084	0.021	60.0	49.5	56.2	58.0	59.2	55.3	6.70	17.3
LWC27	0.033	59.3	52.6	42.4	50.3	42.7	46.2	2.90	18.2
S088	0.125	57.1	51.9	61.5	63.0	53.8	58.4	26.0	34.6
A4	0.367	62.1	64.2	62.2	66.5	63.6	55.2	36.3	39.4
D3	0.399	67.8	64.7	63.3	63.4	62.4	57.4	30.6	38.8

Table 4.9. Disease severity at 36 days after treatment (DAT) of bentgrass inoculated with isolates of *Sclerotinia homoeocarpa*. EC_{50} values are based upon the average EC_{50} value to four DMI fungicides determined previously via a mycelial growth assay. Disease severity is indicated as the proportion of diseased turf/area in each Cone-Tainer™. Values are based upon the mean disease severity at 36 days after treatment of four replications of the isolate treated with the respective rate of four DMI fungicides. Rates include - No fungicide; High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); High label rate (HLR).

Disease Severity – 36 DAT									
		No Fungicide		HSLR		LSLR		HLR	
		Exp Run		Exp Run		Exp Run		Exp Run	
Isolate	**EC50**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
LWC10	0.011	60.8	73.0	62.7	68.2	58.6	67.9	10.4	41.7
S084	0.021	61.8	63.2	62.7	67.4	70.4	69.0	23.7	48.2
LWC27	0.033	65.5	62.9	62.2	65.5	53.7	60.7	9.04	45.6
S088	0.125	62.7	64.5	69.0	71.1	64.2	66.2	37.7	59.2
A4	0.367	72.7	74.4	67.2	72.9	75.4	65.4	50.0	58.0
D3	0.399	75.7	76.4	71.4	72.4	71.8	66.6	43.6	57.6

Figure 4.1. Disease severity in experimental run 1 (A) and experimental run 2 (B) caused by *S. homoeocarpa* inoculum throughout 36 days. No disease was observed at day 1 or day 2. Lines represent DMI fungicide concentrations – High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); No fungicide; High label rate (HLR). Disease severity is based on the means of each isolate throughout individual rating days. Standard error of the mean is 5.01 for run 1 and 8.3 for run 2. Disease severity of inoculum treated with sub-lethal fungicide rates that is significantly different than inoculum with no fungicide treatment is indicated above the respective data points. Significant differences among sub-lethal fungicide rates and no fungicide treatments were evaluated with Fisher's protected LSD (α = 0.05).



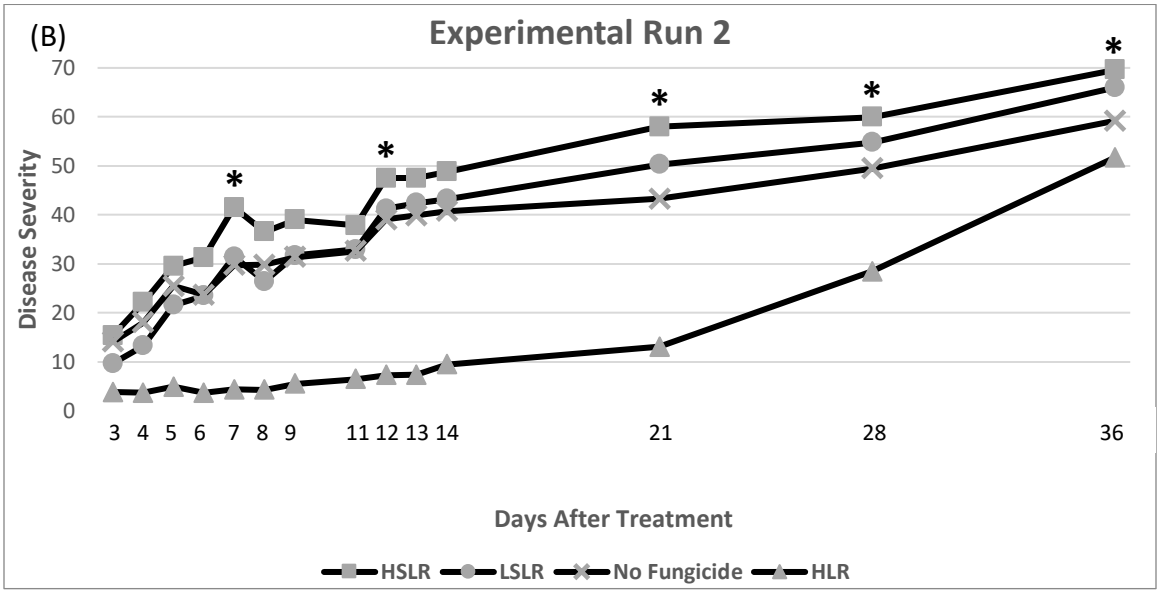


Figure 4.2. Disease severity in experimental run 1 caused by *S. homoeocarpa* inoculum throughout 36 days. No disease was observed at day 1 or day 2. Each panel represents a treatment of a DMI fungicide concentration – No fungicide; High label rate (HLR); High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR). Disease severity is based on the means of the respective sensitivity category throughout individual rating days. Values are averaged across all four fungicides. Error bars represent the standard error of the mean (4.49). No significant differences were observed among sensitivity categories treated with sub-lethal fungicides rates and sensitivity categories with no fungicide treatment. Significant differences among sub-lethal fungicide rates and no fungicide treatments were evaluated with Fisher's protected LSD (α = 0.05).

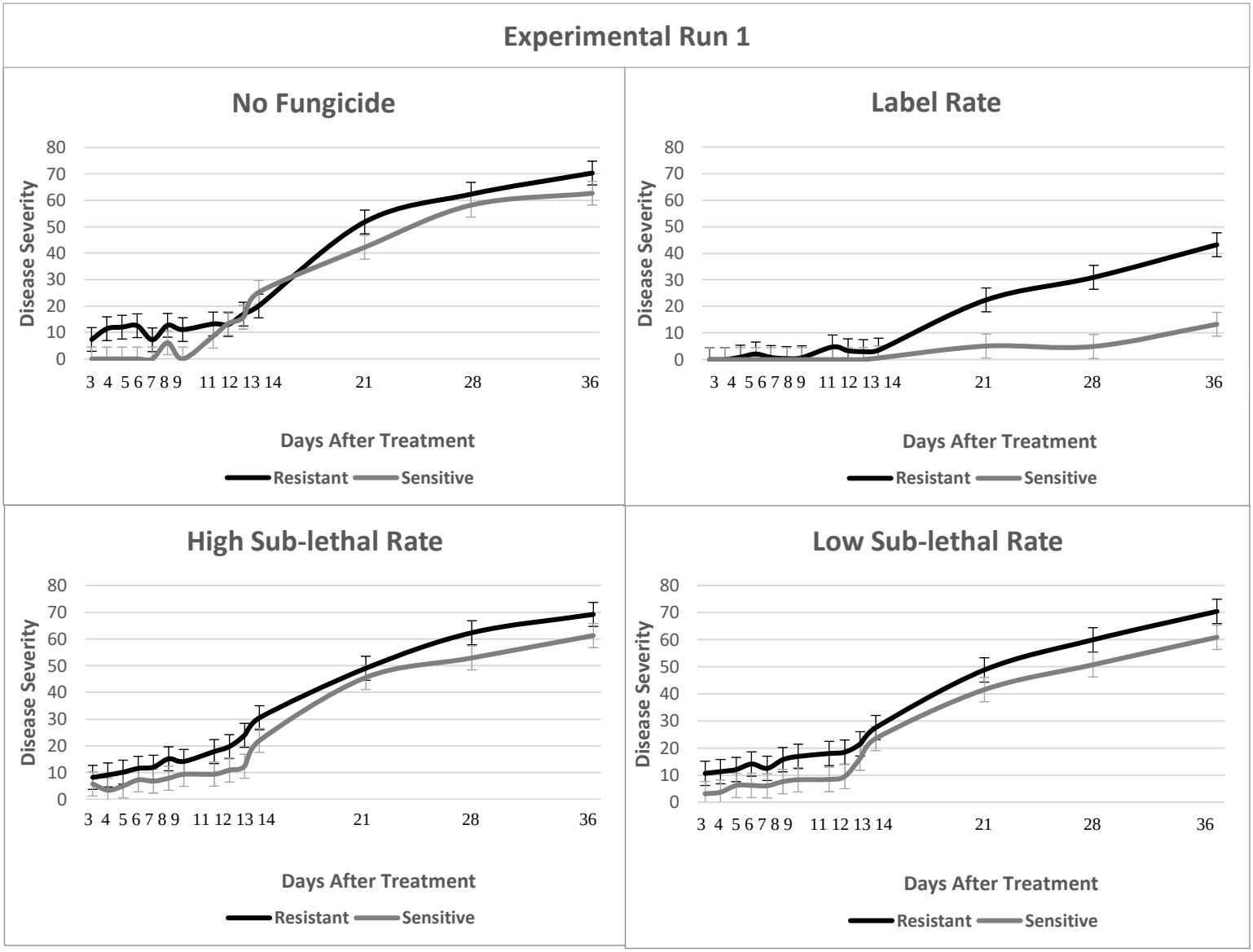


Figure 4.3. Disease severity in experimental run 2 caused by *S. homoeocarpa* inoculum throughout 36 days. No disease was observed at day 1 or day 2. Each panel represents a treatment of a DMI fungicide concentration – No fungicide; High label rate (HLR); High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR). Disease severity is based on the means of the respective sensitivity category throughout individual rating days. Values are averaged across all four fungicides. Error bars represent the standard error of the mean (6.21). No significant differences were observed among sensitivity categories treated with sub-lethal fungicides rates and sensitivity categories with no fungicide treatment. Significant differences among sub-lethal fungicide rates and no fungicide treatments were evaluated with Fisher's protected LSD (α = 0.05).

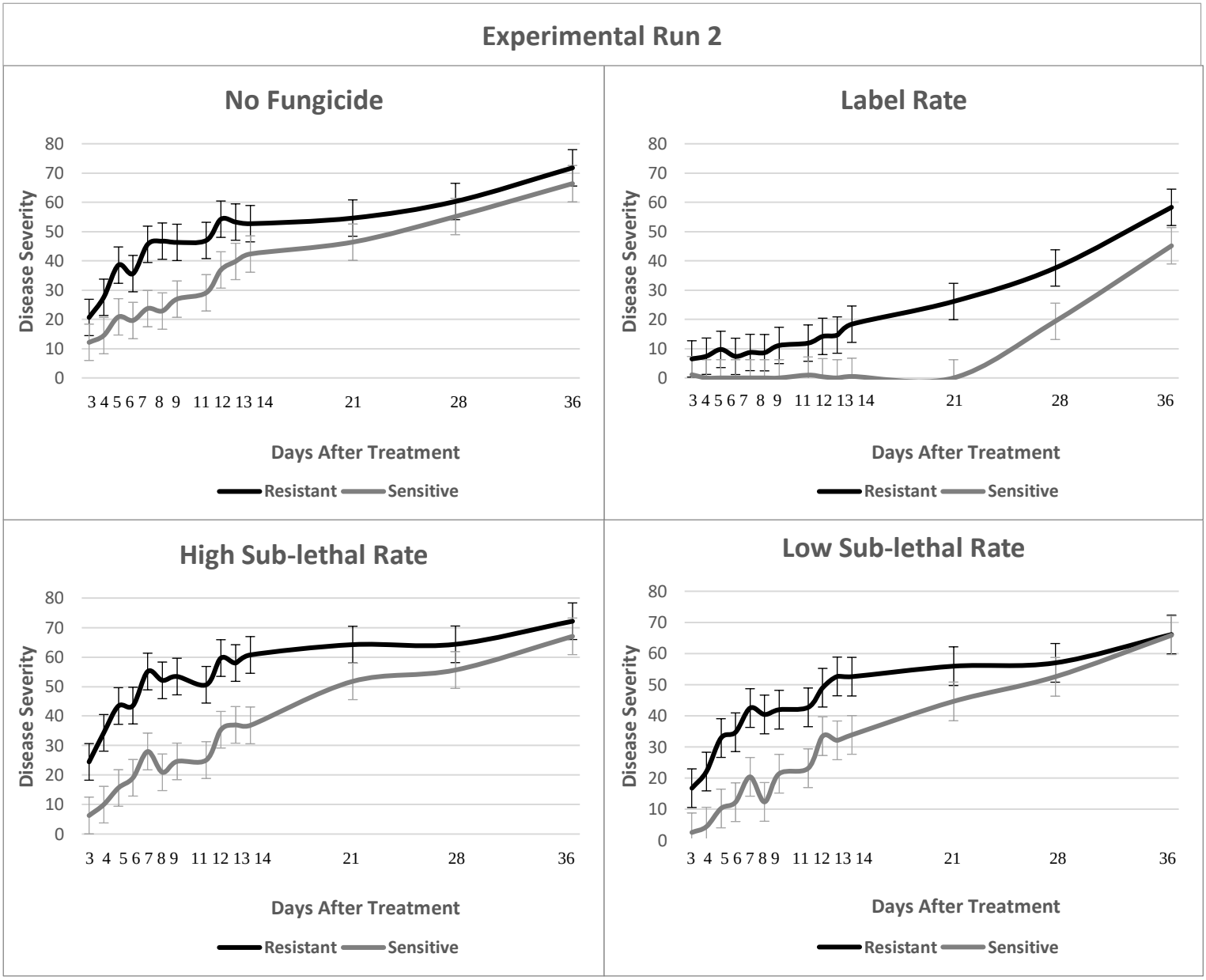
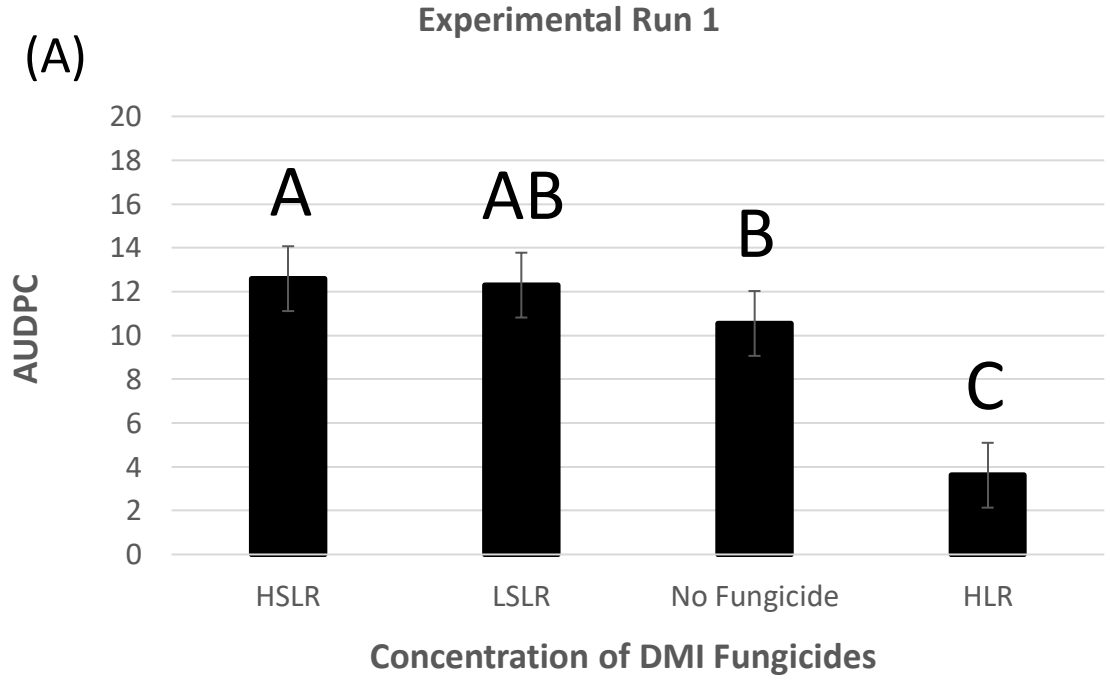


Figure 4.4. Mean AUDPC values in experimental run 1 (A) and experimental run 2 (B) caused by *S. homoeocarpa* inoculum. Bars represent DMI fungicide concentrations – High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); No fungicide; High label rate (HLR). AUDPC values represent the total disease severity calculated over the 36 day period. Mean AUDPC is based on the total AUDPC of each isolate throughout the study. Error bars represent the standard error of the mean (Run 1 – 1.4; Run 2 – 2.1). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).



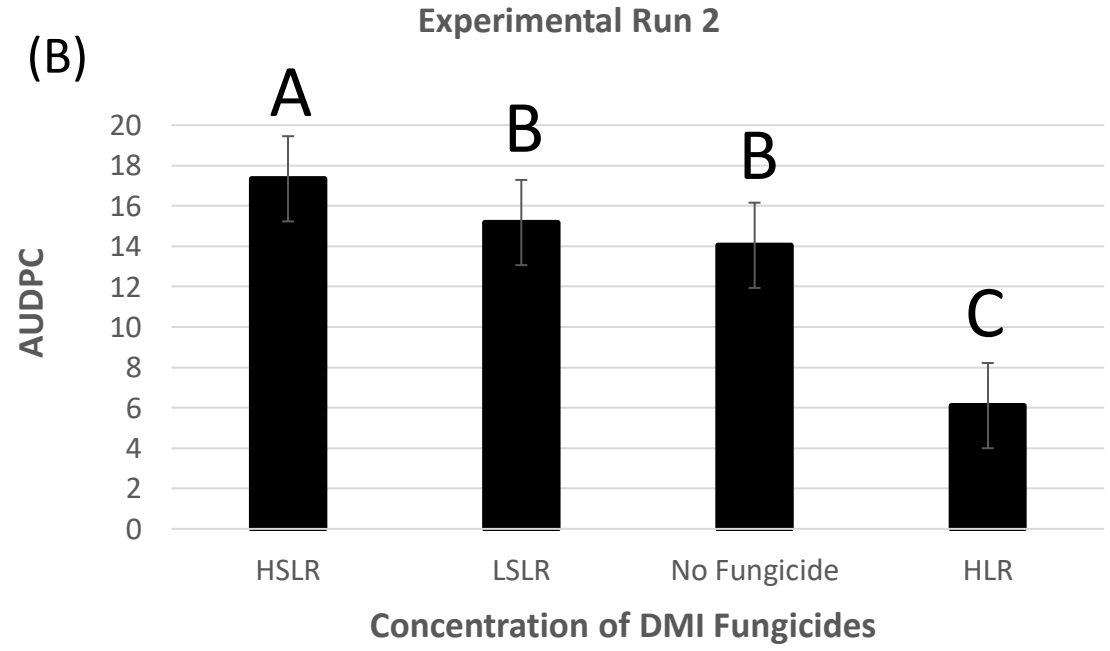


Figure 4.5. Mean AUDPC of inoculum treated with four types of DMI fungicides in the first experimental run (A) and the second experimental run (B). Bars represent different fungicide rates - High sub-lethal rate (HSLR); Low sub-lethal rate (LSLR); Label Rate; No fungicide. AUDPC values represent the total disease severity calculated over the 36 day period. Mean AUDPC is based on the total AUDPC of each replicate throughout the study. Error bars represent the standard error of the mean (Run 1 – 1.4; Run 2 – 2.1). Bars with the same letters are not significantly different from each other according to Fisher's protected LSD (α = 0.05).