SENSITIVITY OF *SCLEROTINIA SCLEROTIORUM* ISOLATES FROM NORTH CENTRAL US TO AZOXYSTROBIN AND BOSCALID

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Title
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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Azoxystrobin and boscalid are fungicides frequently used to manage diseases caused by *Sclerotinia sclerotiorum* (Lib.) de Bary in many broadleaf crops in North Central US. . Continued use of these compounds can induce development of fungicide-resistant strains and thus their sensitivity should be monitored periodically. The objective of this research was to generate baseline sensitivity information of *S. sclerotiorum* to azoxystrobin and boscalid. Salicylhydroxamic acid was toxic to ascospores even at concentrations of 0.1 μg/ml and therefore was not used to estimate sensitivity to azoxystrobin. Azoxystrobin EC_{50} for ascospores and mycelium were 3.05 and 1.01 μg/ml, respectively. Boscalid EC_{50} for ascospores and mycelium were 8.38 and 0.11 μg/ml, respectively. *S. sclerotiorum* isolates collected before 2004 had statistically ($P > 0.05$) similar sensitivity to azoxystrobin but where more sensitive to boscalid ($P = 0.05$) than isolates collected in 2014. Both fungicides continue to be effective tools to manage *S. sclerotiorum*.

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I am very thankful to my parents who supported and encouraged me through my entire career. I am blessed with their unconditional love and understading. Everything I am today is thanks to them.

DEDICATION

This thesis is dedicated to my parents who are the motor of my life.

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LITERATURE REVIEW

Canola: History and Production

Canola (*Brassica napus L.*) is an economically important crop in North America. *Brassica napus* is typically grown in Europe, Australia, and China where it is known as oilseed rape. The crop was introduced from Canada in the early 1980s but its popularity did not take off until Canadian cultivars with low levels of erucic acid and glucosinolate contents were developed in the late 1980s (Lamey and Hershman, 1993). These two compounds are toxic to humans and had limited the use of its oil to industrial applications. Cultivars with similar traits are known in other parts of the world as double-low oilseed rape. Oilseed rape or canola is one of the most popular genetically modified crops in the market due to the high quality of its oil for human consumption as well as for its biodiesel properties (Gunstone, 2004).

Recent data from 2014 indicates that in the US, growers harvested 1.55 million acres of canola with a yield production of 1.14×10^6 metric tons (2.51×10^9 lb.) and a value of approximately US$ 425 million. In 2014, North Dakota (ND) planted 70 percent of the total canola acres planted in the U.S. and produced an average yield of 2.22 tons/ha (1,800 lb/A.) The ND production accounted for 85 percent of the national canola production. In 2014, the market value of this commodity in ND production was estimated at US$ 364 million (USDA-crop production/crop value, 2015).

Taxonomy of *Sclerotinia Sclerotiorum*

The family Sclerotiniaceae is typically characterized by species producing inoperculate asci from stipitate apothecia coming from a sclerotial stroma, which is a melanized aggregate of hyphae (Holst-Jensen et al., 1997b; Whetzel, 1945). This family includes important plant pathogens such as *Sclerotinia, Botrytis and Monilinia* which affect crops worldwide (Holst-Jensen, 1997a). In 1945, Whetzel proposed basic taxonomic traits to diagnose the Sclerotiniaceae

family. Some of these traits were stroma type, ascospore color, presence or absence of conidial state, and type of conidia (Whetzel, 1945). Additional tools for classification of Sclerotiniaceae are the characteristics of sterile tissue, sclerotia and apothecia. The latter is composed of a hymenium, hypothecium, and excipulum (Kohn, 1979; Korf and Dumont, 1972). Sclerotial ontogeny (Willets and Wong, 1980), histochemistry and ultrastructure of sclerotia (Backhouse and Willetts, 1984) were used for delimitation. More recently, biochemical tools (Carbone and Kohn, 1993) and rRNA sequences (Holst-Jensen et al., 1997a, b) have been utilized to further classify Sclerotiniaceae species. The number of species that belong to this genus has changed recently. *Sensu stricto* there only are three species contained in the Sclerotinia genus: *S. minor* Jagger, *S. trifoliorum* Eriks, and *S. sclerotiorum* (Lib) de Bary. DNA analyses of organisms previously identified as *S. asari* and *S. nivalis* were show to not be member of Sclerotinia genus (Kohn et al., 1988). Moreover, S. *homoeocarpa* has also been excluded from this family because it does not produce sclerotia (Rossman et al., 1987).

Sclerotinia sclerotiorum (Lib.) de Bary is taxonomically classified as the following:

Kingdom: Fungi

Phylum: Ascomycota

Class: Discomycetes

Order: Helotiales

Family: Sclerotiniaceae

Genus: *Sclerotinia*

Species: *sclerotiorum*

Sclerotinia Stem Rot

Sclerotinia sclerotiorum (Lib.) de Bary is a major endemic pathogen that affects canola production in North Dakota. *S. sclerotiorum* is a necrotrophic, homothallic, filamentous fungus that causes Sclerotinia stem rot (SSR) on canola and many other crops (Purdy, 1979; Tu, 1997). This cosmopolitan pathogen is very well distributed around the world and affects more than 400 species of plants such as soybean, sunflowers, oilseed rape, among others (Boland and Hall, 1994; Purdy, 1979). In North Dakota, between 1991 and 2002 the annual average SSR incidence was estimated at 13.6% (Lamey, 1995; Lamey et al., 2002; Lamey, 2003, Bradley and Lamey, 2005) with an estimated economic effect of US$94 million (Lamey, 2003). *S. sclerotiorum* has successfully persisted across various geographic regions in part due to its ability to infect diverse plants, including agronomic and horticultural crops, under different environmental conditions as well as its production of sclerotia which survive several years in the soil (Adams and Ayers, 1979). Under North Dakota conditions for example, this pathogen can reduce canola yields at a rate of 0.5% for each percentage point of disease incidence (del Río et al., 2007).

Symptoms and signs

Symptoms occur after the flowering stage has started and typically appear as bleached, water-soaked, and dark lesions on leaves (Figure 1). Water soaked lesions on the leaves expand quickly and move down the petiole into the branches and then stem. Stem lesions develop into necrotic tissues that produce patches of white fluffy mycelium. As the fungus progresses into the main stems, wilting occurs. Infected tissues appear bleached and sometimes become brittle and shredded. The fungus cause lodging and reduces seed production. When the fungal pathogen is well established in the plant, hard black structures called sclerotia, appears inside infected tissue.

White-cottony mycelium and sclerotia are clear signs of the pathogen (Figure 2). Plants above the lesion turn pale green or yellow and die (Bolton et al., 2006; Markell et al., 2009).

Figure 1. Symptoms of *Sclerotinia sclerotiorum* in canola. A) Leaf lesion with watermarks on canola leaves; B) Early stem lesion which appears as a bleached oval area on infected stems; C) Stem bleaching, rotting and lodging caused by SSR; D) Infected stems are weak and can lodge. Source: https://www.agric.wa.gov.au/canola/managing-sclerotinia-stem-rot-canola

Figure 2. Signs of *Sclerotinia sclerotiorum* on canola. A) Stem with white fluffy mycelium and sclerotia; B) Sclerotia inside the stems.
Source: https://www.agric.wa.gov.au/canola/managing-sclerotinia-stem-rot-canola

Sclerotinia Sclerotiorum: Biology and Epidemiology

Disease cycle

Sclerotinia sclerotiorum spends most of its life in the soil as a multicellular, asexual, hard resting structure called sclerotia. Sclerotia have a black, melanized rind surrounding a mass of white hyphae that can stay viable for long periods. Hyphae are hyaline, septate, branched and multinucleate. Mycelium is white to tan *in planta*. There are no asexual conidia produced, but microconidia which do not germinate are produced (Bolton et al., 2006). The life cycle of *S. sclerotiorum* follows very closely that of the disease it causes (Figure 3).

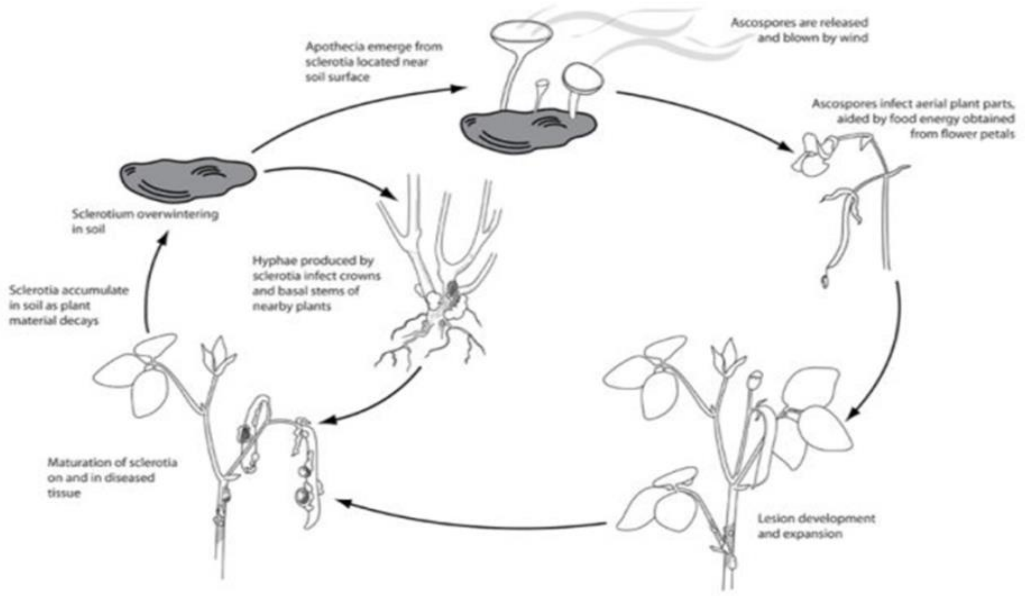


Figure 3. Disease cycle of *Sclerotinia sclerotiorum* in canola. Source: http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/WhiteMold.aspx

Formation of sclerotia

Sclerotia are the primary overwintering structures that allow the fungus to survive in the soil and in/on crop residues for up to 8 years and serve as a primary source of inoculum for the next season (Adams and Ayers, 1979; Willetts and Wong, 1980). A sclerotium is a hyphal aggregate that is produced in response to changes in the quality of the colonized plant substrate

or to changes in environmental conditions. The outer black rind cells around the sclerotia contain melanin which resists microbial degradation (Henson et al., 1999). Each sclerotium has three layers: a thick-walled pigmented rind, a thin-walled cortex, and white medulla (Huang, 1982). High moisture, ventilation, and temperature are important factors for sclerotia survival and influence outbreaks of the disease (Coley-Smith and Cooke, 1971).

Myceliogenic germination of sclerotia

Sclerotia can germinate myceliogenically or carpogenically depending on environmental conditions and crop canopies. In the spring, sclerotia can germinate myceliogenically and produce hyphae that directly infect host roots, resulting in basal stem infections causing crown rot, root rot, basal stalk rot, and wilting. This type of infection is common in sunflowers and carrots. Basal stalk starts when mycelium coming from soil-borne sclerotia contacts the lateral roots of sunflower plants. If there is enough moisture, the fungus grows inside the plant and digests the pith of the stem (Masirevic and Gulya, 1992). Mycelium penetrates the cuticle of the host by using enzymes or mechanical force through appressoria. However, penetration can also occur via stomata (Bolton et al., 2006). Myceliogenic germination is correlated with the melanin pigment within and outside the rind. This type of germination requires high humidity and can occur in the presence of exogenous nutrient, when sclerotia are improperly melanized, or has injured rinds (Bardin and Huang, 2001).

Carpogenic germination of sclerotia

Carpogenic germination is influenced by a number of factors; for example, the geographic origin of the isolates and the temperature at which sclerotia or mycelia are produced (Huang and Kozub, 1991; Huang and Kozub, 1993). Sclerotia produced at cool temperatures (10^{o} C) germinate better than those produced in warmer temperatures (25-30^{o} C). Another

important factor is soil moisture. For optimal germination, sclerotia must be buried at 1 cm with a water capacity of 100 kPa (Bardin and Huang, 2001). Conditioned sclerotia require soil water of 100 kPa for several weeks at temperatures of 10 to 25^{o} C with a photoperiod for apothecia formation (Clarkson et al., 2004). Therefore, irrigation events and high rainfall are key factors for the disease development. If the environmental conditions are favorable, carpogenic germination can occur and mushroom like structures called apothecia are formed. An apothecium is the sexual fruiting body of *S. sclerotiorum* which contain a stipe coming from a sclerotium and a flat to concave hymenial layer. One or more apothecia emerge from a sclerotium. Asci located in the hymenial layer are cylindrical sac-like zygote cells that bear eight hyaline, ellipsoid binucleate ascospores per ascus. Since ascospores are the primary inoculum in Sclerotinia stem rot, carpogenic germination is a large determinant of disease. Carpogenic germination results in stem blight, stalk rot, head rot, pod rot, white mold, and blossom blight of plants (Kohn, 1979; Bardin and Huang, 2001).

Ascospore release and dispersal

Ascospores are the primary inoculum for the development of stem rot disease caused by *Sclerotinia sclerotiorum*. Ascospores colonize petals, leaves and pollen grains (Qandah and del Río, 2011). Ascospores are forcibly discharged from the ascus under optimum conditions in a field at a rate of 1600 spores/h when air temperatures range between 4 and 32^{o} C (Clarkson et al., 2003). The highest peaks of ascospore release during wet and dry years are 11 am and 4 am, respectively (Qandah and del Río, 2011). The forcible discharge of ascospores is known as 'puffing' (Hartill and Underhill, 1976) and depends on changes in relative humidity (RH) of the apothecium created by increments in air temperature and fluctuations in relative humidity (Qandah and del Río, 2011; Bardin and Huang, 2001). A measurement of relative humidity

provides an assessment of risk of occurrence and risk of infection of plant tissues by ascospores. The probability of spore release increases when RH under the canola canopy increases from 85 % to 90 % (Qandah and del Río, 2011). Airborne ascospores can travel with the wind and disperse to further areas and can infect above ground tissue. Carpogenic germination results in stem blight, stalk rot, head rot, pod rot, white mold, and blossom blight of plants (Bardin and Huang, 2001).

Seeds contaminated with sclerotia of *S. sclerotiorum* could also serve as a means of dispersal (Hartman et al., 1998). Contaminated seeds occur when growers used stored grains instead of certified seed. These types of seeds have reduced oil content since its quality and germination has been affected, subsequently causing a lower price in the market (Hoffman et al., 1998). Ascospores are considered the primary inoculum and senescing flowers and favorable weather conditions are required for disease development. *S. sclerotiorum* infects a diverse group of plants under different environmental conditions and has successfully persisted across various geographic regions because of its ability to produce sclerotia which survive in the soil for about eight years (Adams and Ayers, 1979).

Infection of plant tissues by ascospores

Temperature and moisture are key factors that influence stem rot disease. Cool and wet weather are ideal conditions for *S. sclerotiorum* infection (Harikrishnan and del Río, 2006). Ascospores cannot infect healthy green tissue directly. Infection occurs when ascospores contact senescing flowers, germinate and colonize the senescing tissue (Bolton et al., 2006). For an effective germination and infection of flowers, ascospores need water, but disease could develop at relative humidity levels under 25 % (Harikrishnan and del Río, 2006). A sticky mucilage covering of ascospores helps ascospores adhere to the substrate. Ascospores use flower petals as

a source of nutrients to establish infection. The infected petal drops into the canopy and the pathogen moves into healthy leaf and stem tissues (Bolton et al., 2006). Colonization occurs, and the pathogen invades green living tissue close to the flower. (Harikrishnan and del Río, 2006).

Disease Management

S. sclerotiorum causes serious yield losses and has the ability to survive in the soil as sclerotia for a long period of time, affecting susceptible commercial crops. If there is a high amount of inoculum and a dense canopy, stem rot can cause yield losses greater than 50%. There are several disease strategies which primarily focus on the elimination or reduction of the source of inoculum.

Cultural practices

Crop rotation is an option but not quite as effective as others due to the ability of this pathogen to survive in the soil for up to eight years, its broad host range, and its ability to be easily dispersed (Markel et al., 2009). Nevertheless, one to three years between host crops is recommended to reduce the build-up of the primary inoculum in the soil (Peltier et al*.,* 2012). Using a minimum of 3 years of a non-host crop such as corn, cereals (wheat, barley, or oats) and grasses can reduce the number of sclerotia (Garcia-Garza et al*.,* 2002). These non-host crops help interrupt the disease cycle and reduce the accumulation of sclerotia in the field (Zeng et al*.,* 2012). Pathogen-free seed should be used for planting for next season. This is recommended because it restricts the amount of inoculum added to the field and prevents future infestations (Tu, 1989). Wider spacing between rows facilitates and speeds up the drying of leaf surfaces. In addition, it may increase the ventilation within the canopy and reduce the moisture in the upper layer of soil, causing a reduction on the disease (Hoes and Huan, 1985). Deep and constant tillage help to bury sclerotia in the soil. This reduces disease incidence by removing the sclerotia

from the surface avoiding apothecia formation. However, this is no longer practical because most growers nowadays use minimum or no- tillage practices (Markell et al., 2009; Peltier et al., 2012). While each of these practices has some value, their implementation as single measures is not enough to keep the disease below economic levels.

Biological control

Biological control agents help manage stem rot as an alternative disease control strategy of many cropping systems including canola, soybean, celery and dry beans, among others (Zeng et al., 2012). Biological control agents such as mycoparasitic fungi, hypovirulent strains of *S. sclerotiorum*, bacteria, and insects have received the most attention (Bardin, 2001). In the field, sclerotia are targeted and degraded by mycoparasites such as *Coniothyrium minitans* Campbell, *Trichoderma* species and *Sporidesmium sclerotivorum* (Bardin and Huang, 2001). Of these, *C. minitans* is the only one been sold commercially that targets and kills the sclerotia in the soil (Markell et al., 2009; Campbell, 1947). *S. sclerotivorum*, however, may be even more effective since it could reduce by 95% the inoculum density within 10 weeks from application (Adams and Ayers, 1981; Ayers and Adams, 1979; del Río et al., 2002).The use of biological control agents, especially those that destroy sclerotia, constitute an economically feasible practice for reducing the primary inoculum. However, the use of microbial consortia as biocontrol agents to suppress plant disease may constitute the most effective way to manage this disease (Jain et al., 2011). Germination and colonization of sclerotia by its antagonists depend on disease pressure, soil condition, soil temperature, moisture and microbial diversity (Zeng et al., 2012). Highly infested soils are the ones were mycoparasites would work the best. However, at lower infestation levels, biological controls may not be able to eliminate enough sclerotia to reduce

disease. Several other biological control agents and chemical fungicides have been evaluated to manage Sclerotinia diseases in different crops (Fernando et al., 2007).

Host resistance

As of yet, no canola variety exists with resistance to Sclerotinia stem rot. It is challenging to breed for resistance to *Sclerotinia sclerotiorum* because it is controlled by multiple genes (Fuller et al., 1984; Zhao and Meng, 2003). However, numerous sources of resistance have been found within a collection of *Brassica napus* plant introduction (PI) lines. Among them, 7 PI lines have been identified for use in breeding programs based on their agronomic traits (Khot et al., 2011).

Chemical control

Due to the lack of resistant cultivars, the use of fungicides is the ideal management strategy that growers rely on to control stem rot disease. Various chemical fungicides including azoxystrobin (Quadris^{R}), boscalid (Endura^{R}), thiophanate-methyl (Topsin^{R}), and vinclozolin are currently registered for use in the U.S. to manage diseases caused by *S. sclerotiorum* (Bradley et al., 2006). To manage Sclerotinia stem rot in canola it is recommended that fungicides are applied when the crop is at or slightly before the 50% flowering stage (Markell et al., 2009; Spitzer et al., 2012). The ideal fungicide will need to check criteria such as high specificity and activity at low usage rates. Also, it needs to have a systemic and eradicant action to control any phytopathogenic fungi.

The identification of a potential disease risk is important to make an economically justified decision to use fungicides. Forecasting models are being used to increase the efficacy of fungicide applications. While there is a significant positive correlation between disease incidence and the level of petal infestation by *S. sclerotiorum* ascospores at early bloom, the

implementation of a warning system based on infested petals has not been successful. It is extremely critical to spray fungicide for disease control if there are wet conditions at the right temperature and the crop is at early bloom (Turkington and Morrall, 1993). Several forecasting programs have been developed to help growers with spraying decisions. In one of them, the risk of infection is broadcasted via internet in the form of risk maps and risk calculators (del Río, personal comm., 2015). This program estimates risk of infection considering weather variables, as well as cultural practices and field history.

Strobilurin fungicides

In the 1960s, most fungicides available had protectant action and were multi-site inhibitors. Multiple-target site fungicides such as inorganic Bordeaux mixture and organic compounds like phthalimides and aromatic hydrocarbons, among others, were commonly used for disease management. During the 1970s, systemic fungicides like benomyl, morpholine and aminopyrimide compounds offering specific modes of action were introduced in the market. In the 1980s, sterol demethylation inhibitors, DMI, were introduced as systemic products. The development of strobilurin-derived compounds, like azoxystrobin which belong to the class of Quinone outside inhibitors (QoI), was innovative because it was based on molecules that are naturally produced by another fungus. The continued use of these newer compounds, however, has resulted in the development of pathogens strains with resistance to many of these fungicides (Fernández-Ortuño et al., 2008; Morton and Staub, 2008).

QoI fungicides block mitochondrial electron transport chain by binding to the Quinone oxidation site, Qo, in the center of cytochrome *bc1* complex (complex III). The inhibition of mitochondrial respiration causes a disruption of the energy cycle and interferes with ATP synthesis in the fungus (Duan, et al., 2012; Bartlett, et al., 2002; Grasso et al., 2006).

Cytochrome *bc1* is an integral membrane protein complex necessary for fungal respiration. The catalytic core enzyme for a normal respiration is composed by cytochrome *b* and *c1* and Rieske iron-sulfur protein (ISP). This catalytic mechanism needs Q-quinone –binding sites: quinol oxidation site (Qo) and quinone reduction site (Qi) for a complete mitochondrial respiration function (Fernández-Ortuño et al., 2008).

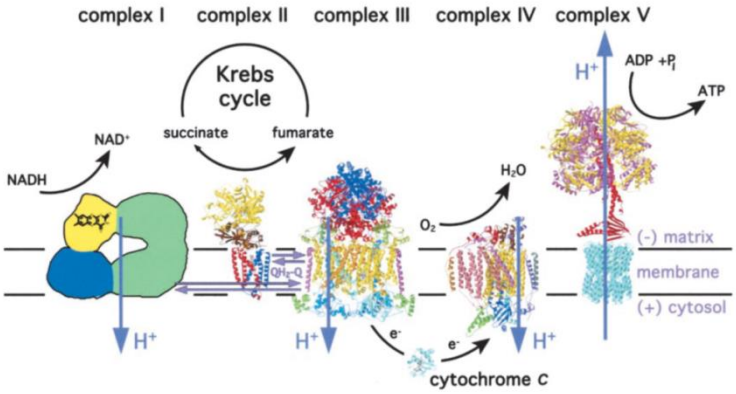
Azoxystrobin comes from natural fungicidal derivatives of β- methoxyacrylic acid such as strobilurin A and oudemansin A. In 1977, strobilurin A produced by *Strobilurus tenacellus* became the first QoI molecule to be identified (Anke et al., 1977). Azoxystrobin is one of the most stable and active fungicides within this group. It has a single mode of action and applies strong selection pressure on the pathogen towards chemical resistance development. Because of the nature of its activity, QoI fungicides are effective against a wide spectrum of pathogens (Fernández-Ortuño et al., 2008). Strobilurin fungicides have a mode of action that interferes with energy production which subsequently affects every growth stage of the fungus. Therefore, azoxystrobin is highly effective in inhibiting spore germination and zoospore motility, stages of fungal development that require high energy. For improved effectiveness, it is recommended to apply strobilurin at early stages of the disease development (Barlett et al., 2002).

Carboxamides fungicides

Carboxamide fungicides were introduced to the market because of their high level of activity against basidiomycetes. The first carboxamide fungicide, carboxin, was introduced to the market in 1966 (Von Schmmeling and Kulka, 1966). Soon after, fungicides such as benodanil, fenfuram, mepronil, flutolanil, furametpyr, and thifluzamide were released. However, their limited spectrum of targets kept them from becoming more popular. It was not until 2003, when new molecules capable of targeting a wider range of pathogens were introduced to the market

that carboxamide fungicides regained popularity. Boscalid is the first active ingredient of this new generation of carboxamide fungicides to be released while penthiopyrad is one of the most recently introduced products (Glättli et al., 2011; Sierotzki and Scalliet, 2013).

Carboxamide fungicides block the activity of the enzyme succinate ubiquinone oxidoreductase (complex II) or succinate dehydrogenase (SDH) which is an essential piece of the tricarboxylic acid cycle (TCA) and the mitochondrial electron transport chain (Figure 4). Their strong fungicidal activity allows inhibition of the cell energy cycle (Broomfield and Hargreaves, 1992; Hägerhäll, 1997; Liu et al., 2009; Myresiotis et al., 2008). This enzyme transfer succinate-derived electrons to the ubiquinone respiratory chain or complex II (Sierotzki and Scalliet, 2013). Chemical reactions of this process include reduction of ubiquinone (UQ) to ubiquinol (UQH2) and the oxidation of succinate to fumarate in the mitochondria (Horsefield et al., 2006).



Mitochondrial oxidative phosphorylation system composed by multisubunit electron transport complexes I, II, III, and IV, and ATP synthase (complex V)

RESPIRATORY COMPLEX II 79

Figure 4. Diagram of respiratory chain of mitochondria (Cecchini, 2003).

The SDH complex II consists of two main membrane domains: peripheral and anchor (Figure 5). The peripheral domain has two hydrophobic subunits SDHA and SDHB where oxidation of succinate to fumarate occurs. SDHA is a flavoprotein and SDHB is an iron-sulfur

protein, which together form a soluble and catalytic site. On the other hand, the anchor domain is made up of two expandable hydrophobic subunits called SDHC and SDHD (Ackrell, 2000). Moreover, in between these subunits a heme b group is localized which has specific binding sites and catalytic properties to transfer electrons to ubiquinone (Avenot and Michailides, 2010). SDH inhibitors can be classified based on binding sites: those linking to the ubiquinone-binding pocket and others to succinate-binding pocket (Sierotzki and Scalliet, 2013). The structure of succinate dehydrogenase inhibitors (SDHIs) consists of four main parts: the core moiety, amide, linker, and hydrophobic rest. The core allows binding and it enters into the active side of SDH. The amide side establishes hydrogen bonds and can develop two components. The linker, is usually a phenyl group and the hydrophobic rest is essential in the biological spectrum and potency of the compound.

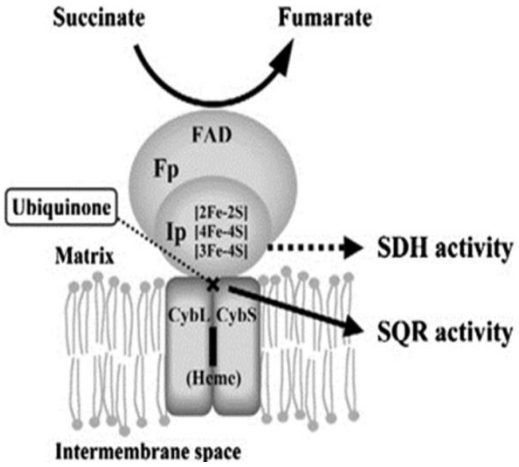


Figure 5. Subunit structure and enzyme activities of complex II (Ōmura and Shiomi, 2007).

Carboxamide fungicides have a specific mode of action that interferes with energy production and inhibit fungal respiration. As consequence it has a high effect on mycelial growth (Fellman and Tourneau, 1983) but does not prevent spore germination.

Fungidice Resistance: Fitness and Mutations

The emergence of fungicide resistant isolates is the result of genetic mutation and/or genetic recombination that occurs during the life of a pathogen. These events could occur even in the absence of selection pressure; however, it is the repeated use of fungicides for the most part that promote their establishment in a population. Fungicide resistance induces a loss of efficacy for the fungicide. Resistant populations can be identified using fungicide assays and molecular techniques that look for specific genetic mutations. There are three phases during the development of fungicide resistance: emergence, selection, and adjustment (van den Bosch et al., 2011). As stated earlier, sexual recombination and mutation create resistant strains during the emergence phase. A mutation is a change in DNA caused during genome replication or cell division which is passed to subsequent generations (Galhargo et al., 2007). A single mutation can overcome resistance and lead to a reduction in field efficacy (Sierotzki and Scalliet, 2013). In the selection phase, the quantity of resistant isolates increases by the selection pressure applied by fungicide exposure (van den Bosch et al., 2011). The successful establishment of these strains will depend on their fitness. Fitness is the ability of an organism to reproduce and survive in the environment through many generations (Antonovics and Alexander, 1989); thus, understanding fitness is important in order to estimate the behavior of the pathogen population in the future and design methods to manage it (Avenot and Michailides, 2010). During the adjustment phase, the resistant population becomes established. Therefore, in this last phase, the dose of the fungicide may be adjusted to maintain an effective control of the disease (van den Bosch et al., 2011). If the selection pressure subsides, the frequency of resistant isolates may decrease if the fitness cost of resistance is onerous. For example, QoI-resistant populations in Oomycetes may decrease while those from *B. graminis f. sp. tritici* remain stable for three generations as resistant isolates

have about the same fitness than sensitive isolates (Chin et al., 2001). The impact of resistance on fitness of isolates is rather species specific since there is a fitness cost on *B. cinerea*, *C. beticola*, and *U. maydis* where dysfunction in fungal development or infection is affected but not in *B. graminis* (Fernández-Ortuño et al., 2008).

Misuse of fungicides facilitates the establishment of resistant strains. Sublethal doses of fungicide may facilitate development of high numbers of resistant mutants but they will have low probability of getting established; due to competition with a high density of sensitive isolates; in contrast, use of excessively high doses, strongly reduces the rate of mutation but increase the probability that a mutant will be able to build up due to the low density of sensitive isolates. The small competition for space allows the resistant strains to survive overtime. The total effect on resistance in a population is affected by the correlation between dose per treatment and how many treatments are required during the emergence and selection phases. Resistance progress starts with sensitive strains in the emergence phase when a mutant spore carrying a resistance mutation develops in the population. The probability of this spore producing progeny will depend on many factors. One of them is the timing of its appearance. If a resistant isolate arises before the fungicide is released in the field, the probability of reproducing will increase as the fungicide is used continuously giving rise to the selection phase. During this phase the use of resistance management strategies is needed to maximize the lifetime of the compound. During selection, the amount of resistant strains is higher than sensitive ones due to effect of the fungicide on sensitive strains. Resistance is determined by the reproduction number of resistant strains in the sensitive pathogen population (van den Bosch et al., 2011). In the field, azoxystrobin and boscalid resistant pathogens under selection pressure can evolve and quickly spread overtime (Avenot and Michailides, 2010).

Resistance to QoI fungicides

Azoxystrobin is an important fungicide for plant disease control because of its efficacy against fungal diseases. Unfortunately, widespread use in combination with its highly specific mode of action that promotes development of resistance in target pests (Fernández-Ortuño et al., 2008). Resistance begins with a point mutation in the mitochondrial cytochrome *b* gene (CYTB) that prevents or reduces fungicide binding. Many mutations that affect sensitivity to these compounds have been identified, for example, the amino acid substitution from glycine to alanine at position 143 (G143A) and from phenylalanine to leucine at position 129 (F129L) (Gisi et al., 2002). Recently, a new change at position 137 (G137R) from glycine to arginine was detected. Isolates with F129L or G137R mutation are considered moderately resistant compared to isolates with G143A mutation which are highly resistant (Sierotzki et al., 2006; Fernandez-Ortuno et al., 2008). Resistance to QoI fungicides have been detected in fungi such as *Alternaria solani* (Pasche et al., 2005; Pasche et al., 2004), *Botrytis cinerea* (Kim and Xiao, 2011; Ishii et al., 2009), *Cercospora beticola* (Malandrakis et al., 2006), *Erysiphe graminis f. sp. tritici* (Chin et al., 2001), *Magnaporthe grisea* (Avila-Adame and Koller, 2003), *M. oryzae* (Ma and Uddin, 2009), *Pyricularia grisea* (Kim et al., 2003), and *Ustilago maydis* (Ziogas et al., 2002). Azoxystrobin inhibits a target site encoded by a mitochondrial gene but the evolution of the fungicide resistance is unclear. One point mutation in a single copy of mtDNA has low impact on fungal survival. Resistance in a population is the combination of many mutations and the selection pressure enforced by the fungicide. However, it is still unknown how many mutations of mitochondrial in a cell are needed to cause a complete QoI resistant phenotype (Fernández-Ortuño et al., 2008).

Lowering the levels of damaging reactive oxygen species and ATP synthesis would give a chance for selection of a point mutation in CYTB gene. Consequently, the development of an alternative respiration pathway could be a transmutation period where sensitive isolates become resistant isolates in the presence of QoI fungicides (Miguez et al., 2004; Wood and Hollomon, 2003). Beside a single mutation, the influence of other mechanisms such as the alternative respiration and efflux transports can contribute to the QoI resistance (Fernández-Ortuño et al., 2008). However, many fungi are capable of using an alternative respiration pathway to avoid the toxic effects of QoI fungicides. Alternative respiration is a metabolic pathway that finds a way around the target site of the QoI fungicides in vitro and provides energy to cells intoxicated with a QoI fungicide (Ziogas et al., 1997). It has been reported that species capable of using the alternative respiration pathway, like *Venturia inaequalis* and *Septoria tritici* are less sensitive to QoI fungicides in vitro but more sensitive in vivo (Vincelli and Dixon, 2002). Xu and collaborators (2012) show that alternative oxidase (AOX) influence in the regulation of growth, development and resistance to oxidative stress of *S. sclerotiorum*.A study demonstrated that AOX respiration reduces mycelium sensitivity of *Sclerotinia sclerotiorum* to azoxystrobin (Xu et al., 2013) by its capacity to shift electron flux from cytochrome pathway (CP) to alternative pathway (AP) when QoI fungicide is used (Tamura et al., 1999) and because AOX can transfer electrons from reduced ubiquinone to oxygen without energy released (Vanlerberghe, 1997).

The ability of these organisms to use an alternative respiration pathway would mask their true sensitivity to QoI fungicides in laboratory toxicity trials. Salicylhydroxamic acid (SHAM) is an inhibitor of an oxidase (AOX) enzyme involved in the alternative respiration pathway used by fungi exposed to QoI fungicides and thus, when added into the culture media it suppresses the alternative respiration pathway. In some fungi, like *Ascochyta rabiei*, adding up to 100 µg ml^{-1}

SHAM to the medium does not seem to have a negative effect on the germination of its spores (Wise et al., 2008); however, the effect of SHAM on spores of *S. sclerotiorum* is not known. While many studies have used SHAM to block the alternate respiration pathway in toxicity trials, the benefit of using it is still debatable (Liang et al., 2015). What seems clear, however, is that as long as the concentrations of SHAM used in a study do not affect spore germination when used alone, they will contribute to produce a more accurate dose response. The advantages of conducting fungicide sensitivity tests *in vitro* include better precision in estimating EC_{50} values; reduction in the space and labor required; and a quicker turn-around time (Vincelli and Dixon, 2002). Therefore, it is important to find the level of SHAM that could be used with azoxystrobin to estimate the real sensitivity of the fungicide. Alternative respiration provides energy to resist QoI's effects *in vitro* but not in vivo. There are two clear limitations for alternative respiration. First, this mechanism gives 40% of energy conservation, where complexes III and IV of the mitochondrial electron transport system are the only ones contributing with proton pump activity. In addition, antioxidants such as flavones inhibit the induction of alternative respiration by reducing reactive oxygen species (ROS) necessary for stimulation of the AOX gene (Wood and Hollomon, 2003).

Resistance to SDHI fungicides

Boscalid is registered for use against *Botrytis cinerea, Sclerotinia spp., Alternaria* spp., *Monilinia* spp., and powdery mildews, among others (Stammler and Speakeman, 2006). Based on previous studies in China, carbendazim (MCB) and dimethachlon were used to control Sclerotinia stem rot and after repeated applications they caused the development of resistant races (Pan, 1998; Shi et al., 2000). Boscalid is a unique fungicide based on its mode of action to control Sclerotinia stem rot. However, frequent use of this compound can result in selection of

resistance among the population (Avenot and Michailides, 2010). Resistance to boscalid is caused by a mutation in the *sdh* genes and has been detected in *B. cinerea* (Stammler et al., 2011; Walker et al., 2011), *Alternaria alternata* (Avenot and Michailides, 2007; Avenot et al., 2008), *Didymella bryoniae* (Stevenson et al., 2008), *Corynespora cassiicola* (Miyamoto et al., 2009), and *Podosphaera xanthii* (Miyamoto et al., 2010). For example, a mutation in a Qo site SDHB causes a change from histidine (H) to tyrosine (Y), resulting in a loss of sensitivity to SDHI fungicides. Moreover, other amino acids, such as leucine and arginine, have been found to have replaced the histidine residue, also resulting in resistance to boscalid. Overall, in various pathogen populations, more than 27 mutations have been reported to confer resistance to SDHI fungicides. For example, *A. alternata* has H134R, S135R mutations in potato; *Botrytis cinerea* H278Y/R mutation in strawberry; *Mycosphaerella graminicola* has T79N mutation in wheat; and *Pyrenophora teres* has H277Y mutation in barley, among others (Sierotzki and Scalliet, 2013). It has been described that there are five unique mutations at three different codons of *sdhB* in *B. cinerea* from strawberries (P225F, N230I, and H278L/R/Y). Therefore, under selection pressure, single or multiple mutations could occur causing a decrease in fungicide sensitivity or increased resistance (Veloukas et al., 2012). Also, indirect exposure of an organism to boscalid or other fungicides could lead to the development of fungicide resistance. This is the case of benzimidazoles, DMIs, and strobilurins. Wheat and barley are used in rotations with canola and receive fungicides applications of two to three times per season to manage diseases not caused by *S. sclerotiorum*. However, *S. sclerotiorum* populations may be indirectly exposed to other SDHI fungicides like carboxin, exerting a selection pressure on the pathogen population to select for beneficial mutations leading to SDHI resistance (Friskop, 2014).

There have been reports of a single case of resistance in *S. sclerotiorum* due to a H132R mutation in the *sdh* gene. Even though the frequency of resistance is not high, without proper mitigation strategies, resistance could develop. The isolates that carry these mutations have a high fitness to survive and reproduce (Sierotzki and Scalliet, 2013). Studies suggest that resistance to carboxin in the same mutation of conserved histidine residue also gives resistance to boscalid in ascomycete fungi. For example, in the field *B. cinerea* isolates have mutations in SDHB subunit, replacing histidine by tyrosine or arginine at position 272, and proline residue was exchange by phenylalanine or leucine at position 225.Variations in the interaction with amino acid in the succinate dehydrogenase determine the different activities between SDHI fungicides. Therefore, understanding the evolution of resistant pathogens is critical to secure the lifetime and sustainability of SDH fungicides (Avenot and Michailides, 2010).

Fungicide Resistance Risk Mitigation

The emergence of resistant strains is always a problem when industries are trying to create an optimized fungicide able to extend its life in the market. Delaying or preventing resistance could be achieved by mixing fungicides in different manners. Mixtures of fungicides could provide a better control of pathogens, insure that an effective control is preserved, and manage resistance for a longer period of time by reducing the level of selection pressure (van den Bosch et al., 2014). For example, simulation studies showed that the application of a mixture of azoxystrobin and chlorothalonil at 25% and 100% of their recommended doses, respectively, for control of *Mycosphaerella graminicola* on wheat increased the effective life of both compounds by 13 years, whereas a mixture of that contains 25% of the dose of azoxystrobin and 20% of the dose of chlorothalonil decreased the effective of life of mixtures by 9 years (van den Berg et al., 2013; van den Bosch et al., 2014). Adding a mixing compound to a high risk-fungicide with and

without decreasing its dose is very important to reduce the build-up of resistance, causing a reduced rate of selection for resistant isolates. By not decreasing the dose, the life of the risk-fungicide will be prolonged, whereas decreasing the dose will maintain an effective disease control of the pathogen. Therefore, as a management strategy, mixtures of fungicides will work ideally by reducing the dose of the risk-fungicide (van den Bosch et al., 2014). Two fungicides with different mode of action can be alternated causing elimination of the amount of applications required of one of them. Consequently, this will bring a delay the development of fungicide resistance in canola fields (Liu et al., 2009).

In addition to genetic mutations, grower practices can contribute to stimulate development of resistance. For example, in an attempt to decrease inputs, growers may use less fungicide in the water resulting in sub-lethal doses or alter spraying pressure to use less water per area which would increase the concentration resulting in overdoses in the tank; or spray at speeds higher than recommended which might result in poor coverage, uneven distribution of the fungicide and eventually to a mixture of sublethal and lethal concentrations on plant tissues. These situations can create mutations and quickly stimulate the evolution of fungicide resistance or cause an increase in disease severity (Bolton et al., 2012; Garzon et al., 2011; Gressel, 2011).

To manage resistant isolates it is important to know the origin, development and spread of resistance. Baseline sensitivity, molecular and genetic approaches are tools used to develop a new fungicide with high activity and specificity of binding to the target site (Avenot and Michailides, 2010). Successful fungicide management of *S. sclerotiorum* to delay resistance development as long as possible includes rotation between the registered chemicals to minimize the selection pressure and development of resistant isolates, ideal timing application of fungicides and coverage, and finally the use of the Sclerotinia forecasting system as an important

tool to predict the development of the disease based on environmental conditions (McLaren et al., 2004; Thomson et al., 1984).

The Fungicide Resistance Action Committee (FRAC) categorizes boscalid and azoxystrobin as medium to high risk and high risk, respectively. For a sustainable and efficient disease control, FRAC recommends not using a single fungicide exclusively, alternate in the mode of action of products, limiting and controlling the number of fungicide applications per season or per crop, mixture with similar fungicides, and spraying fungicides as preventive protectants instead as eradicants (Sieotzki and Scalliet, 2013; Liang et al., 2015). To evaluate fungicides effectiveness it is important to first establish a baseline of information against which the reaction of isolates collected after introduction of the product to the market could be compared. Once this evidence is collected, molecular methods that detect known mutations i.e. PCR-RFLP and Allele Specific-PCR techniques could be used (Avenot and Michailides, 2010).

Importance of Research

Growers rely on fungicide applications as a strategy to control *S. sclerotiorum*, however, isolates can develop resistance to azoxystrobin and boscalid which could lead to control failures and increased economic losses caused by *S. sclerotiorum* in the future. Current levels of sensitivity of *S. sclerotiorum* to these compounds in the North Central region of US are not known. Since canola growers normally apply fungicides once during the canola growing season, we hypothesize that *S. sclerotiorum* isolates have not developed resistance to these compounds. To develop a more effective strategy to manage fungicide resistance, it is necessary to establish a baseline sensitivity of *S. sclerotiorum* to azoxystrobin and boscalid. This information will facilitate future monitoring of resistance risk and act as a point of reference. The baseline sensitivity will be used as a reference to detect early shifts in pathogen sensitivity and also it will

give us evidence that future failures with fungicide applications may be due to the presence of resistance in the population. Moreover, conducting baseline sensitivity will help us to detect any differences in sensitivity between isolates that can cause future resistance problems (Wise et al., 2008). In order to determine the sensitivity of *S. sclerotiorum* to azoxystrobin and boscalid, we will conduct tests in which ascospore germination and mycelium growth will be quantified at different fungicide concentrations. The EC_{50} is the concentration of a fungicide in media that reduces fungal growth or spore germination by 50% compared with the non-amended media. A high EC_{50} value compared to the baseline would indicate a reduction in sensitivity and probably the presence of resistance in the pathogen population to the fungicide. To effectively manage fungicide resistance knowledge of the relationship between reduced sensitivity or efficacy to any fungicide as calculated by EC_{50} values is necessary. The development of resistance will force some compounds out of the market and growers will be limited in their choices of which fungicide to use. Development of fungicide resistance reduces the efficacy of the fungicide and contributes to increases yield losses. Overall, the risk of huge losses in the market will increase and growers and industries may lose millions of dollars in one season.

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SENSITIVITY OF *SCLEROTINIA SCLEROTIORUM* ISOLATES FROM THE NORTH CENTRAL U.S. TO AZOXYSTROBIN

Abstract

Sclerotinia sclerotiorum (Lib.) de Bary causes Sclerotinia stem rot on a wide host range. Growers manage this disease mainly through the use of fungicides like azoxystrobin. The objective of this study was to establish baseline sensitivity of *S. sclerotiorum* to azoxystrobin. Preliminary trials demonstrated that salicylhydroxamic acid (SHAM) was toxic to ascospores even at 0.1 µg/ml and thus was not used to estimate sensitivity to azoxystrobin. The azoxystrobin mean EC_{50} for ascospores was 3.05 µg/ml (range 0.13 to ≥ 28 µg/ml) and 1.01 µg/ml (range 0.32 to ≥9 µg/ml) for mycelium. No resistant isolates were identified and no significant difference in sensitivity was observed between isolates collected prior to azoxystrobin's introduction to the market and isolates collected in 2014. The information generated in this study will be used as baseline. Growers are encouraged to use production practices that help avoid the development of resistant strains.

Introduction

Canola (*Brassica napus* L.) is an economically important crop in North Dakota in the United States. The crop was introduced from Canada as oilseed rape in the early 1980s but it only became popular in the Northern region when cultivars with low erucic acid and low glucosinolates contents entered the market. These cultivars are generically known as canola and produce high quality oil that is acceptable for human consumption and that could be use as biodiesel (Gunstone, 2004; Lamey and Hershman, 1993). The national statistics for canola in U.S. market indicates that in 2014, growers harvested 627,262.7 ha (1.55 million acres) of canola with a yield production of 1.14×10^6 metric tons (2.51×10^9 lb.). In 2014, North Dakota (ND)

planted 70 percent of the area dedicated to canola in the U.S. and produced an average yield of 2.22 tons/ha (1,800 lb/A.) that represented a yield production of 9.71 x 10^5 metric tons (2.14 x 10^9 lb). Moreover, in 2014, ND contributed 85 percent of the national canola production with a value of approximately US$364 million (USDA-crop production/crop value, 2015).

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic, homothallic fungus that belongs to the phylum Ascomycetes. This pathogenic fungus causes Sclerotinia stem rot (SSR) on canola and many other important crops causing large economic losses (Boland and Hall, 1994; Purdy, 1979). *S. sclerotiorum* has a broad geographical distribution; it is prevalent in North Central region of U.S. where seasons tend to be cool and wet during the flowering period (Adams and Ayers, 1979; Bradley and Lamey, 2005). In North Dakota, SSR incidence that affected canola between 1991 and 2002 ranged from 7 to 19% causing a direct economic impact of approximately US$94 million (Lamey, 1995; Lamey et al., 2002; Lamey, 2003; Bradley and Lamey, 2005). A more recent study suggested that under North Dakota conditions, the disease can reduce canola yields at a rate of 0.5% for each percentage point of SSR incidence (del Río et al., 2007).

In North Dakota, canola growers control *S. sclerotiorum* mainly through the use of fungicides such as azoxystrobin (Quadris^R), boscalid (Endura^R), metconazole (Quash^R), prothioconazole (Proline^R), pyraclostrobin (Headline^R), penthiopyrad (Vertisan^R), picoxystrobin (Approach^R), and thiophanate methyl (Topsin^R) (Friskop et al., 2014). These compounds have single modes of action and a history of promoting fungicide-resistance in their target populations (Brent and Hollomon, 2007). Therefore, monitoring the sensitivity of such populations has an important value for resistance management strategies.

Azoxystrobin is a quinone outside inhibitor (QoI) fungicide. This compound belongs to FRAC group 11 and inhibits mitochondrial electron transport chain by binding to the quinone oxidation site, Qo, center of cytochrome *bc1* complex (complex III). Blocking mitochondrial respiration causes a disruption of the energy cycle and interferes with ATP synthesis in the fungus (Duan et al., 2012; Bartlett et al., 2002; Grasso et al., 2006). Azoxystrobin has a site-specific mode of action that causes selection pressure on the target fungal population, increasing its potential to develop fungicide resistance (Fernández-Ortuño et al., 2008). Resistance starts with a point mutation in the mitochondrial cytochrome *b* gene (CYTB) that prevents fungicide binding (Gisi et al., 2002; Sierotzki et al., 2006). Some fungi have the ability to utilize an alternative respiration pathway to circumvent the blocking of the site targeted by the QoI fungicides *in vitro* and provide energy to cells intoxicated with a QoI fungicide (Ziogas et al., 1997). Salicylhydroxamic acid (SHAM) inhibits an oxidase (AOX) enzyme that is responsible for accessing the alternative respiration pathway (Xu et at., 2013). Because of this, SHAM has been used in combination with strobilurin fungicides to help estimate *in vitro* the true toxicity of the fungicides.

The establishment of baseline sensitivity to fungicides is necessary to monitor changes in resistance and help in the early detection of shifts in pathogen sensitivity and the risk of buildup of fungicide-resistant populations in a region (Wise et al., 2008). Among the fungicides registered for use on canola against *S. sclerotiorum*, azoxystrobin, thiophanate methyl and boscalid were registered in North Dakota in 2000, 2003, and 2004, respectively. Of these, thiophanate methyl is the only one for which fungicide sensitivity data has been established in the region (Ameen et al., 2012). The objective of this research was to establish a baseline sensitivity of *S. sclerotiorum* isolates from north central U.S. to azoxystrobin.

Materials and Methods

The following procedures were conducted to estimate the sensitivity of ascospores and mycelium of *S. sclerotiorum* isolates to SHAM, SHAM combined with azoxystrobin and to azoxystrobin without SHAM. For each study, multiple experiments were conducted twice (trials). Each trial had three replications and various numbers of isolates were used for each group. Some isolates were used in more than one experiment. Sensitivity of *S. sclerotiorum* to this fungicide was expressed as EC_{50} or the concentration required to reduce growth or germination by 50%. These values were calculated using water agar and potato dextrose agar (PDA) amended with technical grade of azoxystrobin for ascospores and mycelial trials, respectively. The 116 *S. sclerotiorum* isolates used in this study were collected by various scientists in 13 different states of North Central U.S. between 1980 and 2014 and pure cultures were obtained from Dr. Berlin Nelson from North Dakota State University.

Ascospores and mycelium production

For trials involving sensitivity of ascospores, sclerotia were surface disinfested by immersing them in a 10 % sodium hypochlorite and water solution (v/v) for 3 minutes. Then, the sclerotia were rinsed in sterile, deionized water. Surface disinfested sclerotia were plated on full strength potato dextrose agar (PDA) medium (24 g of Potato Dextrose Broth, 15 g of Bacto Agar, and 1000 ml of distilled water; autoclaved at 121^{o} C for 20 minutes at 103.42 kPa of pressure) in petri dishes and incubated for approximately 3 days at 25^{o} C until the mycelial colony covered approximately one-quarter of the surface of the plate. Two to three successive transfers of hyphal tips into PDA medium were made to obtain pure cultures. Five mm diameter agar plugs containing hyphal tips from these colonies were transferred into flasks containing 200 ml of sterilized diced potatoes. The potatoes had been autoclaved twice, each time for 25 min using the

same temperature and pressure conditions used to prepare the PDA with a 24 h period in between sterilizations. The isolates were incubated in this medium for approximately one month at room temperature (25^{o} C). Sclerotia were harvested from these flasks by wet sieving with sterilized water. Sclerotia 0.5 – 1 cm in length were conditioned by subjecting them to at least nine consecutive freeze/thaw cycles to stimulate carpogenic germination. Each cycle consisted of alternating 24 h at -20^{o} C (~ -4^{o} F) and 24 h at 21^{o} C (~70 ^{o} F). Conditioned sclerotia were incubated under 16 h light at 20^{o} C and 8 h dark at 18^{o} C daily in 45 ml of wet sterile sand with 6 ml of sterile water to create an ideal environment conducive for stipe and apothecia formation. Aerial mycelium that grew on sclerotia and all sclerotia that were soft or deteriorating were discarded from the dishes to avoid the spread of contamination. As mature apothecia discharged ascospores on the dish lids, "loaded lids" were replaced every 2 days with clean lids and stored in a refrigerator at 4^{o} C until used.

For trials involving sensitivity of mycelium, colonies were produced from sclerotia as described earlier. Pure cultures were obtained by transferring two mm diameter agar plugs containing hyphal tips onto fungicide-amended PDA.

Sensitivity of ascospores to SHAM and/or azoxystrobin

Before evaluating the sensitivity of ascospores to azoxystrobin multiple experiments were conducted to determine the concentration of SHAM that would not affect germination significantly and thus interfere with the estimation of the sensitivity of *S. sclerotiorum* to azoxystrobin. For these experiments, all concentrations of both chemicals were derived from stock solutions prepared each time a study was conducted. SHAM stock solutions were prepared by dissolving 100 mg of SHAM (Sigma-Aldrich, St. Louis, MO) in 1 ml methanol. Azoxystrobin stock solutions were prepared by dissolving 1 mg analytical grade azoxystrobin (99%; Sigma-

Aldrich, St. Louis, MO) in 1 ml acetone. Water agar (15 g agar per liter of water and autoclaved as described) was amended with stock solutions of SHAM or azoxystrobin to reach concentrations ranging from 0 to 250 µg/ml (Table 1) and from 0 to 28.8 µg/ml (Table 2), respectively. When SHAM and azoxystrobin were combined their concentrations ranged from 0 to 202 and 0 to 0.6 µg/ml, respectively (Table 3). Fresh ascospores were collected from stored dish lids and suspended using sterile deionized water. Spore concentrations were estimated using a haemocytometer and adjusted to $5x10^4$ spores/ml. Ascospore suspension aliquots (40 µl~ $2x10^3$ spores) were deposited on amended water agar plugs and placed on glass slides. The inoculated plugs were placed on a layer of wet paper towel in plastic containers that were sealed and then incubated in dark at 25° C for 24 h. After incubation, ascospores were stained with lactophenol-cotton blue to quantify germination. Isolate WM 31 was replicated in all trials to be used as a control for homogeneity of variances tests but in some instances isolates WM420, 396, and 118 were used. Spore germination was quantified on 50 spores per treatment and replication combination. An ascospore was considered germinated if its germ tube was at least as long as the length of the ascospore. Data from trials where spore germination on non-amended water agar was < 77% were discarded.

Table 1. Study[1] conducted to determine the sensitivity of *Sclerotinia sclerotiorum* ascospores to salicylhydroxamic acid (SHAM).

Experiments	SHAM concentrations (µg/ml)	Number of isolates used
1	0-100-150-200-250	2
2	0-70-119-202	3
3	0-70-119-202	3
6	0-25-50-75	4
7	0-5-10-20-25-50-75	4
9	0-0.5-1-2-4-8	6
11	0-0.1-0.2-0.4-0.8-1.6-3.2-6.4-12.8-25.6-51.2	5

[1] Spore germination trials conducted using water agar amended with SHAM.

Table 2. Study[1] conducted to determine the sensitivity of *Sclerotinia sclerotiorum* ascospores to azoxystrobin.

Experiments	Azoxystrobin concentrations (µg/ml)	Number of isolates used
2	0-0.05-0.092-0.166	2
6	0-0.05-0.092-0.166-0.3	4
13	0-0.3-0.6-1.2-3.6-7.2	17
14	0-0.3-0.6-1.2-3.6-7.2-14.4	9
15	0-0.3-0.6-1.2-3.6-7.2-14.4-28.8	5
16	0-0.3-0.6-1.2-3.6-7.2-14.4-28.8	8

[1] Spore germination trials conducted using water agar amended with azoxystrobin.

Table 3. Study[1] conducted to determine the sensitivity of *Sclerotinia sclerotiorum* ascospores to azoxystrobin combined with salicylhydroxamic acid (SHAM).

Experiments	SHAM concentrations (µg/ml)	Azoxystrobin concentrations (µg/ml)	Number of isolates used
2	0-70-119-202	0-0.05-0.092-0.166	2
3	0-70-119-202	0-0.05-0.092-0.166-0.3	7
4	0-70	0-0.05-0.092-0.166-0.3	8
5	0-70	0-0.05-0.092-0.166-0.3	11
6	0-25-50-75	0-0.05-0.092-0.166-0.3	3
10	0-5	0-0.05-0.092-0.166-0.3	6
12	0-70	0-0.05-0.092-0.166-0.3-0.6	12
13	0-70	0-0.05-0.092-0.166-0.3-0.6	12

[1] Spore germination trials conducted using water agar amended with SHAM and azoxystrobin.

Sensitivity of mycelium to azoxystrobin

The stock solution and the fungicide-amended PDA used in this study were prepared as described earlier. PDA was amended with azoxystrobin to reach final concentrations of 0, 0.6, 1.2, 2.4, 4.8, and 9.6 µg/ml and poured into petri dishes (85 cm diameter). The experiments 11, 15, 17, 19, and 23 evaluated the effects of azoxystrobin in mycelium growth. A 2 mm plug containing hyphal tips of a *S. sclerotiorum* colony was placed in the middle of the plate and incubated at 25 ^{o} C for 48 h. Each concentration was replicated three times for each of 116 isolates and the entire experiment was conducted twice. Colony diameters were measured in two perpendicular directions. When colony growth was too sparse, i.e. few short hyphae growing

only in one direction as opposed to radiating out in uniform fashion (Figure 6) the diameter of the colony was considered 0.

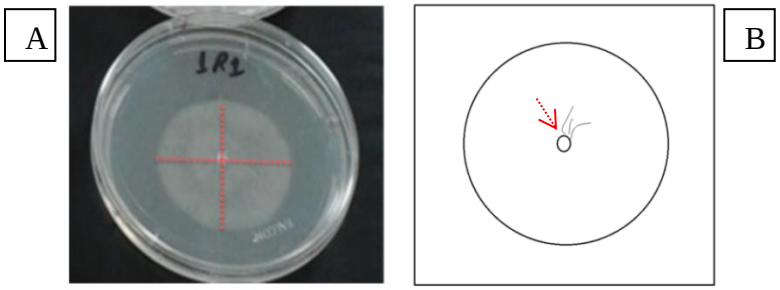

Figure 6. Example of a *Sclerotinia sclerotiorum* colony mycelial growth in PDA amended with azoxystrobin. A) Normal mycelial growth. B) Sparse mycelial growth. The arrow indicates mycelium growing.

Data analyses

Effects of SHAM, azoxystrobin and combinations of both on ascospore germination and mycelium growth

A completely randomized design (CRD) with two factors, azoxystrobin and/or SHAM and isolates, was used in all experiments to evaluate the effect of these chemicals on ascospore germination and mycelium growth. Trials within an experiment had three replications per treatment and each trial was conducted twice. Levene's homogeneity of variances test was used to determine whether trials (within an experiment) or experiments could be combined for analysis. If the experiments could not be combined they were analyzed separately and mean separations were conducted using the least significant difference test (L.S.D) at $P = 0.05$. To further characterize the effect of SHAM on spore germination the data was analyzed using the linear regression procedure (PROC REG) of SAS (SAS version 9.4; SAS Institute, Cary, NC) to quantify the rate of change in suppression of ascospore germination.

Calculation of EC_{50} for ascospores and mycelium

Spore germination and colony growth in absence of fungicide were used as reference points to estimate the percentage of suppression caused by each fungicide concentration. Non-linear regression (PROC NLIN of SAS 9.4; SAS Institute, Cary, NC) was used to estimate the concentration that caused 50% suppression of spore germination or mycelial growth (EC_{50}). To further characterize the reaction of isolates to azoxystrobin with no SHAM, histograms with eight bins established using quartiles and subdivisions of quartiles were prepared and used to arbitrarily classify isolates as sensitive (S), moderately sensitive (MS), moderately tolerant (MT) or tolerant (T) depending on whether the EC_{50} for ascospores was at or below the first quartile, above the first quartile but below the third quartile, in the lower half of the fourth quartile, or in the upper half of the fourth quartile, respectively. A similar approach was used to classify the reaction of ascospores to azoxystrobin combined with 70 μg/ml SHAM and to classify the response of mycelia to azoxystrobin.To determine whether a change in mycelial sensitivity to azoxystrobin had occurred over time, Kruskal-Wallis one-way analysis of variance was conducted to compare the median sensitivity of isolates collected before the introduction of azoxystrobin to the market in 2000, to that of isolates collected in the first eight year after its introduction (2000-2008), and that of isolates collected in 2014.

Results

Sensitivity of ascospores to SHAM and/or azoxystrobin

Sensitivity of ascospores to SHAM

Seven different experiments were conducted to evaluate the toxicity of SHAM to ascospores of *S. sclerotiorum*. Levene's test for homogeneity of variance using isolate WM 31 demonstrated the variances of trials within each experiment were homogenous (P=0.1043 to

P=0.9292) and as a result trials within experiments were combined for analyses. Each successive trial had lower concentrations of SHAM than its predecessor (Table 1) and in all of them statistically significant reductions ($P < 0.0001$) in suppression of ascospores germination were detected even when using the lowest doses (data not shown). Results of the analysis of variance of data from experiment 11, which had the lowest concentrations of SHAM evaluated, were consistent with observations made in previous studies; SHAM suppressed ascospores germination significantly ($P \leq 0.05$) even at the lowest concentration evaluated, in this case 0.1 µg/ml (Table 4.).

Table 4. Effect of salicylhydroxamic acid (SHAM) on suppression of ascospore germination of five *Sclerotinia sclerotiorum* isolates from experiment 11.

SHAM concentrations (µg/ml)[1]	**Mean suppression (%) of ascospore germination[2]**
51.2	26.8 a
25.6	19.5 b
12.8	18.2 b
6.4	14.6 c
3.2	13.6 cd
1.6	12.7 cde
0.8	11.3 de
0.4	10.6 ef
0.2	8.3 f
0.1	5.5 g
0.0	0.0 h

[1] Means value represent 30 observations collected from 5 isolates, each with 3 replications in each of 2 trials.
[2] Means with similar letters are statistically similar to each other at *P*=0.05.

Of the 27 regression models developed to characterize the relation between SHAM and ascospore germination, only one was not statistically significant (Table 5). SHAM had a detrimental effect on spore germination but in general, the rate was related to the range of concentrations used with lowest rates being produced by studies with the widest ranges of concentrations. When concentrations ranged between 0.5 and 8 µg per ml, every unit increase in SHAM increased suppression between 2.3 and 3.5%. However, in trials where SHAM

concentrations ranged between 8 and 250 µg per ml, each unit increase in SHAM increased suppression by less than 1%. This change in rates suggests the relation between SHAM and suppression may not be linear.

Table 5. Regression models that explain the relationship between the suppression of ascospore germination of individual isolates of *Sclerotinia sclerotiorum* by salicylhydroxamic acid (SHAM) alone.

			Regression models[2]					
SHAM concentrations (µg/ml)	**N[1]**	**Isolate I.D[1]**	**Intercept**	**Slope**	**Confidence interval for slope**		**R^2**	**P**
0-100-150-200-250	30	31	1.66	0.06	0.03	0.09	0.41	*
	30	61	2.69	0.12	0.03	0.22	0.19	0.02
0-70-119-202	24	192	1.15	0.08	0.03	0.13	0.34	*
	24	31	3.37	0.21	0.14	0.28	0.66	*
	24	61	2.77	0.13	0.07	0.18	0.50	*
0-70-119-202	24	358	3.47	0.1	0.04	0.16	0.32	*
	24	418	2.50	0.04	-0.01	0.09	0.13	0.08
	24	420	5.03	0.17	0.08	0.27	0.39	*
0-25-50-75	24	31	2.06	0.43	0.32	0.53	0.76	*
	24	412	5.88	0.22	0.08	0.35	0.34	*
	24	418	0.22	0.71	0.64	0.78	0.95	*
	24	420	0.55	0.67	0.53	0.80	0.83	*
0-5-10-20-25-50-75	42	31	10.86	0.46	0.33	0.6	0.54	*
	42	68	13.51	0.42	0.30	0.53	0.56	*
	42	84	12.31	0.41	0.30	0.53	0.56	*
	42	107	9.61	0.3	0.19	0.41	0.44	*
0-0.5-1-2-4-8	36	31	8.76	2.94	1.83	4.06	0.46	*
	36	43	8.70	3.19	2.18	4.20	0.55	*
	36	168	8.25	3.09	1.81	4.36	0.42	*
	36	357	4.52	3.23	2.38	4.09	0.63	*
	36	388	5.73	2.27	1.42	3.12	0.47	*
	36	396	6.46	3.53	2.43	4.62	0.56	*
0-0.1-0.2-0.4-0.8	66	31	6.21	0.24	0.16	0.33	0.34	*
1.6-3.2-6.4-12.8	66	366	7.58	0.19	0.11	0.27	0.24	*
25.6-51.2	66	374	10.49	0.64	0.51	0.78	0.59	*
	66	410	11.29	0.55	0.4	0.71	0.43	*
	66	421	10.90	0.26	0.17	0.36	0.32	*
Grand Total[3]	**1014**		**13.25**	**0.08**	**0.07**	**0.1**	**0.11**	*****

[1]N=number of observations calculated from 3 replications in each of 2 trials per the interaction of isolate and concentration; isolate I.D.=laboratory number.

[2] C.I.=95 % confidence interval; R^2=coefficient of determination; *=≤0.0001 value represents the number of observations indicated in the N. column.

[3] Grand total values represent 1014 observations from the mean of 27 isolates at the same time which are calculated from 3 replications in each of 2 trials per the interaction of isolate and concentration.

Sensitivity of ascospores to azoxystrobin alone

Six different experiments were conducted to evaluate the toxicity of azoxystrobin to ascospores of *S. sclerotiorum*. Levene's test for homogeneity of variance using isolate WM 31 demonstrated that the variances of trials within each experiment were homogenous (P=0.1176 to P=0.8793) and as a result trials within experiments were combined for analyses. However, when comparing experiments 2, 6, 13, 14, 15 and 16, Levene's test indicated they were not homogenous (P<.0001). The LSD test on the means of these experiments (LSD =1.57; P=0.05) separated them in two groups, one composed by experiments 2, 6, 15, and 16 and the other composed by experiments 13 and 14. The experiments within each group were combined for analysis and are presented in Table A.1.

The sensitivity of ascospores of 35 *S. sclerotiorum* isolates to azoxystrobin did not follow a normal distribution and rather had two peaks suggesting a bimodal distribution (Figure 7) with a mean EC_{50} of 3.05µg/ml ranging between 0.13 and >28 µg/ml and a median of 2.62 µg/ml. Approximately 26%, 46%, and 17% of all 35 isolates had $EC_{50} \leq 0.60$, between 0.61 and 5.23, and between 5.24 and 6.62 µg/ml, respectively. Each group was arbitrarily denominated as sensitive, moderately sensitive, and moderately tolerant, respectively. However, 11% of the isolates evaluated had EC_{50} values above 6.62 µg/ml and could be considered tolerant (Table 6). The state mean EC_{50} for North Dakota was 1.19 times numerically greater than the overall mean (Table 7). Only two of the isolates evaluated for sensitivity of their ascospores to azoxystrobin were collected before the introduction of azoxystrobin to the market. North Dakota isolates, WM 68 and WM 73 had a mean EC_{50} value of 1.37±0.94 and 3.15±2.64 µg/ml, respectively. These isolates are considered to be true baseline isolates and fell within the general range of sensitivity observed.

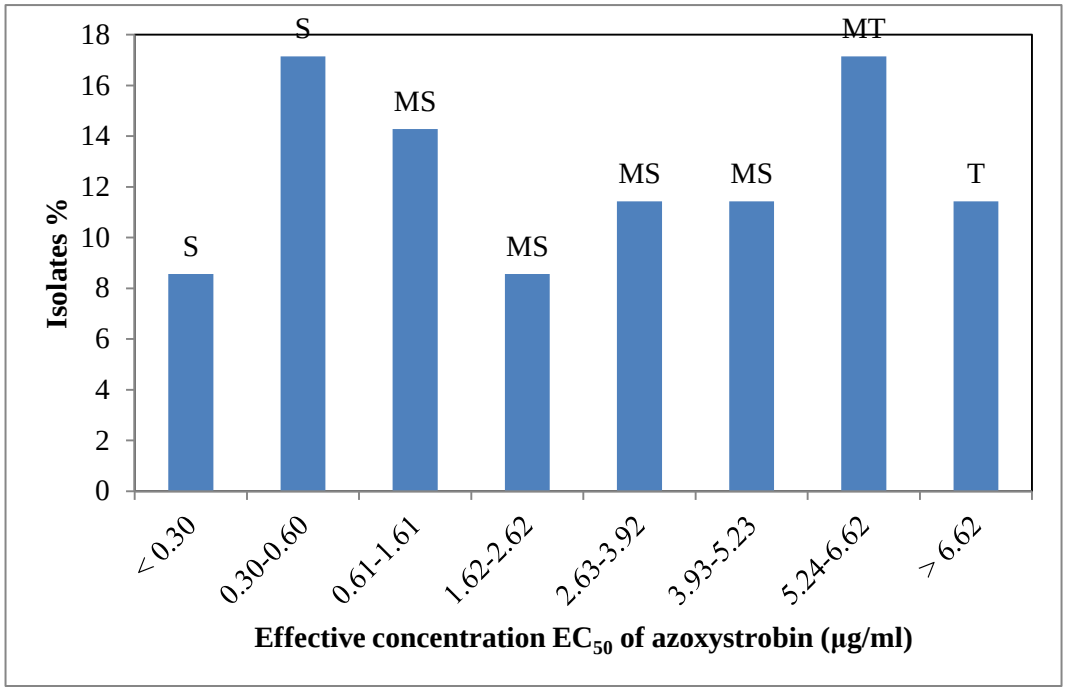

Figure 7. Frequency distribution (%) of the sensitivity of ascospores from 35 *Sclerotinia sclerotiorum* isolates to azoxystrobin alone and expressed as the 50% effective concentration (EC_{50}) in µg/ml. S=susceptible; MS= moderately susceptible; MT=moderately tolerant; T=tolerant.

Table 6. EC_{50} values for ascospore sensitivity of four *Sclerotinia sclerotiorum* isolates considered tolerant to azoxystrobin.

			Azoxystrobin (EC_{50} in µg/ml)[2]					
Isolate I.D.[1]	**Year of collection**	**State**	**Mean**	**S.D.**	**Min**	**Max**	**Median**	**CV**
137	2002	North Dakota	6.72	0.685	6.23	7.20	6.72	10.21
591	2008	North Dakota	7.46	4.367	1.20	12.60	8.28	58.53
189	2004	North Dakota	8.01	3.186	4.50	12.96	7.37	39.79
385	2007	North Dakota	≥28.8	-	-	-	-	-

[1] Isolate I.D.=laboratory number.

[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation; -= not determined.

Table 7. Sensitivity of ascospores of *Sclerotinia sclerotiorum* isolates from 9 states in North Central U.S. region to azoxystrobin. EC_{50} value calculated without salicylhydroxamic acid (SHAM).

		Azoxystrobin (EC_{50} in µg/ml)[1]					
State	**Number of isolates**	**Mean**	**S.D.**	**Min**	**Max**	**Median**	**CV**
Iowa	2	5.52	0.189	5.39	5.66	5.52	3.42
Kansas	1	0.95	-	0.95	0.95	0.95	-
Michigan	1	0.68	-	0.68	0.68	0.68	-
Minnesota	2	0.48	0.127	0.39	0.57	0.48	26.23
Missouri	1	4.12	-	4.12	4.12	4.12	-
Montana	1	0.55	-	0.55	0.55	0.55	-
Nebraska	2	0.95	0.740	0.42	1.47	0.95	78.24
North Dakota	24	3.63	2.448	0.18	8.01	3.15	67.50
Wisconsin	1	0.13	-	0.13	0.13	0.13	-
Grand Total[2]	**35**	**3.05**	**2.464**	**0.13**	**8.01**	**2.62**	

[1] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation; -= not determined.

[2] Grand total means and medians represent 210 observations of 35 isolates which are calculated from 3 replications in each of 2 trials per isolate.

Sensitivity of ascospores to azoxystrobin combined with SHAM

Levene's test for homogeneity of variance using isolate WM 31 demonstrated that the variances of trials within each experiment that evaluated the effect of azoxystrobin combined with 70 µg/ml of SHAM were homogenous (P=0.117 to P=0.6632) and as a result trials within experiments were combined for analysis. However, when comparing experiments 2, 4, 5, and 13 Levene's test indicated they were not homogenous (P=0.0075). The means for the first three experiments were statistically similar to each other (LSD=0.0285; P=0.05) and thus were combined. Further, Levene's test for homogeneity of variance using isolates WM420, 396 and 118 demonstrated that the variances of trials within each experiment were homogenous (P=0.1187 to P=0.6584) and as a result trials within experiments were combined for analysis. Levene's test on variances of experiments 3, 4, 5 and 12 using isolates WM420, 396, and 118 indicated they could also be combined for analysis (P>0.0977).Therefore, based on the

performance of isolates WM 31, WM 420, WM 396, and WM 118; experiments 2, 3, 4, 5 and 12 were combined for analysis. However, experiment 13 could only be combined between trials but not with other experiments and its analysis is presented in Table A.2.

The samples of 38 ascospores of *S. sclerotiorum* isolates exposed to azoxystrobin with 70 µg/ml of SHAM had two different peaks and suggested a bimodal distribution (Figure 8) with a mean EC_{50} value of 0.11 µg/ml (ranging between 0.03 and 0.21µg/ml) and a median of 0.11 µg/ml Approximately 26%, 47%, and 24% of all 38 isolates had $EC_{50} \leq 0.088$, between 0.089 and 0.133, and between 0.134 and 0.173 µg/ml, respectively. Each group was arbitrarily denominated as sensitive, moderately sensitive, and moderately tolerant, respectively. However, one isolate which represents 3 % of the isolates evaluated had EC_{50} values above 0.173 µg/ml and could be considered tolerant. That isolate is WM 363 which was collected in North Dakota in 2008. Isolate WM 363 had a mean EC_{50} value of 0.21±0.004 (minimum and maximum value of 0.21 and 0.22 µg/ml, respectively) and a median of 0.21 µg/ml. The state mean EC_{50} for North Dakota was numerically equal to the overall mean (Table 8). Six baseline isolates collected before the introduction of azoxystrobin to the market were used in this study and had a mean EC_{50} value of 0.10 µg/ml (ranging from 0.07 to 0.13 µg/ml) with a median of 0.09 µg/ml.

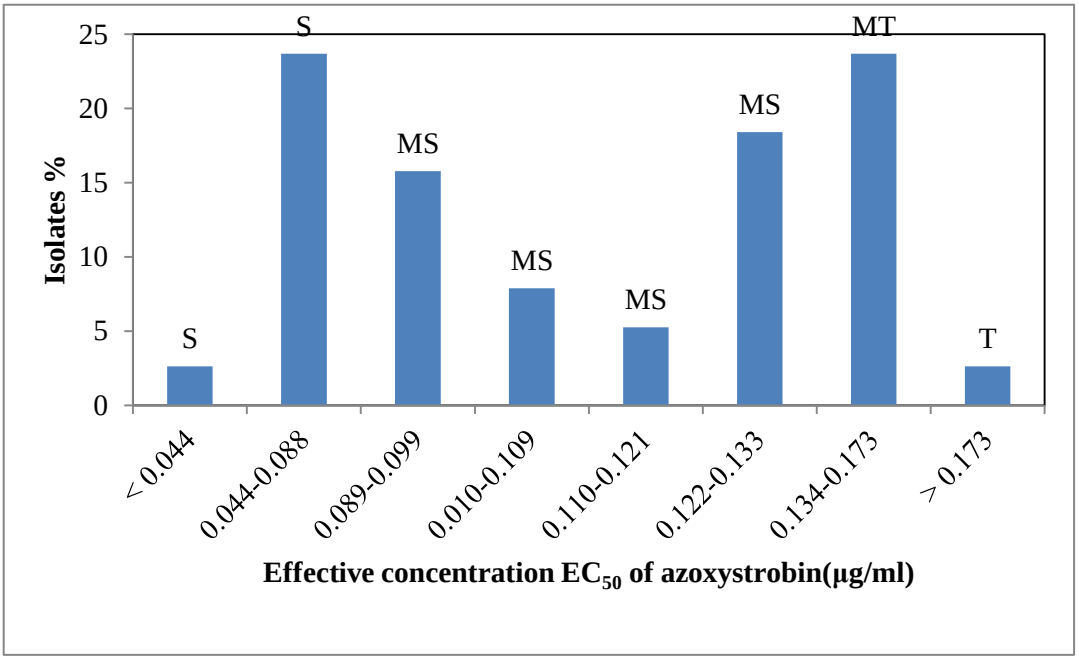

Figure 8. Frequency distribution (%) of the sensitivity of ascospores from 38 *Sclerotinia sclerotiorum* isolates to azoxystrobin combined with 70 (µg/ml) of salicylhidroxamix acid (SHAM) and expressed as 50% effective concentration (EC_{50}) in µg/ml. S=susceptible; MS= moderately susceptible; MT=moderately tolerant; T=tolerant.

Table 8. Sensitivity of ascospores of *Sclerotinia sclerotiorum* isolates from 6 states in North Central U.S. region to azoxystrobin with 70 µg/ml of salicylhydroxamic acid (SHAM).

		Azoxystrobin (EC_{50} in µg/ml)[1]					
State	**Number of isolates**	**Mean**	**S.D.**	**Min**	**Max**	**Median**	**CV**
Illinois	1	0.07	-	0.07	0.07	0.07	-
Iowa	2	0.13	0.010	0.13	0.14	0.13	7.25
Minnesota	2	0.11	0.007	0.10	0.11	0.11	6.33
Missouri	1	0.16	-	0.16	0.16	0.16	-
Nebraska	1	0.10	-	0.10	0.10	0.10	-
North Dakota	31	0.11	0.035	0.03	0.21	0.10	31.25
Grand Total[2]	**38**	**0.11**	**0.033**	**0.03**	**0.21**	**0.11**	

[1]Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation; -= not determined.

[2] Grand total means and medians represent 228 observations of 38 isolates which are calculated from 3 replications in each of 2 trials per isolate.

The mean EC_{50} value of *S. sclerotiorum* ascospores isolates exposed to azoxystrobin alone were on average 31.9 times greater when 70 µg/ml of SHAM were added to the medium (Table 9).

Table 9. Sensitivity (EC_{50}) of ascospores of *Sclerotinia sclerotiorum* to azoxystrobin alone and azoxystrobin combined with 70 µg/ml of salicylhydroxamic acid (SHAM).

	Mean azoxystrobin EC_{50} (µg/ml)[2]	
Isolate I.D.[1]	**0 µg/ml SHAM**	**70 µg/ml SHAM**
420	0.18	0.07
192	0.21	0.03
31	1.47	0.10
107	2.31	0.08
423	3.00	0.14
73	3.15	0.13
381	4.12	0.16
592	4.53	0.12
362	5.27	0.13
412	5.39	0.13
376	5.66	0.14
406	5.90	0.13
363	6.26	0.21
184	6.27	0.15
137	6.72	0.17
385	≥ 28.00	0.13
Total[3]	**4.03**	**0.13**

[1] Isolate I.D.=laboratory number.
[2] Mean values represent observations from 3 replications in each of 2 trials per isolate.
[3] Total means represent 96 observations of 16 isolates which are calculated from 3 replications in each of 2 trials per isolate.

Results of Levene's test for homogeneity of variance from experiment 10 using isolate WM 31 demonstrated that the variances of trials within the experiment that evaluated the effect of azoxystrobin combined with 5 µg/ml of SHAM were homogenous (P=0.1966), therefore trials within the experiment were combined for analysis (Table 10).

Table 10. Sensitivity of ascospores of *Sclerotinia sclerotiorum* to azoxystrobin combined with 5 µg/ml of salicylhydroxamic acid (SHAM) of study 10.

Isolate I.D.[1]	Year of collection[1]	State	Azoxystrobin (EC_{50} in µg/ml)[2]					
			Mean	S.D.	Median	Min	Max	CV
31	2004*	Nebraska	0.19	0.034	0.20	0.15	0.25	17.71
43	1982	North Dakota	0.19	0.061	0.20	0.09	0.24	31.69
168	2004	North Dakota	0.20	0.037	0.20	0.15	0.25	17.92
357	2008	North Dakota	0.18	0.038	0.16	0.15	0.23	21.56
388	2008	North Dakota	0.28	0.033	0.28	0.25	0.30	11.85
396	2008	Minnesota	0.21	0.079	0.18	0.15	0.30	37.67

[1] Isolate I.D.=laboratory number; *=unknown but previous to 2004.
[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.

When comparing the sensitivity to azoxystrobin of isolates evaluated using either 5 or 70 µg/ml, it was observed that the sensitivity of ascospores of *S. sclerotiorum* isolates to azoxystrobin decreased an average of 1.95 times when the concentration of SHAM increased from 5 to 70 µg/ml of SHAM (Table 11).

Table 11. Comparison of sensitivity (EC_{50}) of ascospores of *Sclerotinia sclerotiorum* to azoxystrobin combined with 5 and 70 µg/ml of salicylhydroxamic acid (SHAM).

Isolate I.D.[2]	Mean azoxystrobin EC_{50} (µg/ml)[1]	
	5 µg/ml SHAM	70 µg/ml SHAM
31	0.19	0.10
388	0.28	-
357	0.18	0.13
43	0.19	0.09
31	0.19	0.10
396	0.21	0.11
Total[3]	**0.21**	**0.11**

[1]Mean values represent observations calculated from 3 replications in each of 2 trials per isolate; -= not determined.
[2] Isolate I.D.=laboratory number.
[3] Total means represent 36 observations of 6 isolates which are calculated from 3 replications in each of 2 trials per isolate.

Effect of the interaction of azoxystrobin and SHAM on ascospore germination

Levene's test for homogeneity of variance using isolates evaluated in experiments 2, 3 and 6 demonstrated that the variances of trials within each experiment were homogenous (*P* =0.2825 to *P*=0.9057) and as a result trials within experiments could be combined for analysis. Table 12 shows the effect of SHAM on toxicity of azoxystrobin. The general tendency was that SHAM increased the toxicity of azoxystrobin especially at concentrations of 50 µg per ml or higher.

Table 12. Effect of salicylhydroxamic acid (SHAM) on the sensitivity (EC_{50}) of ascospores of *Sclerotinia sclerotiorum* isolates to azoxystrobin.

Experiment	SHAM (µg/ml)	Mean azoxystrobin EC_{50} in (µg/ml)[2]
2	0	0.10 a
2	70	0.07 b
2	119	0.07 b
2	202	0.05 c
3	0	0.15 ab
3	70	0.10 c
3	119	0.13 b
3	202	0.16 a
6	0	0.19 a
6	25	0.16 a
6	50	0.12 b
6	75	0.11 b

[1] Mean values represent observations from 3 replications in each of 2 trials per isolate for a total of 48 observations from 2 isolates in study 2, 168 observations from 7 isolates in study 3, and 72 observations from the mean of 3 isolates in study 6. Means with similar letters within each study were not significantly different at *P*=0.05.

To further explore the relationship between SHAM and azoxystrobin, regression analyses conducted on two isolates are presented below (Table 13). Levene's test for homogeneity of variance for isolates WM 420 and WM 412 demonstrated that the variances of trials within each experiment were homogenous (P=0.1356 to P=0.5414) and as a result trials within experiments could be combined for analysis. Moreover, Levene's test on variances of experiments 3 and 6 using isolates WM 420 and WM 412 indicated they could also be combined for analysis , P= 0.7299 and P= 0.3710, respectively. When SHAM increased by a unit, the tolerance of ascospores to azoxystrobin lowered, indicating a negative association. Among all the SHAM concentrations with azoxystrobin, the coefficient of determination is close to perfect (R^2>0.97), expect for one (R^2=0.67). That means that there is a strong negative association between the interaction of SHAM and azoxystrobin concentrations on spore germination. SHAM had a negative effect on spore germination but in general, the rate was related to the interaction of azoxytrobin and SHAM concentrations. When we evaluated isolate WM 420, every unit increase in SHAM decreased spore germination between 12.6 and 16%. However, for WM 412, each unit increase in SHAM decreased spore germination between 11 and 19.4%. This change in rates suggests the relation between SHAM and spore germination may not be linear. The general tendency was that SHAM increased the toxicity of azoxystrobin sometimes reducing its EC_{50}. For example, for both isolates the EC_{50} values are statistically different to each other when we use SHAM at concentrations higher than 70 μg/ml.

Table 13. Regression models and the effect of the interaction of salicylhydroxamic acid (SHAM) and azoxystrobin ranging between 0 to 0.3 µg/ml on the sensitivity (EC_{50}) of spore germination of *Sclerotinia sclerotiorum*.

Isolate I.D.[1]	SHAM concentrations (µg/ml)	Mean azoxystrobin EC_{50} (µg/ml)[2]	Regression models[3]		
			Intercept	Slope	R^2
420	0	0.15 a	91.17	-12.93	0.96
	25	0.15 a	77.40	-12.60	0.87
	50	0.11 ab	81.73	-13.07	0.96
	70	0.05 b	72.53	-16.00	0.83
	75	0.10 ab	75.43	-14.10	0.98
	119	0.09 ab	72.10	-14.97	0.98
	202	0.11 ab	69.67	-13.73	0.93
412	0	0.19 ab	99.57	-12.83	0.99
	25	0.21 a	79.60	-11.27	0.67
	50	0.18 abc	87.73	-11.00	0.89
	70	0.12 c	91.83	-17.23	0.99
	75	0.15 abc	85.77	-14.57	0.85
	119	0.14 bc	109.00	-18.80	0.94
	202	0.19 ab	116.63	-19.37	0.82

[1] Isolate I.D.=laboratory number.
[2] Mean values represent observations from 3 replications in each of 2 trials per isolate. Means with similar letters are statistically similar to each other at P=0.05.
[3] All models were highly significant with P<0.001.

Sensitivity of mycelium to azoxystrobin

Levene's test for homogeneity of variance using isolate WM 31 demonstrated that the variances of trials within each experiment were homogenous (P=0.1162 to P=0.5905) and as a result trials within experiments were combined for analysis. However, when comparing experiments, Levene's test indicated they were not homogenous (P=0.0212). The means for experiments 11, 19, and 23 were statistically similar to each other as well as those from experiments 19, 23, 15, and 17 but the means for experiments 11 and 17 were statistically different to each other (LSD=0.25; P=0.05). Therefore, based on the performance of isolate WM 31, experiments were separated into two groups for combined analysis; one included 88 isolates

from experiments 19, 23, 15, and 17 and the other included 29 isolates from 11. Results from experiments 11 and 17 could not be combined and are presented separately in Table A.3.

A total of 116 *S. sclerotiorum* isolates were tested for mycelium sensitivity to azoxystrobin alone. The histogram produced using the percentage frequency distribution of these isolates had two peaks suggesting a bimodal distribution with a mean EC_{50} value of 1.01µg/ml (ranging between 0.32 and >9 µg/ml) and a median of 0.55 µg/ml. Approximately 25 %, 48%, and 19 % of all 116 isolates had $EC_{50} \leq 0.44$, between 0.45 and 0.91, and between 0.92 and 3.24 µg/ml, respectively. Each group was arbitrarily denominated as sensitive, moderately sensitive, and moderately tolerant, respectively (Figure 9.). However, 8% of the isolates evaluated had EC_{50} values above 3.24 µg/ml and could be considered tolerant (Table 14). The state mean EC_{50} for Iowa, Minnesota, Nebraska, North Dakota, and Wisconsin were between 0.81 and 1.85 times numerically greater than the overall mean (Table 15).

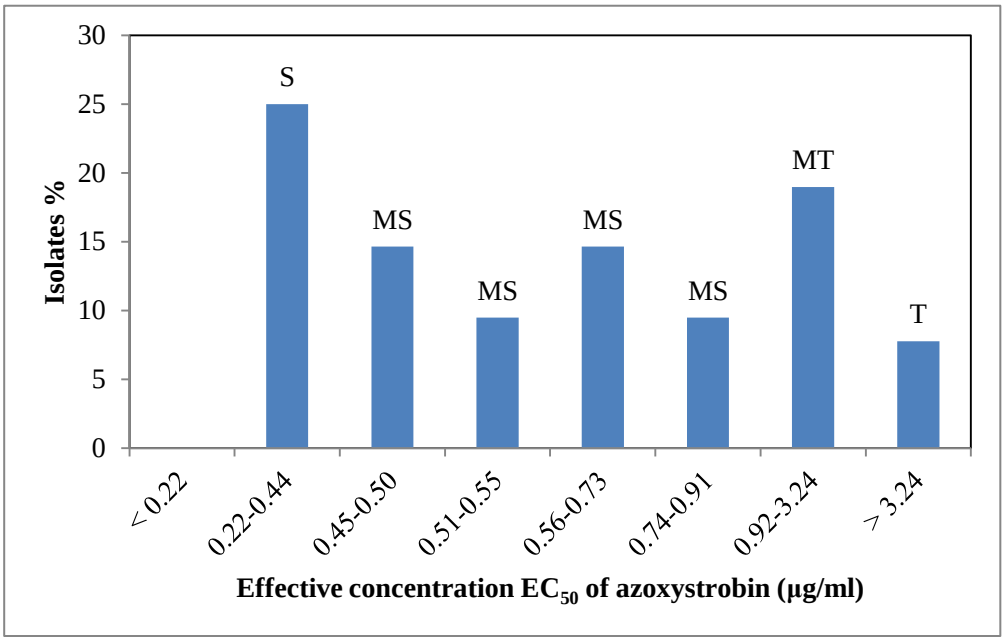

Figure 9. Frequency distribution (%) of the sensitivity of mycelium from 116 *Sclerotinia sclerotiorum* isolates to azoxystrobin alone and expressed as the 50% effective concentration (EC_{50}) in µg/ml. S=susceptible; MS= moderately susceptible; MT=moderately tolerant; T=tolerant.

Table 14. EC_{50} values for mycelium sensitivity of nine *Sclerotinia sclerotiorum* isolates considered tolerant to azoxystrobin.

Isolate I.D.[1]	Year of collection	State	Azoxystrobin (EC_{50} in µg/ml)[2]					
			Mean	S.D.	Min	Max	Median	CV
603	2003	Wisconsin	3.56	5.300	0.50	12.91	1.15	148.98
373	2008	North Dakota	3.84	4.116	0.53	11.87	2.88	107.17
378	2000	Wisconsin	4.23	3.226	0.57	9.90	4.29	76.24
58	1984	North Dakota	5.17	4.451	0.54	11.10	3.28	86.06
410	1998	Illinois	5.38	7.820	0.54	14.40	1.20	145.38
598	2008	Nebraska	5.57	6.350	0.47	14.40	1.58	113.94
596	2008	Nebraska	>9	-	-	-	-	-
398	2008	Minnesota	>9	-	-	-	-	-
601	2002	Ohio	>9	-	-	-	-	-

[1] Isolate I.D.=laboratory number.
[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation; -= not determined.

Table 15. Sensitivity of mycelium of *Sclerotinia sclerotiorum* isolates from 13 states in North Central U.S. region to azoxystrobin. EC_{50} value calculated without salicylhydroxamic acid (SHAM).

State	Number of isolates	Azoxystrobin (EC_{50} in µg/ml)[1]					
		Mean	S.D.	Min	Max	Median	CV
Colorado	1	2.28	-	2.28	2.28	2.28	-
Illinois	2	2.97	3.401	0.57	5.38	2.97	114.36
Indiana	1	0.47	-	0.47	0.47	0.47	-
Iowa	4	1.46	1.308	0.40	3.20	1.13	89.28
Kansas	1	2.32	-	2.32	2.32	2.32	-
Michigan	1	1.54	-	1.54	1.54	1.54	-
Minnesota	8	1.00	0.641	0.32	2.22	0.84	63.90
Montana	1	0.44	-	0.44	0.44	0.44	-
Nebraska	5	1.78	2.53	0.42	5.57	0.57	142.00
North Dakota	83	0.81	0.792	0.34	5.17	0.52	97.47
Ohio	1	-	-	-	-	-	-
South Dakota	1	0.36	-	0.36	0.36	0.36	-
Wisconsin	7	1.86	1.68	0.44	4.23	0.67	90.17
Grand Total[2]	**116**	**1.01**	**1.08**	**0.32**	**5.57**	**0.55**	

[1]Mean and Median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation; -= not determined.
[2] Grand total means and medians represent 696 observations of 116 isolates which are calculated from 3 replications in each of 2 trials per isolate.

To determine whether significant changes in sensitivity had occurred over time, a histogram that compared isolates in three groups was made (Figure 10). Approximately 17% of all samples constitute baseline isolates, 71% represents the sensitivity of isolates that were collected during the first eight years after the introduction of azoxystrobin to the market, and 12 % represents the sensitivity of isolates that were collected after fourteen years following the introduction of the compound in the market. The samples collected before 2000 had a non-normal distribution with a mean EC_{50}value of 1.20 μg/ml (ranging between 0.37 and 5.38 μg/ml) and a median of 0.60 μg/ml. Approximately 15 %, 60% and 15 % of all 20 isolates had $EC_{50} \leq$ 0.44, between 0.45 and 0.91, and between 0.92 and 3.24 μg/ml, respectively. However, 10% of all 20 isolates had EC_{50} values above 3.24 μg/ml and could be considered tolerant. Similarly, the samples collected between 2000 and 2008 also had a non-normal distribution with a mean EC_{50}value of 1.01 μg/ml (ranging between 0.32 and ≥ 9 μg/ml) and a median of 0.57 μg/ml. Approximately, 29%, 40% and 22% of all 82 isolates had $EC_{50} \leq 0.44$, between 0.45 and 0.91, and between 0.92 and 3.24 μg/ml, respectively. However, 9% of isolates evaluated had EC_{50} values EC_{50} above 3.24 μg/ml and could be considered tolerant. On the other hand, while the samples collected in 2014 were not normally distributed, they generally leaned towards sensitivity with a mean EC_{50}value of 0.72 μg/ml (ranging between 0.41 and 2.91 μg/ml) and a median of 0.50 μg/ml. Approximately 14%, 79% and 7% of all 14 isolates had $EC_{50} \leq 0.44$, between 0.45 and 0.91, and between 0.92 and 3.24 μg/ml, respectively. However, no isolate evaluated had EC_{50} values above 3.24 μg/ml.

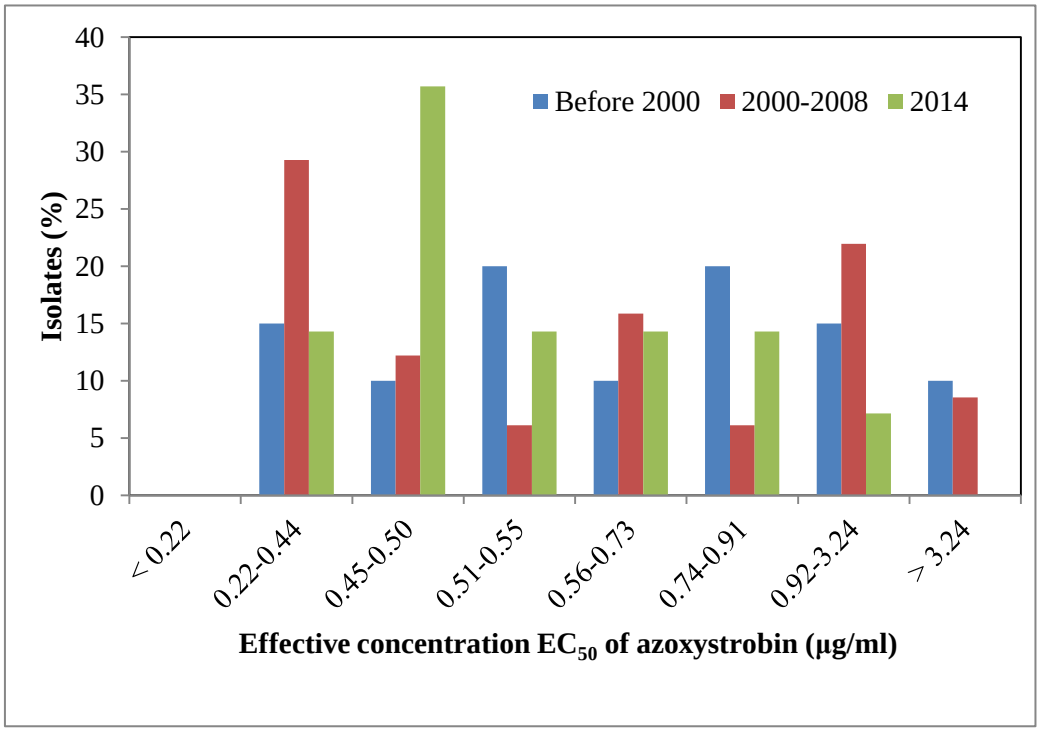

Figure 10. Frequency distribution (%) of the sensitivity of mycelium of 20, 82, and 14 *Sclerotinia sclerotiorum* isolates collected before 2000, between 2000 to 2008, and 2014, respectively, to azoxystrobin alone. Sensitivity expressed as the 50% effective concentration (EC_{50}) in µg/ml.

Because neither of this three groups had a normal distribution, the median described each sample group better than their arithmetic mean. Means are more sensitive to extreme values. Based on Kruskal-Wallis test (P=0.5853), we accepted the null hypothesis which was that the medians of all groups are equal. Therefore, there was not a significant change in sensitivity between years (Table 16).

Table 16. Median EC_{50} for mycelium isolates of *Sclerotinia sclerotiorum* across time period relative to the debut of azoxystrobin in 2000.

Time	Number of isolates	Azoxystrobin (EC_{50} in µg/ml)[1]					
		Mean	Median	S.D.	Min	Max	CV
Before 2000	20	1.20	0.60	1.462	0.37	5.38	121.84
2000-2008	82	1.01	0.57	1.030	0.32	5.57	101.96
2014	14	0.72	0.50	0.645	0.41	2.91	89.59

[1]Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.

Moreover, in this study the mean EC_{50} value of ascospores of 19 *S. sclerotiorum* isolates are 4.20 times greater than the same mycelium isolates, both evaluated with azoxystrobin alone. Therefore, the spore germination for detecting the sensitivity of *S.sclerotiorum* to azoxystrobin is more tolerant than mycelium growth (Table 17).

Table 17. Sensitivity (EC_{50}) of ascospores and mycelium of *Sclerotinia sclerotiorum* to azoxystrobin alone.

			Mean azoxystrobin EC_{50}(µg/ml)[2]	
Isolate I.D.[1]	**Year**	**State**	**Ascospores**	**Mycelium**
68	1994	North Dakota	1.37	0.42
418	2003	Wisconsin	0.13	0.44
175	2004	North Dakota	0.48	0.44
184	2004	North Dakota	6.27	0.45
605	2007	Montana	0.55	0.44
606	2007	North Dakota	3.16	0.70
385	2007	North Dakota	≥ 28	0.45
383	2008	Kansas	0.95	2.32
393	2008	Minnesota	0.39	0.84
596	2008	Nebraska	0.42	≥ 9
356	2008	North Dakota	2.84	0.62
362	2008	North Dakota	5.27	0.35
363	2008	North Dakota	6.26	0.44
388	2008	North Dakota	5.10	0.73
392	2008	North Dakota	1.01	0.43
406	2008	North Dakota	5.90	0.46
412	2008	Iowa	5.39	3.20
591	2008	North Dakota	7.46	0.58
592	2008	North Dakota	4.53	0.35
Total[3]			**3.19**	**0.76**

[1]Isolate I.D. = laboratory number.
[2]Mean values represent observations from 3 replications in each of 2 trials per isolate.
[3]Total means represent 114 observations of 19 isolates which are calculated from 3 replications in each of 2 trials per isolate.

Discussion

This is the first report indicating that SHAM has a detrimental effect on germination of *S. sclerotiorum* ascospores. Even though EC_{50} values for SHAM on ascospores could not be obtained, regression analyses modeled its negative influence on germination of ascospores of *S. sclerotiorum.* SHAM was toxic even at concentrations as low as 0.1 µg/ml. A model generated with all concentrations of SHAM used in these experiments indicated that each microgram of SHAM would increased, the suppression of spore germination by 0.08%. SHAM has been used by many researchers in the past to evaluate the true toxicity of strobilurins because it blocks alternative respiration patwhay when cells are intoxicated with QoI fungicides (Xu et al., 2013; Ziogas et al., 1997). However, its value to estimate the true sensitivity of a fungus seems to be species specific. For example, it has been reported that using 100 µg ml^{-1} of SHAM alone did not interfere significantly in the conidial germination in baseline or suspected resistant *Pyricularia grisea* isolates (Vincelli and Dixon, 2002) or that of *A. solani* spores (Pasche et al., 2004) or of *Ascochyta rabiei* pycnidiospores (Wise et al., 2008). However, it has been shown that using SHAM concentrations of 100 µg ml^{-1} increased the sensitivity of mycelium of *S. sclerotiorum* to azoxystrobin (Xu et al., 2013). Moreover, SHAM concentrations of 20 µg ml^{-1} were toxic to *S. sclerotiorum* mycelial growth (Liang et al., 2015). In their study, Liang and collaborators evaluated sensitivity to pyraclostrobin and concluded that in absence of SHAM the EC_{50} was 3.4 times greater (Liang et al., 2015). However, their team did not evaluate the effect of SHAM on ascospores of *S. sclerotiorum*.

Results of this study support the notion that the alternative respiration pathway that is blocked by SHAM may be more important in *S. sclerotiorum* than in other fungi. This would suggest that *S. sclerotiorum* may naturally be more tolerant to QoI fungicides *in vitro* but not

necessarily *in planta*. Additional research is necessary to characterize the effect of SHAM on both ends of the sensitivity spectrum for *S. sclerotiorum.* This interest may be academic only. Nevertheless, since SHAM has been proved to be toxic to *S. sclerotiorum* ascospores, it should not be used to determine the sensitivity of this fungus to QoI fungicides.

The sensitivity of mycelium of *S. sclerotiorum* to azxystrobin has not changed significantly since its introduction to the market. There could be a number of reasons for this; however, the more likely explanations are: one, there has not been enough pressure on the pathogen to mutate and develop a resistant population; and two, fitness penalties associated with mutations that are resistant could have prevented resistant populations from increasing frequency. For the former, the majority of growers apply the fungicide only once during the growing season and it is possible that in successive seasons they already rotate fungicides. After all, there are more than five different compounds available for Sclerotinia control (Markell et al., 2009; NDSU Extension Service, 2015). For the latter, QoI mutations can affect the ability of the pathogen to reproduce or infect the plant. The mutant isolate may be less fit and suffer from a reduced ability to reproduce. *S. sclerotiorum* undergoes sexual reproduction every growing season which is important for the survival of the pathogen. A fitness penalty negatively affecting this aspect of its life cycle would lead to a reduced number of mutant isolates in the pathogen population due to selection pressure (Fernández-Ortuño et al., 2008). For example, it was reported that fitness penalties in QoI-resistant populations of *B. cinerea* and *C. beticola* interefere in fungal development including the production of fungal infection structures (Malandrakis et al., 2006; Markoglou et al., 2006). Other alternative explanations include the possibility that the methodology used to collect the samples did not allow for resistant isolates to be collected (limited number of samples from several states, as well as within North Dakota).

The work presented in this thesis is the first to report on the sensitivity of *S. sclerotiorum* ascospores to azoxystrobin and the second report that provides information on the sensitivity of *S. sclerotiorum* mycelium to this compound. Our mycelium isolates evaluated with azoxystrobin had EC_{50} ranging from 0.32 to > 9 μg/ml with a mean value of 1.01 μg/ml and a median value of 0.55 μg/ml. These values were calculated from all 116 isolates since the medians of the three individual groups are statistically similar to each other. Contrasting with our results, Chinese isolates evaluated by Duan et al. (2012) had a mean EC_{50} value of 0.293 μg/ml ranging from 0.1127 to 0.6163 μg/ml The sensitivity of mycelium of the US isolates was 3.45 times greater than the Chinese study. We speculate that this difference could be caused by the difference of the genetic populations coming from two different countries. The same organism was under different selection pressure based on the geographic location, resulting in variance of sensitivity to the compound. The characterization of sensitivity of ascospores of this organism to QoI is more informative because QoI fungicides mainly affect the spore germination rather than mycelium growth (Barlett et al., 2002).

In this study, the sensitivity of *S. sclerotiorum* isolates to azoxystrobin was evaluated. Besides demonstrating that most isolates were sensitive to this compound, it was also demonstrated that no significant changes in sensitvity have been observed between isolates collected before the introduction of this compound to the market and isolates collected almost 14 years later. Since it is unlikely that the samples used in this study were already tolerant or resistant to this compound, their reaction to azoxystrobin as a group could be considered akin to baseline sensitivity. Not having detected isolates that could be considered resistant to this compound represents good news for growers who use rely on this compound to manage *S. sclerotiorum*; however, it does not mean one should relax vigilance. Periodical monitoring of

sensitivity is still important because early detection of any shift towards resistance would help reformulate the management strategies to combat this pathogen. In the meantime, practices that contribute to maintain this stability should be used or incorporated into grower's commercial production practices.

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SENSITIVITY OF *SCLEROTINIA SCLEROTIORUM* ISOLATES FROM THE NORTH CENTRAL U.S. TO BOSCALID

Abstract

Sclerotinia sclerotiorum (Lib.) de Bary causes Sclerotinia stem rot and affects canola and more than 400 plants worldwide. This disease is managed through fungicides applications such as boscalid. The objective of this study was to establish baseline sensitivity of *S. sclerotiorum* to boscalid. The boscalid mean EC_{50} for *S. sclerotiorum* ascospores was more tolerant than mycelium with a mean EC_{50} of 8.38 µg/ml (ranging between 3.12 and 14.95 µg/ml) compared to 0.11 µg/ml (ranging from 0.03 and 0.78 µg/ml), respectively. No resistant isolates were identified although a few were considered tolerant. Moreover, a significant difference in sensitivity was observed between isolates collected prior to boscalid's introduction to the market and isolates collected in 2014. However, dose-response in greenhouse trials showed that even the most tolerant isolates were still effectively controlled by boscalid. The sensitivity information generated by this study will be a reference to detect shifts in the future.

Introduction

Canola (*Brassica napus* L.) is a high value crop for North Dakota. Canadians introduced the crop as oilseed rape in 1980s but it only became popular in the Northern region when cultivars with low erucic acid and glucosinolates contents entered the market. These canola cultivars produce high quality oil for human consumption that also could be used as biodiesel (Gunstone, 2004; Lamey and Hershman, 1993). In 2014, U.S. canola growers harvested 627,262.7 ha (1.55 million acres) of canola with a production of 1.14×10^6 metric tons (2.51×10^9 lb.). In that year, North Dakota planted 70 percent of the area dedicated to canola in the U.S. and produced an average yield of 2.22 tons/ha (1,800 lb/A.) representing 85 percent of the

national canola production with an estimated market value ofUS$ 364 million (USDA-crop production/crop value, 2015).

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic, filamentous, homothallic fungus that belongs to the phylum Ascomycota. This pathogenic fungus causes Sclerotinia stem rot (SSR) on canola and many other plant species that result in large economic losses (Boland and Hall, 1994; Purdy, 1979). *S. sclerotiorum* is a cosmopolitan pathogen that has a broad geographic distribution; it is prevalent in North Central region of U.S. where seasons tend to be cool and wet during the flowering period (Adams and Ayers, 1979; Bradley and Lamey, 2005). In North Dakota, SSR incidence that affected canola between 1991 and 2002 ranged from 7 to 19% causing a direct economic impact of approximately US$94 million (Lamey, 1995; Lamey et al., 2002; Lamey, 2003; Bradley and Lamey, 2005). A more recent study suggested that under North Dakota conditions, the disease can reduce canola yields at a rate of 0.5% for each percentage point of SSR incidence (del Río et al., 2007). Moreover, 1% of incidence can result in losses of US$0.486 ha (US$1.2 per acre) at current market prices (del Río, personal comm., 2016).

Boscalid (Endura^{R}) is a succinate dehydrogenase inhibitor (SDHI) fungicide that was registered for use against *S. sclerotiorum* in North Dakota in 2004 (Friskop et al., 2014). This compound belongs to FRAC group 7 and blocks the activity of the enzyme succinate ubiquinone oxidoreductase (complex II) or succinate dehydrogenase (SDH) which is an essential piece of the tricarboxylic acid cycle (TCA) and the mitochondrial electron transport chain. As consequence, SDHI fungicides have strong fungicidal activity (Broomfield and Hargreaves, 1992; Hägerhäll, 1997; Liu et al., 2009; Myresiotis et al., 2008). Boscalid has a translaminar movement property that means that the compound can move short distance. This fungicide is absorbed by leaves and

can move through the leaf but does not have a systemic movement throughout the entire plant. However, this capacity allows the compound to affect mycelium growth during infection.

Like many other compounds that are registered for use against *S. sclerotiorum*, boscalid has a single mode of action and a history to promote fungicide-resistance in its target populations (Brent, and Hollomon, 2007). Resistance to boscalid is caused by mutations in the *sdh* genes (Stammler et al., 2011; Walker et al., 2011). Numerous examples of organisms acquiring resistance against this compound have been published and include species as diverse as *B. cinerea* (Stammler et al., 2011; Walker et al., 2011), *Alternaria alternata* (Avenot and Michailides, 2007; Avenot et al., 2008), *Didymella bryoniae* (Stevenson et al., 2008), *Corynespora cassiicola* (Miyamoto et al., 2009), and *Podosphaera xanthii* (Miyamoto et al., 2010). While some of the more than 27 mutations known to confer resistance to SDHI fungicides may have a fitness cost that significantly impair the ability of isolates to survive and reproduce, others mutations result in isolates that maintain their ability to infect plants (Sierotzki and Scalliet, 2013).

Therefore, to reduce the possibility that resistant populations become established, continuous monitoring of the sensitivity of *S. sclerotiorum* to this fungicide should be conducted. The development of baseline sensitivity information would be the first step in that direction. The objectives of this research were to establish baseline sensitivity information for *S. sclerotiorum* to boscalid and to characterize the dose-response curve of *S. sclerotiorum* mycelium to this compound in greenhouse conditions.

Materials and Methods

The following procedures were conducted to estimate the sensitivity of ascospores and mycelium of *S. sclerotiorum* to boscalid. For each study, multiple experiments were conducted

twice (trials). Each trial has three replications and various numbers of isolates were used for each group. Some isolates were used in more than one experiment. Sensitivity of *S. sclerotiorum* to this fungicide was expressed as EC_{50} or the concentration required to reduce growth or germination by 50%. These values were calculated using water agar and potato dextrose agar (PDA) amended with technical grade fungicide. The 132 *S. sclerotiorum* isolates used in this study were part of the same collection of isolates representing 14 different states of North Central U.S. that were collected between 1980 and 2014 and 1 isolate of from Manitoba, Canada collected in 2008. All isolates pure cultures were obtained by Dr. Berlin Nelson from North Dakota State University.

Ascospores and mycelium production

For trials involving sensitivity of ascospores, sclerotia were surface disinfested by immersing them in a 10% sodium hypochlorite and water solution (v/v) for 3 minutes. Then, the sclerotia were rinsed in sterile, deionized water. Surface-disinfested sclerotia were plated on full strength potato dextrose agar (PDA) medium (24 g of Potato Dextrose Broth, 15 g of Bacto Agar, and 1000 ml of distilled water, autoclaved at 121^{o} C and for 20 minutes at 103.42 kPa of pressure) in petri dishes and incubated for approximately 3 days at 25^{o} C until the mycelial colony covered approximately one-quarter of the surface of the plate. Two to three successive transfers of hyphal tips into PDA medium were made to obtain pure cultures. Five mm diameter agar plugs containing hyphal tips from these colonies were transferred into flasks containing 200 ml of sterilized diced potatoes. The potatoes had been autoclaved twice using the same temperature and pressure conditions used to prepare the PDA but for 25 min each with a 24 h period in between sterilizations. The isolates were incubated in this medium for approximately 1 month at room temperature (25 ^{o} C). Sclerotia were harvested from these flasks by wet sieving

with sterilized water. Sclerotia 0.5 – 1 cm in length were conditioned by subjecting them to at least nine consecutive freeze/thaw cycles to stimulate carpogenic germination. Each cycle consisted of alternating 24 h at -20^{o} C (~ -4^{o} F) and 24 h at 21^{o} C (~70^{o} F). Conditioned sclerotia were incubated under 16 h light at 20^{o} C and 8 h dark at 18^{o} C daily in 45 ml of wet sterile sand with 6 ml of sterile water to create an ideal environment conducive for stipe and apothecia formation. Aerial mycelium that grew on sclerotia and all sclerotia that were soft or deteriorating were discarded from the dishes to avoid the spread of contamination. As mature apothecia discharged ascospores on the dish lids, "loaded lids" were replaced every 2 days with clean lids and stored in a refrigerator at 4^{o} C until used.

For trials involving sensitivity of mycelium, colonies were produced from sclerotia as described earlier. Pure cultures were obtained by transferring two mm diameter agar plugs containing hyphal tips onto fungicide-amended PDA.

Sensitivity of ascospores to boscalid

Experiments evaluated the effects of boscalid on ascospore germination were conducted multiple times. For experiments, 13, 14, 16, and 17, all concentrations of this fungicide were derived from stock solutions that were prepared each time a study was conducted. Boscalid stock solutions were prepared by dissolving 1 mg analytic grade boscalid (99%; Sigma-Aldrich, St. Louis, MO) in 1 ml acetone. Water agar (15 g agar per liter of water autoclaved as described) was amended with stock solutions of boscalid to reach final concentrations ranging from 0 to 16 µg/ml. Fresh ascospores were collected from stored dish lids and suspended using sterile deionized water. Spore concentrations were estimated using a haemocytometer and adjusted to $5x10^{4}$ spores/ml. Ascospore suspension aliquots (40 µl~ $2x10^{3}$ spores) were placed on water agar plugs and placed on glass slides. The water agar ascospores were incubated at 25^{o} C for 24 h in

dark in containers with slides placed on a layer of wet paper to prevent desiccation of the water agar plugs. After incubation, ascospores were stained with lactophenol-cotton blue to quantify germination. Isolate WM 31 was replicated in all trials to be used as a control for homogeneity of variances tests. Spore germination was quantified on 50 spores per treatment and replication combination. An ascospore was considered germinated if its germ tube was at least as long as the length of the ascospore. Data from trials where spore germination on non-amended water agar was < 77% were discarded.

Sensitivity of mycelium to boscalid

The stock solution and the fungicide-amended PDA used in these studies were prepared as described earlier. PDA was amended with boscalid to reach final concentrations of 0, 0.01, 0.1, 1, 4, and 8 μg/ml and poured into petri dishes (85 cm dimeter). The experiments 13, 14, 16, 18, and 22 evaluated the effects of boscalid on mycelium growth. A 2 mm plug containing hyphal tips of a *S. sclerotiorum* colony was placed in the middle of the plate and incubated at 25^{o} C for 48 h. Each concentration was replicated three times for each of 132 isolates and the entire experiment was conducted twice. Colony diameters were measured in two perpendicular directions. When colony growth was too sparse, i.e. few short hyphae growing only in one direction as opposed to radiating out in uniform fashion (Figure 11) the diameter of the colony was considered 0.

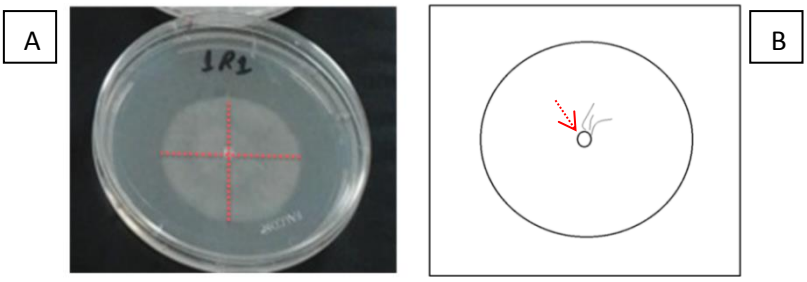

Figure 11. Example of a *Sclerotinia sclerotiorum* colony mycelial growth in PDA amended with boscalid. A) Normal mycelial growth. B) Sparse mycelial growth. The arrow indicates mycelium growing.

Dose response study

The following procedures were conducted in a greenhouse to determine the dose-response curve for mycelium of 6 isolates of *S. sclerotiorum* representing the range of sensitivities to boscalid observed in laboratory trials. Those isolates were divided into three groups using as criterion the quartile and half-quartile divisions as explained in the chapter. Their characteristics regarding sensitivity to boscalid are displayed in Table 18.

Plant materials and inoculum preparation

Canola cultivar "Westar" was used for these studies. Two seeds were sown in plastic pots (12.5 cm length and 9 cm of wide) filled with Sunshine LC1 Professional Growing Mix (Bellevue, WA, USA). After germination, plant populations were thinned to a single plant per pot. Plants were inoculated at approximately one moth old when plants had between 6 and 8 true leaves.

For trials involving sensitivity of mycelium *in planta*, sclerotia from selected isolates were surface disinfested as described earlier and mycelial colonies were produced on PDA as previously described. Pure cultures were initially inoculated on *B. carinata* leaves and incubated for 36 hours. After that time, the isolates were re-isolated on PDA. This step was conducted to

make sure all isolates were equally "fresh". "Fresh" mycelial colonies were plated on PDA and incubated for 24 h at 25° C before being used for inoculations.

Table 18. Characteristics of the reaction of six *Sclerotinia sclerotiorum* isolates to boscalid that were used in greenhouse trials to establish dose response associations.

Isolate I.D.	Year of collection	State	Boscalid (EC_{50} in µg/ml)[2]							Lesion size[3]
			Mean	S.D.	Min	Max	Median	CV	S.E.	
596	2008	Nebraska	0.08	0.007	0.07	0.09	0.08	9.32	MS	11.45
409	2008	Indiana	0.08	0.004	0.07	0.08	0.08	5.45	MS	21.80
358	2008	North Dakota	0.30	0.249	0.08	0.62	0.25	82.46	MT	22.85
603	2003	Wisconsin	0.37	0.505	0.09	1.38	0.13	137.47	MT	20.45
385	2007	North Dakota	0.46	0.281	0.13	0.84	0.44	60.71	T	14.95
607	2007	North Dakota	0.78	0.073	0.67	0.87	0.78	9.32	T	17.30

[1] Isolate I.D.=laboratory number.
[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation. S.E.=Sensitivity of the isolates. Moderately Sensitive (MS) isolate had a mean EC_{50} value between 0.073 and 0.091 µg/ml., Moderate tolerant (MT) had a mean EC_{50} between 0.092and 0.435 µg/ml. and tolerant (T) isolates had a mean EC_{50} value above 0.435 µg/ml.
[3] Values represent the average of the lesion size from 5 replications in each of 2 trials per isolate obtained in the absence of fungicide.

Spraying and inoculation procedures

The evaluation of the sensitivity of mycelium in planta to boscalid was tested in trials 1 and 2. For these studies, all concentrations for this chemical were derived from a stock solutions prepared each time a study was conducted. Each stock solution of boscalid was prepared by dissolving 1 g of Endura (a commercial formulation that contains 70% of boscalid; BASF Corporation, Florham Park, NJ) in 1000 ml deionized water and mixed with 0.5% of non-ionic surfactant adjuvant (NSI, Bayer Crop Science, Durham, NC). NSI was used to provide a uniform spray and better surface coverage. Fungicide final concentrations of 0, 0.1, 1, 4, and 8 ppm were evaluated. Sixty, single leaves with a size ranging from 6 to 12 cm were detached from canola plants. Older leaves which were located on the lower half of the plant and younger leaves coming from the upper half were used for trial 1 and 2, respectively. This division was done to

create natural blocks for greenhouse studies. Spraying procedures were conducted in an automated spraying booth (Generation III Research Track Sprayer from Devries Manufacturing; Hollandale, MN) calibrated to deliver fungicide at 193 kPa (28 psi) and 4.8 km/h (3 mph) with a flat TeeJet 8002 nozzle (TeeJet Technologies, Springfield, IL) set 45 cm (18 in) above the samples. Leaves received four passes of the sprayer to ensure a complete foliar coverage and air dried for four hours at 22^{o} C. Then leaves were placed in plastic containers with a layer of wet paper towels to maintain high humidity. A 2 mm agar plug containing hyphal tips of a *S. sclerotiorum* colony was immediately placed on the middle of each detached leaf with the mycelial side in contact with the leaf surface. After sealing the containers, the inoculated leaves were incubated for 36 h in the dark at room temperature (25^{o} C). Lesion diameters were measured in two perpendicular directions after the incubation period. The level of control provided by each fungicide concentration was expressed as percentage of disease suppression from their respective non-protected plants.

Data analyses

Effect of boscalid on ascospores germination and mycelium growth

A completely randomized design (CRD) was used in all experiments that evaluated the sensitivity of isolates to boscalid. Trials within an experiment had three replications per treatment and each trial was conducted twice. Levene's homogeneity of variances test was used to determine whether trials within an experiment could be combined for analysis as well as to see whether experiments could be combined for analysis. In cases where Levene's test showed experiments could not be combined, the least significant different test (P=0.05) was used to determine which experiments could be combined.

Calculation of EC_{50} for ascospores and mycelium

Spore germination and colony growth in absence of fungicide were used as reference points to estimate the percentage suppression caused by each fungicide concentration. Non-linear regression (PROC NLIN of SAS 9.4; SAS Institute, Cary, NC) was used to estimate the concentration that caused 50% suppression of spore germination or mycelial growth (EC_{50}). To further characterize the reaction of the isolates to boscalid, a histogram was produced with eight bins established using quartiles and subdivisions of quartiles, and their respective EC_{50} was used to arbitrarily denominate each isolate as sensitive (S), moderately sensitive (MS), moderately tolerant (MT) or tolerant (T) depending on whether their EC_{50} for ascospores was at or below the first quartile, above the first quartile but below the third quartile, in the lower half of the fourth quartile, or in the upper half of the fourth quartile. A similar approach was used to classify the response of mycelia to azoxystrobin. Isolates evaluated for mycelium sensitivity to boscalid were separated in three groups, isolates collected before boscalid was introduced to the market, isolates collected within 2004 to 2008 after market introduction, and isolates collected in 2014. The median values of these isolates were compared using the Kruskal-Wallis one way analysis of variance to determine whether significant differences existed among them. Likewise, to determine which medians were statistically different the Dwass-Steel-Critchlow-Fligner multiple comparison test at P=0.05 was used.

Dose response study

For greenhouse study, a randomized complete block design (RCBD) with two factors, boscalid and isolates, was used. Trials within one experiment had five replications per treatment and each trial was conducted twice. Levene's homogeneity of variances test was used to determine whether trials could be combined for analysis. If they could not, then each trial was

analyzed separately. Analysis of variance (ANOVA) was conducted using the general linear model procedure (PROC GLM) of SAS (SAS 9.4, SAS, Institute, Cary, NC) to determine if there were significant differences between isolates and the interaction of concentration and isolates. In the absence of a significant effect of isolates ($P \geq 0.05$), the average of all 6 isolates was calculated and the analysis of variance was conducted again with concentrations as the only factor. Means for concentrations were compared to one another using the least significant difference test (L.S.D.) at $P=0.05$ to determine which treatments are statistically different from each other.

Results

Sensitivity of ascospores to boscalid

Four different experiments were conducted to evaluate the toxicity of boscalid to ascospores of *S. sclerotiorum*. Levene's test for homogeneity of variance using isolate WM 31 demonstrated the variances of trials 1 and 2 within each experiment were homogenous ($P=0.1314$ to $P=0.144$) and as a result, trials within experiments were combined for analyses. However, Levene's test also indicated the variances of trials within experiment 17 were not homogenous ($P \leq 0.0001$) and as result, trials in that experiment were analyzed separately. When comparing experiments 14 and 16, Levene's test demonstrated they were homogenous ($P=0.05$). Based on the performance of isolate WM 31, experiments 14 and 16 were combined for analysis. However, experiment 13 does not have isolate WM 31 and any other repeated isolate; therefore, trials for this experiment were analyzed separately and are presented in Table A.4.

The sensitivity of ascospores of 20 *S. sclerotiorum* isolates to boscalid did not follow a normal distribution and rather had two peaks suggesting a bimodal distribution (Figure 12) with a mean EC_{50} value of 8.38 µg/ml (ranging between 3.12 and 14.95 µg/ml) and a median of 8.22

µg/ml. Approximately 25%, 50%, and 10% of all 20 isolates had $EC_{50} \leq 6.79$, between 6.80 and 9.40, and between 9.41 and 12.17 µg/ml, respectively. Each group was considered sensitive, moderately sensitive, and moderately tolerant, respectively. However, three isolates representing 15% of the isolates evaluated had EC_{50} above 12.17 µg/ml and were considered tolerant. The characteristics of the reaction of these isolates to boscalid are presented in Table 19. The state mean EC_{50} for North Dakota was not statistically different to that of other states (Table 20). Six isolates collected before the introduction of boscalid to the market were included in this study and had a mean EC_{50} value of 6.75 µg/ml (ranging from 3.12 to 14.95 µg/ml) with a median of 5.30 µg/ml. These isolates are considered to be true baseline isolates.

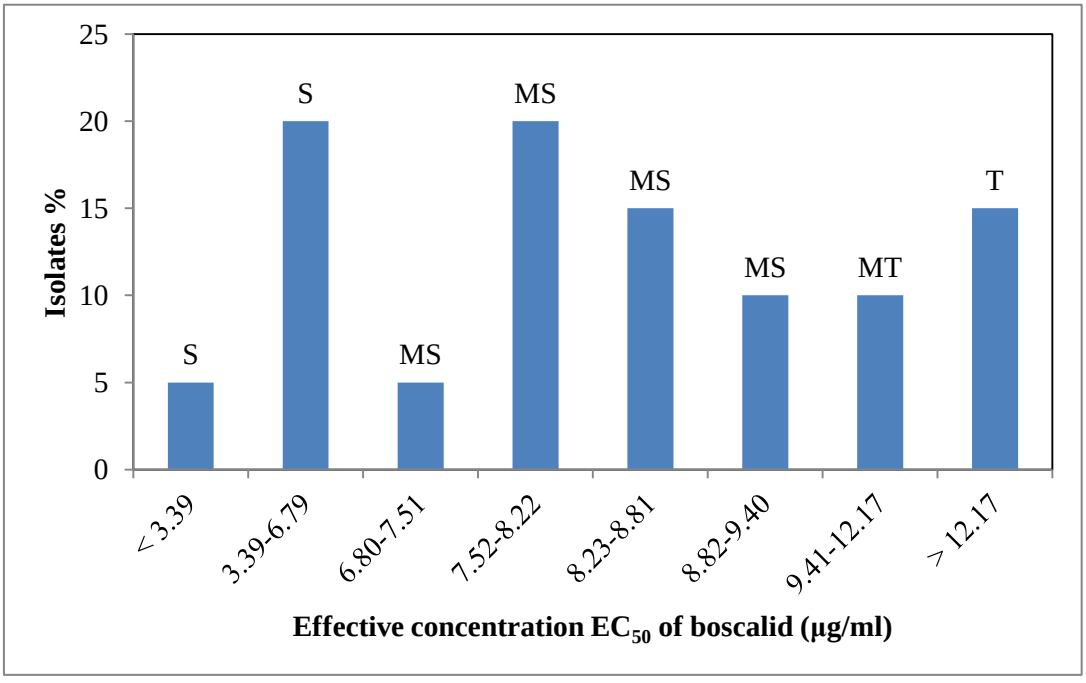

Figure 12. Frequency distribution (%) of the sensitivity of ascospores from 20 *Sclerotinia sclerotiorum* isolates to boscalid and expressed as the 50% effective concentration (EC_{50}) in µg/ml. S=susceptible; MS= moderately susceptible; MT=moderately tolerant; T=tolerant.

Table 19. EC_{50} values for ascospores sensitivity of three *Sclerotinia sclerotiorum* isolates considered tolerant to boscalid.

			Boscalid (EC_{50} in µg/ml)[2]					
Isolate I.D.[1]	**Year of collection**	**State**	**Mean**	**S.D.**	**Min**	**Max**	**Median**	**CV**
363	2008	North Dakota	12.71	3.201	7.60	16.00	13.54	25.19
586	2008	North Dakota	13.70	2.562	10.18	16.00	14.31	18.71
380	2003	Wisconsin	14.95	0.990	13.71	16.00	15.05	6.62

[1] Isolate I.D.=laboratory number.
[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D. = standard deviation; Min=minimum, Max=maximum; CV= coefficient (%) of variation.

Table 20. Sensitivity of ascospores of *Sclerotinia sclerotiorum* isolates from 6 states in North Central U.S. region to boscalid.

		Boscalid (EC_{50} in µg/ml)[1]					
State	**Number of isolates**	**Mean**	**S.D.**	**Min**	**Max**	**Median**	**CV**
Iowa	3	7.84	0.740	7.02	8.46	8.05	9.44
Minnesota	1	9.53	-	9.53	9.53	9.53	-
Montana	1	8.53	-	8.53	8.53	8.53	-
Nebraska	1	8.40	-	8.40	8.40	8.40	-
North Dakota	12	8.05	3.496	3.12	13.70	7.81	43.45
Wisconsin	2	10.52	6.270	6.09	14.95	10.52	59.61
Grand Total[2]	**20**	**8.38**	**3.142**	**3.12**	**14.95**	**8.22**	

[1]Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation; -= not determined.
[2] Grand total means and medians represent 120 observations of 20 isolates which are calculated from 3 replications in each of 2 trials per isolate.

Sensitivity of mycelium to boscalid

Levene's test for homogeneity of variance using isolate WM 31 demonstrated that the variances of trials within each experiment were homogenous (P=0.1697 to P=0.3560) and as a result trials within experiments were combined for analysis. When comparing experiments 13, 14, 16, 18, and 22, Levene's test indicated they were homogenous (P=0.2303). Therefore, based

on the performance of isolate WM 31, all experiments were combined for analysis (see Table A.5. for individual isolate data).

A total of 132 *S. sclerotiorum* isolates were tested for sensitivity of their mycelia to boscalid. The percentage frequency distribution of these isolates had two peaks suggesting a bimodal distribution with a mean EC_{50} value of 0.11 µg/ml (ranging between 0.03 and 0.78 µg/ml) and a median of 0.08 µg/ml. Approximately 25%, 50%, and 23% of all 132 isolates had $EC_{50} \leq 0.072$, between 0.073 and 0.091, and between 0.092 and 0.435 µg/ml, respectively. Each group was arbitrarily denominated as sensitive, moderately sensitive, and moderately tolerant, respectively (Figure 13.). However, 2% of the isolates evaluated had EC_{50} above 0.435 µg/ml and were considered tolerant. The characteristics of the response of these isolates to boscalid are presented in Table 21. The state mean EC_{50} for Iowa, Minnesota, Nebraska, North Dakota and Wisconsin were between 0.73 and 1.38 times numerically greater than the overall mean (Table 22). For both North Dakota and the rest of the other states, the distribution is non-normal.

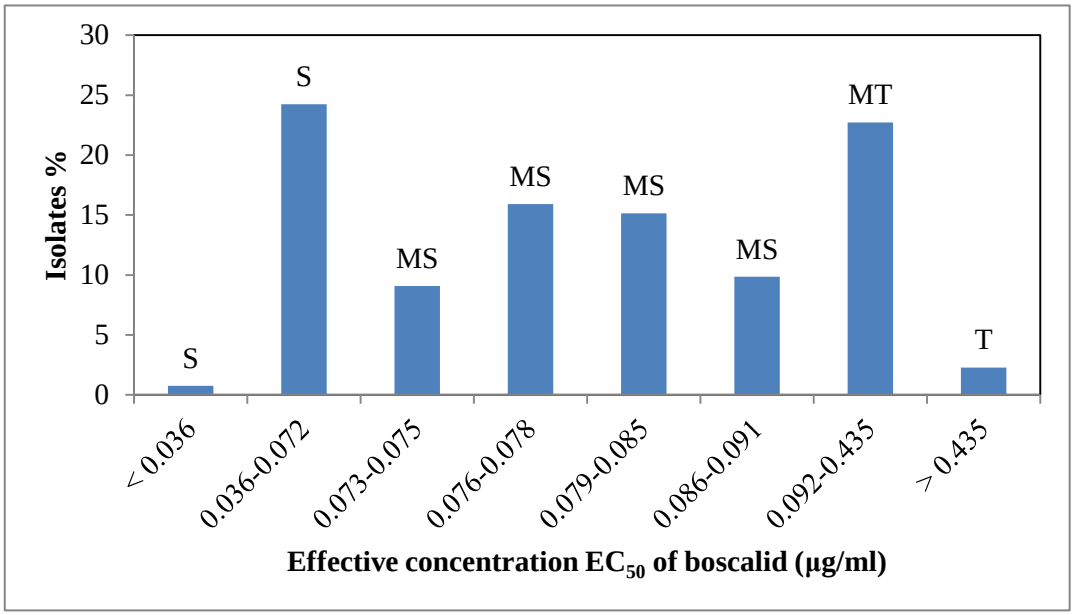

Figure 13. Frequency distribution (%) of the sensitivity of mycelium from 132 *Sclerotinia sclerotiorum* isolates to boscalid and expressed as the 50% effective concentration (EC_{50}) in µg/ml. S=susceptible; MS= moderately susceptible; MT=moderately tolerant; T=tolerant.

Table 21. EC_{50} values for mycelium sensitivity of three *Sclerotinia sclerotiorum* isolates considered tolerant to boscalid.

Isolate I.D.[1]	Year of collection	State	Boscalid (EC_{50} in µg/ml)[2]					
			Mean	S.D.	Min	Max	Median	CV
385	2007	North Dakota	0.46	0.281	0.13	0.84	0.44	60.71
411	2008	Iowa	0.61	0.248	0.30	0.88	0.61	40.49
607	2007	North Dakota	0.78	0.073	0.67	0.87	0.78	9.32

[1] Isolate I.D. =laboratory number.
[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D. = standard deviation; Min=minimum, Max=maximum; CV= coefficient (%) of variation

Table 22. Sensitivity of mycelium of *Sclerotinia sclerotiorum* isolates from 14 states in North Central U.S. region and 1 isolate from Manitoba, Canada to boscalid.

State	Number of isolates	Boscalid (EC_{50} in µg/ml)[1]					
		Mean	S.D.	Min	Max	Median	CV
Colorado	1	0.06	-	0.06	0.06	0.061	-
Illinois	2	0.08	0.01	0.07	0.16	0.082	0.14
Indiana	1	0.08	-	0.08	0.08	0.078	-
Iowa	4	0.25	0.25	0.07	0.99	0.154	1.02
Kansas	1	0.07	-	0.07	0.07	0.067	-
Manitoba	1	0.06	-	0.06	0.06	0.061	-
Michigan	2	0.08	0.00	0.08	0.15	0.076	0.02
Minnesota	10	0.08	0.02	0.06	0.82	0.082	0.22
Missouri	1	0.07	-	0.07	0.07	0.074	-
Montana	1	0.08	-	0.08	0.08	0.077	-
Nebraska	5	0.11	0.08	0.05	0.53	0.075	0.79
North Dakota	95	0.10	0.10	0.03	9.87	0.079	0.93
Ohio	1	0.08	-	0.08	0.08	0.076	-
South Dakota	1	0.10	-	0.10	0.10	0.104	-
Wisconsin	6	0.15	0.11	0.08	0.92	0.115	0.72
Grand Total[2]	**132**	**0.11**	**0.10**	**0.03**	**14.05**	**0.078**	

[1] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.
[2] Grand total means and medians represent 792 observations of 132 isolates which are calculated from 3 replications in each of 2 trials per isolate.

Approximately, 28% of the isolates evaluated were collected before the introduction of boscalid to the market and 61% were collected in the first four years after its introduction. The remaining 11 % were isolates collected ten years after the introduction of the compound to the market. A closer look at these three groups showed that samples collected before 2004 had a non-normal distribution with a mean EC_{50}value of 0.10 µg/ml (ranging between 0.05 and 0.37 µg/ml) and a median of 0.08 µg/ml. Approximately 27%, 49% and 24% of all 37 isolates had $EC_{50} \leq 0.072$, between 0.073 and 0.091, and between 0.092 and 0.435 µg/ml, respectively. However, no isolate evaluated had EC_{50} values above 0.435 µg/ml. Samples collected between 2004 and 2008 had a non-normal distribution with a mean EC_{50}value of 0.12 µg/ml (ranging between 0.03 and 0.78 µg/ml) and a median of 0.08 µg/ml. Approximately 21%, 50% and 25% of all 81 isolates had $EC_{50} \leq 0.072$, between 0.073 and 0.091, and between 0.092 and 0.435 µg/ml, respectively. However, 4% of isolates had EC_{50} above 0.435 µg/ml and were considered tolerant. On the other hand, the samples collected in 2014 generally leaned towards sensitivity with a mean EC_{50}value of 0.07 µg/ml (ranging between 0.05 and 0.13 µg/ml) and a median of 0.07 µg/ml. Approximately 43%, 50% and 7% of all 14 isolates had $EC_{50} \leq 0.072$, between 0.073 and 0.091, and between 0.092 and 0.435 µg/ml, respectively. Nevertheless, no isolate had EC_{50} above 0.435 µg/ml (Figure 14).

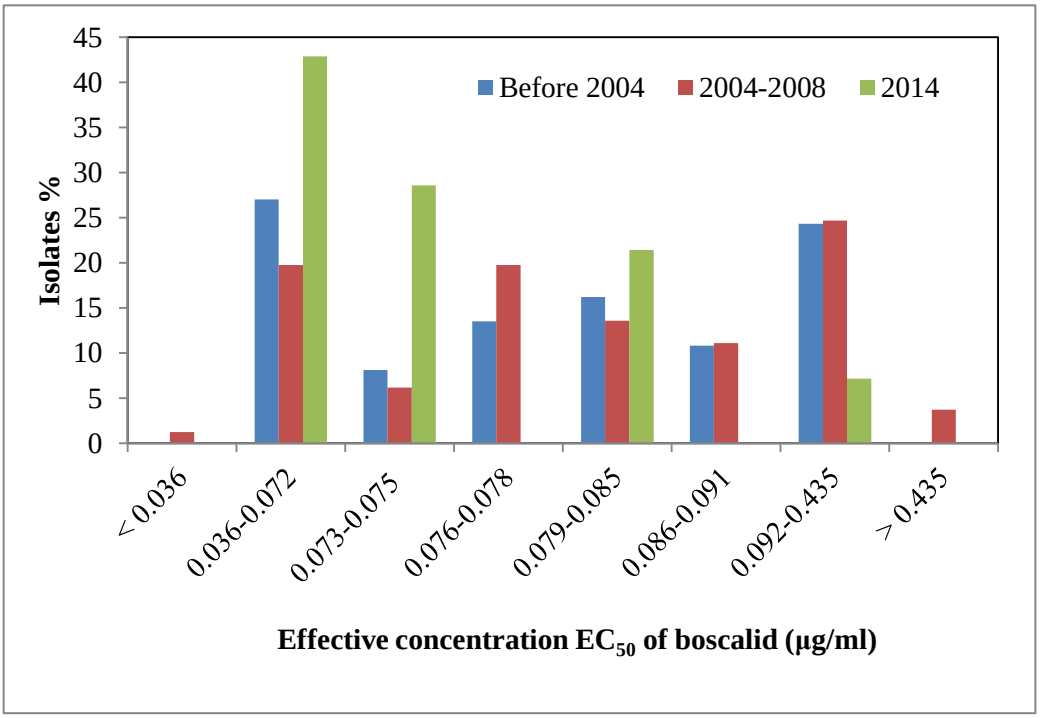

Figure 14. Frequency distribution (%) of the sensitivity of mycelium of 37, 81, and 14 *Sclerotinia sclerotiorum* isolates collected before 2004, between 2004 to 2008, and 2014, respectively, to boscalid. Sensitivity expressed as the 50% effective concentration (EC_{50}) in µg/ml.

Based on Kruskal-Wallis test (P= 0.0318), we rejected the null hypothesis and accepted the alternative hypothesis which is that at least the median of one group is different from the median of at least one other group. Therefore, there is a significant change in sensitivity between time groups (Table 23). Based on Dwass-Steel-Critchlow-Fligner multiple comparison test, isolates before 2004 and between 2004 and 2008 are statiscally similar to each other. However, recent isolates from 2014 are statiscally different (P<0.0475) from the first two periods of time.

Table 23. Median EC_{50} for mycelium isolates of *Sclerotinia sclerotiorum* across time period relative to the debut of boscalid in 2004.

Time	Number of isolates	Boscalid (EC_{50} in µg/ml)[1]						
		Mean	Median	S.D.	Min	Max	CV	Pr>DSCF
Before 2004	37	0.10	0.08	0.064	0.05	0.37	65.55	0.9922
2004-2008	81	0.12	0.08	0.117	0.03	0.78	100.75	0.0475
2014	14	0.07	0.07	0.020	0.05	0.13	27.52	0.0289

[1]Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D. = standard deviation; Min=minimum, Max=maximum; CV= coefficient (%) of variation.

Dose response study

Based on non fungicide treated leaves, Levene's test for homogeneity of variance demonstrated that the variances of the 2 trials within one experiment were homogenous (*P*=0.0658) and as a result the trials could be analyzed combined. Based on that, an LSD test on the mean of the isolates (LSD=4.21; *P*=0.05) separated them in four groups, one with isolates 3, 5, and 6; the second group with isolates 2 and 5; the third with isolates 1 and 2; and the last group by 1, and 4 (Table 20). Besides the differences in the isolate aggressiveness, the method used to evaluate the efficacy of boscalid is still valid, because each concentration was compared in the absence of the fungicide per each isolate.

One experiment was conducted to evaluate the greenhouse trials. Levene's test for homogeneity of variance demonstrated that the variances of the 2 trials within one experiment were not homogenous (*P*=0.0241) and as a result the trials were analyzed separately. Analysis of variance (ANOVA) for trial 1 and 2 indicate that there was no significant difference between isolates (*P*=0.3670 and *P*=0.1067) and between the interaction of concentration and isolates (*P*=0.9567 and *P*=0.2613), respectively. However, there was a significant difference between concentrations for both trials (*P*<.0001). Therefore, the average of all six isolates per concentration and replication was calculated and the data reanalyzed. Based on the results of the

ANOVA, an LSD test on the mean of the treatments was conducted. The LSD test (LSD=9.94; *P*=0.05) indicated that, for trial 1, concentrations 8 ppm and 4 pppm and 1 ppm and 0.1 ppm are statistically similar to each other. Whereas, for trial 2, the LSD test on the means of concentrations (LSD=7.07; *P*= 0.05) indicates that 8 ppm and 4 ppm are statistically similar to each other. However, the means for the other concentrations were statistically different from each other (Figure 15). This study also demonstrated that isolates with different levels of sensitivity observed in the laboratory trials, moderately sensitive, moderately tolerant and tolerant, were equally controlled by boscalid. Selected isolates caused similar lesion lengths regardless of the isolate sensitivity. Therefore, boscalid is still effective in controlling the disease caused by moderately sensitive or tolerant isolates.

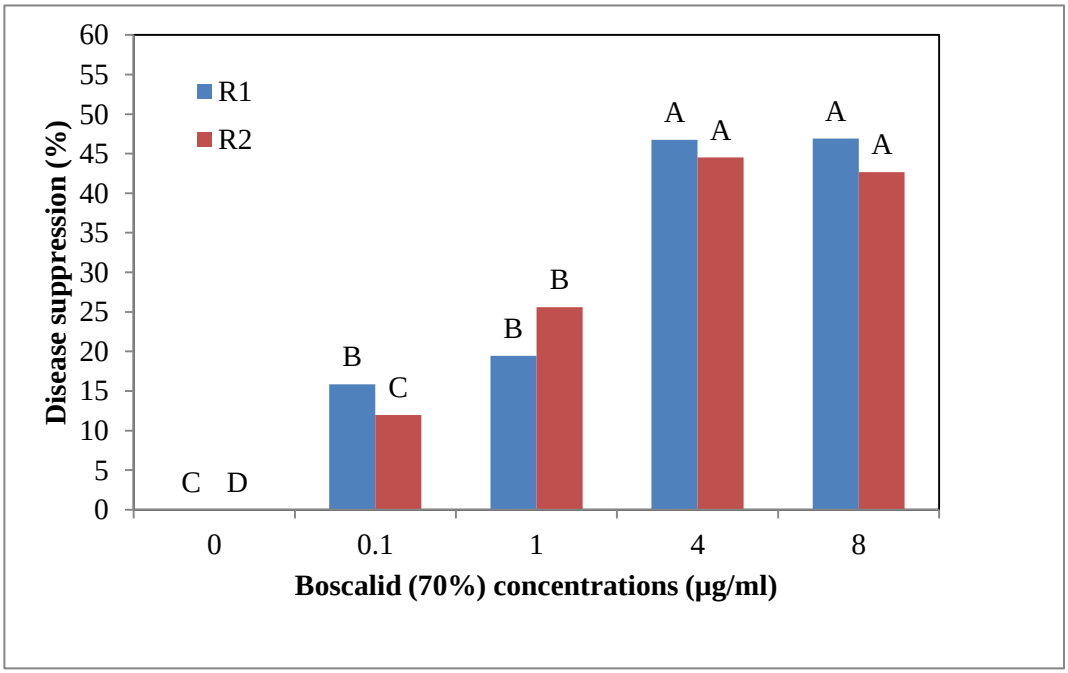

Figure 15. Dose response curve of boscalid for control of Sclerotinia stem rot of canola in greenhouse detached leaf assays. R1=trial 1; R2=trial 2. Means with similar letters on columns of the same color are statistically similar to each other at *P*=0.05.

Discussion

This is the first report of ascospore sensitivity to boscalid in *S. sclerotiorum*. The values estimated were not in the range of sensitivity obtained by other researchers for other species. For example, in New Zeland, conidia of *Spilocaea oleagina* baseline isolates had a mean EC_{50} value of 0.031 µg/ml ranging from 0.005 to 0.5 µg/ml (Obanor et al., 2005). Also, in China 228 baseline isolates of *B. cinerea* had a mean EC_{50} for spores of 0.42 µg/ml ranging from 0.02 to 1.68 µg/ml (Zhang et al., 2007). Another study assessed two sets of isolates of conidia of *A. alternata*. The first set evaluated 49 baseline isolates and found a mean EC_{50} value of 1.515 µg/ml ranging from 0.089 to 3.435 µg/ml. Whereas, the second set evaluated 59 isolates exposed to boscalid for two consecutive years at a rate of two sprays per season. These isolates had a mean EC_{50} value of 1.214 µg/ml ranging from 0.055 to 4.222 µg/ml (Avenot and Michailides, 2007). The baseline sensitivity of conidia of 71 *A. rabiei* isolates had a mean EC_{50} of 0.1903 µg/ml ranging from 0.0177 to 0.4960 µg/ml (Wise et al., 2008); and conidia of 137 *B. cinerea* baseline isolates ranged from 0.01 to 0.021 µg/ml with a mean EC value of 0.06 µg/ml (Stammler and Speakman, 2006). Even though few isolates were tested in our study, *S. sclerotiorum* ascospores from north central U.S. seem to be 3 to 218 times more tolerant to boscalid than to species listed above. These wide differences in reponse to boscalid could be species-related and geographic origin.

The sensitivity of mycelium of *S. sclerotiorum* to boscalid has increased significantly since its introduction to the market. We could speculate that the difference in sensitivity between years may have been caused by indirect exposure to other SDHI fungicides that were used in U.S. agricultural practices before boscalid was introduced to the market such as carboxin (Mc Mullen and Markell, 2009). We also speculate that the application of other SDHI fungicides

previously may cause indirect selection pressure and as a result organisms could develop some tolerance to boscalid. This would be more feasible with fungicides that interefere with different complex sites of the mitochondrial respiration such as pyrimidin-amines, pyrazole-MET1, QoI, and Qil (Friskop et al., 2014). While cross-resistance between compounds from the same chemical group exist (Kim et al., 2003; Sierotzki et al., 2000), cross resistance between chemicals from different groups has not been proven. Pasche and collaborators (2005) found a negative cross-sensitivity between boscalid and QoI fungicides such as azoxystrobin, famoxamode, and fenamidone in *A. solani*. However, this is still unkown in *S.sclerotiorum*. The bimodal nature of the distributions observed on isolates collected before 2004 and between 2004 and 2008 could be explained by a number of factors; among them, the sampling procedures used to collect them, their geographic origin, as well as potential differences in indirect selection pressure produced by application of other SDHI fungicides as well as differences in the number of isolates from each state. In many cases states were represented by less than four isolates while others, for example North Dakota were represented by 9-23 times more isolates. In contrast, isolates collected in 2014 showed a distribution leaning towards sensitivity. All isolates in this group were collected from North Dakota. It is safe to assume that the latter were more representative of the populations that one would expect to find when evaluating the sensitivity of isolates to a fungicide they have limited exposure to.

Few studies in the literature show a baseline sensitivity of mycelium of *S. sclerotiorum* to boscalid because this is a relatively new chemical compound used to control stem rot. The characterization of sensitivity of mycelium of this organism to SDHI is more informative because SDHI fungicides mainly affect the mycelium growth (Fellman and Tourneau, 1983). For example, in China two studies were conducted prior the legal use of boscalid in the market. In

the first study, 120 isolates of *S. sclerotiorum* collected from oilseed rape fields in the province of Jiangsu had a mean EC_{50} of 0.17 μg/ml with a range from 0.028 to 0.398 μg/ml for mycelium growth (Wang et al., 2009). Whereas in the second study, the baseline sensitivity of mycelium of 161 *S. sclerotiorum* isolates had a mean EC_{50} value of 0.042 μg/ml ranging from 0.002 to 0.391 μg/ml (Liu et al., 2009). In this study we showed that 37 *S. sclerotiorum* baseline isolates collected from North central U.S. had EC_{50} ranging from 0.05 to 0.37 μg/ml, with a mean of 0.10 μg/ml and a median value of 0.08 μg/ml. Despite, the few isolates evaluated with boscalid, the sensitivity of mycelium of baseline *S. sclerotiorum* isolates from north central U.S. ranged from 0.59 to 2.38 times greater than the research data reported in China. We could speculate that the difference in EC_{50} values is because of the difference in the genetic population as a result of a different geographic distribution (Attanayake et al., 2013).

The greenhouse study showed that the tolerance observed *in vitro* did not translate into loss of control *in planta*. Therefore, boscalid is still an effective tool to manage stem rot on canola. As a matter of fact, all concentrations evaluated significantly reduced disease severity when compared to the non-protected controls and no differences between isolates were detected even at the lowest concentrations. Moreover, there was no significant change in disease control when the boscalid concentration was increased from 4 to 8 μg/ml, for both trials. However, there was a significant difference between 0.1 and 1 μg/ml, only for trial 2 but not for trial 1. Based on these results, it is recommended that growers rotate fungicides with different modes of action to reduce the possibility that resistance develops in the region. The use of fungicides mixes could be another good alternative approach to reduce the risk of development of resistance.

The establishment of the baseline sensitivity to boscalid will be a valuable reference point for future studies that look for changes in sensitivity of *S. sclerotiorum* to this compound. The

evidence provided in this study indicates that no resistant isolates were found; however, one should not lose sight of the potential of this fungicide to promote development of resistance in its target population. It is recommended that periodic surveys are conducted to monitor the sensitivity of *S. sclerotiorum* populations in the region to boscalid.

As a conclusion, the sensitivity of isolates to boscalid has changed significantly in the ten years after its introduction to the market. Only four isolates had a mycelium $EC_{50} > 0.435$ μg/ml and could be considered tolerant. In greenhouse experiments, it was found that tolerant isolates *in vitro* are not tolerant *in planta* and boscalid is still a good source of control for Sclerotinia stem rot disease.

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APPENDIX

Table A.1. Six experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* ascospores germination to azoxystrobin alone.

				Azoxystrobin EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min**	**Max**	**CV**
2,6,15,16	31	2004*	Nebraska	0.35	0.314	0.19	0.09	1.20	88.67
	175	2004	North Dakota	0.48	0.353	0.39	0.16	0.92	73.39
	192	2005	North Dakota	0.21	0.055	0.21	0.15	0.28	25.71
	383	2008	Kansas	0.95	0.606	0.67	0.46	1.94	63.76
	386	2008	North Dakota	0.52	0.217	0.51	0.30	0.83	41.45
	393	2008	Minnesota	0.39	0.209	0.28	0.22	0.68	52.92
	395	2008	Minnesota	0.57	0.12	0.58	0.42	0.77	20.92
	415	2008	Michigan	0.68	0.329	0.56	0.51	1.35	48.30
	418	2003	Wisconsin	0.13	0.039	0.13	0.09	0.19	30.23
	420	2002	North Dakota	0.18	0.028	0.17	0.15	0.22	15.14
	596	2008	Nebraska	0.42	0.226	0.41	0.19	0.72	53.49
	605	2007	Montana	0.55	0.226	0.48	0.27	0.84	41.11
	606	2007	North Dakota	3.16	1.812	3.36	0.90	5.18	57.37
13,14	31	2004*	Nebraska	3.70	1.822	3.85	0.75	6.05	49.27
	68	1994	North Dakota	1.37	0.938	1.13	0.46	3.6	68.27
	73	1996	North Dakota	3.15	2.636	3.24	0.59	5.66	83.59
	107	2002	North Dakota	2.31	1.025	2.16	1.05	3.90	44.35
	128	2002	North Dakota	5.06	3.455	4.73	1.20	10.8	68.35
	137	2002	North Dakota	6.72	0.685	6.72	6.23	7.20	10.21
	138	2002	North Dakota	2.39	2.121	1.89	0.41	6.12	88.69
	139	2002	North Dakota	2.23	0.874	2.40	1.15	3.20	39.28
	184	2004	North Dakota	6.27	0.738	6.24	5.40	7.20	11.77

Table A.1. Six experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* ascospores germination to azoxystrobin alone (continued).

				Azoxystrobin EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min**	**Max**	**CV**
	189	2004	North Dakota	8.01	3.186	7.37	4.50	12.96	39.79
	356	2008	North Dakota	2.84	1.346	2.77	1.20	4.58	47.33
	362	2008	North Dakota	5.27	1.135	5.40	4.00	6.69	21.54
	363	2008	North Dakota	6.26	0.478	6.30	5.66	6.98	7.63
	376	2008	Iowa	5.66	-	5.66	5.66	5.66	-
	381	2004	Missouri	4.12	3.066	5.7	0.59	6.07	74.42
	385	2007	North Dakota	≥ 28	-	-	-	-	-
	388	2008	North Dakota	5.10	1.181	5.34	3.60	6.17	23.18
	392	2008	North Dakota	1.01	0.45	0.96	0.58	1.80	44.64
	406	2008	North Dakota	5.90	0.386	5.90	5.63	6.17	6.55
	412	2008	Iowa	5.39	1.061	5.14	4.32	6.69	19.69
	423	2007	North Dakota	3.00	1.623	2.70	1.09	5.40	54.18
	591	2008	North Dakota	7.46	4.367	8.28	1.20	12.6	58.53
	592	2008	North Dakota	4.53	1.965	4.14	2.64	7.20	43.39

[1] Isolate I.D. = laboratory number;*=Previous 2004.

[2] Mean and median values represent observations which are calculated from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.

Table A.2. Six experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* ascospores germination to azoxystrobin combined with 70 µg/ml of salicylhydroxamic acid (SHAM).

				Azoxystrobin EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min.**	**Max.**	**CV**
2,3,4,5,12	31	2004*	Nebraska	0.09	0.026	0.08	0.05	0.15	30.22
	43	1982	North Dakota	0.09	0.010	0.09	0.07	0.10	11.44
	62	1993	North Dakota	0.08	0.013	0.08	0.07	0.11	15.89
	72	1996	North Dakota	0.09	0.016	0.09	0.07	0.11	17.26
	78	1996	North Dakota	0.12	0.014	0.13	0.10	0.14	11.21
	84	2001	North Dakota	0.13	0.013	0.13	0.11	0.15	9.79
	86	2001	North Dakota	0.14	0.007	0.14	0.13	0.15	4.55
	94	2001	North Dakota	0.10	0.046	0.11	0.03	0.15	43.84
	105	2002	North Dakota	0.09	0.018	0.09	0.08	0.11	19.19
	107	2002	North Dakota	0.08	0.015	0.07	0.07	0.11	18.71
	113	2002	North Dakota	0.09	0.016	0.08	0.07	0.11	18.56
	118	2002	North Dakota	0.09	0.019	0.09	0.07	0.12	20.63
	119	2002	North Dakota	0.10	0.018	0.11	0.08	0.12	17.80
	137	2002	North Dakota	0.17	0.027	0.17	0.14	0.21	15.61
	192	2005	North Dakota	0.03	0.001	0.03	0.03	0.03	3.73
	357	2008	North Dakota	0.13	0.003	0.13	0.12	0.13	2.59
	358	2008	North Dakota	0.14	0.095	0.13	0.03	0.23	69.43
	361	2008	North Dakota	0.09	0.025	0.09	0.07	0.12	27.06
	363	2008	North Dakota	0.21	0.004	0.21	0.21	0.22	1.98
	364	2008	Minnesota	0.10	0.020	0.10	0.09	0.13	18.71
	367	2008	North Dakota	0.12	0.070	0.11	0.05	0.19	59.81
	382	2008	North Dakota	0.08	0.010	0.08	0.07	0.09	12.38
	391	2008	North Dakota	0.09	0.019	0.09	0.07	0.11	22.01

Table A.2. Six experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* ascospores germination to azoxystrobin combined with 70 µg/ml of salicylhydroxamic acid (SHAM) (continued).

				Azoxystrobin EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min.**	**Max.**	**CV**
	396	2008	Minnesota	0.11	0.030	0.12	0.07	0.18	26.49
	410	1998	Illinois	0.07	0.005	0.07	0.07	0.08	6.99
	420	2002	North Dakota	0.07	0.026	0.07	0.03	0.10	39.91
	421	2008	North Dakota	0.08	0.009	0.08	0.07	0.10	11.20
	580	2008	North Dakota	0.09	0.019	0.09	0.08	0.13	20.78
13	31	2004*	Nebraska	0.12	0.007	0.13	0.11	0.13	5.67
	73	1996	North Dakota	0.13	0.013	0.13	0.12	0.16	9.65
	184	2004	North Dakota	0.15	0.003	0.15	0.14	0.15	1.83
	362	2008	North Dakota	0.13	0.006	0.13	0.13	0.14	4.81
	376	2008	Iowa	0.14	0.012	0.14	0.13	0.16	8.69
	381	2004	Missouri	0.16	0.039	0.15	0.13	0.21	24.16
	385	2007	North Dakota	0.13	0.012	0.13	0.11	0.14	8.83
	406	2008	North Dakota	0.13	0.006	0.13	0.13	0.14	4.30
	412	2008	Iowa	0.13	0.006	0.13	0.12	0.13	4.72
	423	2007	North Dakota	0.14	0.02	0.14	0.12	0.18	14.29
	592	2008	North Dakota	0.12	0.017	0.13	0.10	0.14	13.48

[1]Isolate I.D.=laboratory number; *=Previous 2004.

[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.

Table A.3. Five experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* mycelium growth to azoxystrobin.

				Azoxystrobin EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min**	**Max**	**CV**
11	31	2004*	Nebraska	0.78	0.420	0.54	0.51	1.50	53.61
	43	1982	North Dakota	0.37	0.042	0.37	0.32	0.44	11.46
	68	1994	North Dakota	0.42	0.056	0.44	0.33	0.48	13.47
	78	1996	North Dakota	0.39	0.080	0.36	0.31	0.51	20.66
	85	2001	North Dakota	0.37	0.092	0.33	0.30	0.52	24.47
	89	2001	North Dakota	2.04	3.766	0.51	0.33	9.72	184.79
	113	2002	North Dakota	0.50	0.194	0.43	0.33	0.81	39.18
	175	2004	North Dakota	0.44	0.059	0.42	0.36	0.54	13.50
	184	2004	North Dakota	0.45	0.156	0.38	0.31	0.66	34.84
	356	2008	North Dakota	0.62	0.234	0.55	0.47	1.09	37.52
	361	2008	North Dakota	0.39	0.063	0.41	0.30	0.46	16.12
	362	2008	North Dakota	0.35	0.099	0.30	0.30	0.55	28.34
	363	2008	North Dakota	0.44	0.046	0.45	0.38	0.50	10.46
	366	2008	North Dakota	0.43	0.053	0.43	0.36	0.51	12.54
	367	2008	North Dakota	0.38	0.046	0.38	0.31	0.44	12.05
	369	2008	North Dakota	0.77	0.400	0.67	0.38	1.47	51.79
	377	2008	Iowa	0.40	0.073	0.39	0.30	0.50	18.48
	387	2008	North Dakota	0.35	0.064	0.33	0.30	0.47	18.10
	392	2008	North Dakota	0.43	0.091	0.48	0.30	0.51	21.02
	394	2008	Minnesota	0.32	0.032	0.30	0.30	0.38	9.97
	401	2008	North Dakota	3.13	3.497	2.23	0.54	9.82	111.59
	409	2008	Indiana	0.47	0.139	0.45	0.30	0.67	29.49
	579	2008	North Dakota	0.36	0.038	0.36	0.30	0.42	10.60
	583	2008	Minnesota	0.47	0.136	0.44	0.30	0.71	29.05
	587	2008	Nebraska	0.48	0.091	0.49	0.37	0.59	18.76

Table A.3. Five experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* mycelium growth to azoxystrobin (continued).

				Azoxystrobin EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min**	**Max**	**CV**
	590	2008	North Dakota	0.41	0.062	0.42	0.32	0.49	15.10
	591	2008	North Dakota	0.58	0.294	0.48	0.34	1.15	50.53
	592	2008	North Dakota	0.35	0.065	0.32	0.30	0.45	18.55
	595	2008	North Dakota	0.34	0.058	0.32	0.30	0.46	17.12
15,17,19,23	31	2004*	Nebraska	0.62	0.146	0.57	0.40	0.90	23.69
	35	1980	North Dakota	0.74	0.400	0.59	0.53	1.55	54.22
	36	1980	North Dakota	0.91	0.578	0.57	0.47	1.71	63.47
	37	1980	North Dakota	0.49	0.049	0.49	0.44	0.56	9.94
	39	1981	North Dakota	0.49	0.053	0.48	0.44	0.56	10.81
	40	1982	North Dakota	0.52	0.018	0.51	0.50	0.54	3.52
	41	1982	North Dakota	0.60	0.109	0.56	0.54	0.82	18.21
	46	1983	North Dakota	0.52	0.060	0.52	0.43	0.59	11.62
	47	1983	North Dakota	0.50	0.075	0.49	0.43	0.64	14.98
	49	1983	North Dakota	1.40	1.092	0.98	0.56	3.36	78.03
	50	1983	North Dakota	0.61	0.124	0.58	0.49	0.85	20.39
	51	1983	North Dakota	0.96	0.577	0.60	0.57	1.80	60.21
	53	1983	North Dakota	0.55	0.120	0.53	0.43	0.75	21.88
	54	1983	North Dakota	2.29	4.130	0.65	0.44	10.72	180.10
	55	1983	North Dakota	0.87	0.516	0.68	0.55	1.89	59.38
	56	1983	North Dakota	0.82	0.482	0.72	0.42	1.63	58.73
	58	1984	North Dakota	5.17	4.451	3.28	0.54	11.10	86.06
	358	2008	North Dakota	0.78	0.287	0.64	0.56	1.16	36.67
	359	2008	North Dakota	1.53	1.479	0.65	0.51	3.74	96.88
	360	2008	North Dakota	0.45	0.042	0.44	0.41	0.52	9.37
	365	**	Nebraska	0.42	0.079	0.43	0.3	0.54	18.88
	368	2008	North Dakota	0.92	1.077	0.49	0.31	3.09	117.20

Table A.3. Five experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* mycelium growth to azoxystrobin (continued).

Experiments	Isolate I.D.[1]	Year of collection[2]	State	Azoxystrobin EC_{50}(µg/ml)[3] Mean	S.D.	Median	Min	Max	CV
	371	2008	North Dakota	0.84	0.176	0.82	0.58	1.05	20.91
	372	2008	North Dakota	0.39	0.077	0.39	0.30	0.51	19.73
	373	2008	North Dakota	3.84	4.116	2.88	0.53	11.87	107.17
	375	2008	North Dakota	1.42	0.851	1.02	0.79	3.00	59.71
	378	2000	Wisconsin	4.23	3.226	4.29	0.57	9.90	76.24
	379	2000	Wisconsin	3.08	5.505	0.70	0.41	12.92	178.78
	380	2003	Wisconsin	0.67	0.392	0.55	0.30	1.39	58.17
	382	2008	North Dakota	0.44	0.061	0.43	0.35	0.52	13.92
	383	2008	Kansas	2.32	1.617	1.92	0.53	4.20	69.72
	385	2007	North Dakota	0.45	0.027	0.45	0.41	0.49	5.92
	388	2008	North Dakota	0.73	0.288	0.60	0.54	1.30	39.54
	389	2008	North Dakota	1.40	0.695	1.41	0.60	2.23	49.69
	390	2008	North Dakota	0.49	0.024	0.49	0.46	0.52	4.94
	393	2008	Minnesota	0.84	0.155	0.85	0.57	1.03	18.49
	396	2008	Minnesota	1.13	0.419	1.12	0.49	1.80	37.14
	397	2008	Minnesota	2.22	-	2.22	2.22	2.22	-
	398	2008	Minnesota	>9.6	-	-	-	-	-
	399	2008	North Dakota	0.41	0.041	0.41	0.37	0.48	10.00
	400	2008	North Dakota	1.08	0.491	0.90	0.52	1.80	45.46
	402	2008	North Dakota	0.54	0.032	0.54	0.50	0.59	5.87
	403	2008	North Dakota	0.69	0.224	0.64	0.51	1.13	32.21
	404	2008	North Dakota	0.57	0.172	0.53	0.43	0.90	30.16
	405	2008	North Dakota	0.43	0.031	0.43	0.39	0.46	7.32
	406	2008	North Dakota	0.46	0.065	0.44	0.39	0.57	14.10
	408	2008	Minnesota	1.33	1.240	0.87	0.53	3.82	93.03
	410	1998	Illinois	5.38	7.820	1.20	0.54	14.4	145.38

Table A.3. Five experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* mycelium growth to azoxystrobin (continued).

Experiments	Isolate I.D.[1]	Year of collection[2]	State	Azoxystrobin EC_{50}(µg/ml)[3]					
				Mean	S.D.	Median	Min	Max	CV
	411	2008	Iowa	1.75	0.802	1.64	0.95	3.05	45.91
	412	2008	Iowa	3.20	1.603	3.58	0.60	4.80	50.11
	413	2008	Iowa	0.51	0.098	0.54	0.34	0.60	18.99
	414	2008	Michigan	1.54	0.854	1.40	0.58	2.80	55.56
	416	2000	Wisconsin	0.52	0.027	0.52	0.49	0.57	5.27
	418	2003	Wisconsin	0.44	0.07	0.43	0.33	0.55	16.04
	419	2002	North Dakota	0.47	0.052	0.47	0.40	0.53	11.13
	421	2008	North Dakota	0.35	0.068	0.32	0.30	0.47	19.59
	422	**	North Dakota	0.76	0.270	0.65	0.59	1.29	35.69
	578	2008	North Dakota	0.62	0.166	0.57	0.53	0.96	26.53
	580	2008	North Dakota	1.82	1.342	1.75	0.55	3.47	73.82
	581	2008	North Dakota	0.65	0.178	0.57	0.52	0.98	27.42
	585	2008	Illinois	0.57	0.095	0.55	0.48	0.75	16.63
	588	2008	North Dakota	0.57	0.193	0.54	0.38	0.94	33.68
	596	2008	Nebraska	>9.6	-	-	-	-	-
	597	2006	Colorado	2.28	1.197	2.57	0.60	3.40	52.55
	598	2008	Nebraska	5.57	6.35	1.58	0.47	14.4	113.94
	599	2008	South Dakota	0.36	0.051	0.36	0.30	0.44	14.21
	600	2006	Minnesota	0.72	0.622	0.44	0.34	1.95	86.28
	601	2002	Ohio	>9.6	-	-	-	-	-
	602	2000	Wisconsin	0.54	0.036	0.56	0.50	0.58	6.64
	603	2003	Wisconsin	3.56	5.300	1.15	0.50	12.91	148.98
	604	2008	North Dakota	0.48	0.065	0.46	0.41	0.60	13.47
	605	2007	Montana	0.44	0.091	0.45	0.30	0.55	20.70
	606	**	North Dakota	0.70	0.205	0.59	0.51	1.00	29.29
	607	2007	North Dakota	1.36	1.059	1.17	0.39	2.90	78.07

Table A.3. Five experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* mycelium growth to azoxystrobin (continued).

Experiments	Isolate I.D.[1]	Year of collection[2]	State	Azoxystrobin EC_{50}(µg/ml)[3]					
				Mean	S.D.	Median	Min	Max	CV
	712	2014	North Dakota	0.41	0.066	0.42	0.33	0.48	15.97
	713	2014	North Dakota	0.49	0.038	0.50	0.42	0.52	7.84
	714	2014	North Dakota	0.79	0.163	0.77	0.60	0.99	20.69
	715	2014	North Dakota	0.42	0.067	0.44	0.30	0.49	16.05
	716	2014	North Dakota	0.66	0.183	0.59	0.56	1.04	27.52
	718	2014	North Dakota	0.46	0.011	0.46	0.45	0.48	2.39
	722	2014	North Dakota	0.48	0.034	0.47	0.43	0.51	7.05
	724	2014	North Dakota	2.91	3.814	1.35	0.93	10.63	131.28
	727	2014	North Dakota	0.51	0.026	0.51	0.48	0.56	5.15
	730	2014	North Dakota	0.47	0.037	0.48	0.42	0.51	7.81
	731	2014	North Dakota	0.46	0.028	0.46	0.44	0.51	6.01
	743	2014	North Dakota	0.62	0.084	0.58	0.56	0.77	13.58
	792	2014	North Dakota	0.88	0.210	0.87	0.58	1.20	23.78
	846	2014	North Dakota	0.51	0.086	0.54	0.38	0.59	16.78

[1] Isolate I.D.=laboratory number; *=Previous 2004; **=unknown year of collection

[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV= coefficient (%) of variation.

Table A.4. Four experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* ascopores germination to boscalid.

				Boscalid EC_{50}(µg/ml)[3]					
Experiments	**Isolate I.D.[1]**	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min**	**Max**	**CV**
13	31-R1	2004*	Nebraska	7.72	1.985	6.75	6.40	10.00	25.73
	31-R2			-	-	-	-	-	-
	363-R1	2008	North Dakota	14.65	1.249	14.40	13.54	16.00	8.53
	363-R2			9.80	3.111	9.80	7.60	12.00	31.75
	376-R1	2008	Iowa	11.00	1.010	11.00	10.29	11.71	9.18
	376-R2			4.37	2.625	2.89	2.82	7.40	60.07
	385-R1	2007	North Dakota	12.36	0.514	12.36	12.00	12.73	4.16
	385-R2			11.23	6.750	11.23	6.45	16.00	60.12
	388-R1	2008	North Dakota	6.97	1.935	6.00	5.71	9.20	27.76
	388-R2			8.15	7.084	6.22	2.23	16.00	86.90
	412-R1	2008	Iowa	10.37	1.175	10.37	9.54	11.20	11.33
	412-R2			7.18	0.501	7.00	6.80	7.75	6.97
	423-R1	2007	North Dakota	10.41	0.806	10.00	9.88	11.33	7.75
	423-R2			4.78	2.792	3.38	2.98	8.00	58.36
17	31-R1	2004*	Nebraska	12.18	2.314	12.18	10.55	13.82	19.00
	31-R2			10.30	0.416	10.50	9.82	10.57	4.04
	374-R1	2008	North Dakota	13.42	2.671	13.60	10.67	16.00	19.90
	374-R2			4.64	1.248	5.00	3.25	5.67	26.91
	380-R1	2003	Wisconsin	14.67	-	14.67	14.67	14.67	-
	380-R2			15.05	1.190	15.43	13.71	16.00	7.91
	413-R1	2008	Iowa	4.01	0.714	3.80	3.42	4.80	17.83
	413-R2			12.10	2.007	12.29	10.00	14.00	16.59
	418-R1	2003	Wisconsin	3.60	1.129	3.42	2.56	4.80	31.39
	418-R2			8.58	3.849	6.73	6.00	13.00	44.88

Table A.4. Four experiments that evaluated the sensitivity of *Sclerotinia sclerotiorum* ascopores germination to boscalid (continued).

Experiments	Isolate I.D.[1]	Year of collection[2]	State	Boscalid EC_{50}(µg/ml)[3]					
				Mean	S.D.	Median	Min	Max	CV
	586-R1	2008	North Dakota	12.93	2.513	13.50	10.18	15.11	19.44
	586-R2			16.00	-	16.00	16.00	16.00	-
14,16	31	2004*	Nebraska	7.29	3.204	6.90	3.44	14.67	43.93
	68	1994	North Dakota	3.12	1.291	2.69	1.64	5.00	41.38
	128	2002	North Dakota	4.52	2.477	3.36	2.95	9.33	54.84
	139	2002	North Dakota	3.40	0.940	3.35	2.41	4.67	27.66
	392	2008	North Dakota	5.75	1.708	5.50	4.00	8.00	29.70
	395	2008	Minnesota	9.53	3.83	8.57	4.96	15.43	40.17
	591	2008	North Dakota	8.03	5.164	7.49	3.00	14.67	64.30
	605	2007	Montana	8.53	3.331	7.31	6.17	13.33	39.07
	606	2007	North Dakota	9.35	2.267	9.35	6.10	12.00	24.25

[1] Isolate I.D.=laboratory number; R1=trial 1; R2=trial 2; *=Previous 2004.

[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.

Table A.5. Sensitivity of *Sclerotinia sclerotiorum* mycelium growth to boscalid.

			Boscalid EC_{50}(µg/ml)[3]					
Isolate I.D.[1]	**Year of collection[2]**	**State**	**Mean**	**S.D.**	**Median**	**Min**	**Max**	**CV**
31	2004*	Nebraska	0.07	0.011	0.07	0.05	0.10	14.95
35	1980	North Dakota	0.07	0.003	0.07	0.07	0.08	4.61
36	1980	North Dakota	0.08	0.004	0.08	0.07	0.08	4.69
37	1980	North Dakota	0.08	0.008	0.08	0.07	0.09	9.93
39	1981	North Dakota	0.07	0.004	0.07	0.06	0.08	5.44
40	1982	North Dakota	0.07	0.024	0.07	0.02	0.10	35.5
41	1982	North Dakota	0.09	0.010	0.09	0.08	0.10	11.52
43	1982	North Dakota	0.32	0.131	0.28	0.18	0.49	40.90
46	1983	North Dakota	0.09	0.007	0.09	0.07	0.09	8.30
47	1983	North Dakota	0.06	0.005	0.06	0.06	0.07	7.73
49	1983	North Dakota	0.08	0.011	0.08	0.07	0.10	13.65
50	1983	North Dakota	0.06	0.010	0.06	0.05	0.07	15.95
51	1983	North Dakota	0.07	0.006	0.07	0.07	0.08	7.60
53	1983	North Dakota	0.08	0.008	0.08	0.06	0.08	10.63
54	1983	North Dakota	0.05	0.010	0.05	0.03	0.06	19.88
55	1983	North Dakota	0.08	0.003	0.08	0.08	0.08	3.97
56	1983	North Dakota	0.09	0.006	0.09	0.09	0.10	6.17
58	1984	North Dakota	0.12	0.050	0.10	0.08	0.21	41.00
61	1985	Minnesota	0.06	0.015	0.06	0.05	0.08	23.72
68	1994	North Dakota	0.09	0.006	0.09	0.08	0.10	6.42
78	1996	North Dakota	0.09	0.005	0.09	0.08	0.10	6.03
84	2001	North Dakota	0.09	0.009	0.09	0.08	0.10	9.60
85	2001	North Dakota	0.07	0.003	0.07	0.07	0.08	4.32
89	2001	North Dakota	0.08	0.004	0.08	0.08	0.09	4.66
113	2002	North Dakota	0.14	0.125	0.09	0.07	0.40	87.48
135	2002	North Dakota	0.07	0.011	0.07	0.06	0.08	15.89
145	2003	North Dakota	0.08	0.010	0.08	0.07	0.09	12.05
175	2004	North Dakota	0.29	0.172	0.28	0.07	0.48	58.42
184	2004	North Dakota	0.08	0.008	0.08	0.06	0.08	11.11
189	2004	North Dakota	0.06	0.006	0.06	0.05	0.07	9.26
356	2008	North Dakota	0.07	0.002	0.07	0.07	0.08	2.42
357	2008	North Dakota	0.10	0.044	0.08	0.08	0.19	43.39
358	2008	North Dakota	0.3	0.249	0.25	0.08	0.62	82.46
360	2008	North Dakota	0.09	0.005	0.09	0.08	0.09	6.08
361	2008	North Dakota	0.37	0.080	0.34	0.28	0.47	21.62
362	2008	North Dakota	0.08	0.006	0.08	0.07	0.09	7.06
363	2008	North Dakota	0.14	0.104	0.09	0.08	0.34	75.92

Table A.5. Sensitivity of *Sclerotinia sclerotiorum* mycelium growth to boscalid (continued).

Isolate I.D.[1]	Year of collection[2]	State	Boscalid EC_{50}(µg/ml)[3]					
			Mean	S.D.	Median	Min	Max	CV
365	2008	Nebraska	0.26	0.214	0.17	0.10	0.65	83.67
366	2008	North Dakota	0.13	0.070	0.10	0.09	0.26	52.90
367	2008	North Dakota	0.07	0.006	0.07	0.06	0.08	8.82
368	2008	North Dakota	0.09	0.035	0.08	0.06	0.16	37.67
369	2008	North Dakota	0.09	0.004	0.09	0.09	0.09	4.18
372	2008	North Dakota	0.09	0.008	0.09	0.08	0.10	8.77
373	2008	North Dakota	0.09	0.009	0.09	0.07	0.10	10.78
374	2008	North Dakota	0.08	0.003	0.08	0.07	0.08	3.96
375	2008	North Dakota	0.09	0.017	0.08	0.08	0.12	19.26
377	2008	Iowa	0.07	0.002	0.07	0.07	0.08	3.27
378	2000	Wisconsin	0.13	0.064	0.09	0.09	0.22	49.45
380	2003	Wisconsin	0.17	0.095	0.17	0.07	0.28	56.10
381	2004	Missouri	0.07	0.010	0.07	0.06	0.09	12.99
382	2008	North Dakota	0.05	0.017	0.05	0.03	0.08	33.75
383	2008	Kansas	0.07	0.007	0.07	0.06	0.08	11.10
385	2007	North Dakota	0.46	0.281	0.44	0.13	0.84	60.71
386	2008	North Dakota	0.08	0.004	0.08	0.07	0.08	5.02
387	2008	North Dakota	0.19	0.198	0.09	0.08	0.58	105.01
388	2008	North Dakota	0.08	0.008	0.08	0.07	0.09	9.98
389	2008	North Dakota	0.08	0.013	0.08	0.06	0.09	16.90
390	2008	North Dakota	0.07	0.004	0.07	0.07	0.08	5.16
391	2008	North Dakota	0.09	0.005	0.09	0.09	0.10	5.09
392	2008	North Dakota	0.08	0.008	0.08	0.06	0.09	10.50
393	2008	Minnesota	0.10	0.021	0.09	0.08	0.14	20.58
394	2008	Minnesota	0.09	0.005	0.09	0.08	0.10	5.67
395	2008	Minnesota	0.06	0.007	0.06	0.06	0.07	10.25
396	2008	Minnesota	0.08	0.013	0.08	0.06	0.09	16.37
397	2008	Minnesota	0.08	0.006	0.08	0.08	0.09	7.81
398	2008	Minnesota	0.08	0.008	0.08	0.07	0.09	9.45
399	2008	North Dakota	0.08	0.004	0.08	0.07	0.08	5.12
400	2008	North Dakota	0.09	0.004	0.09	0.08	0.09	4.46
401	2008	North Dakota	0.08	0.011	0.08	0.07	0.09	13.61
402	2008	North Dakota	0.06	0.006	0.06	0.05	0.07	10.24
403	2008	North Dakota	0.07	0.010	0.07	0.06	0.08	14.75
404	2008	North Dakota	0.08	0.006	0.08	0.07	0.08	7.63
405	2008	North Dakota	0.19	0.113	0.16	0.10	0.38	60.32
406	2008	North Dakota	0.10	0.027	0.09	0.08	0.15	28.62

Table A.5. Sensitivity of *Sclerotinia sclerotiorum* mycelium growth to boscalid (continued).

Isolate I.D.[1]	Year of collection[2]	State	Boscalid EC_{50}(µg/ml)[3]					
			Mean	S.D.	Median	Min	Max	CV
407	2008	North Dakota	0.08	0.005	0.08	0.07	0.08	6.84
408	2008	Minnesota	0.09	0.005	0.09	0.08	0.10	5.32
409	2008	Indiana	0.08	0.004	0.08	0.07	0.08	5.45
410	1998	Illinois	0.07	0.006	0.08	0.06	0.08	8.50
411	2008	Iowa	0.61	0.248	0.61	0.30	0.88	40.49
412	2008	Iowa	0.08	0.014	0.07	0.06	0.09	18.20
413	2008	Iowa	0.23	0.143	0.23	0.09	0.40	61.48
414	2008	Michigan	0.08	0.013	0.08	0.06	0.09	16.87
415	2008	Michigan	0.08	0.007	0.08	0.07	0.09	8.98
416	2000	Wisconsin	0.10	0.030	0.09	0.08	0.16	30.5
417	2002	Wisconsin	0.08	0.006	0.08	0.07	0.09	7.71
418	2003	Wisconsin	0.08	0.017	0.08	0.04	0.09	22.69
419	2002	North Dakota	0.07	0.011	0.07	0.05	0.08	16.24
420	2002	North Dakota	0.08	0.008	0.08	0.07	0.09	9.83
421	2008	North Dakota	0.08	0.009	0.08	0.07	0.09	11.49
422	2008	North Dakota	0.10	0.022	0.09	0.08	0.14	22.91
423	2007	North Dakota	0.07	0.004	0.07	0.07	0.08	5.72
576	2008	North Dakota	0.08	0.003	0.08	0.07	0.08	4.37
577	2008	Manitoba	0.06	0.014	0.06	0.04	0.08	22.36
579	2008	North Dakota	0.03	0.023	0.03	0.01	0.07	75.95
581	2008	North Dakota	0.11	0.092	0.08	0.05	0.29	85.19
582	2008	North Dakota	0.07	0.008	0.07	0.06	0.08	10.89
583	2008	Minnesota	0.06	0.006	0.06	0.05	0.07	11.51
585	2008	Illinois	0.09	0.024	0.08	0.08	0.14	26.16
586	2008	North Dakota	0.08	0.002	0.08	0.08	0.09	1.82
587	2008	Nebraska	0.07	0.011	0.07	0.06	0.09	14.97
588	2008	North Dakota	0.04	0.015	0.04	0.01	0.05	40.27
589	2008	North Dakota	0.15	0.198	0.08	0.02	0.55	134.01
590	2008	North Dakota	0.06	0.012	0.07	0.04	0.07	19.72
591	2008	North Dakota	0.08	0.003	0.07	0.07	0.08	3.66
592	2008	North Dakota	0.08	0.007	0.08	0.07	0.09	9.39
593	2008	North Dakota	0.11	0.026	0.10	0.09	0.15	23.51
595	2008	North Dakota	0.08	0.004	0.08	0.08	0.09	4.38
596	2008	Nebraska	0.08	0.007	0.08	0.07	0.09	9.32
597	2006	Colorado	0.06	0.011	0.06	0.04	0.07	18.34
598	2008	Nebraska	0.05	0.014	0.05	0.03	0.07	25.88
599	2008	South Dakota	0.10	0.031	0.09	0.08	0.17	30.26

Table A.5. Sensitivity of *Sclerotinia sclerotiorum* mycelium growth to boscalid (continued).

Isolate I.D.[1]	Year of collection[2]	State	Boscalid EC_{50}(µg/ml)[3]					
			Mean	S.D.	Median	Min	Max	CV
600	2006	Minnesota	0.11	0.089	0.08	0.07	0.3	78.43
601	2002	Ohio	0.08	0.006	0.08	0.07	0.08	7.29
603	2003	Wisconsin	0.37	0.505	0.13	0.09	1.38	137.47
604	2008	North Dakota	0.07	0.007	0.07	0.06	0.08	11.08
605	2007	Montana	0.08	0.006	0.08	0.07	0.09	8.31
606	2007	North Dakota	0.08	0.005	0.08	0.07	0.09	6.24
607	2007	North Dakota	0.78	0.073	0.78	0.67	0.87	9.32
712	2014	North Dakota	0.07	0.005	0.07	0.06	0.08	6.81
713	2014	North Dakota	0.06	0.019	0.06	0.03	0.08	34.77
714	2014	North Dakota	0.08	0.008	0.08	0.08	0.09	9.34
715	2014	North Dakota	0.05	0.011	0.05	0.04	0.07	20.96
716	2014	North Dakota	0.13	0.074	0.09	0.08	0.27	56.12
718	2014	North Dakota	0.08	0.004	0.08	0.08	0.09	4.55
722	2014	North Dakota	0.06	0.005	0.06	0.06	0.06	7.64
724	2014	North Dakota	0.07	0.006	0.07	0.06	0.07	9.02
727	2014	North Dakota	0.05	0.007	0.05	0.04	0.06	14.15
730	2014	North Dakota	0.08	0.009	0.08	0.06	0.09	11.46
731	2014	North Dakota	0.07	0.004	0.07	0.07	0.08	5.88
743	2014	North Dakota	0.07	0.005	0.08	0.07	0.08	6.09
792	2014	North Dakota	0.07	0.004	0.07	0.06	0.08	6.01
846	2014	North Dakota	0.07	0.007	0.07	0.06	0.08	9.39

[1] Isolate I.D.=laboratory number; *=Previous 2004.

[2] Mean and median values represent observations from 3 replications in each of 2 trials per isolate; S.D.=standard deviation; Min=minimum, Max=maximum; CV=coefficient (%) of variation.