CHARACTERIZATION OF SENSITIVITY OF *SCLEROTINIA SCLEROTIORUM*

ISOLATES FROM NORTH CENTRAL US TO THIOPHANATE-METHYL AND

METCONAZOLE

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary causes Sclerotinia stem rot on canola and many other crops of economic importance in the U.S. SSR is primarily controlled with fungicides applied at flowering time. Most fungicides currently used to control SSR can promote resistance buildup in their target populations making monitoring of sensitivity important. In this study the reaction of *S. sclerotiorum* to thiophanate-methyl (TM) and metconazole (MTZ) was characterized. Samples collected in several states of north central U.S. were used. Three and ten isolates were considered to be moderately insensitive to TM and MTZ, respectively. Greenhouse trials indicated, however, that diseases caused by these isolates could be effectively controlled using currently recommended doses of each compound. *In vitro* sensitivity to TM was temperature dependent. A previously unreported mutation at codon E111D in the β-tubulin gene of a TM-moderately insensitive isolate was identified.

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DEDICATION

This thesis is dedicated to my parents Mr. V.T. Haneef and Mrs. S. Bano who believed in me

and supported my every decision and encouraged me to be a better person every day.

This thesis is also dedicated to would be husband, Shyam Solanki, who has inspired,

encouraged and motivated me to succeed.

Last but not the least, this thesis is dedicated to all who have love of learning.

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CHAPTER 1. LITERATURE REVIEW

Introduction

Canola

Canola (*Brassica napus* L.) is a crop with a short history in North Dakota although its economic importance continues to increase very rapidly. The crop was introduced from Canada as oilseed rape probably in the early 1980s. The development of canola cultivars, oilseed rape genotypes with low erucic acid and glucosinolates contents, in the late 1980s, helped popularize this crop in the region. By 1991, approximately 8,000 ha were planted in the northeastern corner of the state (Lamey and Hershman, 1993). By 2012, the area planted to canola had increased to 348,000 ha statewide (USDA/NASS, 2011). Along with increased hectarage, the market value of this commodity has increased from less than US$ 5 million in 1991 to US$ 297 million in 2011 (USDA/NASS, 2011). By 2006 more than 90% of the canola planted in the United States was located in North Dakota (del Rio *et al.,* 2007).

Sclerotinia stem rot

Sclerotinia sclerotiorum (Lib.) de Bary is the necrotrophic plant pathogenic fungus that causes Sclerotinia stem rot (SSR) on canola and many other important crops (Boland and Hall 1994, Purdy 1979). This pathogen has a broad geographical distribution, and is prevalent in many US states including those in the North Central region (Bradley and Lamey, 2005). SSR epidemics that affected soybeans in 1997, 2004 and 2009 resulted in losses estimated at more than US$1,000 million (USDA/NASS, 2011). SSR is endemic in canola-growing regions of North Dakota. Between 1991 and 2002, the annual average SSR incidence was 13.6% (Bradley and Lamey, 2005) with an estimated direct economic impact of approximately US$94 million

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(Lamey, 2003). Virtually, all commercial canola genotypes grown in the region are susceptible to the disease and lose an average of 0.5% of their yield potential for every percentage unit of SSR incidence (del Rio *et al.*, 2007). In addition to causing yield losses SSR reduces seed quality. Seed contaminated with sclerotia may receive lower prices and also serves as an important source of inoculum if planted into fields with no history of SSR (Kull *et al.*, 2004). Infected seeds can have reduced germination and in some cases also oil and protein concentrations may be reduced (Danielson *et al.*, 2004). The ability of *S. sclerotiorum* to infect a diverse group of plants under different climatic conditions and its ability to produce sclerotia that persist in the soil for almost eight years, explain in part its successful persistence and wide geographic distribution (Adams and Ayers, 1979).

Taxonomy of the pathogen

Taxonomically, *Sclerotinia sclerotiorum* (Lib.) de Bary belongs to the Kingdom Fungi as it has cell walls made of chitin and glucans. Its thallus is of mycelial type, no motile cells, with septate hyphae and produces sexual spores, ascospores, inside ascus, characteristics that rest it in the Phylum Ascomycota (Alexopoulos *et al.,* 1996). Ascomycota is the largest phylum of fungi with 3,400 genera and more than 32,000 species; fungi in this phylum, have septa with simple pores and Woronin bodies (Alexopoulos *et al.,* 1996). The fruiting bodies, ascomata, are cup-shaped structures called apothecia. In the apothecia, sexual reproduction occurs by the fusion of two nuclei from compatible mating types that form a zygote. Meiotic division of the dikaryon and subsequent crozier formation and mitosis results in production of 8 ascospores per ascus (Alexopoulos *et al.,* 1996). This fungus is homothallic or self-compatible, which means the sexual reproduction occurs in a single thallus; both mating type idiomorphs are present in the same nucleus (Alexopoulos *et al.,* 1996). Production of apothecium, a disc or cup-shaped fruiting

body, places *S. sclerotiorum* within the Discomycetes (Alexopoulos *et al.,* 1996). The cup's interior surface or hymenium has asci along with few sterile elements or hamathecium called paraphysis. The ascus is inoperculate, which means it does not have an opening to release ascospores; instead, ascospores are released through a pore or canal, by rupturing of the ascus apex or by disintegration of the ascus wall. The new classification system assigns *Sclerotinia* in the Leotiomycetes within the orders Helotiales (Maddison and Schulz, 2007). The order Helotiales is composed by fungi that produce unitunicate (one layer) inoperculate asci and consists of 13 families, one of them being the Sclerotiniaceae. Whetzel established the family Sclerotiniaceae in 1945. The other taxonomic characters used for delimiting Sclerotiniaceae since Whetzel's, include the characteristics of sterile tissues of both apothecia and sclerotia (Kohn, 1979a,b; Korf and Dumont, 1972); sclerotial ontogeny (Willetts and Wong, 1980); histochemistry and ultrastructure of sclerotia (Backhouse and Willetts, 1984); biochemical characteristics (Carbone and Kohn, 1993) and *r*RNA gene sequences (Holst-Jensen *et al.,* 1997a,b). Fungi belonging to this family often form sclerotia, which are melanized hyphal aggregates (Alexopoulos *et al.,* 1996). The melanized sclerotia plays a key role in the life cycle of the fungus because it can germinate both vegetatively, producing mycelium to colonize locally, or carpogenically, producing apothecia in which approximately 7 million ascospores will be formed. *S. sclerotiorum* do not produce asexual spores or conidia. Currently, 33 genera within the Sclerotiniaceae are recognized (Willets, 1997). For species separation the characters used are size of sclerotia (Jagger, 1920), host association (Kreitlow, 1949), ascus and ascospores sizes (Ramsey, 1924), and nuclear and mitochondrial RFLP analyses (Kohn *et al.,* 1988). *S. sclerotiorum* is a homothallic (self-fertile) fungus with only occasional outcrosses.

Previous studies showed that the difference in the breeding system is a key reason of an organization of MAT genes at one or more MAT loci (Debuchy, 2010). Thus the classification based on tree of life project is (Maddison and Schultz, 2007)

Kingdom: Fungi

Phylum: Ascomycota

Class: Leotiomycetes

Order: Helotiales

Family: Sclerotiniaceae

Genus: *Sclerotinia*

Species: *sclerotiorum*.

Pathogen biology and disease cycle

Disease cycle

Sclerotia overwinter in the soil, and may germinate the following summer either by producing mycelium or apothecium. Not all sclerotia will germinate the following summer, however. Myceliogenic germination of sclerotia produces hyphae that can directly infect plant tissue (Brandin and Huang, 2001). When soils are shaded, moist and cool (40-60° F; 4-16° C), sclerotia within the top 4-5 cm of the soil profile can germinate to produce apothecia (Adams and Ayers, 1979; Wu and Subbarao, 2008; Peltier *et al.,*, 2012). During carpogenic germination one or more apothecial initials, known as stipes, are produced by sclerotia; this stipes later develops into cup-shaped apothecia where millions of ascospores are formed (Abawi and Grogan, 1979; Willets and Wong, 1980). Ascospores are the primary source of inoculum for most diseases caused by this pathogen (Abawi and Grogan, 1979). In field conditions, ascospores are released

during a period of five to ten days depending on weather conditions (Phillips, 1987). Ascospores infect the above ground portion of the host plant. Ascospores require exogenous nutrient sources and a film of water to germinate (Bolton *et al.*, 2006). Senescing flower parts serve as primary sources of nutrients for ascospores as they fall on leaves, petioles or stem (Turkington and Morrall, 1993). Infection by *S. sclerotiorum* is favored by cool to moderate daily temperatures with a maximum $< 85^o$ F or 29° C, and moisture from rain, fog, dew, or high relative humidity (Workneh and Yang, 2000). High disease incidence could occur in dense crop canopies powered by favorable micro environmental conditions (Turkington and Morrall, 1993). A dense plant canopy during flowering, growth stages 4.1 (beginning of flowering) and 4.2 (beginning of pod formation) in the growth scale of Harper and Berkenkamp (1975), increase the likelihood of Sclerotinia stem rot development (Grau and Hartman, 1999).

Signs and symptoms of sclerotinia stem rot

Sclerotinia stem rot symptoms are observed normally at the end of the flowering season. Leaves usually have water-soaked lesions that expand rapidly and move through the petiole and into the stem. Infected stems develop water-soaked lesions which turn into necrotic tissues that could be covered by white fluffy mycelium and/or sclerotia (Bolton *et al.*, 2006).

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Disease management

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Some of the most commonly recommended practices for disease management, like the use of resistant cultivars and crop rotations that alternate one season of the target crop with two or three seasons of non-host crops, are of limited benefit when managing SSR. Most commercial canola, soybean, and sunflower cultivars are susceptible to *S. sclerotiorum* and the benefic effects of crop rotations are reduced by the ability of sclerotia to survive in the soil for long periods of time (Adams and Ayers, 1979). When used alone, other cultural practices like the use

of clean seed, maintaining adequate plant population densities within recommended row and plant spacing, and use of moderate fertilization programs are not enough to effectively control this disease. The efficacy of fungicide use could be increased when growers scout their fields for the presence of apothecia one to two weeks prior to flowering and check the available risk maps to make better informed spraying decisions (National Sclerotinia Initiative, 2012). Breeding for resistance against *S. sclerotiorum* is difficult as resistance to this disease is governed by multiple genes (Fuller *et al.*, 1984). Biological control of Sclerotinia diseases is pursued as an alternative disease control strategy that is partially associated with crop rotations. Under field conditions, sclerotia are attacked and degraded by a number of mycoparasites, such as *Coniothyrium minitans* and *Sporidesmium sclerotivorum* (Ayers and Adams, 1981). Biological activity in the soil could reduce sclerotial numbers by as much as 95% and SSR incidence by 10 to almost 70% (Sesan and Csep, 1992; Boland 1997; Zeng *et al.,* 2012). Since biological control would not eliminate all sclerotia, plants in heavily infested fields would continue to become infected by *S. sclerotiorum* (Peltier *et al.*, 2012). Therefore use of fungicides is the utmost important management strategy growers rely on for control of *S. sclerotiorum*. Different biological and chemical fungicides have been studied for the efficient management of Sclerotinia diseases (Bradley *et al.,* 2006; del Rio *et al.,* 2002; Fernando *et al.*, 2007).

Chemical control

Use of fungicides has been the primary method of controlling SSR. Field trials conducted in North Dakota and Minnesota from 2000 to 2004 resulted in a consistent reduction of SSR when plants were treated with azoxystrobin, benomyl, boscalid, iprodione, prothioconazole, tebuconazole, thiophanate-methyl, trifloxystrobin or vinclozolin (Bradley *et al.*, 2006). Since then vinclozolin and benomyl have been retired from the market due to health concerns and

development of fungicide-resistant strains. Fungicides registered for control of Sclerotinia stem rot in North Dakota are: azoxystrobin (Quadris[R]), boscalid (Endura[R]), metconazole (Quash[R]), prothioconazole (Proline[R]), pyraclostrobin (Headline[R]), and thiophanate-methyl (Topsin[R]) (McMullen and Markell, 2010). Also, the biological control compounds Serenade and Polyversum are registered for use against Sclerotinia in foliar applications (McMullen and Markell, 2010). The efficacy of a fungicide application depends on the timing of its delivery to match plant development and environmental conditions that favor infection (McMullen and Markell, 2010). Fungicides should be applied when plants are somewhere between 25% bloom (Dueck *et al*., 1983) and 50% bloom (Thomson *et al.,* 1984). To increase the efficacy of applications, fungicide should be used only when the risk of infection is high in a field where apothecia are present and the plants have not passed the 50% flowering stage.

Fungicide resistance

Repeated use and/or misuse of fungicides may result in the development of targeted populations that are no longer sufficiently sensitive to it. These populations may also appear in response to repeated use of another fungicide which is related to it chemically and/or biochemically through a common mechanism of antifungal action (FRAC, 2007). Fungicide resistance can be conferred by various mechanisms including: (I) an altered target site, which reduces the binding of the fungicide; (II) the synthesis of an alternative enzyme capable of substituting the target enzyme; (III) the overproduction of the fungicide target; (IV) an active efflux or reduced uptake of the fungicide; and (V) a metabolic breakdown of the fungicide (Ma and Michailides, 2005; Gisi *et al.,* 2000; Gullino *et al.,* 2000; Fluit *et al.,* 2001; MgGrath, 2001). Some of the compounds registered for control of *S. sclerotiorum* in North Dakota are also used to manage diseases that affect crops grown in rotation with canola such as smut, rust and root rot

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(Markell and Khan, 2012). This action could lead to the unintended exposure of *S. sclerotiorum* apothecia that sprout under their canopies as well as of the sclerotia on a more regular basis. This unintended exposure could exert selection pressure on the pathogen with the consequent buildup of tolerance over a period of time (FRAC, 2007). This insensitivity of the pathogen could lead to a condition where the fungicide is no longer economically viable to control the disease. Thus, evaluation of fungicide sensitivity of a pathogen is highly recommended on timely basis.

Fungicide description and mechanism of resistance

TM is the oldest fungicide in use for SSR control in North Dakota (McMullen and Bradley, 2003). The benzimidazole compound was registered in 1973 and reregistered as a systemic fungicide in Nov., 1984 due to the adoption of new standards by the United States Environment Protection Agency (USEPA, 2004). The primary metabolite in TM is carbendazim (methyl 2-benzimidazole carbamate) or MBC. TM falls under FRAC code 1 and acts on the mitosis and cell division in the target fungi. TM is considered a high resistance risk compound.

MTZ is one of the most recently registered compounds for control of *S. sclerotiorum* in canola; however, MTZ was initially registered in the US in 2007 for use on turf and ornamentals as systemic fungicide (USEPA, 2006). The FRAC code for MTZ is 3 and the compound acts as demethylation inhibitor (DMI) of 24-methylenedihydrolanosterol at C14, a precursor of ergosterol in fungi (Brent, 1995). According to EPA (USEPA, 2006), MTZ may be applied at a rate of 219 ml/ha as ground sprays only.

Resistance to thiophanate-methyl

Problems of resistance to benzimidazoles (benomyl) were first reported in *Monilinia fructicola* in 1975 in Australia (Whan, 1976), Michigan (Jones, 1976; Zehr, 1982) and New York

(Szkolnik and Gilpatrick, 1977; Zehr, 1982) and later in 1976 in South Carolina (Ogawa, 1981; Zehr, 1982) and then in California (Ogawa *et al.,* 1981; Zehr, 1982). Resistance was first developed in orchards where benomyl was used exclusively to control decay (Zehr, 1982). *Sclerotinia fructicola*, which causes brown rot of stone fruits, was reported to have resistance against benzimidazoles in field conditions (Whan, 1976).

Sclerotinia sclerotiorum falls under a medium fungicide resistance risk pathogen group (FRAC, 2012). The first report of fungicide resistance in *S. sclerotiorum* was against benomyl (Benlate) and was observed in Canada (Gossen, 2001). Resistant isolates had $EC_{50} < 8$ µg/ml, but two isolates were very resistant ($EC_{50} > 200$ µg/ml). Another *Sclerotinia* species, *S. homoeocarpa* was reported to be insensitive if the isolates grew on potato dextrose agar medium amended with 1µg/ml of benomyl (Detweiler *et al.,* 1983)

DNA sequencing permits exploration of the structure of a gene encoding a target protein, and its influence on resistance risk (FRAC, 2007). The development of molecular detection technologies is a rapidly advancing field (McCartney *et al.,* 2003; FRAC, 2007), allowing detection of rare fungicide resistant mutations in plant pathogens at frequencies as low as 1 in 10,000 (FRAC, 2007). To detect a point mutation causing resistance, PCR (Polymerase Chain Reaction) assays can be devised using either allele-specific primers or appropriate probes or restriction digests to interrogate amplified DNA fragments (FRAC, 2007). Resistance to benzimidazole fungicides has been correlated with point mutations in the β-tubulin gene, which result in altered amino acid sequences at the benzimidazole-binding site (Ma and Michalides, 2005). Diagnostic methods are available for detection of target-site resistance to benzimidazoles and to a limited degree also for DMI fungicides, although in the latter case, a direct correlation between resistance and occurrence of specific mutations in the

target site is not so obvious due to the polygenic nature of the resistance (FRAC, 2007). Results from numerous studies have shown changes at codons 6, 50, 167, 198, 200, and 240 in the β-tubulin gene could cause benzimidazole resistance in field isolates of plant pathogenic fungi (Ma and Michalides, 2005). Biochemical confirmation was presented by Hollomon *et al.* (1998) who expressed β-tubulin as a fusion with a maltose binding protein (this fusion protein is soluble) and observed that benzimidazoles bound to the recombinant maltose binding protein fusion b-tubulin, and that this binding was reduced by the mutation at codon 198 from glutamic acid to glycine. Mutations at different codons in the β-tubulin gene may result in different resistance levels to benzimidazoles. In *Monilinia fructicola*, the mutation at codon 6 and 198 led to a low and a high resistance level, respectively (Ma *et al.,* 2003b, Ma and Michalides, 2005). In *Venturia inaequalis*, the mutations at the codon 198 and 200 caused a medium and a high resistance level to benzimidazole, respectively (Ma and Michailides, 2005). Additionally, different substitutions at the same codon may also cause different resistance levels. Mutants of *Tapesia yallundae* with changes at the codon 198 from Glu to Ala, Gly, Lys, and Gln had 50% effective concentration (EC$_{50}$) values to carbendazim ranging from 500 µg/ml to more than 2500 µg/ml (Albertini *et al.,* 1999).

Resistance to metconazole

The first DMI compounds introduced to the market in the 1970s were triflorine, triadimefon and imazalil (FRAC, 2007). Since then at least 30 more DMI compounds have been used in agriculture. At the time the FRAC Working Group formed, in 1982, there were very few reports of resistance to DMI compounds. These fungicides have a site-specific mode of action, and resistant mutants were easily obtained by mutagenic treatment in the laboratory. However, such mutants had reduced pathogenicity and other fitness attributes, so that development of

practical resistance was deemed unlikely (Fuchs and Drandarevski, 1976; FRAC, 2007). Practical resistance did in fact develop in several pathogens during the 1980s (e.g. powdery mildews, *Venturia inaequalis*, *Mycosphaerella fijiensis* var. *difformis*), but relatively slowly and with fluctuating severity, as is considered to be characteristic of polygenic resistance (FRAC, 2007)*. Sclerotinia homoeocarpa* isolates were found to be resistant to DMIs triadimefon (Bayleton), fenarimol (Rubigan), and propiconazole (Banner) in 1992 (Vargas *et al,.* 1992).

Field resistance against metconazole has not been reported yet. However, biochemical evidence for polygenic resistance to azole (DMI) fungicides obtained in laboratory trials indicates involvement of at least two resistance mechanisms (FRAC, 2007). Resistance can be developed due to many reasons, like due to inhibition of sterol demethylation in sterol biosynthesis pathway, which is due to point mutation of Y136F in *CYP51* gene which is due to in *Erysiphe graminis* f. sp. *hordei* (Delye *et al.,* 1998) and *Uncinula necator* (Delye *et al.,* 1997). Second reason for resistance development is due to over expression of *CYP51* gene as reported in *Penicillium digitatum* (Hamamoto *et al.,* 2000) and *Venturia inaequalis* (Schnabel and Jones, 2001).

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CHAPTER 2. CHARACTERIZATION OF SENSITIVITY OF *SCLEROTINIA SCLEROTIORUM* ISOLATES FROM NORTH CENTRAL U.S. TO THIOPHANATE-METHYL AND METCONAZOLE

Abstract

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic plant pathogenic fungus that causes Sclerotinia stem rot (SSR) of canola and many other important broadleaf crops. The absence of effective levels of genetic resistance in commercial canola genotypes have resulted in farmers depending largely on fungicide applications for its control. Thiophanate-methyl and metconazole are the oldest and most recently registered fungicides for control of *S. sclerotiorum*, respectively. Both fungicides have single-site modes of action and the chemical families they belong to have a history of resistance buildup making the monitoring of *S. sclerotiorum* sensitivity to them a necessity. In this study, the sensitivity of *S. sclerotiorum* isolates to thiophanate-methyl and metconazole was assessed in fungicide-amended potato dextrose agar (PDA). Isolates evaluated had been collected from 13 states in North Central US and the province of Manitoba in Canada. EC_{50} for thiophanate-methyl and metconazole ranged from 0.34 ± 0.06 to 1.62 ± 0.75 µg/ml and from 0.06 ± 0.01 to 1.74 ± 0.11 µg/ml, respectively. Isolates with $EC_{50} > 2$ µg/ml or > 1 µg/ml, are considered resistant to thiophanate-methyl and metconazole, respectively. Detection of a few resistant isolates in the region is a warning sign to adopt strategies that may help delay proliferation of resistant strains in the near future.

Introduction

Canola (*Brassica napus* L.) is a crop with a short history in North Dakota although its economic importance continues to increase very rapidly. The crop was introduced from Canada as oilseed rape probably in the early 1980s. The development in the late 1980s of canola

cultivars, oilseed rape genotypes with low erucic acid and glucosinolates contents, helped popularize this crop in the region. By 1991, approximately 8,000 ha were planted to this crop in the northeastern corner of the state (Lamey and Hershman, 1993). By 2012, the area planted to canola had increased to 348,000 ha statewide (USDA/NASS, 2011). Along with increased hectarage, the market value of this commodity has increased from less than US$ 5 million in 1991 to US$ 297 million in 2011 (USDA/NASS, 2011). By 2006, more than 90% of the canola planted in the United States was located in North Dakota (del Rio *et al.,* 2007).

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic plant pathogenic fungus which causes Sclerotinia stem rot (SSR) of canola and many other important crops (Boland and Hall, 1994; Purdy, 1979). This pathogen, which has a broad geographical distribution, is prevalent in many US states including those in the North Central region (Bradley and Lamey, 2005), where it has been responsible for large economic losses. SSR epidemics that affected soybeans in 1997, 2004 and 2009 resulted in losses estimated at more than US$1 billion (USDA/NASS, 2011). In North Dakota, SSR epidemics that affected canola between 1991 and 2002 caused an estimated direct economic impact of approximately US$94 million (Lamey, 2003). Virtually, all commercial canola genotypes grown in the region are susceptible to the disease and their yield potential is reduced by an average of 0.5% for every percentage unit of SSR incidence (del Rio *et al.,* 2007). In addition to causing yield losses SSR reduces seed quality. Seed contaminated with sclerotia may receive lower prices and also serves as an important source of inoculum if planted into fields with no history of SSR (Hartman *et al.,* 1998). Infected seeds have reduced germination and in some cases also lower oil and protein concentrations (Danielson *et al.,* 2004). The ability of *S. sclerotiorum* to infect a diverse group of plants under different climatic conditions and its ability to produce sclerotia that persist in the soil for almost eight years,

explain in part its successful persistence and wide geographic distribution (Adams and Ayers, 1979). SSR management in North Dakota is conducted mainly through fungicide applications. Azoxystrobin (Quadris[R]), boscalid (Endura[R]), metconazole (Quash[R]), prothioconazole (Proline[R]), pyraclostrobin (Headline[R]), and thiophanate-methyl (Topsin[R]) are the fungicides most commonly used for control of *S. sclerotiorum* in North Dakota and the North Central region (McMullen and Markell, 2010). These compounds have single mechanisms of action and some have a history of promoting fungicide-resistance buildup in their target populations (FRAC, 2007). Thus, continued monitoring of the sensitivity of such populations is necessary.

Thiophanate-methyl and problem of resistance

TM is the oldest fungicide in use against *S. sclerotiorum* in North Dakota. TM has been registered in the US since 1973 (USEPA, 2004). However, registration for use in canola in North Dakota dates back only to 2003 (McMullen and Bradley, 2003); TM is a benzimidazole and belongs to FRAC group 1, which is considered a high risk fungicide-resistance group (FRAC, 2012). Resistance against benzimidazoles is caused by changes in the β-tubulin protein (FRAC, 2007). The first practical resistance case against compounds in this group was observed in Australia in 1975 after 2 years of commercial use of benomyl against *Monilinia fructicola* (Whan, 1976); this was quickly followed by reports in Michigan (Jones and Ehret, 1976; Zehr, 1982) and New York (Szkolnik and Gilpatrick, 1977; Zehr, 1982); and in South Carolina (Ogawa *et al.*, 1981; Zehr, 1982), and California (Ogawa *et al.*, 1981; Zehr, 1982). TM and benomyl have carbendazim as their primary metabolite (Vonk and Sijpesteijn, 1971). The first case of resistance of *S. sclerotiorum* to a benzimidazole was reported on benomyl (Benlate) in 2001 and occurred in the Canadian prairies (Gossen *et al.*, 2001). Gossen *et al.* (2001) reported finding isolates with $EC_{50} > 200$ μg/ml. Pan *et al,.* (1997) established a carbendazim

baseline sensitivity for *S. sclerotiorum* and proposed that *S. sclerotiorum* isolates with $EC_{50} \geq 2$ μg/ml be considered resistant. Researchers working on *S. sclerotiorum* use the same threshold to separate TM-sensitive from insensitive isolates. In contrast, isolates from a related species, *S. homoeocarpa*, are considered sensitive if they fail to grow on potato dextrose agar (PDA) medium amended with 1 μg/ml of benomyl (Detweiler *et al.*, 1983).

Metconazole and problem of resistance

MTZ is a triazole belonging to the demethylation inhibitors (DMI) chemical group. Fungicides in this group act on 24-methylenedihydrolanosterol at C14, a precursor of ergosterol in fungi (Brent, 1995). MTZ's FRAC code is 3. The first DMI fungicides, triforine, triadimefon, and imazalil, were introduced to the market in the early 1970s (FRAC, 2007). Since then at least 30 more DMIs have been used in agriculture. MTZ was registered in the US in 2007 for use on turf grass and ornamentals (USEPA, 2006) and in 2010 it was registered for use against *S. sclerotiorum* in canola in North Dakota (McMullen and Markell, 2010).

DMI fungicides have a single site mode of action and belong to medium fungicide-resistance risk group. While resistant strains were easily obtained by mutagenic treatment in the laboratory, such mutants had reduced pathogenicity and other fitness disadvantages, thus development of practical resistance was deemed unlikely (Fuchs and Drandarevski, 1976; FRAC, 2007). Resistance to DMI fungicides was first observed soon after the Fungicide Resistance Action Committee Working Group (FRAC) was formed. The first report was produced in 1982 after 7 years of commercial use against powdery mildews on cucurbits and barley (FRAC, 2007). Numerous cases of resistance were reported during the 1980s (e.g. powdery mildews, *Venturia inaequalis*, *Mycosphaerella fijiensis* var. *difformis*), but resistance

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buildup occurred relatively slowly and with fluctuating severity, as is considered to be characteristic of polygenic resistance (FRAC, 2007). Resistance of *S. homoeocarpa* to triadimefon (Bayleton), fenarimol (Rubigan), and propiconazole (Banner) was reported in 1992 (Vargas *et al.,* 1992). Based on EC_{50} value determination, *Cercospora beticola* isolates were characterized as having low ($EC_{50} < 0.01$ µg/ml), medium (EC_{50} of 0.01 to 1.0 µg/ml), or high ($EC_{50} > 1.0$ µg/ml) resistance to DMI fungicides (Bolton *et al.*, 2011). Therefore, we are using the same scale for threshold of sensitive and insensitive isolates. Resistance of *S. sclerotiorum* to TM has not been reported in the US whereas resistance of this pathogen to MTZ has not been reported yet. Periodical monitoring of the sensitivity of *S. sclerotiorum* to these compounds will help adjust fungicide application programs to prevent or reduce buildup of fungicide resistance in the region. The objective of this study was to characterize the sensitivity of *S. sclerotiorum* isolates collected from several states in North Central US to TM and MTZ.

Materials and methods

Sensitivity of *S. sclerotiorum* to these fungicides was expressed in terms of their 50% effective concentration (EC_{50}). These values were calculated using potato dextrose agar (PDA) amended with technical grade fungicides (Figure 2.1).

Isolates

S. sclerotiorum isolates collected from 13 states of North Central US and the province of Manitoba in Canada, between 2000 and 2008 were used in this study. A total of 81 and 91 *S. sclerotiorum* isolates were evaluated for their sensitivity to TM and MTZ, respectively. Sclerotia from all isolates were surface disinfested by immersing them in a 10% NaOCl and water solution (v/v) (Clorox Sales Co., Oakland, CA, USA) for 1 min and then rinsed in sterile distilled water. Surface-disinfested sclerotia were plated on quarter-strength PDA medium (6 g of Potato

Dextrose Broth (HiMedia Laboratories Pvt. Ltd, India), 15 g Bacto Agar (Becton, Dickinson and Company, Sparks, MD USA) and 1000 ml of distilled water, autoclaved at 121° C and 103.4 kPa for 20 minutes) and incubated at room temperature (21° C) until the mycelial colony covered about one-quarter of the surface area of the dish.

Fungicide concentrations

Thiophanate-methyl

TM (technical grade=95% a.i.) (Ceraxagri, King of Prussia, PA, USA) was diluted in acetone to make a 1000 μg/ml stock solution. Luke-warm quarter-strength PDA was amended with the stock solution to final concentrations of 0, 0.1, 0.5, 1, 1.5, 2 and 5 μg/ml. These media were poured into Petri dishes at a rate of approximately 25 ml per dish. Agar plugs from all 81 isolates obtained as described in the previous paragraph were plated in duplicates for each concentration. Plates were incubated for 48 hours in dark at 21° C; at the end of the incubation period two colony diameters were measured perpendicular to each other. The study was conducted twice, and each time all isolates had two replications per concentration.
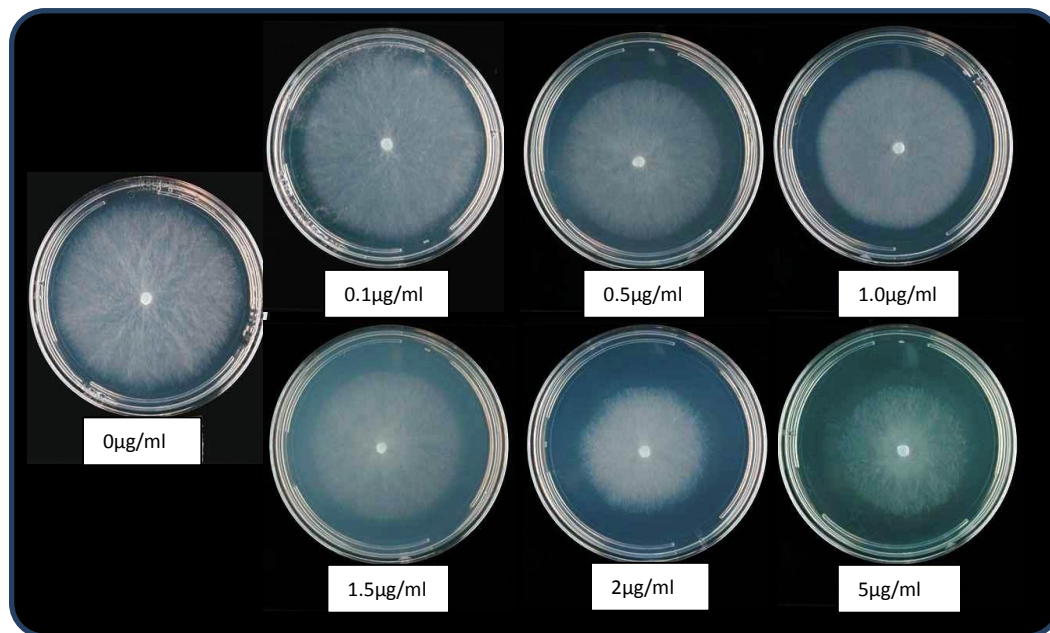
Figure 2.1. *Sclerotinia sclerotiorum* **colonies growing on potato dextrose agar amended with technical grade thiophanate-methyl at various concentrations.**

Metconazole

Technical grade MTZ with 99.5% a.i (Sigma-Aldrich, Co., St. Louis, MO, USA) was diluted with acetone to make a 1000 μg/ml stock solution. Quarter- strength PDA was amended with the stock solution to final concentrations of 0, 0.1, 0.5, 1.0, and 2 μg/ml. Agar plugs, 4 mm in diameter and containing hyphal tips of a colony were plated on these media. Each concentration was replicated twice and a total of 91 isolates were evaluated. Seeded plates were incubated for 48 hours in dark at 21° C. The sensitivity test for MTZ was conducted twice. Two readings of colony diameters perpendicular to each other were measured.

Data analyses

Colony growth in absence of fungicide was used as reference point to estimate growth reduction in presence of fungicide. This suppression data expressed as percentage was used to

evaluate effective concentration of fungicide or the EC_{50} values to reduce colony growth of fungus by 50% for all isolates using SAS 9.2 (SAS Institute, Cary, NC). Levene's test for homogeneity of variances was conducted using SAS 9.2 to compare trials' variances. Upon confirmation that the variances were similar, a combined analysis of variance was conducted using the General Linear Models procedure (PROC GLM) of SAS 9.2. Comparison between all isolates across two replications and two trails were made by running protected least significant difference value (l.s.d.). Sources of variation for the combined analysis were trial, isolate and trial*isolate. *S. sclerotiorum* isolates with TM $EC_{50} \geq 2$ μg/ml and MTZ $EC_{50} \geq 1$ μg/ml were considered resistant (Pan, 1997; Bolton, 2011). To further characterize the reaction of *S. sclerotiorum* isolates to each fungicide, the range of EC_{50} values in the populations were divided into four quartiles. In this way, isolates with $EC_{50} \leq 0.60$ μg/ml or ≤ 0.26 were considered sensitive to TM and MTZ, respectively. Isolates with $EC_{50} \geq 0.69$ μg/ml or ≥ 0.38, but below the resistance thresholds, were considered moderately insensitive to TM and MTZ, respectively. Isolates in between these categories were considered moderately sensitive to each compound.

Results

Thiophanate-methyl

Homogeneity of variance test indicated that variances were homogeneous ($P = 0.49$) and therefore could be analyzed in a combined ANOVA. In the combined ANOVA, no significant differences were observed between trials; however, the effect of isolates and their interaction with trials were statistically significant (Table 2.1). The samples evaluated leaned in general towards sensitivity (Figure 2.2) with mean EC_{50} values for isolates ranging between 0.34 and 1.62 μg/ml. Approximately 14% of all 81 isolates had $EC_{50} > 1.06$ μg/ml and could be considered moderately insensitive. However, the range of EC_{50} values produced by three of these

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isolates, WM413 collected in Iowa, WM414 collected in Michigan, and WM365 collected in

Nebraska, included values above the resistance threshold (Table 2.2).

Table 2.1. Analysis of variance table of combined analysis of the study on sensitivity of *Sclerotinia sclerotiorum* **isolates from 13 U.S. states and the province of Manitoba, Canada to thiophanate-methyl.**

Sources of variation	DF[1]	Mean Square	F-value	Pr > F
Trials	1	0.21	2.03	0.1559
Isolates	80	0.21	2.04	< 0.0001
trials*isolates	80	0.20	1.9	0.0003

[1] DF= degrees of freedom

The mean EC_{50} for these isolates were 1.43 ± 2.04, 1.18 ± 0.72, and 1.62 ± 0.80 µg/ml,

respectively. The means, medians, and minimum and maximum EC_{50} values for each state are

presented in Table 2.3. States that provided five or more isolates had statistically similar mean

EC_{50}. The overall mean EC_{50}, calculated across all isolates rather than across states, was 0.76.

The l.s.d. ($P = 0.05$) value calculated for the entire population was 0.23 µg/ml (Table 2.3).
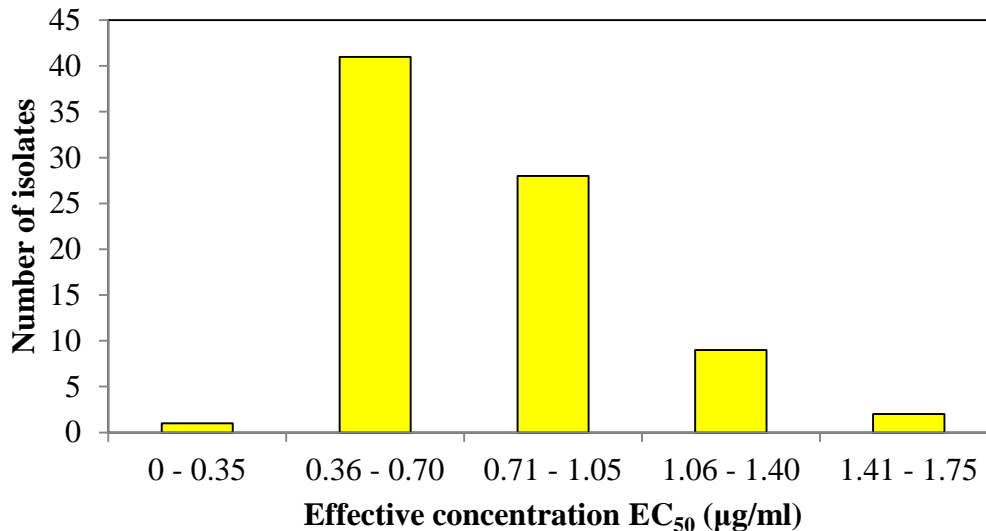
Figure 2.2. Frequency distribution of the response of *Sclerotinia sclerotiorum* **isolates from North Central US and the province of Manitoba in Canada to thiophanate-methyl in fungicide-amended agar trials.**

Table 2.2. Thiophanate-methyl EC$_{50}$ values of few moderately insensitive *Sclerotinia sclerotiorum* isolates which are close to being resistant.

Isolates	EC$_{50}$ (µg/ml)		
	Mean	**Median**	**Range[1]**
WM606	1.07	1.09	0.73 – 1.36
WM389	1.10	1.11	0.95 – 1.22
WM363	1.10	1.08	0.82 – 1.43
WM586	1.11	1.11	0.93 – 1.28
WM604	1.15	1.26	0.47 – 1.63
WM414	1.18	1.11	0.49 – 2.00
WM599	1.19	1.18	1.05 – 1.33
WM392	1.24	1.30	0.60 – 1.76
WM380	1.24	1.20	0.67 – 1.92
WM413	1.43	0.58	0.08 – 4.46
WM365	1.62	1.49	0.79 – 2.70

[1] Values are based on four observations per isolate (two trials and two replications within each trial).

Table 2.3. Sensitivity of *Sclerotinia sclerotiorum* isolates from 13 US states and the province of Manitoba in Canada to thiophanate-methyl.

Origin	Number of Isolates	Fungicide sensitivity (EC$_{50}$ in µg/ml) [1]		
		Mean	**Median**	**Minimum-Maximum**
Colorado	1	0.70	0.70	0.66 – 0.74
Illinois	5	0.84	0.68	0.08 – 4.46
Indiana	1	0.65	0.63	0.55 – 0.80
Iowa	1	0.85	0.94	0.35 – 1.17
Kansas	1	0.88	0.75	0.50 – 1.51
Manitoba	1	0.89	0.90	0.42 – 1.31
Michigan	2	0.87	0.65	0.43 – 2.00
Minnesota	9	0.64	0.60	0.37 – 1.04
Missouri	1	0.78	0.72	0.57 – 1.11
Montana	1	0.67	0.62	0.52 – 0.90
Nebraska	2	1.19	0.91	0.64 – 2.70
North Dakota	48	0.74	0.70	0.01 - 1.76
South Dakota	1	1.19	1.18	1.05 – 1.33
Wisconsin	7	0.80	0.70	0.39 – 1.92
Grand values[2]	81	0.76	0.69	0.01 – 4.46
l.s.d ($P = 0.05$)[3]		0.23		

[1] Values represent mean, median, and minimum and maximum values based on EC$_{50}$ two trials and two replication within each trial for every isolate.

[2] Total number of isolates; mean and median values calculated on all replications within isolates rather than on values by state.

[3] l.s.d. = Protected least significant difference value.

Metconazole

Homogeneity of variance test indicated the variances of both trials were homogeneous ($P = 0.0901$) and therefore trials could be combined for analysis. In the combined ANOVA, the effect of trials was not statistically significant, but the effects of isolates and their interaction with trials were significant (Table 2.4). The frequency distribution of EC$_{50}$ values for the 91 isolates evaluated is presented in Figure 2.3 and shows that the population was skewed towards sensitivity. The means, medians, and minimum and maximum EC$_{50}$ values for are presented in Table 2.5. The overall mean EC$_{50}$, calculated across all isolates rather than across states, was 0.36. The l.s.d. ($P = 0.05$) value calculated for the entire population was 0.13 µg/ml.

The state mean EC_{50} for Illinois, Michigan, Nebraska, North Dakota, Ohio, and Wisconsin were between 1.4 and 2.2 times greater than that of the overall mean. Among the 91 *S. sclerotiorum* isolates evaluated, ten isolates had EC_{50} values above the threshold for resistance ($EC_{50} > 1.0$ µg/ml) to DMI fungicides (Table 2.6).

Table 2.4. Analysis of variance table of combined analysis of the study on sensitivity of *Sclerotinia sclerotiorum* isolates from 14 U.S. states and the province of Manitoba, Canada to metconazole.

Sources of variation	DF[1]	Mean Square	F value	Pr > F
Trials	1	0.04	2.20	0.1399
Isolates	90	0.30	16.68	<0.0001
Trials*isolates	90	0.05	2.52	<0.0001

[1]DF= degree of freedom
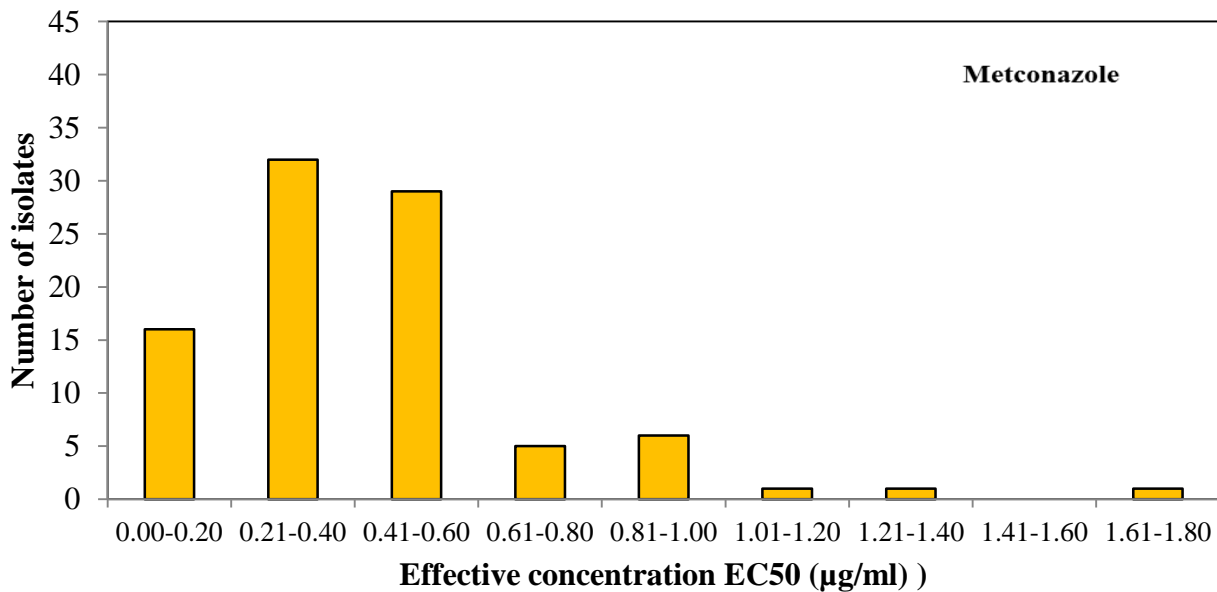
Figure 2.3. Frequency distribution of the response of *Sclerotinia sclerotiorum* isolates from 14 North Central US and the province of Manitoba, Canada to metconazole in fungicide-amended agar trials.

Table 2.5. Sensitivity of *Sclerotinia sclerotiorum* isolates from 14 US states and the province of Manitoba in Canada to metconazole.

Origin	Number of Isolates	Fungicide sensitivity (EC$_{50}$) in µg/ml		
		Mean	**Median**	**Minimum-Maximum**
Colorado	1	0.20	0.23	0.08 – 0.27
Illinois	1	0.56	0.40	0.38 – 1.05
Indiana	1	0.39	0.39	0.32 – 0.47
Iowa	5	0.38	0.39	0.08 - 0.73
Kansas	1	0.09	0.08	0.07 – 0.14
Manitoba	1	0.06	0.06	0.05 – 0.07
Michigan	2	0.60	0.48	0.09 – 1.43
Minnesota	10	0.38	0.36	0.06 - 0.90
Missouri	1	0.38	0.38	0.32 – 0.43
Montana	1	0.38	0.40	0.27 – 0.46
Nebraska	3	0.80	0.43	0.23 – 1.85
North Dakota	54	0.43	0.40	0.06 – 1.50
Ohio	1	0.53	0.54	0.27 – 0.78
South Dakota	1	0.18	0.18	0.07 – 0.29
Wisconsin	8	0.51	0.35	0.08 – 1.20
Grand values[1]	91	0.36	0.36	0.07 – 0.98
l.s.d ($P = 0.05$)[2]		0.13		

[1] Total number of isolates; mean and median values calculated on all isolates rather than on values by state.

[2] l.s.d. = Protected least significant difference value.

Table 2.6. *Sclerotinia sclerotiorum* isolates with highest EC$_{50}$ values (µg/ml) and regarded as resistant to metconazole.

| Isolate | 50% effective concentration (EC$_{50}$) in µg/ml | | |
	Mean	Standard deviation	Range
WM591	0.77	0.24	0.54 – 1.08
WM576	0.89	0.17	0.69 – 1.09
WM595	0.82	0.28	0.48 – 1.17
WM401	0.92	0.33	0.63 – 1.20
WM415	0.91	0.37	0.55 – 1.43
WM388	0.85	0.46	0.43 – 1.28
WM379	0.96	0.53	0.38 - 1.50
WM607	1.28	0.25	0.95 - 1.50
WM608	1.20	0.42	0.87 – 1.77
WM596	1.74	0.11	1.63 – 1.85

[1]Values are based on four observations per isolate (two trials and two replications within each trial).

Discussion

TM has been registered in the US since 1973 (US-EPA, 2004). However, registration for use in canola in North Dakota dates back only to 2003 (McMullen and Bradley, 2003). Literature on sensitivity of *S. sclerotiorum* to TM in the US is scarce and may not accurately represent the sensitivity of the isolates evaluated. The first report of fungicide resistance in *S. sclerotiorum* was associated with its reaction to the benzimidazole benomyl in the Canadian prairies in 2001 (Gossen *et al.,* 2001). Benomyl, marketed by DuPont as Benlate[R], had been in use in the prairies since 1968. Gossen *et al.* (2001) found isolates with EC$_{50}$ < 8 µg/ml), but two isolates were very resistant (EC$_{50}$ > 200 µg/ml). In 2002, Li *et al.* reported a benomyl EC$_{50}$ < 1 µg/ml for a single *S. sclerotiorum* isolate collected in 1989 in Alberta. While the study covered a large set of concentrations, only one isolate was evaluated. Mueller *et al.* in 2002 evaluated 91

isolates collected from multiple US states and including single isolates from Canada, Argentina, and Switzerland. In their study, Mueller *et al.* (2002) used TM concentrations of 0, 0.1, 10, 50, 100 and 500 µg/ml by diluting commercial fungicide formulations rather than the technical or analytical grade active ingredient. The inclusion of adjuvants and other compounds that frequently accompany the active ingredient in these formulations may affect the response of the fungus to the active ingredient; further, the large gap in the concentrations at the lower end may have also reduced the accuracy of the estimation of the sensitivity to the fungicide. Mueller *et al.* (2002) reported a mean EC_{50} of 2.2 µg/ml for TM. Pan *et al.* (1997) established a carbendazim baseline sensitivity for *S. sclerotiorum* and proposed that *S. sclerotiorum* isolates with $EC_{50} \geq 2$ µg/ml be considered resistant. TM and benomyl have carbendazim as their primary metabolite (Vonk and Sijpesteijn, 1971). These three isolates could therefore be considered resistant to TM. While the number of isolates considered resistant to TM in this study is relatively small, 3.7% of all isolates evaluated, their presence is an indication that tolerance to this compound may be building up in the region. Thus, measures that could help reduce the risk of resistance buildup such as not using the product exclusively, avoiding multiple and/or unnecessary applications within the same growing season, and alternating the use of this compound with fungicides of different mode of action, should be implemented.

The information on sensitivity of *S. sclerotiorum* to MTZ generated by this study is considered a baseline for sensitivity to this compound since all isolates were collected before MTZ was registered for use against *S. sclerotiorum* (McMullen and Markell, 2010). MTZ EC_{50} values for all isolates ranged from 0.06 to 1.74 µg/ml with an overall mean of 0.43 µg/ml. Establishing a threshold value to identify *S. sclerotiorum* isolates that are MTZ-insensitive is difficult since there is no precedent information for this species and the values can vary widely

even between species within the same genus. The average baseline sensitivity of *S. homoeocarpa* to the DMI fungicide propiconazole was estimated at 0.0049 μg/ml; whereas isolates that had been exposed repeatedly to propiconazole had an average EC_{50} of 0.0283 μg/ml (Miller *et al.*, 2002). This represents an almost six-fold increase in tolerance. A more recent study that evaluated the sensitivity of *S. homoeocarpa* to metconazole revealed EC_{50} values ranging between 0.008 and 0.91 μg/ml (Ok *et al.*, 2011). In our study the mean EC_{50} was almost 88-fold that of the baseline estimated by Miller *et al*. (2002) with three isolates having mean $EC_{50} \geq 1.2$ μg/ml and approximately 11% of the *S. sclerotiorum* population tested that could be considered resistant. In the absence of data from samples from the same species one has to consider the possibility that *S. sclerotiorum* is naturally more tolerant to metconazole than *S. homoeocarpa* and other unrelated species like *Fusarium oxysporum*, *F. graminearum*, and *Fusarium sp. nov.* which had MTZ EC_{50} values ranging between 0.02 and 0.04 μg/ml (Burlakoti *et al.*, 2010).

The interaction between trial and isolate was significant for both TM and MTZ. One of the *S. sclerotiorum* isolate behaved differently in both the trials of TM and had a large standard deviation (2.04), which could be the reason of interaction between trials by isolate. Sensitivity to MTZ did not have any such *S. sclerotiorum* isolate with a striking difference in two trials.

None of the isolates considered moderately insensitive or resistant to TM were also considered moderately insensitive or resistant to MTZ. Therefore, there is absence of any cross sensitivity which is essential to address whether or not one fungicide can control strains of the target pathogen that are resistant to other fungicide. Alternatively, one could speculate that the unintended exposure of *S. sclerotiorum* isolates to DMI fungicides targeting cereal diseases grown in rotation with canola in the region may have created enough selection pressure to increase the tolerance of the entire population to the levels observed in this study. In either case,

it is imperative that measures intended to delay fungicide resistant buildup be implemented in the region before higher levels of resistance to these compounds develop.

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CHAPTER 3. EFFECT OF ENHANCED INSENSITIVITY OF *SCLEROTINIA SCLEROTIORUM* ISOLATES TO THIOPHANATE-METHYL AND METCONAZOLE ON EFFICACY OF DISEASE CONTROL

Abstract

A laboratory study that evaluated the *in vitro* sensitivity of more than 80 *S. sclerotiorum* isolates to thiophanate-methyl (TM) and metconazole (MTZ) identified several isolates that could be considered moderately insensitive or resistant to these compounds. The objective of this study was to determine if the levels of resistance observed in laboratory trials would result in reduced disease control in greenhouse conditions. Sunflower plants were sprayed with the recommended doses of TM (1X = 4168 μg per ml) or MTZ (1X = 1038 μg per ml) and also with 0.5X and 2X solutions. Thirty hours later, plants were inoculated with isolates considered sensitive, moderately insensitive or resistant to either TM or MTZ in replicated trials. Trials were conducted twice using a randomized complete block design each time. In general, the application of TM and MTZ at the recommended doses (1X) reduced disease severity by approximately 58 and 52%, respectively, compared to non-protected plants. Differences in disease control between TM-moderately sensitive and moderately insensitive isolates were not too clear. In contrast, the levels of control of diseases caused by MTZ-resistant isolates were almost one half of that observed for MTZ-sensitive isolates. These results suggest that disease control may be adequate at the present finding of level of insensitivity.

Introduction

Diseases caused by *Sclerotinia sclerotiorum* (Lib.) de Bary are managed mainly through use of fungicides in North Central US. Two of the fungicides registered for use against *S. sclerotiorum* in the region are thiophanate-methyl (TM) and metconazole (MTZ). TM was

registered as a fungicide in 1973 (USEPA, 2004) and reregistered due to United States Environment Protection Agency's new standards in the US in November 1, 1984 as a systemic fungicide to be used on a variety of trees, vines, root crops, wheat and canola (USEPA, 2005). TM falls under FRAC code 1 and acts on the mitosis and cell division in the target fungi. TM is considered a high resistance-risk fungicide. MTZ was registered in the US in 2007 for use on turf and ornamentals as systemic fungicide (USEPA, 2006). MTZ was registered for use in canola against Sclerotinia stem rot in the region in 2010 (McMullen and Markell, 2010). MTZ has a single site mode of action and belongs to the medium-risk fungicide group (FRAC, 2007).

Buildup of resistance against single-mode-of-action fungicides will occur at some point during their commercial life (Brent and Hollomon, 2007). Resistance generally appear as a response to repeated use of the fungicide, or to repeated use of another fungicide which is related to it chemically and/or biochemically through a common mechanism of antifungal action (FRAC, 2007). Fungicide resistance can be conferred by various mechanisms (Ma and Michailides, 2005; Gisi *et al.*, 2000; Gullino *et al.,* 2000; Fluit *et al.,* 2001; MgGrath, 2001), including: (I) an altered target site, which reduces the binding of the fungicide; (II) the synthesis of an alternative enzyme capable of substituting the target enzyme; (III) the overproduction of the fungicide target; (IV) an active efflux or reduced uptake of the fungicide; and (V) a metabolic breakdown of the fungicide.

Since the widespread use of systemic site-specific fungicides began in the early 1970s, fungicide resistance in fungal plant pathogens has become an important problem. Typically, a low frequency of resistance genes that is present in the target population because of mutations increases in frequency when the population is exposed to the fungicide (Mavroeidi and Shaw, 2006). The high selection pressure exerted by the fungicide increases the number of insensitive

isolates more quickly than sensitive types. Finally, after repeated fungicide applications most of the population becomes resistant and the chemical has no effect (Mavroeidi and Shaw, 2006). Therefore the first important factor in the development of field resistance is the initial frequency of the resistant phenotypes in the pathogen population (Mavroeidi and Shaw, 2006).

Problems of resistance to benzimidazoles were first reported in *Monilinia fructicola* in Australia (Whan, 1976), Michigan (Jones and Ehret, 1976; Zehr, 1982) and New York (Szkolnik and Gilpatrick, 1977; Zehr, 1982) in 1975, and later in 1976 in South Carolina (Ogawa *et al.*, 1981; Zehr, 1982) and then in California (Ogawa *et al.,* 1981; Zehr, 1982). Resistance was first developed in orchards where benomyl was used exclusively to control decay (Zehr, 1982). According to FRAC, 2012; *Sclerotinia fructicola* which causes brown rot of stone fruits was reported to have resistance against benzimidazole in 1976 (Zehr, 1982). The first report of resistance in *S. sclerotiorum* was related to benomyl fungicide (Benlate), which is a benzimidazole, and was observed on the Canadian prairies (Gossen *et al.,* 2001). They found isolates with benomyl $EC_{50} < 8$ µg/ml, but two isolates were very resistant ($EC_{50} > 200$ µg/ml). *S. homoeocarpa* was reported to be sensitive if the isolates failed to grow on medium amended with 1 µg/ml of benomyl (Detweiler *et al.*, 1983) and later resistance was found in the field conditions by Wong in 2003 (FRAC, 2012). Pan *et al.* (1997) established a carbendazim baseline sensitivity for *S. sclerotiorum* and proposed that *S. sclerotiorum* isolates with $EC_{50} \geq 2$ µg/ml be considered resistant. Therefore, using Pan *et al.*'s threshold three of the isolates evaluated in this study were considered to be resistant.

DMIs were first used in the 1970s, triforine, triadimefon and imazalil being early representatives (FRAC, 2007). Since then at least 30 more DMIs have been used in agriculture. At the time the FRAC Working Group formed, in 1982, there were very few reports of DMI

resistance. They have a single site mode of action, and resistant mutants were easily obtained by mutagenic treatment in the laboratory. However, such mutants had reduced pathogenicity and other fitness attributes, so that development of practical resistance was deemed unlikely (Fuchs and Drandarevski, 1976; FRAC, 2007). Practical resistance did in fact develop in several pathogens during the 1980s (e.g. powdery mildews, *Venturia inaequalis*, *Mycosphaerella fijiensis* var. *difformis*), but relatively slowly and with fluctuating severity, as is considered to be characteristic of polygenic resistance (FRAC, 2007). *S. homoeocarpa* isolates resistant to triadimefon (Bayleton), fenarimol (Rubigan, and propiconazole (Banner) were detected in 1991 (Vargas *et al.,* 1992).

The existence and efficacy of selection for fungicide resistance depend on many factors. These may be either pathogen-dependent (biology, genetics, and epidemiology); fungicide-dependent (type of compound, dose, frequency of application); or host-dependent (plant architecture, host resistance) (Mavroeidi and Shaw, 2006). Based on the generally accepted view of the influence of various fungicide-dependent factors on the strength of selection for resistance, various anti-resistance strategies have been proposed, including control of fungicide doses, and mixing of fungicides with different modes of action.

However, the theories and experimental data for how fungicide doses affect the strength of selection are inconsistent. The main guidelines from the Fungicide Resistance Action Committee (www.frac.info) are to limit the number of applications; to use the full recommended dose; and to mix fungicides with different modes of action (Brent, 1995; Mavroeidi and Shaw, 2006). This advice is based on the hypothesis that both high doses and mixtures will kill isolates of all sensitivities maximally and therefore minimize the build-up of resistant genotypes. A contrasting hypothesis is that a reduced dose would decrease the evolution of resistance by

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allowing relatively more sensitive isolates to survive. If mixtures of fungicides are applied to as anti-resistance strategy, attention has to be paid. If choosing partners of different mobility, the more mobile one will accumulate in the tip. This will end up in different fungicides present in different locations of the plant. So, independent selection for resistance can result in the different places (Metcalfe *et al.,* 2000). Decreasing the dose of DMI fungicides from one-quarter to one-eighth of the full recommended dose was found to reduce resistance development in *Mycosphaerella graminicola* (Metcalfe *et al.,* 2000; Mavroeidi and Shaw, 2006). Periodical monitoring of the sensitivity of *S. sclerotiorum* to these compounds will help adjust fungicide application programs to prevent or reduce buildup of fungicide resistance in the region.

A study that characterized the sensitivity of *S. sclerotiorum* isolates from North Central US to TM and MTZ revealed that several isolates had EC_{50} values very close to the thresholds that separate sensitivity from resistance to these compounds. Whether the levels of insensitivity observed in that study were high enough to reduce the efficacy of currently recommended fungicide doses is not clear and needs to be assessed. The objective of this study was to evaluate the impact the levels of resistance detected, have on the efficacy of TM and MTZ to control the disease caused by these isolates.

Material and methods

Greenhouse tests were conducted to determine whether the levels of insensitivity to TM and MTZ observed in *S. sclerotiorum* isolates used in previous experiments had any effect on the efficacy of the fungicides to protect plants against *S. sclerotiorum* infection.

Plant materials

Sunflower oilseed hybrid Mycogen 8H288 seeds were sown in 10.16 cm pots filled with soilless media Sunshine LC1 mix Professional Growing Mix (Bellevue, WA, USA). After germination of sunflower seeds only one plant was retained in each pot for one month. Plants with similar height were selected to create blocks of 36 sunflower plants each for the TM studies and 20 plants for the MTZ studies.

Inoculum production

Thiophanate-methyl

Six *S. sclerotiorum*, two moderately sensitive and four moderately insensitive isolates were selected for this study based on similar levels of aggressiveness (Table 3.1). The aggressiveness of these isolates had been measured on six different crops (Dr. Berlin Nelson, personal communication). Sclerotia were surface disinfested by immersing them in a 10% NaOCl and water solution (v/v) (Clorox Sales Co., Oakland, CA, USA) for 1 min and then rinsed in distilled water. Surface-disinfested sclerotia were plated on quarter-strength potato dextrose agar (PDA) medium (6 g of Potato Dextrose Broth (HiMedia Laboratories Pvt. Ltd, India), 15 g Bacto Agar (Becton, Dickinson and Company, Sparks, MD 21152 USA) and 1000 ml of distilled water, autoclaved at 121° C and 100 kPa for 20 minutes) and incubated at room temperature (21° C) until the mycelial colony covered about one-quarter of the surface area of the dish. Six 250 ml flasks were each filled with 50 g wheat seeds and 50 ml of distilled water.

Table 3.1. *Sclerotinia sclerotiorum* **isolates used for evaluating the effect of thiophanate-methyl enhanced insensitivity; Sensitive isolate had $EC_{50} < 0.6$, moderately sensitive (MS) EC_{50} 0.6 - 0.75 and moderately insensitive (MI) isolates had $EC_{50} > 0.75$ µg/ml.**

| Isolate | Origin | EC_{50} µg/ml[1] | | Sensitivity[2] |
		Mean	Std. dev.	
WM376	Iowa	0.64	0.03	MS
WM600	Minnesota	0.65	0.01	MS
WM596	Nebraska	0.76	0.06	MI
WM576	Wisconsin	0.87	0.78	MI
WM577	Manitoba	0.89	0.10	MI
WM414	Michigan	1.18	0.31	MI

[1] Mean EC_{50} based on two trials and two replications per trial. Std. dev. = standard deviation of mean.
[2] MS= moderately sensitive, MI= moderately insensitive. These labels were assigned based on quartile distribution of the 81 *Sclerotinia sclerotiorum* isolate sensitivity to thiophanate-methyl.

After overnight soaking the flasks were autoclaved at 121° C and 100 kPa for 20 minutes two times at an interval of 24 hrs. Ten agar plugs, 4 mm diameter each, containing hyphal tips of the colony were used to inoculate the wheat seeds. These medium was incubated for 6 days at $21°$ C. Every day the medium was stirred to make sure no clumps were created. When the mycelium colonized nearly every wheat seed, the inoculum was removed from the flask and dried in laminar airflow for 24 hours. This inoculum was then stored at $4°$ C in airtight bags until used. At the time of inoculation the colonized seeds were crushed and 160-180 mg was used per plant.

Metconazole

Four *S. sclerotiorum* isolates, two of them considered resistant to TMZ and two considered sensitive were used in this study (Table 3.2). Sclerotia and mycelium for plant inoculations were prepared as described for the TM study. The sensitive isolates, WM577 and WM593 had known aggressiveness with mean lesion length 35 and 28mm, respectively.

Table 3.2. *Sclerotinia sclerotiorum* isolates used for evaluating effect of enhanced insensitivity of metconazole on disease control; Sensitive isolate had mean $EC_{50} < 0.26$, resistant had mean $EC_{50} \geq 1.0$ µg/ml $\pm$ standard deviation.

| Isolate | Origin | EC_{50} µg/ml[1] | | Sensitivity[2] |
		Mean	Std. dev.	
WM 577	Manitoba	0.06	0.013	Sensitive
WM593	North Dakota	0.07	0.004	Sensitive
WM388	North Dakota	0.85	0.450	Resistant
WM596	Nebraska	1.74	0.108	Resistant

[1] Mean EC50 was based on value for two trials and two replications per trial. Std. dev = standard deviation of mean.

[2] Sensitivity based on were assigned based on quartile distribution of the 81 *Sclerotinia sclerotiorum* isolate sensitivity to metconazole.

Inoculation and spraying procedures

The experiment was set up using a randomized complete block design with 6 and 4 replications for the TM and MTZ studies, respectively. One month old sunflower plants were sorted by height into six blocks of 36 plants each for the TM studies and four blocks with 20 plants each for the MTZ studies. Each block was sprayed with a given fungicide dose. The fungicides used were Topsin M 70WP, a.i. 70% thiophanate-methyl, (United Phosphorus, Inc., King of Prussia, PA, U.S.A.) at the recommended field doses (1x=1.12 kg/ha) and at 0x, 0.25x, 0.5x, and 2x and Quash 50WDG, a.i. 50% MTZ, (Valent U.S.A. Corporation, Walnut Creek, CA, U.S.A.) at the recommended field doses (1x= 219 mg/ha) and at 0x, 0.25x, 0.5x, and 2x. Each fungicide was applied in separate experiments. Sprayings were conducted in an automated spraying booth calibrated to deliver 140.25 liter at 279.8 kPa. Plants received three passes of the sprayer to make sure complete foliar coverage had been achieved. After spraying the plants were transferred to a greenhouse room; 30 hours later, plant stem diameters were measured using a Vernier caliper at the middle point between the first two pairs of true leaves. The stems were then inoculated at that mid-point by making a small puncture with a needle and wrapping it with

a moist cotton plug containing 160-180 mg inoculum of *S. sclerotiorum* isolates. Inoculated plants were incubated in moist chamber for 44 hours at humidity cycle of 15 sec after every 15 minutes. Lesion length was measured at 44, 86 and 110 hours after inoculation (Figure 3.1).
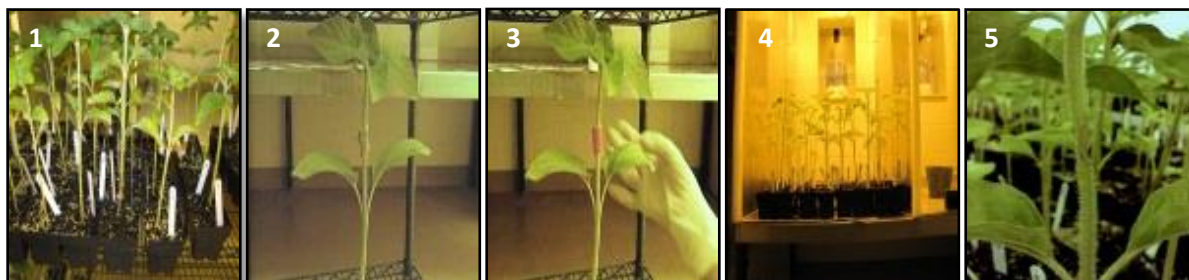
Figure 3.1. Greenhouse experiment showing thiophanate-methyl sprayed sunflower plants (1), inoculation by wheat seeds infected with S. sclerotiorum (2, 3), plants in humidity chambers (4) and plants with lesion (5).

Data collection and analyses

Trials evaluating each fungicide were conducted twice. Each time, lesion lengths were measured 44, 86 and 110 hours after inoculation. The area under the disease progress curve (AUDPC) was calculated for each treatment and replication. AUDPC total accumulated disease for the duration $t=t_i = \Sigma\,(y_i+y_i+1)/2(t_{i+1}-t)$, where y is lesion length, t=time (APSnet website). The efficacy of control provided by each fungicide concentration was expressed as percentage of disease control from their respective non-protected controls. The equation used was: [1 − (AUDPC of plants protected with a particular fungicide concentration/ AUDPC of not protected control) × 100]. Levene's tests were conducted to determine if variances of the two trials for each fungicide were homogeneous. Upon confirmation of a non-significant Levene's test, the experiments were combined in single sets for each compound. Variance analyses were conducted using the General Linear Procedure, PROC GLM, of SAS (Statistical Analyses System, Version 9.2, Cary, North Carolina). Mean percent disease severity and control were compared using Fisher's protected LSD test ($\alpha = 0.05$).

Results

Thiophanate-methyl

The variances were homogenous in the two trials as the Levene's value was $P = 0.74$ and thus the two trials were combined. The analysis of covariance was not significant ($P > 0.05$). The analysis of variance detected significant effects of isolates and concentrations (Table 3.3). Significant differences in AUDPC were found between isolates inoculated on plants that were not sprayed with fungicide (Table 3.4). Isolates WM600 and WM376, considered moderately sensitive to TM were considered the least and most aggressive isolates with the latter having an AUDPC approximately 3.7 times greater than the former. Isolate WM596, considered moderately insensitive to TM was as aggressive as WM376 and significantly more aggressive than WM 600 whereas isolate WM414 was as aggressive as WM600 but significantly less aggressive than WM596. The levels of disease control obtained with TM at 1X and 2X (main effects) were significantly better than that of 0.5X but were not different from each other (Figure 3.2). Significant differences were also observed in the main effect of isolates. The highest levels of control were obtained with isolate WM600 followed by WM596 (Figure 3.3). No differences ($P = 0.05$) were detected between the other isolates. While control of all isolates increased with increasing fungicide concentrations, the rate of control increase was smaller for WM600 compared to the other isolates (Figure 3.4). In general, the application of TM at the recommended doses, 1X, reduced disease by an average of 38% compared to the non-protected controls (Figure 3.5). However, when individual isolates were compared, the levels of control observed for the moderately sensitive isolate WM600 were almost one-half of that observed for the other isolates (Figure 3.5).

Table 3.3. Combined analysis of variance of the effect of thiophanate-methyl on control of Sclerotinia stem rot of sunflower caused by *Sclerotinia sclerotiorum* isolates with different levels of sensitivity to thiophanate-methyl[1].

Sources of variation[2]	Degrees of freedom	Sum of squares	F Value	Pr>F
Trial	1	1236	0.67	0.4141
Rep(trial)	10	38803	2.10	0.0244
Isolate	5	48105	5.21	0.0001
Trial*isolate	5	17418	1.89	0.0967
Conc	4	206550	27.95	<0.0001
Trial*Conc	4	1403	0.19	0.9436
Conc*Isolate	20	50433	1.36	0.1384
Trial*Conc*Isolate	20	22770	0.62	0.9001

[1] Trials conducted in greenhouse conditions.

[2] Rep=replication, Conc= thiophanate-methyl concentrations.

Table 3.4. Area under the disease progress curve for Sclerotinia stem rot of sunflower caused by *Sclerotinia sclerotiorum* isolates with different sensitivities to thiophanate-methyl and metconazole.

Isolate	Area under disease progress curve[1]	
	Thiophanate-methyl	Metconazole
WM376	4767.9 [bc]	-[2]
WM414	2609.0 [bc]	-
WM576	2960.7 [a]	-
WM577	2895.4 [c]	2878.1 [b]
WM596	4485.4 [ab]	5352.4 [a]
WM600	1431.0 [c]	-
WM593	-	2131.9 [b]
WM388	-	3075.0 [b]
l.s.d.	1766.3	1629.6

[1] Areas are means across two runs of experiments with xx replications obtained in greenhouse trials.

[2] dash mark (-) means isolates were not tested against this fungicide.

Lowercase letters behind treatment means show which treatment means are significantly different.

Figure 3.2. Effect of three concentrations of thiophanate-methyl (TM) on Sclerotinia stem rot severities caused by Sclerotinia sclerotiorum isolates with different sensitivities to TM. Disease severities are expressed as percentages of control caused by these isolates across all dosage of TM compared with disease on non-protected plants. Bars represent standard error of the means.

Figure 3.3. Mean severity of Sclerotinia stem rot caused by Sclerotinia sclerotiorum isolates with different sensitivities to thiophanate-methyl (TM) on plants protected with different TM concentrations. Disease severity is expressed as percentages of control caused by these isolates across all dosage of TM compared with the disease on non-protected plants. TM moderately-sensitive isolates are represented in light green columns. TM moderately-insensitive isolates are represented in yellow columns. Error bars represent standard error of the means.

Figure 3.4. Dose-response associations between *Sclerotinia sclerotiorum* isolates with different sensitivities to thiophanate-methyl and different concentrations of thiophanate-methyl.

Figure 3.5. Severity of Sclerotinia stem rot caused by Sclerotinia sclerotiorum isolates with different sensitivities to thiophanate-methyl (TM) on plants protected with the recommended TM doses (1X= 4168 µg per ml of water). Disease severities are expressed as percentages of control caused by these isolates at 1X TM compared to disease on non-protected plants. TM moderately-insensitive isolates are represented in orange columns. TM moderately-insensitive isolates are represented in yellow columns. Error bars represent standard error of the means.

Metconazole

The variances of the two trials evaluating the effect of MTZ on disease control were homogenous according to the Levene's test ($P = 0.64$) and thus the two trials were combined. The combined analysis detected significance differences between isolates and concentrations (Table 3.5). Significant differences in AUDPC were found between isolates at no fungicide application (Table 3.4). Isolate WM596, considered resistant to MTZ, was significantly more aggressive ($P = 0.05$) that the rest of isolates evaluated including the other resistant isolate, WM388. WM596 produced an AUPDC that was between 1.75 and 2.5 times greater than that produced by the other isolates. No differences in aggressiveness were detected between the MTZ-sensitive isolates and the resistant isolate WM388. MTZ controlled disease equally well at all concentrations (Figure 3.6). In general, control of disease caused by the resistant isolate WM596 was significantly lower than that caused by sensitive isolates WM 593 and WM577 (Figure 3.7). This significance could be due to decreasing disease control of WM596 with increasing MTZ concentrations (Figure 3.8). Least significant difference value (l.s.d.) to compare between isolates across four replications and two trials was 12.9. However, another resistant isolate, WM388 was not statistically different from the sensitive isolates. When comparing between isolates sprayed with the recommended field doses (1X) of MTZ, isolate WM596 had significantly lesser disease control. For the other resistant isolate, WM388, disease control was not significant from the two sensitive isolates (Figure 3.9).

Table 3.5. Combined analysis of variance of the effect of metconazole on control of by Sclerotinia stem rot of sunflower caused by *Sclerotinia sclerotiorum* isolates with different levels of sensitivity to metconazole[1].

Sources of variation[1]	Degrees of freedom	Sum of squares	F Value	Pr>F
Trial	1	785	0.92	0.3388
Rep(trial)	6	1164	1.37	0.2339
Isolate	3	2921	3.43	0.0195
Trial*isolate	3	189	0.22	0.8813
Conc	4	16338	19.19	<0.0001
Trial*conc	4	309	0.36	0.8347
Conc*isolate	12	987	1.16	0.3204
Trial*conc*isolate	12	456	0.54	0.8876

[1] Trials conducted in greenhouse conditions.

[2] Rep=replication and Conc= metconazole concentrations.
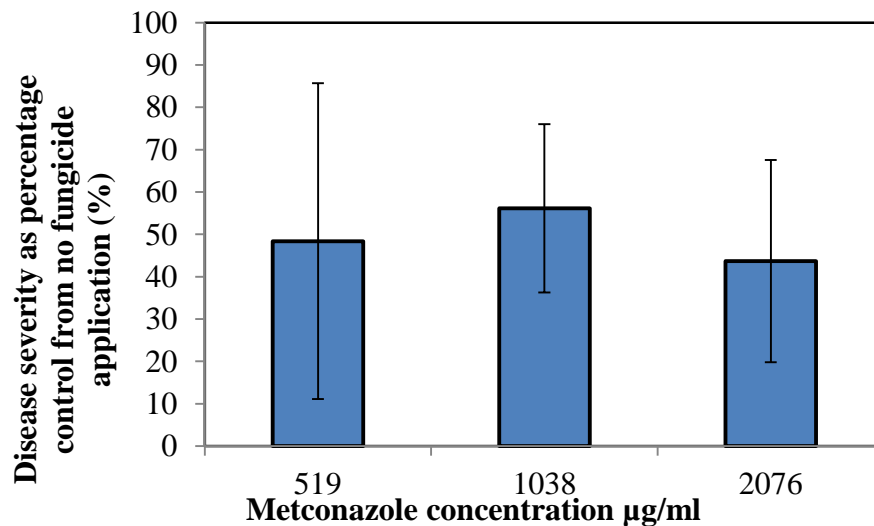
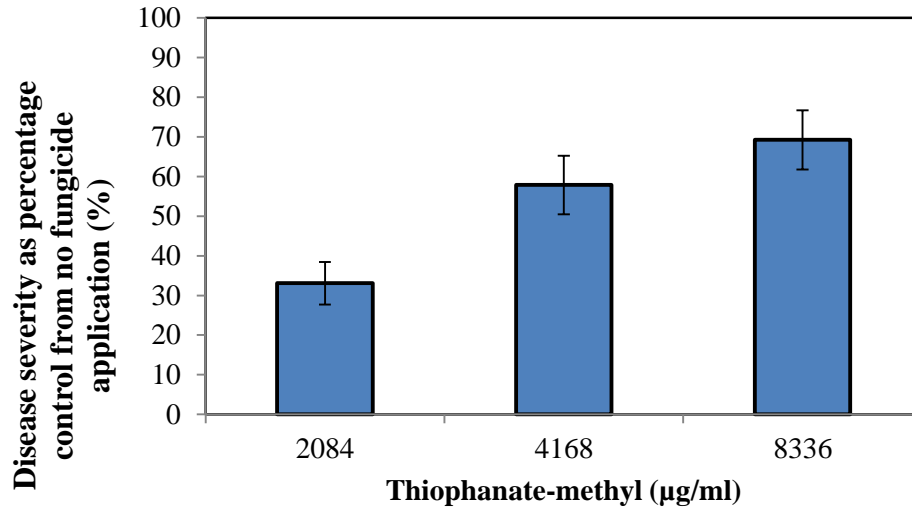**Figure 3.6. Effect of three concentrations of metconazole (MTZ) on Sclerotinia stem rot severities caused by Sclerotinia sclerotiorum isolates with different sensitivities to MTZ. Disease severities are expressed as percentages of control caused by these isolates across all dosage of MTZ compared with disease on non-protected plants. Bars represent standard error of the means.**
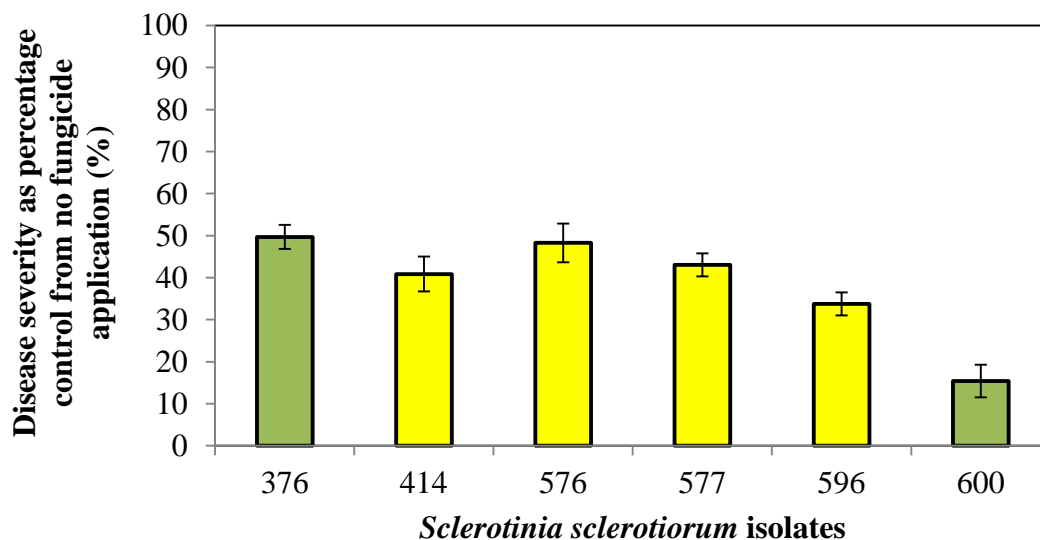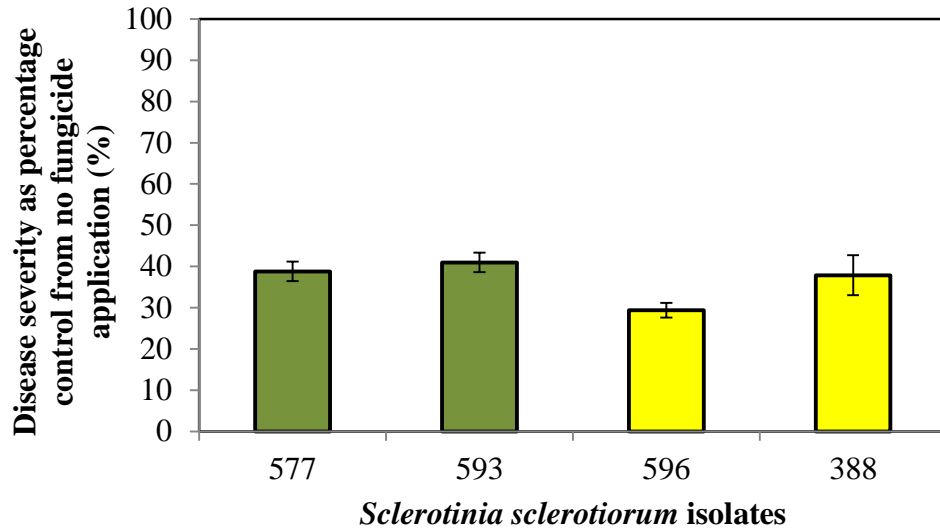
Figure 3.7. Severity of Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* isolates with different sensitivities to metconazole (MTZ) on plants protected with various concentrations of MTZ. Disease severities are expressed as percentages of control caused by these isolates across all dosage of MTZ compared with disease on non-protected plants. MTZ-sensitive isolates in green columns and resistant isolates in yellow columns. Bars represent standard error of the means.
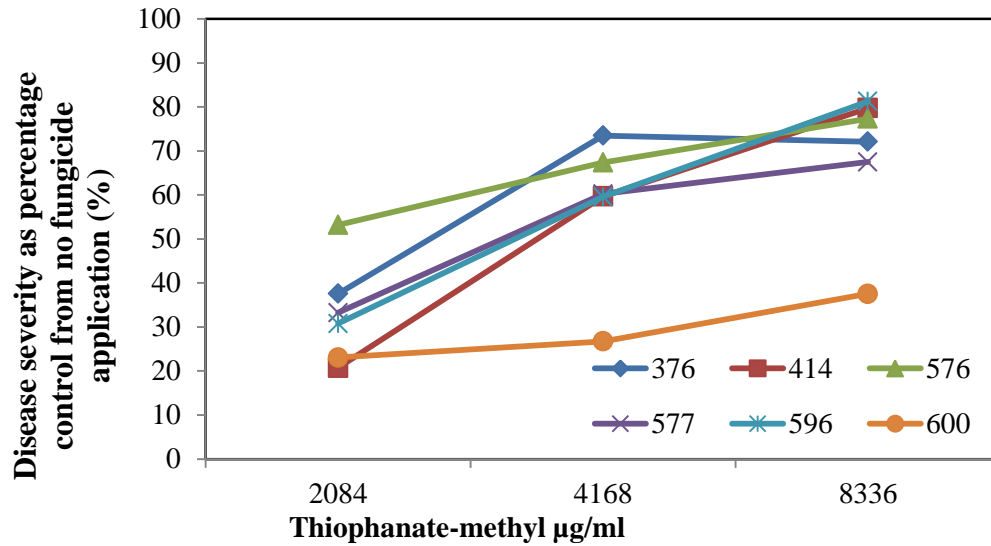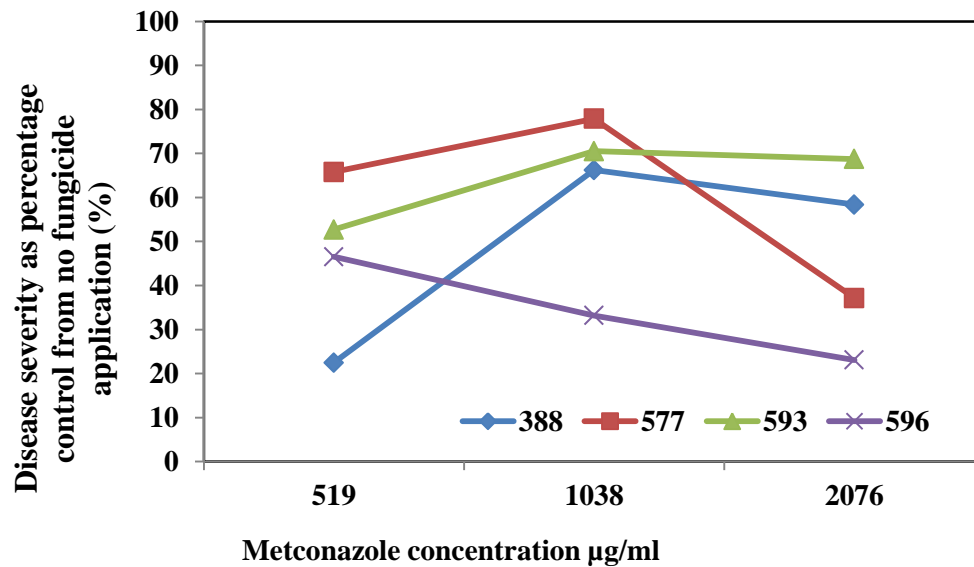

Figure 3.8. Dose-response associations between *Sclerotinia sclerotiorum* isolates with different sensitivities to metconazole and different concentrations of metconazole.
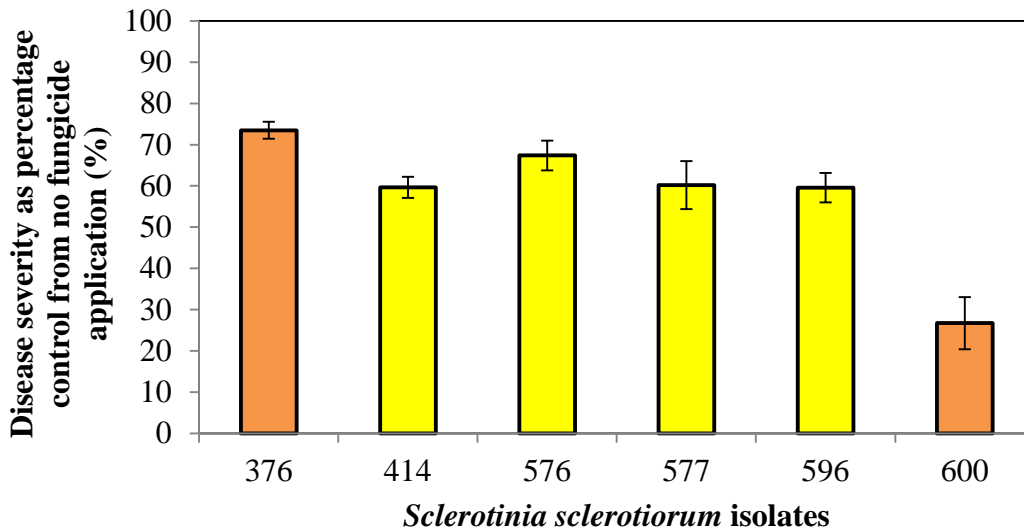
52
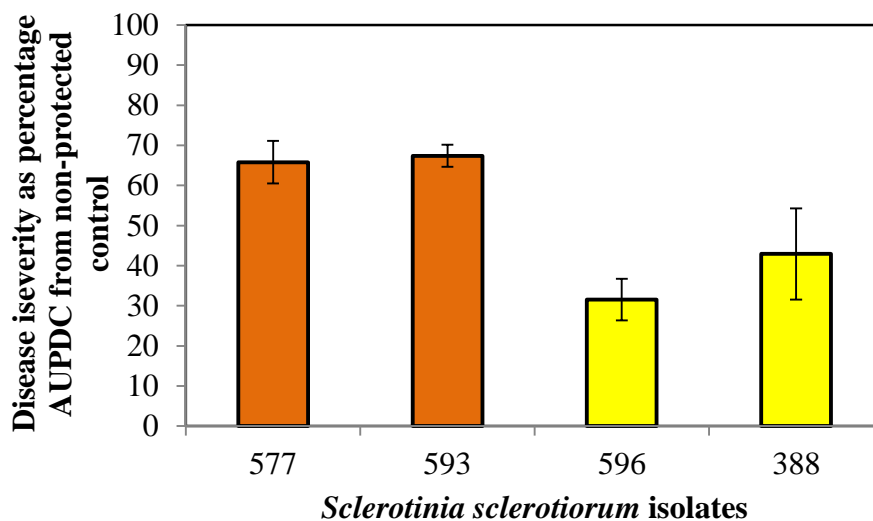
Figure 3.9. Severity of Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* isolates with different sensitivities to metconazole (MTZ) on plants protected with the recommended MTZ doses (1X= 1038 µg per ml of water). Disease severities are expressed as percentages of control caused by these isolates at 1X MTZ compared with disease on non-protected plants. Sensitive isolates are presented in orange columns and resistant isolates in yellow columns. Bars represent standard error of the means.

Discussion

The application of the recommended commercial doses (1X) of TM was effective in reducing the severity of the disease caused by all isolates independently of their aggressiveness. The amount of disease severity caused by the two most aggressive isolates, the moderately insensitive WM596 and the moderately sensitive WM376, were reduced by 60 to 73% compared to the non-protected controls when plants were sprayed with the recommended doses of TM. The moderately sensitive isolate WM600 seemed to be the less affected by increasing concentrations of TM. This apparently sensitive reaction observed across all the fungicide concentration could have been an artifact created by its less aggressive nature. After all, the efficacy of control was a proportion of the amount of disease observed in the non-protected plants. From these results it is clear that even though some levels of insensitivity are already present in the population, the

current recommendations (McMullen and Markell, 2010) are still effective. Situation similar, enhanced levels of insensitivity are detected but there is limited or no loss of efficacy has been reported for other organisms (Babij *et al.,* 2000). Nevertheless, the main effects of isolates showed that the four moderately insensitive isolates of *S. sclerotiorum* produced significantly greater disease than one of the moderately sensitive isolate. Complete control of the disease was not observed even at the highest concentration evaluated a situation that is different than what has been reported for other organisms like *Botrytis cinerea* (Fernandez-Ortuno and Schnabel, 2012). It is possible that complete control could have been achieved if isolates more sensitive than the ones used would have been chosen for the study. In another study on *Botrytis cinerea* (Myresiotis *et al.,* 2008), although belonging to the same chemical group, benzimidazoles; carbendazim failed to control sufficiently the benzimidazole resistant isolates in the greenhouse experiment.

MTZ sensitivity results represent a baseline data of MTZ in north central US. When plants were not protected with MTZ, the resistant isolate WM596 produced higher amounts of disease or was more aggressive than other isolates. The main effect of testing efficacy of disease control due to enhanced level of insensitivity of *S. sclerotiorum* to metconazole showed that the resistant isolates of *S. sclerotiorum* had less than 50% disease control than the moderately sensitive isolates across all the treatments. When comparing between different isolates sprayed with recommended dosage (1X) of MTZ, we observed that resistant isolates had lower disease control than moderately sensitive isolates. In a previous study conducted on *Alternaria alternata* disease control by QoI fungicide, sensitive and reduced sensitive isolates was not significantly different (Pasche *et al.,* 2004). The same group working on *Alternaria solani* found that moderate level of resistant isolates (5 - 20 µg/ml) were controlled better than high level of

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resistant isolates (>20 µg/ml). They indicated that level of disease control that boscalid provided for moderately resistant isolates was very similar to that reported for QoI fungicides azoxystrobin and pyraclostrobin controlling *A. solani* isolates with the F129L mutation (Gudmestad *et al.,* 2013). Reason for development of resistance in DMI; suggest that accumulation of several resistance mechanisms may be needed before practical disease control difficulties emerge, which probably accounts for the slow stepwise development of azole resistance in many plant pathogens. The inability of MTZ to reduce disease caused by resistant isolates needs to be carefully handled by following the resistance management at farm level. Spraying of MTZ or any other compound sharing the same mechanism of action should be rotated with fungicide of other mode of action. This information should be used irrespective of crop but with respect to the farm. There is need of evaluating fungicide mixes for better resistance management.

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CHAPTER 4. EFFECT OF INCUBATION TEMPERATURE ON THE FUNGICIDE SENSITIVITY OF *SCLEROTINIA SCLEROTIORUM* TO THIOPHANATE-METHYL

Abstract

Laboratory estimation of EC_{50} values are conducted most of the time at "room temperature" or somewhere around 20° C. Sometimes, however, this temperature is not the optimum temperature of the target organism. So, the objective of this study was to evaluate the effect of incubation temperature on sensitivity of *Sclerotinia sclerotiorum* to thiophanate-methyl (TM). The study was conducted in growth chambers using three temperatures, 15, 21, and 25° C, and 15 isolates with varying degrees of sensitivity to TM. The study had three replications and the entire experiment was conducted three times. A highly significant ($P < 0.001$) and positive association was detected between tolerance to TM and incubation temperature. This association was tighter for insensitive (MI) isolates which had a temperature dependent response. With increase in temperature from 15 to 21°C all the isolates, irrespective of level of sensitivity had better tolerance to thiophanate-methyl but temperature increase from 21 to 25°C, the insensitive isolates (NS) had reduced tolerance to thiophanate-methyl. This study addresses an important factor in chemical control of plant pathogens. Information on the temperature sensitivity of plant pathogens would directly impact the fungicide application strategies.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic plant pathogenic fungus which causes Sclerotinia stem rot (SSR) of canola and many other important crops (Boland and Hall 1994; Purdy 1979). This pathogen has a broad geographical distribution, and is prevalent in many US states including those in the North Central region (Bradley and Lamey, 2005). Use of fungicides has been the primary method of controlling SSR in canola as well as in many other

crops where this pathogen is important. Fungicides registered for control of Sclerotinia stem rot include azoxystrobin (Quadris[R]), boscalid (Endura[R]), metconazole (Quash[R]), prothioconazole (Proline[R]), pyraclostrobin (Headline[R]), and thiophanate-methyl (Topsin[R]) (McMullen and Markell, 2010). Thiophanate-methyl (TM) is perhaps the oldest fungicide in use for SSR control. A recent study with 81 isolates of *S. sclerotiorum* collected from 13 states in North Central US showed EC_{50} values ranging from 0.34 to1.6 µg/ml with a 0.76 µg/ml mean and 0.69 µg/ml median (chapter 2 of this thesis). These EC_{50} values were obtained by incubatingthe isolates at 21° C in medium amended with TM. While this temperature is within what is considered optimum for this pathogen (Harikrishnan and del Rio, 2006), a quick review of literature pertaining to estimation of EC_{50} values for other organisms, revealed that most tests are conducted at around 20° C independently of what the optimum temperatures for the organisms studied are. For example, *Ulocladium cucurbitae* has optimal spore germination at 30° C, although germination can occur between 9 and 36° C, yet EC_{50} were calculated at 21° C (Zitter, 1992). A study monitoring resistance of *Cercospora beticola* to fungicides was conducted at "room temperature" which in their situation was approximately 21° C (Secor *et al.,* 2010), while favorable temperatures for *C. beticola* development are around 27 $^{\circ}$C (Darouux, *et al.* 1960). While these "room temperatures" may have been used to facilitate comparison of results between laboratories and related organisms, they may have also contributed to the creation of artificial information. Information on the sensitivity of an organism to a fungicide developed at temperatures that are optimal for pathogen/disease development may be misleading.

Temperatures can have a profound effect on the response of an organism to fungicides. Highly resistant isolates of *Monilinia fructicola* (EC_{50} benomyl >50 µg/ml) became sensitive when incubated at 31°C, while moderately resistant isolates (EC_{50} benomyl ranging between

2.38 and 6.42 μg/ml) became sensitive at temperatures below 15° C (Ma and Michailides, 2003b). Triadimefon-resistant strains of *Uncinula necator* were less sensitive to the fungicide when incubated at 15° C than at 25° C (Ypema and Gubler, 1997). If fungicide sensitivity is affected by temperature, it is in our best interest to determine its effect on the species one works with. So, the objective of this study was to evaluate the effect of incubation temperature on *S. sclerotiorum* sensitivity to TM.

Materials and method

Isolates and inoculum preparation

Twelve *S. sclerotiorum* isolates with varying degrees of sensitivity to TM were used in this study. Three arbitrary groups were created: a moderately insensitive (MI) group with $EC_{50} \geq$ 0.75 μg/ml was represented by six isolates; a very sensitive (VS) group with $EC_{50} < 0.6$ μg/ml was represented by four isolates. An intermediate group, moderately sensitive (MS), was represented by five isolates. Sclerotia from each isolate were surface disinfested by immersing them in a 10% NaOCl and water solution (v/v) (Clorox Sales Co., Oakland, CA, USA) for 1 min. After disinfestation, sclerotia were rinsed in sterile distilled water once and plated on quarter-strength potato dextrose agar (PDA) medium to generate mycelial colonies that would be used for the study. Isolates were incubated at 21° C until the colonies covered approximately one-fourth of the dishes' surface. At that time, agar plugs 4 mm in diameter and containing hyphal tips of actively growing *S. sclerotiorum* colonies were cut to be used as inoculum the fungicide test.

Media preparation

Quarter-strength PDA used to produce inoculum from each isolate as well as the medium to be amended with fungicides was prepared by combining 6 g of Potato Dextrose Broth (HiMedia Laboratories Pvt. Ltd, India), 15 g agar (Bacto Agar, Becton, Dickinson and Co., Sparks, MD, USA) and 1000 ml of distilled water and autoclaved at 121° C and 103.4 kPa for 20 minutes. Technical grade TM (95% a.i, Ceraxagri, King of Prussia, PA, USA) was diluted with acetone to make 1000 μg/ml stock solution. Luke-warm quarter-strength PDA was amended with the stock solution to final concentrations of 0, 0.1, 0.5, 1, 1.5, 2, 2.5 and 5 μg/ml with three replications. The amended media were poured into Petri dishes at a rate of approximately 25 ml per dish. Dishes were used immediately after preparation.

EC_{50} estimation and data analyses

Inoculated plates were incubated for 48 h at 15, 21 or 25° C in dark. For each experimental unit, growth was expressed as the average of two perpendicular readings of colony diameters. Percentage of growth suppression for each isolate was calculated based on the growth observed in the corresponding non-amended medium dishes. Negative values of growth suppression, situation in which colonies growing in fungicide-amended medium were slightly larger than colonies growing in non-amended medium, were equaled to zero for estimation of sensitivity of isolates to the fungicide. Fungicide sensitivity was expressed in terms of the concentration required to reduce growth of each isolate by 50% (EC_{50}). These EC_{50} values were calculated using PROC NLIN (non-linear regression) using SAS 9.2 (SAS Institute, Cary, NC) and were expressed in μg per ml. This study was conducted three times. Levene's test for homogeneity of variances was conducted on the EC_{50} values of each of the times the entire experiment was conducted. The test was conducted using SAS 9.2. Upon confirmation that the

trials could be combined, an analysis of variance was conducted using the general linear models procedure, PROC GLM, of SAS 9.2 with sources of variation as trials, replication within trials, isolate, temperature and isolate*temperature. Single-degree-of-freedom contrast analyses were conducted to determine whether the means of different groups of treatments were statistically significant or not; these comparisons included the means for isolates considered VS vs. MS or vs. MI at specific incubation temperatures, were different from each other. Linear regression analyses were conducted to describe the effect of temperature on the sensitivity of each of these groups.

Results

Homogeneity of variance test indicated that variances were homogeneous ($P = 0.28$) and therefore could be analyzed in a combined ANOVA. In the combined ANOVA, the effects of isolates, temperatures, and the isolate*temperature interactions were statistically significant but the effect of trial and replications within trial were not (Table 4.1). Significant differences ($P < 0.0001$) in sensitivity to TM were observed between isolates across incubation temperatures. The mean EC_{50} for isolates considered very sensitive (VS), moderately sensitive (MS), and moderately insensitive (MI) to TM were 0.79, 0.91, and 1.0 µg/ml, respectively. These means were significantly different from each other ($P = 0.05$) (Figure 4.1). The main effect of incubation temperature on sensitivity to TM across all the 12 isolates of *S. sclerotiorum* isolates was significant ($p < 0.0001$). The mean EC_{50} for 15° C, 21 and 25° C across all the 12 isolates were 0.68, 1.01 and 1.04 µg/ml, respectively. These means were significantly different for 15° C only ($P = 0.05$) (Figure 4.2).

Table 4.1 Combined analysis of variance for the effect of incubation temperature on the sensitivity of *Sclerotinia sclerotiorum* to thiophanate-methyl.

Sources of variation[1]	Degrees of Freedom	Mean Square	F Value	Pr > F
trial	2	0.25	2.93	0.0552
rep(trial)	6	0.03	0.35	0.9108
iso	11	2.08	24.73	<0.0001
temp	2	4.29	50.93	<0.0001
iso*temp	22	0.32	3.74	<0.0001

[1] rep(trial)= interaction between replications and trials; iso=isolate; temp=temperature and iso*temp=interaction between isolates and temperature.
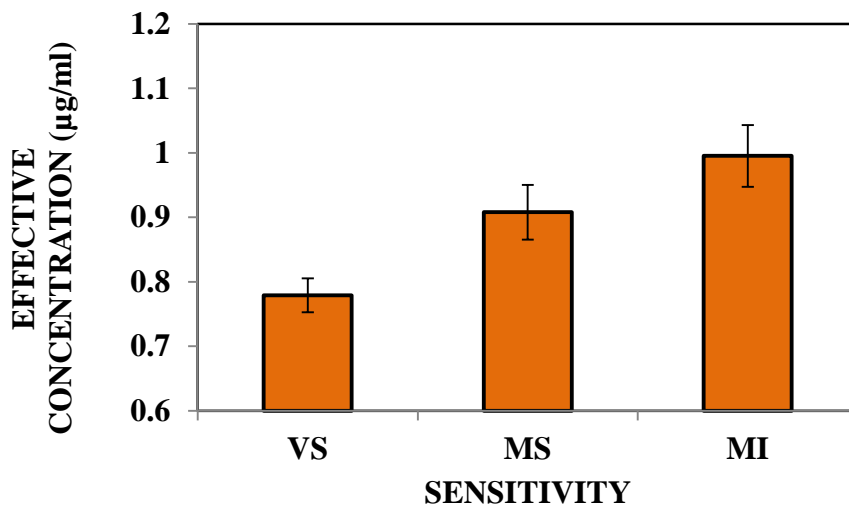
Figure 4.1. Mean fungicide sensitivity, expressed as 50% effective concentration (EC$_{50}$), of *Sclerotinia sclerotiorum* isolates considered very sensitive (VS), moderately sensitive (MS), and moderately insensitive (MI) to thiophanate-methyl across three incubation temperatures. Bars in columns represent standard error.
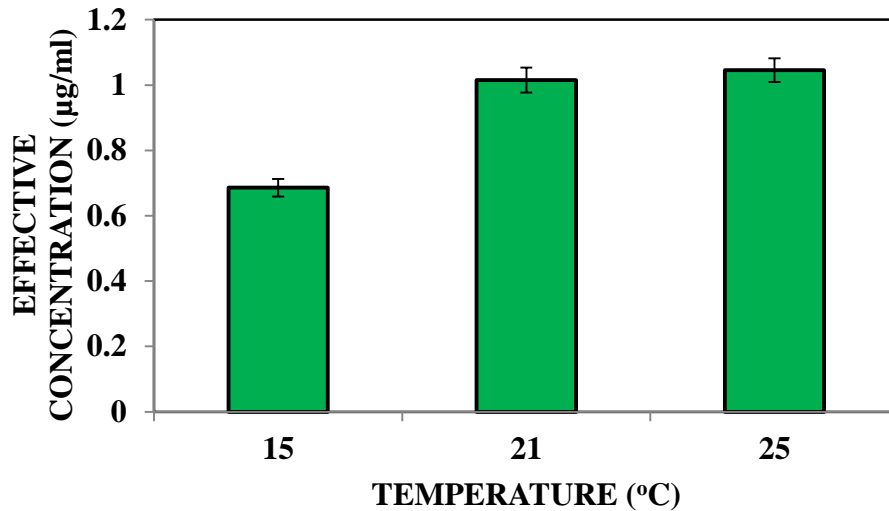
Figure 4.2. Main effect of incubation temperature on sensitivity to thiophanate-methyl, expressed as 50% effective concentration (EC$_{50}$), of 12 *Sclerotinia sclerotiorum* isolates. Bars in columns represent standard error

The incubation temperature had a profound effect on the sensitivity of *S. sclerotiorum* isolates to TM (Table 4.2). When comparing MI vs. VS, the MI group had a mean EC$_{50}$ that was 45% greater than that of the VS group when incubated at 15° C ($P = 0.0063$) or at 21° C ($P < 0.0001$); however, when incubated at 25° C both groups were equally sensitive ($P = 0.6882$). When the MS and VS groups were compared, their differences were greatest at 15° C than at 21 or 25° C; at 15° C, the mean EC$_{50}$ for MS was 35% greater ($P = 0.0247$) than that of VS, but only 18% greater ($P = 0.0451$) when incubated at 21° C. When incubated at 25° C, both groups were equally sensitive to the fungicide ($P = 0.5447$). When MI and MS were compared, their differences were greatest at 21° C than at 15 or 25° C; at 21° C, the mean EC$_{50}$ for MI was 23% greater ($P = 0.0004$) than that of MS, but only 7% greater ($P = 0.0451$) when incubated at 15° C and not significantly different at 25° C (Table 4.2).

Table 4.2. Contrast analyses to determine the effect of fungicide sensitivity and incubation temperature of 12 *Sclerotinia sclerotiorum* isolates considered very sensitive (VS), moderately sensitive (MS) or moderately insensitive (MI) to thiophanate-methyl.

Contrast	Incubation temperature (°C)	Pr > F	Means [1]	
			A	B
MI vs. VS	15	0.0063	0.75	0.52
	21	< 0.0001	1.19	0.81
	25	0.6882	1.04	1.01
MS vs. VS	15	0.0247	0.70	0.52
	21	0.0451	0.97	0.81
	25	0.5447	1.06	1.01
MI vs. MS	15	0.4042	0.75	0.70
	21	0.0004	1.19	0.97
	25	0.8197	1.04	1.06

[1] Means A and B refer to the means of the first and second terms in each contrast, respectively.

In general, the incubation temperature had a significant (P=0.008) and positive association with sensitivity of *S. sclerotiorum* to TM (Figure 4.3). When evaluated separately, the association between VS and MS isolates and temperature were almost linear with the EC_{50} for VS isolates increasing 0.049 units for every unit increase of temperature (Fig. 4.3A) and that of the MS isolates increasing by 0.037 units (Fig. 4.3B); but the association between MI isolates and temperature was not. The Ec_{50} of MI isolates increased with temperature when temperature changed from 15 to 21°C but when the temperature increased to 25° C, the EC_{50} did not increase at the same rate (Fig. 4.3C) Although, there exists a positive and strong relation between VS and MS isolates and temperature, we found weak relation between the MI isolates and temperature, which was about 25.3%.
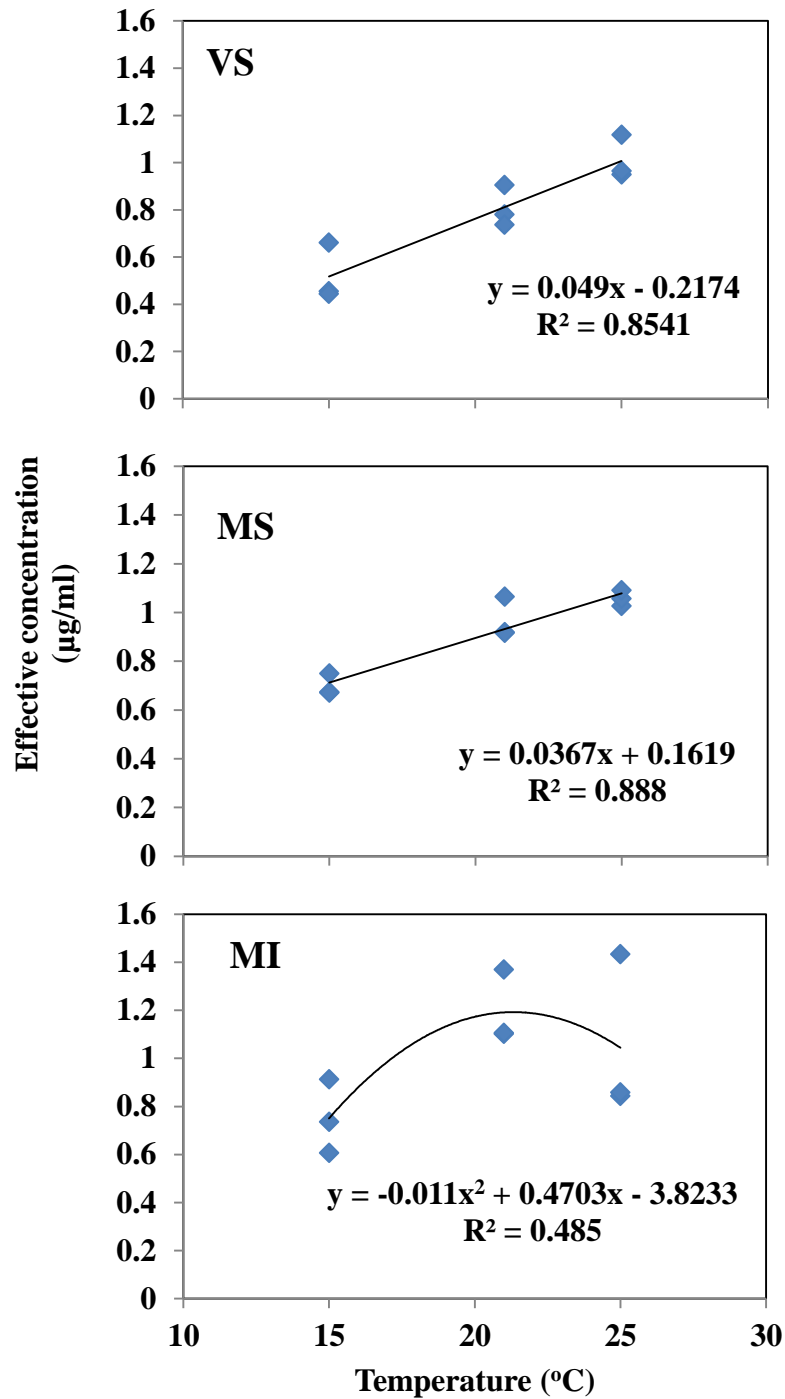
Figure 4.3. Changes in sensitivity of *Sclerotinia sclerotiorum* isolates considered A) moderately sensitive (MS); B) moderately insensitive (MI); and C) very sensitive (VS) to thiophanate-methyl as a function of incubation temperature.

Discussion

The response of all isolates of *S. sclerotiorum* to TM was temperature dependent. EC_{50} values increased with varying degrees of magnitude when temperatures increased from 15 to 25° C. TM-sensitive and moderately insensitive isolates increased their tolerance to the fungicide by almost 50% and 37%, respectively. This could be due to temperature regulated molecular changes at the site of action of the fungicide. However, we noticed a relatively slower rate of mycelial growth of the fungus in the media at lower temperatures too. The increased tolerance by the insensitive isolates with an increase of temperature leads us to assume that there could be role of temperature sensitive mutations which does not affect phenotypically after a specific temperature in the pathogen. For example, *Monilinia fructicola* ($EC_{50} > 50$ µg/ml) were heat sensitive and did not show high resistance to benomyl at 31° C, while the low resistant isolates (EC_{50} ranging from 2.38 to 6.42 µg/ml) were cold sensitive and did not show resistance to benomyl at temperatures below 15 °C (Ma and Michailides, 2003b). The rate of metabolism of the fungicide in the plant system may also play a role; at higher temperature the insensitive pathogen was rather sensitive because the fungus could not degrade the fungicide molecule fast enough. Lacey, 1990, reported that drug resistance in any organism is a change in the chemicals pharmacodynamics (absorption, distribution, metabolism, excretion and site of action). Resistance therefore need not necessarily occur at the site of action.

Information on the effect of temperature on sensitivity of plant pathogens to fungicides is scarce but the existing literature suggests the response is species dependent and an increase in temperature not always results in increased sensitivity. In *S. sclerotiorum* prefers temperatures in the range of 12 to 28° C (Harikrishnan and del Rio, 2006; Sun and Yang, 2000). To get a more complete picture of the effect of temperature on sensitivity of this organism to TM, temperatures

like 12° C should be included since the pathogen can develop at that temperature too. The overall picture of the effect of temperature on sensitivity of plant pathogens to fungicides would help us standardize the sensitivity evaluation studies in the laboratory. Therefore, it is highly recommended to look for the effect of temperature on sensitivity on other pathogens. Reports of resistance in a pathogen population obtained at room temperature may change if lower or higher temperatures are used. Information on the effect of temperature on reaction of insensitive isolates to the fungicide could directly impact fungicide application strategies. For instance, if resistant isolates show sensitivity to the fungicide at high temperatures, hot days may be more appropriate times for the application of the fungicide in field where resistant isolates have been detected (Ma and Michailides, 2005). In the same token, lower field temperatures that may favor Sclerotinia stem rot development may also reduce the efficacy of fungicide applications thus requiring the use of higher concentrations. Thus, EC_{50} values reported at lab temperatures may not be the true representative of sensitivity of a pathogen present in its natural environment. Epidemiological models for timely fungicide sprays should also consider pathogen sensitivity to temperature for evaluation of the most effective temperature for field spray to target the insensitive isolates and finally reduce resistance build up.

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CHAPTER 5. GENETIC CHARACTERIZATION OF RESISTANCE OF SCLEROTINIA SCLEROTIORUM TO THIOPHANATE-METHYL

Abstract

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic plant pathogenic fungus that causes Sclerotinia stem rot (SSR) of canola and many other important broadleaf crops. Thiophanate-methyl (TM) which is a benzimidazole fungicide registered for control of *S. sclerotiorum* in canola has a single-site mode of action and a history of resistance buildup on target populations. In a recent study in which 81 *S. sclerotiorum* isolates collected from North Dakota and 12 other states in North Central US were evaluated for their sensitivity to TM, one isolate, WM365, was considered resistant with an EC_{50} value of 1.62 ± 0.75 µg/ml. Since resistance to benzimidazoles has been correlated with point mutations in the β-tubulin gene, DNA was extracted from 81 isolates with EC_{50} ranging between 0.34 and 1.62 µg/ml, so the sequence of their β-tubulin gene could be examined. To amplify the β-tubulin gene of *S. sclerotiorum*, the gene's sequence information was downloaded from the Broad Institute website. Two primer pairs were constructed using the Basic Local Alignment Sequence Tool to amplify the 1927 bp long β-tubulin gene. The amplicons from individual PCR reactions were sent to McLab for sequencing in both directions and then aligned to the reference sequence using BioEdit Sequence Alignment software. Isolate WM365 had a previously unreported mutation that is due to change from glutamic acid (E) to aspartic acid (D) at codon 111 in the polypeptide chain. This change may be responsible for its enhanced tolerance to TM. None of the other isolates had changes in their gene sequence.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary causes Sclerotinia stem rot on canola and many other broad leaf crops including soybean, dry bean, and sunflowers in North Central US (Bradley and Lamey, 2005). In the absence of effective genetic resistance, the disease is managed mainly through the use of fungicides (Bradley *et al.,*, 2006). Fungicides registered for control of Sclerotinia stem rot in North Dakota are: azoxystrobin (Quadris[R]), boscalid (Endura[R]), metconazole (Quash[R]), prothioconazole (Proline[R]), pyraclostrobin (Headline[R]), and thiophanate-methyl (Topsin[R]) (McMullen and Markell, 2010). Of this group, thiophanate-methyl (TM) is perhaps the oldest compound in use; TM was registered as a fungicide in 1973 (USEPA, 2004) and reregistered in 1996 due to United States Environment Protection Agency's revised standards as a systemic fungicide to be used on a variety of trees, vines, root crops, wheat and canola (USEPA, 2005). Chemically, TM does not belong to the benzimidazole family, however, its primary metabolite is carbendazim (methyl 2-benzimidazole carbamate) or MBC (Vonk and Sijpesteijn, 1971), thus for practical purposes it is considered a benzimidazole. TM falls under FRAC code 1 and acts on the mitosis and cell division in the target fungi (FRAC, 2007). The first benzimidazole to be used in plants was benomyl, which was introduced to the market in the late 1960's (Delp, 1995). TM and benomyl have carbendazim as their primary metabolite (Vonk and Sijpesteijn, 1971). Pan *et al*. (1997) established a carbendazim baseline sensitivity for *S. sclerotiorum* and proposed that isolates with $EC_{50} \geq 2$ μg/ml be considered resistant. Therefore, using Pan et al.'s threshold, one isolates of *S. sclerotiorum* evaluated for sensitivity to TM as part of this thesis (see chapter 2) would be considered close to resistant.

Resistance to benzimidazoles has been associated with a reduction in the ability of the fungicides to bind to the β-tubulin gene. Point mutations in this gene at codons 6, His to Tyr; 50,

Tyr to Cys; 167, Phe to Tyr; 198, Glu to Ala/Gln/Gly/Lys/Val; 200, Phe to Tyr; and 240, Leu to Phe, have been identified as responsible for the resistance to benzimidazoles (Ma and Michailides, 2005). These mutations have been confirmed by site-directed mutagenesis followed by gene replacement (Li *et al.,* 1996). Not all mutations, however, lead to severe increases in resistance and in some instances different mutations in the same codon can result in different levels of resistance (Li *et al.,* 1996; Ma and Michailides, 2005). In *Monilinia fructicola*, the mutation at codon 6 led to a low resistance level while a mutation at codon 198 led to a high level of resistance (Ma and Michailides, 2003b). In *Venturia inaequalis*, mutations at codons 198 and 200 caused a medium and high resistance level, respectively (Koenraadt *et al.,* 1992). In a study described in chapter 2 of this thesis in which we evaluated 81 *S. sclerotiorum* isolates for their sensitivity to TM, we found one isolate, WM365, whose EC_{50} included the 2 μg/ml threshold for resistance and few others with EC_{50} that were very close to it. Thus, the objective of this study was to determine whether the β-tubulin genes of these isolates had changed.

Materials and methods

Isolates and media preparation

Eighty one *S. sclerotiorum* isolates were used in this study. *S. sclerotiorum* isolates were selected from the study described in chapter 2, based on their sensitivity to TM. The mean TM EC_{50} values for isolates ranged between 0.34 and 1.62 μg/ml. (Table 5.1).

Table 5.1. Sensitivity of representative *Sclerotinia sclerotiorum* isolates to thiophanate-methyl used in the evaluation of genetic basis of mutation.

Isolate	EC$_{50}$ (µg/ml)		Sensitivity	Origin
	Mean	Standard error of mean		
WM398	0.57	0.08	S	Minnesota
WM600	0.65	0.07	MS	Minnesota
WM605	0.67	0.08	MS	Montana
WM596	0.76	0.09	MI	Nebraska
WM 577	0.88	0.23	MI	Manitoba
WM365	1.62	0.40	MI	Nebraska

[1]S=Sensitive isolate had EC$_{50}$ < 0.6, MS=moderately sensitive with EC$_{50}$ 0.6 - 0.75 MI=moderately insensitive isolates with EC$_{50}$ > 0.75 µg/ml.

One quarter-strength potato dextrose agar (PDA) medium was used to produce mycelial colonies of each isolate. The medium, which was prepared by combining 6 g of Potato Dextrose Broth (HiMedia Laboratories Pvt. Ltd, India), 15 g Bacto Agar (Becton, Dickinson and Company, Sparks, MD, USA) and 1000 ml of distilled water, was autoclaved at 121° C and 103.4 kPa for 20 minutes before being poured into plastic Petri dishes. Sclerotia from each of these 81 isolates were surface disinfested by immersing them in a 10% aqueous solution of NaOCl (v/v) (Clorox Sales Co., Oakland, CA, USA) for 1 min. Sclerotia were rinsed in distilled water. Surface-disinfested sclerotia were plated and incubated at room temperature (21° C) until the mycelial colony covered about one-quarter of the surface area of the dish.

DNA extraction and sequence analyses

Agar plugs containing hyphal tips from each isolate were collected and transferred into 250 ml flasks containing each 150 ml of sterilized full strength potato dextrose broth. The broth, which was made by combining 24 g of Potato Dextrose Broth (HiMedia Laboratories Pvt. Ltd, India) and 1000 ml of distilled water, was autoclaved at 121° C and 103.4 kPa for 20 minutes.

Inoculated media were incubated in a horizontal shaker (Lab Line Orbit Environ, IL, USA) at 100 rpm for 3 days at 21° C. Mycelia from each colony were then harvested by vacuum filtration using Whatman filter paper # 1 to retain the mycelia. Mycelia were then blotted dry in a laminar flow hood. Approximately 100 mg of dry mycelia was used to extract genomic DNA using DNeasy Plant Mini Kit (Qiagen, Chatsworth, CA) following manufacturer's instructions. DNA was quantified using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Products, Wilmington, DE). The DNA concentration was adjusted to 30 ng per µl by adding adequate amounts of TE buffer (Tris HCl 10mM, EDTA 1 mM) and stored at -20° C until used.

To develop primers that could be used to amplify the β-tubulin gene of *S. sclerotiorum*, the gene's sequence information was downloaded from the Broad Institute website (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/Regions.html). The primer pairs were designed to amplify overlapping fragments of the β-tubulin gene (Figure 5.1).
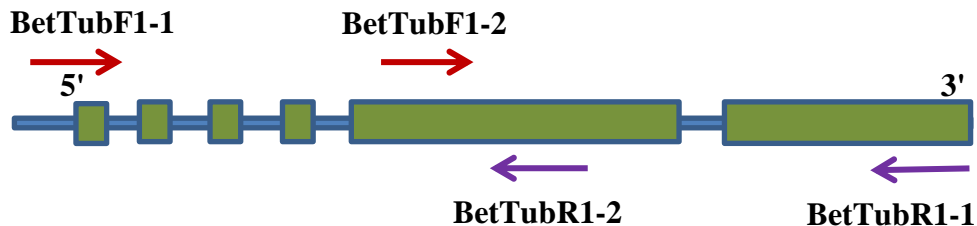
Figure 5.1. Schematic representation of *Sclerotinia sclerotiorum* β –tubulin gene with primers pairs used in the evaluation of genetic basis of fungicide mutation.

Two forward and two reverse primers were used to amplify the 1927 bp length of β-tubulin gene (Table 2). The specific temperature for annealing primers was identified by running PCR reactions on two temperature gradients, starting from 49.1 to 57.8 (12 different temperatures) and 53 to 62.9° C (12 different temperatures) and running the amplicons on 1%

TBE agarose gel (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) to select the temperature that resulted in distinct bands.

The PCR was conducted in a PCR Thermocycler (Eppendorf North America, Hauppauge, NY, USA) under the following conditions: denaturation at 94° C for 5 min followed by 94° C for 1 min, annealing at 58° C for 30 sec, extension at 72° C for 80 sec. These conditions were used for 30 cycles followed by a final 10 min extension period at 72° C. The PCR products were stored at 4° C until used on. Amplicons from PCR reactions from each isolate were sent to McLab (San Francisco, CA, U.S.A.) for sequencing in both directions using the same two primer pairs, BetTubF1-1 and BetTubR1-2, and BetTubF1-2 and BetTubR1-1. Sequences received were trimmed and compared to reference sequences using BioEdit Sequence Alignment editor manually. The nucleotide sequences were converted to their corresponding amino acid sequences and aligned using Clustal W accessory application in the BioEdit program (Hall, 1999) to determine whether changes had occurred.

Results

The β-tubulin gene sequence of *S. sclerotiorum*, was downloaded from the Broad Institute website and primer pairs designed to amplify overlapping fragments of the β-tubulin gene are as follows (Table 5.2). The determination of temperature for annealing primers by running PCR reactions on two temperature gradients, starting from 49.1 to 57.8 (12 different temperatures) and 53 to 62.9^o C (12 different temperatures) and running the amplicons on 1% TBE agarose gel (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) resulted in distinct bands for temperature around 58 ^{o} C based on the specificity of band amplification (Fig 5.2 and Fig 5.3).

Table 5.2. Primers and primer sequences used for amplification of the β –tubulin gene of *Sclerotinia sclerotiorum* **isolates in this study.**

Primers:	5' → 3'
BetTubF1-1	ACATCTCAGCAATCCTTAACCTCC
BetTubR1-1	AACCAGTCCGTCGTTTCTTC
BetTubF1-2	GCCGGTATGGGTACGCTTTT
BetTubR1-2	GCGATGGGACGACGGAGAAG
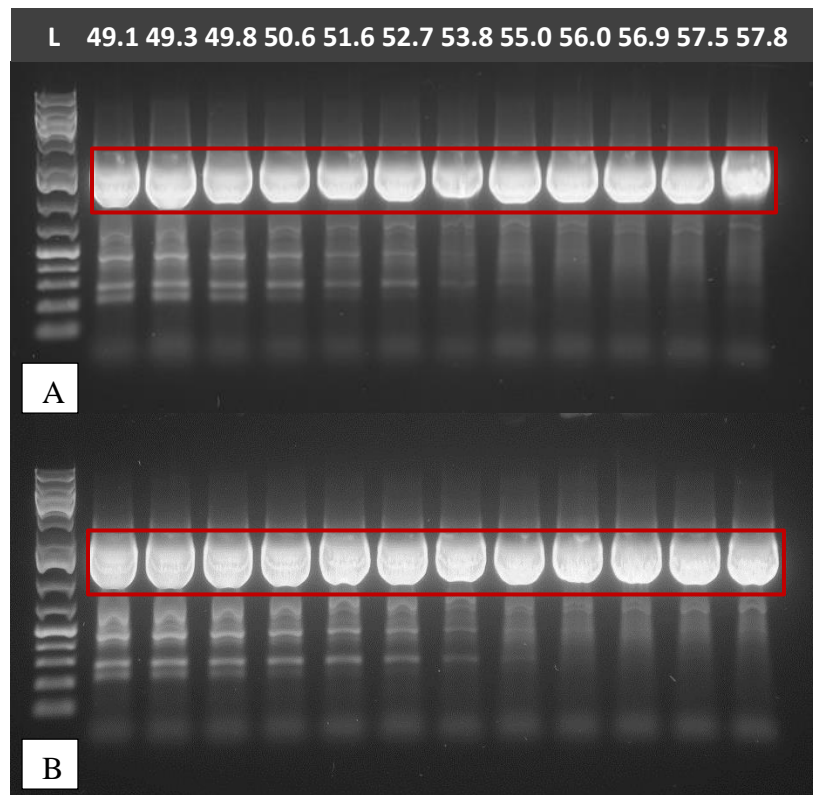
Figure 5.2. *Sclerotinia sclerotiorum***'s β-tubulin gene bands amplified using A) primer pair BetTubF1-1 and BetTubR1-2 at annealing temperature gradient from 49.1 to 57.8 ^{o} C, and B) primer pair BetTubF1-2 and BetTubR1-1 at annealing temperature gradient from 49.1 to 57.8 ^{o} C.**
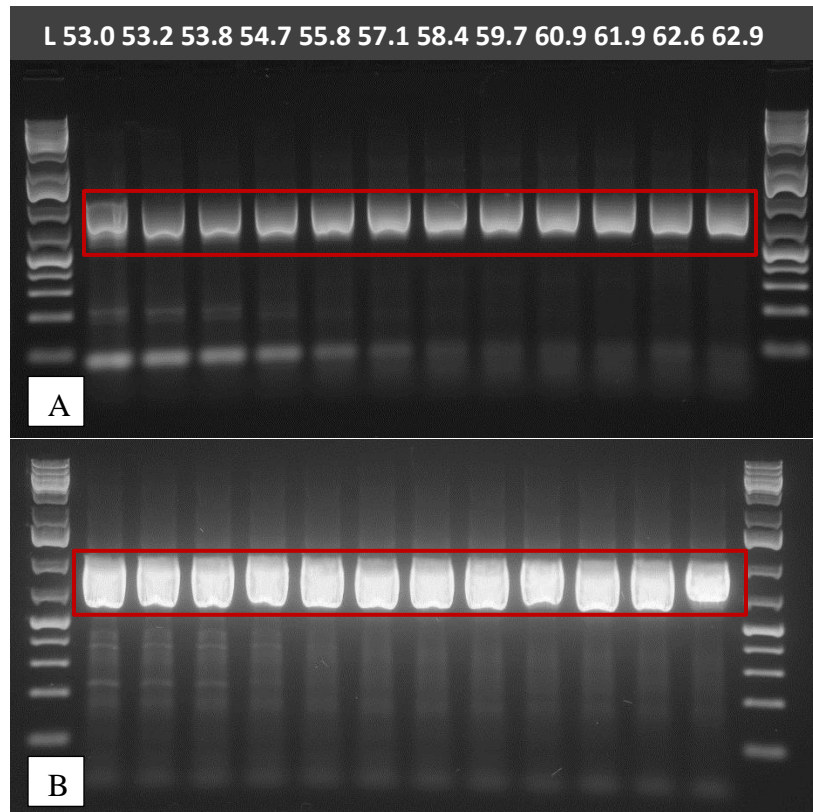
Figure 5.3. *Sclerotinia sclerotiorum***'s β-tubulin gene bands amplified using A) primer pair BetTubF1-1 and BetTubR1-2 at annealing temperature gradient from 53.0 to 62.9 °C, and B) primer pair BetTubF1-2 and BetTubR1-1 at annealing temperature gradient from 53.0 to 62.9 °C.**

A missense mutation was detected at nucleotide position 333 in isolate WM365 which is resistant to TM. This mutation changed the base from guanine (G) to cytosine (C) (Fig. 5.4). Sequences of β-tubulin of the rest 80 isolates did not have any mutations. This change, which was unique to this isolate, translated into Aspartic acid (D) rather than Glutamic acid (E) at codon 111 in the polypeptide chain of the β-tubulin gene (Fig.5.5)

Nucleotide sequence with corresponding position number in the β-tubulin gene							
ISOLATE	280	290	300	310	320	330	340	350
WM365	GTCAATCCGGTGCTGGTAACAACTGGGCTAAGGGTCATTACACTGAGGGTGCTGACCTTGTCGACCAAGTTCTTG
WM398	....G....................
WM577	....G....................
WM596	....G....................
WM600	....G....................
WM605	....G....................

Figure 5.4. Change in nucleotide sequence of β-tubulin gene of *Sclerotinia sclerotiorum* isolate WM365, change of guanine to cytosine is present at position 333.

Amino acid sequence with corresponding position number in the β-tubulin gene					
ISOLATE	90	100	110	120	130	140
WM365	FRPDNFVFGQSGAGNNWAKGHYTEGADLVDQVLDVVRREAEGCDCLQGFQITHSLGGG
WM398	..............................E.........................
WM577	..............................E.........................
WM596	..............................E.........................
WM600	..............................E.........................
WM605	..............................E.........................

Figure 5.5. In the polypeptide chain of β-tubulin gene of, WM365 isolate of *Sclerotinia sclerotiorum* the mutation translated into change of amino acid at 111codon from Glutamic acid (E) to Aspartic acid (D).

Discussion

In this study we report a new mutation at codon 111. This mutation was detected only in one isolate, WM365, and might be the cause of the increased insensitivity of this isolate to TM. Isolate WM365 had an EC_{50} 1.62 µg/ml with standard error of 0.40, which means it would cross the threshold proposed by Pan *et al.* (1997) that *S. sclerotiorum* isolates with $EC_{50} \geq 2$ µg/ml should be considered resistant. No other isolate had any other mutation. Mutations at the site of action of fungicides which translates into insensitivity of pathogen to that fungicide are a stable genetic trait. In the case of resistance to benzimidazole fungicides, this trait is associated with point mutations in the β-tubulin gene. Previous reports indicated that mutations at codons 6, 50, 167, 198, 200, and 240 were responsible for resistance to benzimidazoles (Ma and Michailides,

2005). In *Aspergillus nidulans,* amino acid at codon 198 was changed from glutamic acid to aspartic acid confer benomyl resistance (Jung *et al.*, 1992). Not all mutations, however, have the same effect (Koenraadt *et al.,* 1992; Ma and Michailides, 2003b) and different mutations of the same codon could result in different responses (Ma and Michailides, 2005). An excellent example of the latter are changes from Glutamic acid to Alanine, Glycine, Lysine, or Glutamine in the β-tubulin gene of *Tapesia yallundae* which result in carbendazim IC_{50} that ranged from >25, to 0.5 µg/ml (Albertini *et al.,* 1999).

The change at codon 111 in the β-tubulin gene of isolate WM365 could be the reason for its increased tolerance to TM. However, the change of Glutamic acid (E) to Aspartic acid (D) will likely have minimal effects on the structure and function of the protein as these two amino acids have nearly same properties being acidic and similar in structure (Lehninger, *et al*., 2008) This change could be responsible for the low level of resistance in the isolate. No attempts were made in this study, however, to validate the role of this mutation. Validation could be achieved by creating the same mutation in the β-tubulin gene of a sensitive isolate and observe if it results in a change in sensitivity to the fungicide.

The identification of a single isolate with a mutation that may have increased its tolerance to TM slightly is an indication that other mutations with greater impact on its sensitivity could occur. *S. sclerotiorum* infects a large and diverse group of plant species and while fungicide applications are conducted usually once during the crop life cycle, unintentional exposure of the fungus to the fungicide while it is applied to other crops could result in constant but light selection pressure. If mutations do occur, the ecological fitness of resistant isolates would determine its persistence after selection. As long as the selection pressure is present, the resistance is a benefit for the pathogen survival. If fungicide applications are discontinued,

however, the population of sensitive isolates may increase again as the benefit of the mutation becomes irrelevant (Secor *et al.*, 2010). However, if the resistant isolates are as fit as the sensitive isolates, resistance will persist for long time even when the fungicide is not in use to control the disease. Resistance to TM has been reported to revert slowly toward sensitivity, but continued fungicide application will likely increase or at least maintain the frequency of resistant isolates within the population (Dovas *et al.*, 1976).

The homothallic nature of *S. sclerotiorum* could help increase the probability of perpetuating an inheritable mutation in the population in a region; after all, every time an epidemic develops is usually because ascospores have been produced; after all, ascospores are the primary inoculum for many Sclerotinia epidemics (Phillips, 1987). In this study we evaluated 81 isolates, which had one whose EC_{50} were close to the threshold of resistance. Screening of a larger set of isolates with varying levels of sensitivity to TM would help us better understand benzimidazole resistance in *S. sclerotiorum* populations in the region. Characterization of the association between different mutations and their effect on the sensitivity of the fungus to TM could help us develop protocols for rapid detection of insensitive isolates in a larger population of *S. sclerotiorum* population in the region.

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APPENDIX A. *SCLEROTINIA SCLEROTIORUM* ISOLATES SENSITIVITY TO

THIOPHANATE-METHYL

S. No.	Isolate	Origin	EC_{50}	Std. Deviation
1	WM356	ND	0.58	0.20
2	WM360	ND	0.69	0.19
3	WM361	ND	0.66	0.22
4	WM362	ND	0.76	0.04
5	WM363	ND	1.10	0.28
6	WM364	MN	0.63	0.14
7	WM365	NE	1.62	0.80
8	WM367	ND	0.63	0.28
9	WM368	ND	0.67	0.15
10	WM369	ND	0.86	0.51
11	WM371	ND	0.73	0.12
12	WM372	ND	0.68	0.13
13	WM373	ND	0.68	0.09
14	WM374	ND	0.86	0.26
15	WM375	ND	0.87	0.19
16	WM376	IA	0.64	0.07
17	WM377	IA	0.79	0.21
18	WM378	WI	0.80	0.17
19	WM379	WI	0.97	0.45
20	WM380	WI	1.24	0.59
21	WM381	MO	0.78	0.24
22	WM382	ND	0.62	0.02
23	WM383	KS	0.88	0.48
24	WM385	ND	0.74	0.10
25	WM386	ND	0.47	0.16
26	WM387	ND	0.59	0.35
27	WM388	ND	0.64	0.07
28	WM389	ND	1.10	0.12
29	WM390	ND	0.92	0.52
30	WM391	ND	0.70	0.13
31	WM392	ND	1.24	0.59
32	WM393	MN	0.85	0.13
33	WM394	MN	0.59	0.08
34	WM395	MN	0.50	0.05
35	WM396	MN	0.70	0.26

S. No.	Isolate	Origin	EC$_{50}$	Std. Deviation
36	WM398	MN	0.57	0.16
37	WM399	ND	0.63	0.20
38	WM400	ND	0.67	0.20
39	WM402	ND	0.99	0.20
40	WM404	ND	0.41	0.29
41	WM405	ND	0.63	0.44
42	WM406	ND	0.75	0.19
43	WM407	ND	0.61	0.14
44	WM408	MN	0.52	0.10
45	WM409	IN	0.85	0.38
46	WM410	IL	0.65	0.12
47	WM411	IA	0.58	0.13
48	WM412	IA	0.78	0.05
49	WM413	IA	1.43	2.04
50	WM414	MI	1.18	0.72
51	WM415	MI	0.56	0.14
52	WM416	WI	0.65	0.18
53	WM417	WI	0.58	0.18
54	WM418	WI	0.76	0.35
55	WM419	ND	0.64	0.34
56	WM420	ND	0.59	0.17
57	WM421	ND	0.75	0.31
58	WM422	ND	0.60	0.57
59	WM423	ND	0.59	0.25
60	WM470	ND	0.96	0.53
61	WM576	ND	0.87	0.58
62	WM577	MB	0.89	0.45
63	WM579	ND	0.85	0.22
64	WM580	ND	0.42	0.32
65	WM582	ND	0.34	0.06
66	WM583	MN	0.78	0.08
67	WM586	ND	1.11	0.19
68	WM588	ND	0.82	0.30
69	WM589	ND	0.54	0.22
70	WM590	ND	0.62	0.11
71	WM593	ND	0.56	0.23
72	WM594	ND	0.56	0.16
73	WM595	ND	0.95	0.13
74	WM596	NE	0.76	0.18

S. No.	Isolate	Origin	EC$_{50}$	Std. Deviation
75	WM597	CO	0.70	0.03
76	WM599	SD	1.19	0.12
77	WM600	MN	0.65	0.15
78	WM603	WI	0.56	0.15
79	WM604	ND	1.15	0.49
80	WM605	MT	0.67	0.17
81	WM606	ND	1.06	0.26

APPENDIX B. *SCLEROTINIA SCLEROTIORUM* ISOLATES SENSITIVITY TO

METCONAZOLE

S. No.	Isolate	Origin	Mean EC_{50}	Std. Deviation
1	WM356	ND	0.41	0.06
2	WM357	ND	0.47	0.02
3	WM358	ND	0.42	0.08
4	WM359	ND	0.14	0.07
5	WM360	ND	0.27	0.07
6	WM361	ND	0.37	0.14
7	WM362	ND	0.37	0.13
8	WM363	ND	0.17	0.08
9	WM364	MN	0.37	0.06
10	WM365	NE	0.24	0.01
11	WM366	ND	0.2	0.06
12	WM367	ND	0.47	0.19
13	WM368	ND	0.39	0.03
14	WM369	ND	0.46	0.07
15	WM371	ND	0.53	0.11
16	WM372	ND	0.41	0.02
17	WM373	ND	0.37	0.09
18	WM374	ND	0.46	0.10
19	WM375	ND	0.37	0.09
20	WM376	IA	0.29	0.16
21	WM377	IA	0.45	0.09
22	WM378	WI	0.34	0.13
23	WM379	WI	0.96	0.53
24	WM380	WI	0.08	0.01
25	WM381	MO	0.37	0.06
26	WM382	ND	0.09	0.01
27	WM383	KS	0.09	0.03
28	WM385	ND	0.48	0.08
29	WM386	ND	0.16	0.10
30	WM387	ND	0.46	0.13
31	WM388	ND	0.85	0.46
32	WM389	ND	0.38	0.11
33	WM391	ND	0.13	0.06
34	WM392	ND	0.58	0.05
35	WM393	MN	0.56	0.12

S. No.	Isolate	Origin	Mean EC_{50}	Std. Deviation
36	WM394	MN	0.68	0.17
37	WM396	MN	0.31	0.21
38	WM397	MN	0.53	0.10
39	WM398	MN	0.47	0.27
40	WM399	ND	0.45	0.11
41	WM400	ND	0.34	0.08
42	WM401	ND	0.92	0.33
43	WM402	ND	0.31	0.06
44	WM403	ND	0.29	0.12
45	WM404	ND	0.45	0.06
46	WM405	ND	0.59	0.14
47	WM407	ND	0.09	0.01
48	WM408	MN	0.24	0.12
49	WM409	IN	0.39	0.08
50	WM411	IA	0.22	0.16
51	WM412	IA	0.41	0.03
52	WM413	IA	0.52	0.22
53	WM414	MI	0.28	0.15
54	WM415	MI	0.91	0.37
55	WM416	WI	0.76	0.11
56	WM417	WI	0.37	0.24
57	WM418	WI	0.21	0.11
58	WM419	ND	0.26	0.14
59	WM420	ND	0.16	0.09
60	WM421	ND	0.54	0.11
61	WM422	ND	0.38	0.04
62	WM423	ND	0.33	0.01
63	WM576	ND	0.89	0.17
64	WM577	MB	0.06	0.01
65	WM578	ND	0.35	0.03
66	WM579	ND	0.23	0.02
67	WM580	ND	0.51	0.20
68	WM582	ND	0.5	0.09
69	WM583	MN	0.21	0.09
70	WM584	MN	0.07	0.01
71	WM585	IL	0.56	0.33
72	WM587	NE	0.43	0.02
73	WM588	ND	0.47	0.17
74	WM589	ND	0.53	0.40

S. No.	Isolate	Origin	Mean EC$_{50}$	Std. Deviation
75	WM590	ND	0.66	0.30
76	WM591	ND	0.77	0.24
77	WM592	ND	0.3	0.05
78	WM593	ND	0.07	0.00
79	WM594	ND	0.41	0.16
80	WM595	ND	0.82	0.28
81	WM596	NE	1.74	0.11
82	WM597	CO	0.2	0.09
83	WM599	SD	0.18	0.11
84	WM600	MN	0.38	0.03
85	WM601	OH	0.53	0.27
86	WM603	WI	0.15	0.06
87	WM604	ND	0.74	0.23
88	WM605	MT	0.38	0.08
89	WM606	ND	0.38	0.10
90	WM607	ND	1.28	0.25
91	WM608	WI	1.20	0.42