ABSTRACT

Title of Dissertation: *SCLEROTINIA SCLEROTIORUM* DIVERSITY AND MANAGEMENT OF WHITE MOLD ON LIMA BEAN IN MID-ATLANTIC REGION, USA

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Lima bean (*Phaseolus lunatus* L.) is one of the most important vegetables grown in the mid-Atlantic region of the US. Delaware has more acreage of land per year allocated for lima bean production, primarily used for processing, than any other state. The yield of lima bean is severely affected by white mold caused by *Sclerotinia sclerotiorum*. Currently, there is limited information on the population diversity of *S. sclerotiorum* in the mid-Atlantic region compared to other production regions, such as New York. Due to lack of research conducted in mid-Atlantic region, there are no specific fungicide application guidelines for lima beans. Improved understanding of the diversity within or among *S. sclerotiorum* isolates obtained from different geographical regions and various hosts will assist selecting representative isolates for use in developing improved disease management strategies including development of host resistance and effective fungicide guidelines. The main objectives of this research includes 1) studying the diversity of *S. sclerotiorum* isolates from lima bean

and other crops in the mid-Atlantic and other regions and 2) improving disease management guidelines for white mold.

Forty-two *S. sclerotiorum* isolates were collected from ten crops within eight different states in the US. The diversity of the collected isolates was evaluated for, a) lesion length and oxalic acid production on nine cultivars (five lima bean, two soybean, and two common bean), b) mycelial compatibility groupings (MCGs) and molecular characterization, and c) fungicide sensitivity (*in-vitro*) to two concentrations of boscalid, cyprodinil, fludioxonil, fluazinam, prothioconazole, and thiophanate-methyl . A field study also evaluated six application timings of boscalid, at 20% flowering, 100% flowering, two weeks and three weeks after 20% flowering, a double applications, and non-treated control for management of white mold in lima bean.

The collected isolates produced different lesion lengths, which were dependent on the crops and cultivars tested. Isolate 13, which was obtained from soybean, NJ, was the most aggressive in causing the longest lesions. Isolate 6, which was obtained from snap bean, DE, was the least aggressive isolate in causing the shortest lesion. Isolates were also significantly differed in oxalic acid production. Isolate 13 and isolate 4 were the highest oxalic acid producers. Seventy-five percent of the MCGs interactions were incompatible. The Shannon index (*Ho*) values of the MCGs were between 0 - 0.35 indicate that there is high diversity among the *S. sclerotiorum* isolates tested and that the isolates may reproduce sexually rather than via vegetative reproduction. The molecular characterization of the sequences examined at the ITS region and β-tubulin gene provided high sequence similarities among our isolates. The low variability did not allow us to evaluate differences among isolates. The molecular/genetic variability within population was 1 - 2%. To evaluate fungicide sensitivity of isolates, the percent reduction in mycelial growth (PRMG) of each isolate in presence of Dimethyl Sulfoxide (DMSO) and the fungicide was compared to the control (the isolate grown in the presence of DMSO). The collected isolates varied in PRMG to all six fungicides. The PRMG of the isolates differed at the two concentrations, except for cyprodinil and fludioxonil. There was a significant interaction between the concentrations and isolates sensitivity

to all fungicides except boscalid and thiophanate-methyl. Correlations were conducted to identify associations between fungicide sensitivities, lesion length, and oxalic acid production. Isolates' sensitivity to boscalid was negatively correlated to lesion length (r=-0.28397; *P*=0.0004) and oxalic acid production (r=-0.23370; *P*=0.0040). In addition, fungicide sensitivity to fluazinam was positively correlated to fungicide sensitivity to prothioconazole (r=0.35695; *P*<.0001) and thiophanate methyl (r=0.46247; *P*=<.0001). Likewise, fungicide sensitivity to fludioxonil was positively correlated to fungicide sensitivity to boscalid (r=0.19309; *P*=0.0179) and thiophanate methyl (0.28760; *P*=0.0004). However, fluazinam sensitivity was negatively correlated to boscalid sensitivity (r= -0.20119; *P*=0.0136). In the fungicide timing evaluation, the disease incidence was reduced by 6.4%, 5.4%, 3.9%, and 7.6% compared to no treatment when fungicides were applied at 20% flowering (*P*<0.0001), 100% flowering (*P*<0.0001), one week after 100% flowering (*P*<0.0128), or at 20% and 100% flowering (*P*<0.0001), respectively. These application timings also reduced the disease severity by 5.7%, 8.0%, 6.0%, and 7.0% compared to no treatment, respectively. Earlier, within 2 weeks of 20% flowering and double fungicide treatment reduced disease incidence and disease severity and improved yield of lima bean.

This research improves our understanding of the diversity of the mid-Atlantic *Sclerotinia sclerotiorum* population and suggests that, during selection of resistant lima bean cultivars, plants should be challenged by an array of *S. sclerotiorum* isolates, not just one putatively aggressive or susceptible isolate. My research also establishes guidelines for timing of fungicide management of white mold and developed baseline data on isolate sensitivity to fungicides.

SCLEROTINIA SCLEROTIORUM DIVERSITY AND MANAGEMENT OF WHITE MOLD ON LIMA BEAN IN MID-ATLANTIC REGION, USA

by

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Dedication

I would like to dedicate this dissertation to my late father, Bekele Demissie, and late grandmother Mulu Girma.

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List of Abbreviations and Acronyms

°C = Degrees Celsius

°F = Degrees Fahrenheit

g = gram(s)

Kg= Kilograms

a.i. = active ingredient

OA= Oxalic Acid

DI = Disease Incidence

DS = Disease Severity

PDA = Potato Dextrose Agar

DS = Dianna Sermon's

PRMG = Percent Reduction in Mycelial Growth

DMSO = Dimethyl Sulfoxide

Chapter 1: Introduction

1.1. Lima bean (*Phaseolus lunatus* L.)

Phaseolus lunatus L., also known as lima bean, butter bean (BSBI 2007), sieva bean (USDA 2019), double bean or Madagascar bean, is a tender annual leguminous plant grown for its flat, crescent-oval-shaped edible seeds. *P. lunatus* originated in Meso-and South America and diverged into two distinct gene pools upon domestication, the Mesoamerican and Andean type of lima bean (Kee et al 1997). The Mesoamerican type lima bean is small seeded (Seiva type) and is distributed in the neotropical lowlands. The Andean type lima bean is large seeded (lima type) and originated in the western Andes region. The Mesoamerican gene pool encompasses baby lima types and has broad environmental adaptation and small seed size, whereas the Andean gene pool includes Fordhook type lima bean and is characterized by narrow environmental adaptation and large seed size (Nienhuis et al 1995). Native Americans grew both varieties in what is now the southern US. Immigrants and people who were enslaved later cultivated them and exported them to Europe (Park et al. 2016; Kee et al 1997). The term "butter bean" is widely used in North and South Carolina for a large, flat and yellow/white variety of lima bean (*P. lunatus* var. *macrocarpus*, or *P. limensis*).

In the US in 2018, lima beans for fresh market and processing were planted to 10,156 hectares of land, and yield and total production was 1,519 kg per hectare and $1{,}5x10^6$ kg, respectively (USDA, 2019). In 2018, California planted 1,942 hectares; the yield was 1,626 kg per hectares, with a total production was $7.8x10^6$. Similarly, in Illinois total hectares planted, yield, and total production were 202 hectares, 1,829 kg per hectare, and $8.2x10^6$ kg; in Maryland it was 971 hectares, 1,524 kg per hectare, and $3.2x10^6$ kg; in Virginia, 162 hectares, 356 kg per hectare, and 2.9 $x10^6$ kg; in Washington state 890 hectares, 2,540 kg per hectare, and $4.7x10^6$ kg. In all other states, a total 2,671 hectares were planted, 1,219 kg per hectare were produced and reported a total of $8.6x10^6$ kg production.

Lima bean is an important crop grown in the mid-Atlantic region, especially in Delaware. In Delaware, lima bean is the cornerstone crop of the vegetable processing industry (Kee et. al 1997) and in recent years, production in Delaware was the largest of any state in the US. In 2018,

lima beans were grown on more than 3,318 hectares of land. The yield and total production in 2018 were 2,887 kg per hectare, and 179.6 $x10^6$ kg, respectively (USDA NASS vegetables report, 2019; DASB, 2018; USDA, 2018). Lima bean production averaged 3.4 tons ha^{-1} in Delaware and an annual production of 12,500 metric tons was estimated in 2017 and sold at a price of $0.6 kg-1 (DASB, 2018). The production is primarily concentrated in Sussex County (Whalen et al. 2007). In Maryland, production of lima bean for processing is expanding annually (Johnson 2014). Despite its regional importance, yield per hectare of lima bean in the mid-Atlantic is lower than in other growing regions (Johnson, 2014). For example, in 2013 Delaware yield was 1,278 kg ha^{-1} compared to 2,802 kg ha^{-1} in California (USDA NASS vegetables report, 2013).

Optimum conditions for growing lima beans is temperatures between 15° and 21°C (or 60° and 70°F) and a soil temperature of 18°C (or 65°F) or higher. There are two growth habits of lima beans: bush and pole or vine types. Bush types which include baby lima and Fordhook types grow to approx. 0.6 m tall, 60 to 80 days from sowing, and produce small seeds of approx. 1 cm long. Pole lima beans have viney growth habit, can grow 3 to 3.7 m tall, and produce large seeds of approx. 3 cm long and reaches for harvest from 85 to 90 days from sowing (Albert 2020; Erickson 1992). The bush types require plant spacing, and the pole types require lower plant spacing but need support to grow vertically. The pole type flower indeterminately and require short photoperiod (Kee et al 1997; Erickson 1992), however most commercial cultivars of baby and Fordhook types of lima bean flower determinately (Johnson 2014; Maynard and Hochmuth, 2007; Kee et al 1997). Both determinate and indeterminate types of lima bean flowers are produced on raceme; three flowers appear at each node along the raceme and the flowers are generally pubescent on its outer face and colored white, pink, pink to purple, or violet (Kee et al. 1997; Raj et al. 1993). Lima bean is a self-pollinating plant having a “perfect” or “complete” flower, meaning that each individual flower contains both the male (anther) and female (stigma) flowering parts necessary for fertilization and seed production (Albert, 2020). Lima bean cultivars varying in their flowering time (Dohle, 2017) and under field conditions, each cultivar may flower at different times (Self-observation).

Planting, spacing, and plant population per area depends on the type of lima beans. For example, baby lima type can be planted in a row spacing of 0.8 to 0.9 m apart with 10 to 13

plants per m. Seeding rate of baby lima is approximately 55 kg ha^{-1} of seed, 4 cm deep (deeper if soil is dry) if it is non-irrigated. For irrigated fields, the row spacing varies between 46 to 76 cm apart with 10 to 13 cm between plants and a seeding rate of 111 kg ha^{-1} of seed at 46 cm spacing and 90 kg ha^{-1} at 76 cm spacing, respectively (Wyenandt and van Vuuren 2019).

Harvesting of lima bean also depends on type. In general, the bush lima beans will be ready for harvest 60 to 80 days after sowing; pole beans will be ready for harvest 85 to 90 days after sowing. Harvesting of baby lima for processing is done when the highest percentage of full pods can be obtained and when plants have approximately 10% dry pods if it is mechanical picking. Otherwise if they are hand-harvested, pods can be picked at the full green seed stage (Wyenandt and van Vuuren 2019; Nienhuis et al 1995). In the Mid-Atlantic region, Fordhook types are often planted between May 15 to July 10, and harvested August 1 to October 20. Pole types are planted between May 15 to June 15 and harvested in July 15 to October 30. Baby lima types are planted between May 15 to July 20 and harvested in August 1 to October 30, usually after a pea or small grain crop (Wyenandt and van Vuuren 2019; Johnson 2014).

Factors that influence lima bean yields include weather conditions that affect flower bud growth, pollination and pod ripening, abscission of flowers and pods. High temperatures, low relative humidity and poor soil moisture contribute to decreased pod set and retention. Temperatures of 32 °C or above limit pollination and pod development (Whalen et al. 2007). Prolonged drought (7 days or more with less than 2.54 cm of water) often negatively affect yield. High nighttime temperatures often adversely affect yield, as energy is used for respiration, thereby restricting plant physiological ability to set and retain pods (Johnson, 2014; Whalen et al. 2007). In the mid-Atlantic region, diseases such as anthracnose and web blight (*Rhizoctonia*), root rots (*Rhizoctonia solani*, *Fusarium solani f. sp phaseoli* and several *Pythium* species), bacterial brown spot (*Pseudomonas syringae pv. syringae*), soybean rust (*Phakopsora pachyrhizi*), lima bean downy mildew (*Phytophthora phaseoli*), lima bean pod blight (*Phytophthora capsici*), gray mold (*Botrytis cinerea*), and white mold (*Sclerotinia sclerotiorum*) limit lima bean production (Saharan and Mehta, 2008; Heffer Link and Johnson, 2007; Whalen et al. 2007; PMSP, 2003).

White mold, which is caused by the necrotrophic fungus *Sclerotinia sclerotiorum*, is the number one disease of lima bean in Delaware (PMSP, 2003). The pathogen, *S. sclerotiorum*, colonizes senescent blossoms of lima bean, establishing in developing pods, and reduces yield up to 40% in highly infested fields in some years (Everts, 2006; PMSP, 2003). Taxonomically *S. sclerotiorum* is classified under the kingdom Fungi, phylum Ascomycota, class Discomycetes, order Helotiales, family Sclerotiniaceae, and genus *Sclerotinia* (Saharan and Mehta, 2008; Heffer Link and Johnson, 2007; Bolton et al., 2006). *Sclerotinia minor* (Jagger), *S. trifoliorum* (Eriks), *S. fructigena*, *S. laxa* are some other members of *Sclerotinia* species in the same phylum and order as *S. sclerotiorum* (Saharan and Mehta, 2008; Heffer Link and Johnson, 2007). *S. sclerotiorum* affects more than 400 plant species worldwide (Boland and Hall, 1994). *S. sclerotiorum* is soil inhabiting and attacks and greatly reduces the yields of several economically important legumes including common bean (*Phaseolus vulgaris* L.; Del Río et al., 2004, Miklas et al., 2013), soybean (*Glycine max* (L.) Merr; Mueller et al., 1999), oilseed (*Brassica napus* L.; Xiaojia et al., 2005), chickpea (*Cicer arietinum* L.; Mandal and Dubey, 2012), lima bean (*P. lunatus*; Everts, 2016). It also reduces seed and pod quality of several grain legumes (Miklas et al., 2013).

1.1. Disease Distribution

Epidemics caused by *S. sclerotiorum* occur worldwide (Kull et al. 2004; Kull et al. 2003; Sun et al. 2005; Hartman et al. 1999; Boland and Hall, 1994). It is the most important disease of most bean crops in the temperate zones of the northern and southern hemisphere including in North and South America, and Europe (Schwartz and Steadman, 1989). White mold is also problematic in tropical and semi-arid climates, especially during cool seasons.

Australia (Jones et al. 2011) and some African and Asian (Allen, 1983) countries are among the major bean growing regions and epidemics have occurred on both continents in recent years. For example, white mold occurs in many Asian countries such as Iran, India, Bangladesh, and China (Nahar et al. 2019; Ojaghian 2009; Dutta et al. 2009; Zhou et al. 2014). In North America *S. sclerotiorum* is widespread including in Canada (Schwartz and Singh, 2013; Li et al. 2010; Bardin and Huang, 2001; Miklas et al. 2013, 1999; Huynh et al.2010; Del Río et al. 2005; Atallah and Johnson 2004; Workneh et al. 2000). White mold is high in common bean in South American countries such as in Brazil and Argentina (Lehner et al. 2015; Juliatti et al. 2013;

Schwartz and Singh, 2013; Petrofeza et al. 2012). The pathogen is also common and causes widespread disease on several crops in European countries such Bulgaria (Sofkova et al. 2010) and Serbia (Vidic and Jasnic, 2008). White mold is also major problem in the Middle East in Palestine (Al-Masri et al. 2010), and is one of the most common bean diseases in Africa (Onaran, 2009). In general, white mold epidemics are most frequent in temperate zones of higher elevations in humid and sub-tropical regions (Miklas et al., 2013; Schwartz and Steadman 1989). In the mid-Atlantic region of the US environmental and climatic conditions favor the development of white mold in both spring and fall. Average temperature of 16 - 22^{O}C, relative humidity 76 - 80 %, soil temperature 17 - 24°C, and soil volumetric water content 0.074 - 0.217 % were recorded between July and October in the years 2014 to 2016, conditions that are favorable for disease development.

1.2. Life cycle of *Sclerotinia sclerotiorum*

S. sclerotiorum produces vegetative propagules, or sclerotia, that enable the pathogen to survive during adverse conditions and serves as the primary source of inoculum for the next cropping season. Sclerotia are 2 to 5 mm in diameter and up to 25 mm in length, black, long-lived, and melanized survival structures (Yue et al., 2010; Erental et al. 2008; Heffer and Johnson, 2007). The sclerotia over-winter and germinate in few days to produce mycelia (asexual reproduction) or apothecia (sexual reproduction). Optimum conditions for apothecia formation are soil moisture levels of about 50% field capacity and temperatures of 15 to 18 °C (60 to 65°F) (Hagedorn and Inlis, 1986). The myceliogenic (hyphal) germination of sclerotia occurs when apothecia germinate by the direct emergence of hyphae (termed ‘myceliogenic’ or ‘eruptive’ germination) from sclerotia (Heffer Link and Johnson. 2007). Whereas, the apothecial (or carpogenic) germination of sclerotia produce fleshy-colored mushroom-like fruiting bodies termed apothecia, which measure 4 to 8 mm in diameter and germinate at a soil depth of up to 2 cm to reach to the soil surface (Bolton et al. 2006; Bardin and Huang, 2001; Steadman, 1979). One or several apothecia can emerge from a single sclerotium and can produce ascospores under favorable environmental conditions (Warmington and Clarkson, 2015; Bolton et al. 2005; Jones et al. 2004; Kull et al. 2004). Each apothecium may release more than 10 million ascospores over a period of several days, which are blown by wind to the aerial portions of plants. Ascospores are hyaline (clear or non-pigmented), unicellular, and thin-walled spores that can survive for only a

few days after release (Bolton et al. 2005). Spores from apothecia infect senescing lima bean blossoms. The infection in the leaf appears initially as a water-soaked lesion, which later on becomes necrotic, and the fungus eventually grows to the stem or toward to the root (Mueller et al. 2015) (Figure 1.1).

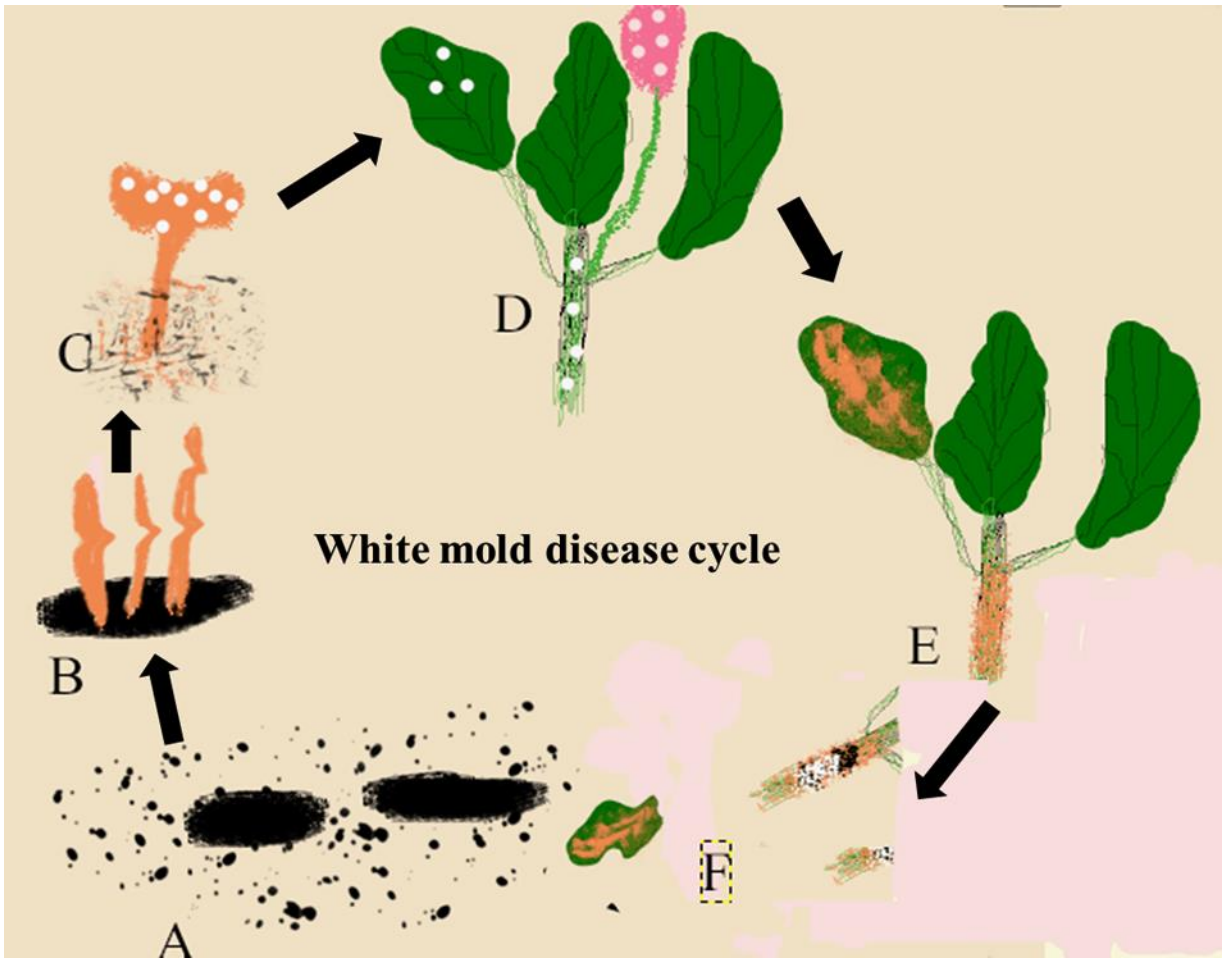


Figure 1.1. White mold disease cycle. A=Sclerotia of *S. sclerotiorum* survive in the soil; B=Sclerotia germinate to produce apothecia; C=Apothecia produces ascospores; D= Ascospores colonize senescing flowers and leaves; E=Symptom of white mold on leaves and infection can spread into the stem at the node; and F=Signs of *S. sclerotiorum* include sclerotia and tufts of white mycelium (sclerotia form inside and outside stems and pods and are dropped to the soil during harvest) (Adapted from Mueller et al. 2015).

1.4. White mold disease symptom

Disease symptoms caused by *S. sclerotiorum* occur on leaves, stems, roots, pods, and seeds of lima bean at different developmental stages (Sylvester-Bradley and Donald, 1984). Under favorable conditions such as cool maximum daily temperatures (lower than 30°C/85°F) and moisture from rain, fog, dew, or high relative humidity, the pathogen spreads on the soil as mycelia (Figure 1.2c). The leaves of infected plants show water-soaked lesions that can expand rapidly and spread to the stem. The infected stem will then develop dark and/or water-soaked lesions and subsequently aerial mycelia will develop on the infected portion of the stem. The infected stem eventually becomes girdled and mummified (Figure 1.2b). The most noticeable signs of *S. sclerotiorum* occur after the lesions become necrotic; patches of fluffy white cottony mycelium develop (Heffer and Johnson, 2007; Bolton et al., 2006). Infected pods also turn into a soft, watery mass, dry out and turn brown (Figure 1.2a). The brown areas will be covered with a dense white fungal growth, which later turns gray and is dotted with small, hard black bodies called sclerotia (Figure 1.2d; Mueller et al. 2015; Harikrishnan and Del Río 2006; Clarkson et al., 2004; Abawi and Grogan, 1979). Wilting, bleaching, and shredding may also be observed on aboveground tissues and are considered secondary symptoms (Heffer and Johnson, 2007).

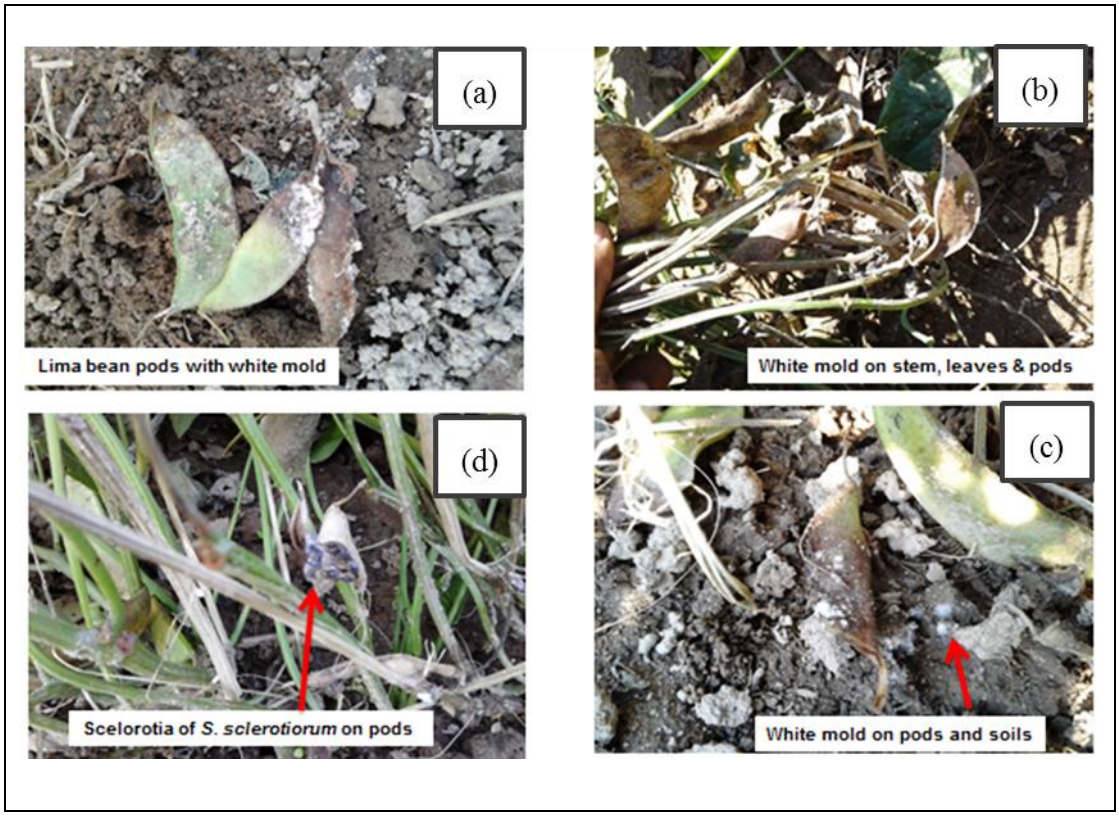


Figure 1.2. Symptom of white mold on (a) pods; (b) stem and leaves; (c) *Sclerotinia sclerotiorum* on pods and soil; and (d) sclerotia on developed on pods of lima bean (From grower's field in Lewes, DE in the summer of 2016).

1.3. Host Range of *Sclerotinia sclerotiorum*

The pathogen, *S. sclerotiorum*, has a broad host range of more than 400 plant species (Manjunatha et al 2014; Sun et al. 2005; Mullen, 2001; Boland and Hall, 1994). Many of the hosts are dicotyledonous and include peas (*Pisum sativum*), cabbage (*Brassica oleracea var. capitata*), canola (*Brassica napus*), lettuce (*Lactuca sativa*), peanut (*Arachis hypogaea*), sunflower (*Helianthus annuus*), forage legumes, vegetables, and ornamentals, cucumber (*Cucumis sativus*), soybean and common bean, potato (*Solanum tuberosum*), country bean (*Lablab purpureus* L.), Echium (*Echium vulgare*), Carnation (*Dianthus caryophyllus* L.), tomato (*Solanum lycopersicum*), and lima bean (Warmington and Clarkson, 2015; Zhou et al. 2014; Hegedus and Rimmer, 2005; Mueller et al.; 1999; Farr et al., 1989; Grau, 1988; Schwartz and Singh, 2013; Li et al. 2010; Bardin and Huang, 2001; Al-Masri et al. 2010; Lehner et al. 2015; Miklas et al. 2013; Petrofeza et al. 2012; Miklas et al. 1999; Huynh et al.2010; Sofkova et al.

2010; Onaran, 2009; Vidic and Jasnic, 2008; Del Río et al. 2005; Atallah and Johnson 2004; Kull et al., 2003; Workneh et al. 2000; Nahar et al. 2019; Ojaghian 2009; Dutta et al. 2009; Juliatti et al. 2013; Selvaraj et al. 2015; De Aguiar et al. 2014; Schwartz and Steadman, 1989; Johnson, 2014; Everts, 2016 and self-field observation).

1.4. Diversity of *Sclerotinia sclerotiorum*

Determining the diversity of *S. sclerotiorum* isolates is an important initial step to understand variability among isolates obtained from different geographical locations and different crops. Understanding the diversity or variability of *S. sclerotiorum* populations in a given region can guide the selection of representative isolates for use in developing disease management strategies including testing for host resistance and evaluation of effective fungicides (Aban et al.2018; Dunn et al. 2017; Petrofeza et al. 2012; Li et al. 2009). Diversity analysis of *S. sclerotiorum* populations would also assist in monitoring the changes occurring in the pathogen population and enhance our knowledge about the epidemiology of disease.

Diversity of *S. sclerotiorum* can be identified using growth characteristics, mycelial compatibility groups (MCG), aggressiveness, and the production of virulence/pathogenicity factors such as oxalic and other organic acids. It is generally assumed that local populations of *S. sclerotiorum* are likely to have low diversity because of their life style, i.e. that they are homothallic and spore dispersal is limited (i.e. spores generally remain within 100 m of the dispersal site) and limited long distance wind dispersal of ascospores (Winton et al. 2006; Kull et al. 2004; Abawi and Grogan, 1979). Previous research has examined the diversity of *S. sclerotiorum* using different techniques, and the result of that research is presented under this section.

1.4.1. Mycelial compatibility groups (MCGs)

S. sclerotiorum is predominantly homothallic, which may result in a clonal population structure. This clonal population may be separated into distinct mycelial compatibility groups (Clarkson et al. 2013; Atallah et al. 2004; Kohn et al. 1990). However, there are certain reports of outcrossing and genetic exchange between isolates (Atallah et al. 2004). Mycelial compatibility grouping (MCG) is a "macroscopic assay of the self-non-self-recognition system common in fungi and is determined using a side-by-side pairing system" (Kull et al. 2004; Kohn et al. 1990).

Mycelial incompatibility is described as a failure of different strains to fuse and form one colony and is characterized by the formation of a barrage of dead cells between two incompatible colonies (Kohn et al. 1990). In this research project, MCG was used to assess *S. sclerotiorum* isolates and a few to many MCGs were identified and recorded from different crops.

Based on multiple studies, *S. sclerotiorum* populations have been grouped into large numbers of MCGs. In general, these MCGs have low diversity of isolates (Aldrich-Wolfe et al. 2015; Litholdo et al. 2011; Otto-Hanson et al. 2011; Mo et al. 2007; Durman et al. 2003). For example, approximately 50% of the MCGs within *S. sclerotiorum* populations from canola from China and Washington State in USA were represented by single *S. sclerotiorum* isolate, respectively and there were no shared MCGs between the two populations (Attanayake et al. 2012). Moreover, Durman et al. (2003) reported that some MCGs differed among crops and approximately 60% of the MCGs were unique for each crop. These results may indicate the clonal nature, and low diversification of MCG in the *S. sclerotiorum* populations from the same crop. However, Mandal and Dubey, (2012) demonstrated that among 24 isolates from six different crops and 10 regions in India there was greater heterogeneity in MCG, and none of the isolates were compatible or incompatible with all the isolates and each MCG was found in isolates from different locations and hosts. This result may indicate that there could be high diversity among isolates from different crops, or indicate that sexual reproduction occurs commonly in the pathogen in India.

1.4.2. Aggressiveness of Sclerotinia sclerotiorum

"Pathogen aggressiveness" is defined as the relative ability of the pathogen to colonize the host and cause damage, and "virulence" as the relative capacity to produce disease (Kull et al. 2004; Agrios, 1999; Shurtleff and Averre, 1997). *S. sclerotiorum* isolates on different hosts show variation in degree of aggressiveness of pathogenicity, which is dependent on host species or cultivars. For example, diverse isolates of *S. sclerotiorum* differed in aggressiveness on alfalfa cultivars, and experiment-cultivar and experiment-isolate interactions were observed, but no isolate-cultivar interaction was observed (Pratt and Rowe, 1995). Kull et al. (2004) reported that no isolate-cultivar interaction was detected in soybean cultivars; however effective separation of resistant and susceptible cultivars was dependent on isolate selection. Significant variations in

aggressiveness between *S. sclerotiorum* isolates were reported in sunflower (Ekins et al. 2007; Li et al. 2008), in common bean (Silva et al. 2014; Otto-Hanson et al. 2011; Pascual et al. 2010), in soybean (Willbur et al. 2017). The aggressiveness of *S. sclerotiorum* to its hosts might be related to variation in isolate oxalic acid production or due to the geographical location or crop hosts where isolates are obtained. However, Peili and Huazhi, Y. (2006) reported that even though isolates of *S. sclerotiorum* varied in aggressiveness on rapeseed; the aggressiveness was not related to geographic distribution and host origin. Similarly, Lehner et al. (2016) reported that there was similarity in aggressiveness between *S. sclerotiorum* isolates collected from common bean fields from four Brazilian states. Variation in aggressiveness, which occurs often, can be assessed by measuring variables associated with the disease sign and symptoms, such as lesion size and/or length., has not been reported. Currently, little is known about the variation in aggressiveness among *Sclerotinia* isolates from lima bean or from different crops in the mid-Atlantic, particularly from Delaware and Maryland.

1.4.3. Production of virulence/pathogenicity or oxalic acid

Several virulence factors, including oxalic acid, fumeric acid, pectolytic enzymes, glycosidases, cellulases, xylanases, melanin, and other organic and inorganic acids produced by *S. sclerotiorum* have been used to study variation among *S. sclerotiorum* isolates from different regions and crops (Davidson et al. 2016; Petrofeza et al. 2012; Ziman et al. 1998; Errampalli and Kohn, 1995; Lumsden, 1976). The ability to generate the cell wall degrading enzyme, oxalic acid, and its involvement in the pathogenicity or virulence of *S. sclerotiorum* is the subject of several studies. Oxalic acid disrupts host defenses and regulates the host redox climate, leading to cell death and disease establishment in the host tissues. (Willbur et al. 2019; Fagundes-Nacarath et al. 2018; Davidson et al. 2016; Bolton et al. 2016; Kabbage et al. 2015; Koga et al. 2014; Kabbage et al. 2013; Attanayake et al. 2013; Petrofeza et al. 2012; Kim et al. 2008; Durman et al. 2005; Kull et al. 2004; Kohn et al. 1991; Godoy et al. 1990; Marciano et al. 1989). Oxalic acid promotes pathogenesis through acidification of host tissues and sequestration of calcium from host cell walls. Oxalic acid is also involved in stomatal opening during infection, which increases the transpiration rate, decreases biomass, and contributes to wilting (Guimaraes and Stotz 2004).

Durman et al. (2005) reported that there were significant differences among *S. sclerotiorum* isolates of different MCGs in both oxalic acid and organic acids produced, ranging from the mean production of 18 to 110 μg oxalic acid mg^{-1} dry wt. The same report showed that isolates obtained from soybean produced the highest oxalic acid concentration (71 μg oxalic acid mg^{-1} dry wt) compared to sunflower and lettuce. Ziman et al. (1998) also reported that in resistance in oilseed rape cultivars was dependent on the intensity of oxalic acid production. More evidence showed the involvement of oxalic acid in pathogenicity or aggressiveness of *S. sclerotiorum*. Virulence is lost in transgenic soybean plants that express oxalic acid degrading enzymes, i.e. oxalate oxidase (Davidson et al. 2016), and Godoy et al. (1990) demonstrated that oxalic acid deficient mutants were non-pathogenic. Mo et al. (2007) showed that aggressiveness was positively related to oxalic acid concentration (r=0.74) in oilseed rape. However, there are also reports that these virulence factors, particularly oxalic acid production are not involved, or at least are not the primary determinant factors, in the pathogenicity or aggressiveness of *S. sclerotiorum* (Attanayake et al. 2013; Petrofeza unpublished; Morrall et al. 1972).

S. sclerotiorum isolates collected from Delaware and Maryland have not been characterized using oxalic acid as a virulence factor to enable infection of crops grown in the region. Moreover, no comparison, in terms of oxalic acid productions has been done between *S. sclerotiorum* isolated from lima bean vs. other crops in the Mid-Atlantic region.

1.4.4. Genetic diversity of Sclerotinia sclerotiorum

Diversity of *S. sclerotiorum* can also be studied using genetic or molecular approaches. A number of diversity studies have been done in isolates collected from several crops using various molecular techniques. These techniques include sequence-related amplified polymorphism (SRAP; Li et al. 2009) in sunflower, restriction fragment length polymorphism (RFLP; Hambleton et al. 2002) in soybean, amplified fragment length polymorphism (AFLP; Cubeta et al. 1997) in cabbage, RFLP (Yli-Mattila et al. 2010) in clover, microsatellite (SSR) markers (Lehner et al. 2015) in common bean, internal transcribed spacers (ITS) sequences (Mandal and Dubey, 2012) in chickpea, and RFLP in peas and lentil (Malvárez et al. 2007), and in canola (Kohli et al. 1992). The genetic diversity or variability of *S. sclerotiorum* isolates collected from different countries has also been investigated using different sets of molecular markers. Simple

sequence repeats (SSR) (Lehner et al. 2017) from tropical and temperate regions, SSR (Malvárez et al. 2007) from North America (Washington State and Canada), microsatellite marker (Meinhardt et al. 2002) from Brazil, were some of the molecular techniques used to study genetic diversity of *S. sclerotiorum*.

Some genetic markers are powerful and able to detect genetic variability within the species *S. sclerotiorum*. However, some markers do not sufficiently differentiate within the species and do not provide adequate information among isolates. For example, Dunn et al. (2017) showed that SSR markers using hyphal tips of *S. sclerotiorum* isolates from New York States showed that there were twenty-four multilocus genotypes (MLGs) detected within the population which was mainly dominated by only one MLG. Li et al. (2009) showed that *S. sclerotiorum* isolates from the UK formed a population that was significantly distinct compared to populations from Canada and Inner Mongolia China. Atallah et al (2007) reported that using microsatellite markers 92% of the variability among 167 isolates was found within subpopulations from potato plant in Columbia Basin of Washington State. Gomes et al. (2011) used 10 microsatellite loci to characterize the genetic diversity and structure of 79 *S. sclerotiorum* isolates from four Brazilian dry bean populations and observed that eight of them were polymorphic within populations. The same report identified 102 different haplotypes ranging from 6 to 18 per locus and analyses based on genetic diversity indicated variability among and within populations of 28.79% and 71.21%, respectively. However, the same research, after restriction fragment length polymorphism (PCR-RFLP) analysis, reported that the ITS1-5.8S-ITS2 regions failed to show any sequence polymorphism among the *S. sclerotiorum* isolates. Using the DNA fingerprinting technique, which involves Southern blotting using a repetitive cloned sequence, pLK44.20, as a probe, a high degree of variation was identified both within and between populations of *S. sclerotiorum* collected from four locations in the South Island of New Zealand (Margaret et al. 1999). However, using RFLPs, RAPDs, and MCGs, Ekins et al. (2011) reported that there was no significant genetic variations among *S. sclerotiorum* isolates collected from different fields in eastern Australia located within approximately 350 km of each other. Atallah et al. (2004) also reported that field was not a significant source of genetic variation in *S. sclerotiorum* collected in Washington State, US. Similarly, Hemmati et al. (2009) reported that no genetic variation was detected among *S. sclerotiorum* populations in different provinces in Iran (Winton et al. 2006).

Availability of the complete genome has opened unlimited possibilities for omics studies in *S. sclerotiorum* (Derbyshire et al. 2017; Schwartz and Singh, 2015; Amselem et al. 2011). The extent of genetic diversity or genetic variability within isolates of *S. sclerotiorum* infesting lima bean fields has not been reported, yet. In addition, there is no information on genetic variability among isolates of *S. sclerotiorum* collected from different crops in Delaware and Maryland.

1.4.5. Sensitivity of Sclerotinia sclerotiorum to active ingredients (a.i.s) of fungicides

Information on the sensitivity of *S. sclerotiorum* to different active ingredients (a.i.s) of fungicides can also be used to characterize variability among isolates of *S. sclerotiorum.* Application of fungicides is the major, or only, strategy to manage white mold disease in many crops plants, including lima bean. For example, in lima bean, growers in the mid-Atlantic region depend heavily on the use of fungicides due to non-availability of resistant cultivars. However, the intensive use of fungicides can select for resistant isolates and may lead to control failures (Brent and Hollomon, 2007). Sometimes, up to six fungicide sprays are applied to manage the disease, especially in highly infested areas and under favorable weather conditions (Lehner et al. 2017). There are several reports of the development of resistant isolates of *S. sclerotiorum* to active ingredients of fungicides used to control white mold in many crops. For example, Lehner et al. (2015) reported one isolate, out of 282, obtained from common bean from Brazil was resistant to thiophanate-methyl. Some isolates of *S. sclerotiorum* from oilseed rape and soybean from China were shown resistant to dicarboximide fungicides dimethachlon, iprodione, and carbendazim (MBC) (Wang et al. 2014; Zhou et al. 2014; Liu et al. 2009). MBC resistance in *S. sclerotiorum* is widespread in most oilseed rape cropping areas in the center to north-eastern France, and a few cases of *S. sclerotiorum* that were less susceptible to dicarboximide fungicide were also detected (Penaud et al. 2003).

However, in the US in general and in the mid-Atlantic region in particular, there are no reports of the development of resistance by *S. sclerotiorum* to any currently registered fungicide active ingredients used to manage *S. sclerotiorum* in any crop. Nevertheless, assessing *S. sclerotiorum* tolerance and sensitivity to the most frequently used fungicides is essential for the development of management practices for white mold.

We lack information on diversity of *S. sclerotiorum* populations in the mid-Atlantic region. There has been no research on the population of *S. sclerotiorum* in lima bean in the region and the current project was aimed at identifying and documenting the diversity among the *S. sclerotiorum* population.

1.5. Management of white mold

White mold is difficult to manage due to its survival in soil as sclerotia and because of its wide host range (Willbur et al. 2019; Lehner et al. 2017). Despite the difficulty, however, a number of approaches have been used to control *S. sclerotiorum* in different crops including chemical fungicides, biological controls, host resistance, and various cultural practices including crop rotation, canopy management, irrigation, and tillage practices.

1.5.1. Chemical fungicides

Several registered chemical fungicides have been used to control white mold disease in the past. Chemical fungicides remain one of the most reliable management options to control *S. sclerotiorum*. Several FRAC (fungicides resistant action committee) groups or classes of fungicides, based on mode of actions (MOA) in the biosynthetic pathways of plant pathogens and resistance risk, are used to control *S. sclerotiorum* in different crops. Numbers and letters are used to distinguish the FRAC groups. The numbers are assigned primarily according to the time of product introduction to the market. The letters refer to P = host plant defense inducers, M = multi-site inhibitors, and U = recent molecules with unknown mode of action and unknown resistance risk (U is a transient status, usually lasting no longer than 8 years, until information about mode of action and mechanism of resistance becomes available) (FRAC code list, 2018). The inherent risk for resistance evolution to a given fungicide group is estimated to be low, medium or high according to the principles described in FRAC monographs. Resistance management is driven by intrinsic risk of each fungicide, pathogen risk, and agronomic risk (FRAC, 2018).

Several fungicides are used to control *S. sclerotiorum* in different crops at different locations. The lists of chemical fungicides, FRAC code and their risk label are given in Table 1.1. In addition to these chemicals, biofumigant volatiles, such as from *Brassica juncea* ‘Vittasso’ (Warmington and Clarkson, 2015) have been used to control *S. sclerotiorum*. However, there are no specific fungicide application guidelines developed for white mold on lima beans. Growers

currently use guidelines developed for snap beans or soybean (Everts, 2016; Steadman, 1979; Hunter et al. 1978) presuming that lima bean shares some similarities with snap bean and soybean in terms of crop canopy. However, some lima bean cultivars flower indeterminately, and all cultivars have a longer growing season as compared to the determinate flowering snap bean which has shorter growing season.

395 Table 1.1. Examples of fungicides used to manage white mold caused by *Sclerotinia sclerotiorum* on several crops, their FRAC code
396 and resistance risk.

FRAC Code	Active ingredients	Chemical group	Target site	Resistance risk	Crops	Sources
12	Fludioxonil	PhenylPyrroles (PP)	MAP/Histidine-Kinase in osmotic signal transduction (*Os-2*, *HOG1*) gene	Low to medium risk	Soybean	Mueller et al., 2002; Duan et al. 2013; Matheron et al. 2004; Kuang et al. 2011
7	Boscalid	Carboxamides	Succinate dehydrogenase	Medium risk	Dry bean, Soybean, Oilseed rape, Dry bean, Snap bean, Lima bean	Mahoney et al., 2014; Bradely et al. 2006; Spitzer et al. 2017; Kee et al. 2004;
29	Fluazinam	2,6 dinitroanilines	histidine kinase gene (*Shk1*)	Low risk	Dry bean, Tomato, Snap bean	Lehner et al. 2017; Mahoney et al. 2014
1	Thiophanate-methyl	Methyl Benzimidazole Carbamates (MBC)	ß-tubulin gene	High risk	Common bean, dry bean, Tomato, Snap bean	Mahoney et al., 2014; Bradely et al. 2006; Huzar-Novakowiski et al. 2017; McCreary et al. 2016
3	Prothioconazole	Demethylation inhibitors (DMI)	*Cyp51* gene	Medium risk	Oilseed rape, Dry bean	Bradely et al. 2006; Muller et al. 2002; Spitzer et al. 2017; McCreary et al. 2016
9	Cyprodinil	Anilino-Pyrimidines (AP)	Cystathionine *γ-synthase* and *β-lyase* genes	Medium risk	Oilseed rape	FRAC 2006. Hou et al. 2018

Preliminary studies conducted in Delaware have confirmed that fungicides applied when pods are up to 3.8 cm in length result in substantial yield increase (Everts, 2016 unpublished). Direct application of fungicide to flower petals is a more efficient method of fungicide application in beans to control white mold. Muller et al. (2002) recommended applying fungicide directly on the flower petals of snap bean and soybean, especially the lower portion of crop canopy as this is the main entry point of the pathogen.

1.5.2. Biological controls

Several biocontrol agents such as *Bacillus subtilis* species (Xiaojia et al., 2005; Xiaojia et al., 2014) and *Pseudomonas* spp. (Savchuk and Fernando, 2004), *Gliocladium virens*, *Sporidesmium sclerotivorum*, and *Trichoderma viride* (Schwartz and Singh, 2015) reduced the incidence of disease caused by *S. sclerotiorum* on oilseed rape, canola, and other crops. However, in most cases the reduction in disease incidence caused by *S. sclerotiorum* was not adequate or not statistically significant (Agrios, 2005; Schwartz and Steadman, 1989; Steadman and Boland, 2005). Onaran and Yanar, (2011) also showed the use of bacterial species including *Serratia*, *Burkholderia*, and *Pseudomonas* were significantly reduced the mycelial growth of *S. sclerotiorum* under *in-vitro* condition. *Coniothyrium minitans*, sold commercially as Contans, WG, is also a well-documented biocontrol agent of *S. sclerotiorum*, which infects and degrads the sclerotia in soil (Patridge et al. 2006; Fiume and Fiume, 2005; Jones et al. 2004). *Coniothyrium minitans* has demonstrated season-long efficacy for white mold in lima bean (Everts, 2003, unpublished). Although, Contans, by using the sclerotia as a food source, reduces initial inoculum of the white mold pathogen, multi-year reductions in inoculum have not been studied. Direct application of Contans to the soil and incorporation in advance of planting or immediately after seed germination may significantly reduce disease development and thus should be further investigated.

1.5.3. Use of resistant plant materials

Use of resistant plant materials is also used as a management strategy to control white mold or stem rot on several crops. Vuong et al. (2004) evaluated soybean, dry bean and sunflower cultivars and demonstrated that there were significant differences among cultivars in disease development. The use of resistant cultivars to manage *S. sclerotiorum* in different crops is

widespread. Host resistance is available in soybean (Willbur et al. 2017; Zhao et al. 2015; Arahana et al. 2001), canola (Bradely et al. 2006; Hu et al. 2005), common bean (Antonio et al. 2008), potato (Ojaghian, 2010), sunflower (Vuong et al. 2004), lettuce (Fiume et al. 2005; Grube and Ryder, 2004), and rapeseed (Xu et al. 2014). In lima bean, on the other hand, there are no identified resistant genotypes to *S. sclerotiorum*.

1.5.4. Cultural practices

Cultural practices to modify the canopy in a way that reduces the intensity and duration of a disease-favorable microclimate include row spacing and orientation, modification of nitrogen fertilizer application, and cultivar selection, and can lessen white mold severity (Heffer Link and Johnson, 2007). Crop rotation with non-host crops such as corn, wheat, barley, or oats reduces the number of sclerotia in the soil by loss of viability over time (Heffer Link and Johnson, 2007; Rousseau et al. 2007; Gracia-Garza et al. 2002). No-till fields facilitate fewer apothecia and lower disease severity (Gracia-Garza et al. 2002; Kurle et al. 2001; Workneh and Yang 2000). However, some researches showed that deep tillage reduces disease incidence by removing sclerotia from the upper soil profile, which will reduce the number of apothecia produced (Mueller et al. 2002b). High plant populations contribute to dense, closed canopies and increased Sclerotinia stem rot incidence in soybean (Lee et al. 2005; Kurle et al. 2001). Lima bean should be planted at recommended minimum seeding rates that maintain yield potential, and high plant populations should be avoided, especially in fields with a history of white mold. Narrow plant row spacing may lead to faster and more complete canopy closure in soybean (Peltier et al. 2012) and in lima bean fields as well. Therefore wider row spacing may reduce levels of white mold in lima bean or other crop fields.

1.6. Rationale of the research

There is currently no information on the diversity and aggressiveness of *S. sclerotiorum* that infects lima bean in mid-Atlantic region. Nor is there information on how populations of *S. sclerotiorum* on lima bean in Delaware and Maryland and other production regions such as New York compare in diversity and aggressiveness. In addition, no comparison, in terms of MCGs, oxalic acid productions, etc, has been done between *S. sclerotiorum* isolated from lima bean vs. other crops in the mid-Atlantic. There are no specific fungicide application guidelines for lima

beans in the region because little research has been conducted so far. Currently, the growers are using the spray guidelines developed on green or snap bean along with intensive scouting. Studies of white mold on snap bean cannot be directly applied to lima bean since the two crops respond differently. The growing season of lima bean is approximately 30 days longer than that of snap beans and also lima bean and snap bean have different growth habit. Although, *C. minitans* provides season-long efficacy for white mold in lima bean, multi-year reductions in inoculum have not been studied yet and thus adoption of Contans by growers in mid-Atlantic region is very low. To our knowledge, there is no available study on the molecular diversity of *S. sclerotiorum* isolated from different crops and locations in mid-Atlantic regions.

1.7. Statement of research objectives

The overall goal of the research was to develop strategies to study the diversity of *S. scleoritorum* isolates from lima bean and other crops in the region and to manage white mold disease on lima bean.

The first objective was to determine optimum time of application of Endura (boscalid) fungicide spray for white mold using a fungicide disease response. Provide growers with information on the optimum time to spray for white mold under continuous disease pressure.

The second objective was to determine aggressiveness (measured as stem lesion length) and oxalic acid production among isolates of *S. sclerotiorum* collected from different fields of lima bean and other crops in the mid-Atlantic region.

The third objective was to determine MCGs and molecular diversity among isolates of *S. sclerotiorum* collected from different fields of lima bean and other crops in the mid-Atlantic region.

The fourth objective was to determine the sensitivity of *S. sclerotiorum* isolates collected from different fields of lima bean and other crops in the mid-Atlantic region to the fungicide a.i.'s boscalid, fludioxonil, cyprodinil, thiophanate-methyl, prothioconazole, and fluazinam.

Chapter 2: White mold incidence, severity and lima bean yield response to fungicide application timing in the mid-Atlantic Region

ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary causes white mold on lima bean (*Phaseolus lunatus*) and significant yield and quality loss. Fungicides are widely used to manage white mold, yet there are no application-timing guidelines for lima bean. The knowledge gap prevents research for effective fungicide efficacy and integrated management that combines fungicides, biological, and cultural practices. Trials to determine the optimum timing for fungicide application were conducted from 2014 – 2017 with endura (0.76 kg ha^{-1}) at four locations in Delaware. Fungicides were applied (i) approximately 30 days after planting (DAP) when at least 20% of plants had opened flowers; (ii) approximately 37 DAP when 100% of plants had opened flowers; (iii) approximately 44 DAP; (iv) 51-55 DAP; (v) at 20% flowering plus one week later [30+37 DAP]; and (vi) non-sprayed [non-treated control (NTC)]. Disease incidence as number of infected plants m^{-1} of row, disease severity as percentage of infected tillers per plant m^{-1} of row and yield (kg) from 2.3 m^2 subsection of row area were recorded. Data were analyzed using Proc GLIMMIX. Disease incidence was reduced by 6.4%, 5.4%, 3.9%, and 7.6% compared to NTC when fungicides were applied 30 DAP (P<0.0001), 37 DAP (P<0.0001), 44 DAP (P<0.0128), and 30+37 DAP (P<0.0001, respectively. These application timings also reduced disease severity by 5.7%, 8.0%, 6.0%, and 7.0% compared to NTC, respectively. Earlier (i.e. 30 to 44 DAP) or within 2 weeks of 20% flowering and double treatment of boscalid reduced Disease incidence and Disease severity and improved yield of lima bean.

2.1. Introduction

Lima bean (*Phaseolus lunatus*) is an important crop grown in the mid-Atlantic region of the U.S. In 2017 and 2018, lima beans were grown on more hectares in Delaware, 3,889 and 3,318 hectares, respectively than any other vegetable crop, an area greater than any other state in the U.S. (DASB 2018; USDA-NASS 2019). Lima bean production averaged 3.4 tons ha^{-1} in Delaware and an annual production of 12,500 metric tons was estimated in 2017 and sold at a price of \$0.6 kg^{-1} (DASB 2018). Similarly, in Maryland production of lima bean for processing is expanding annually (Johnson 2014). Despite its regional importance, yield per hectare of lima bean in the mid-Atlantic is lower than in other growing region (Johnson 2014). For example, in 2013 Delaware yield was 1,278 kg ha^{-1} compared to 2,802 kg ha^{-1} in California (USDA-NASS 2013). High temperatures, especially night temperatures (> 32^{o}C) in Delaware lead to high flower abscission and reduced pod set and pod loss (Ernest et al. 2017). Diseases such as white mold also result in significant yield loss in lima bean (ANR 2014; Ernest et al. 2017; Johnson 2014; Kee et. al. 1997; Wootten 1994).

White mold is caused by the necrotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, which colonizes senescent blossoms of lima bean, establishes in developing pods, and reduces yield up to 40% in highly infested fields (Everts 2006). White mold is endemic in the region where common rotational crops are also hosts, including common bean (*Phaseolus vulgaris* L.) (del Río et al. 2004; Miklas et al. 2013), soybean (*Glycine max*) (Mueller et al. 1999), canola (*Brassica napus*) (Xiaojia et al. 2005), and pea (*Pisum sativum*) (Portera et al. 2009). In addition to reducing yield, white mold reduces seed quality of lima bean (Everts 2006; Kee et al. 2004).

Lima bean growers and processors in the eastern U.S. ranked white mold as their top disease concern in an industry panel-working group (Everts 2006, 2002). In addition to reducing yield through pod destruction, the presence of sclerotia may contaminate the frozen or canned products, where the tolerance limit for sclerotia is zero (Everts et al. 2002).

Moderate to cool temperatures of 19 - 24°C and high moisture or a relative humidity greater than 80% are highly favorable for white mold development (Harikrishnan and Del Río 2006; Heran et al. 1999; Schwartz and Steadman 1989; Weiss and Steadman 1980). Therefore, the epidemics are most frequent in humid temperate and sub-tropical regions (Miklas et al. 2013;

Schwartz and Steadman 1989). In the mid-Atlantic region, favorable environmental conditions for white mold development exist during both spring and fall seasons. Lima bean grown in fields near the Atlantic coast often experience long periods of fog that increase the length of periods with high soil moisture. Conditions that favor white mold, average temperature of 20°C, >80% relative humidity, 19°C soil temperature, and soil volumetric water content of 0.171 m^3/m^3, between field capacity and plant available water content, frequently occurred between July to October in the years 2014 to 2017 (Figure 2.1).

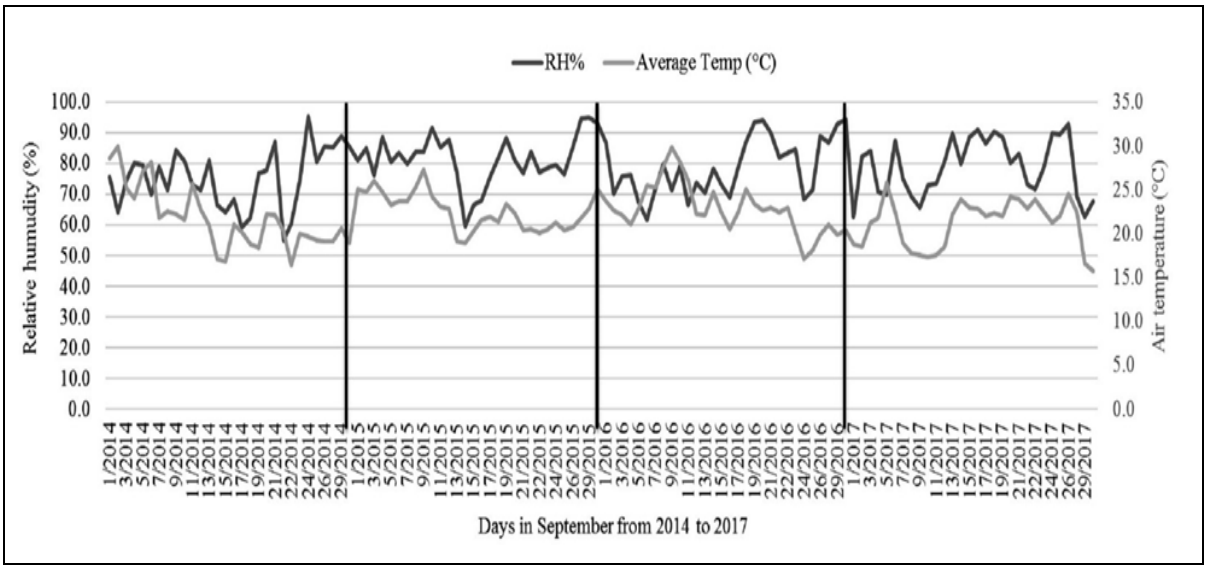


Figure 2.1. A graphical representation of weather conditions representing average temperature (°C), relative humidity (%), and soil temperature (°C) in Lewis, Delaware from 2014 - 2017.

The ability of *S. sclerotiorum* to form sclerotia that survive up to 5 years in the soil and its wide host range of 400 plant species make white mold difficult to manage (Boland and Hall 1994; Bolton et al. 2006; Fernando et al. 2004; Heffer Link and Johnson, 2007). White mold incidence and severity are highly variable and difficult to predict (Mahoney et al. 2014; McDonald and Boland 2004) and management of *S. sclerotiorum* varies among crops, where different approaches are used. Host resistance is an effective disease management strategy in soybeans (Huzar-Novakowiski et al. 2017; Vuong et al. 2004) and there are a few resistant or tolerant snap bean and dry bean cultivars (Bolton et al. 2006; Lehner et al. 2017; Schwartz and Singh 2013), however no resistant or tolerant cultivars have been reported in lima bean. The cover crop *Brassica juncea* 'Vittasso' produces biofumigant volatiles, which reduce germination of sclerotia *in vitro*, but has not been successful under field conditions (Warmington and

554 Clarkson 2016). Cultural practices such as increasing row spacing greater than 50 cm, which
555 reduces plant populations and disease (Heffer Link and Johnson, 2007; Peltier et al. 2012),
556 cannot be used in a lima bean crop because row spacing is dictated by the communal use of
557 harvest equipment.

558 Fungicides with active ingredients (a.i.) fludioxonil, boscalid, fluazinam, picoxystrobin,
559 fluopyram, benomyl, tebuconazole, thiophanate-methyl, and vinclozolin were evaluated to
560 control disease caused by *S. sclerotiorum* in different crops (Hunter et al. 1978; Mahoney et al.
561 2014; Morton and Hall 1989; Mueller et al. 1999, 2002, and 2004 Steadman 1979). Willbur et al.
562 (2019), for example, showed that boscalid and picoxystrobin provided significant reductions in
563 Sclerotinia stem rot severity and best yield benefit in soybean and dry bean.

564 Growers in the mid-Atlantic region rely on monitoring guidelines developed to schedule
565 fungicide applications on snap beans presuming that lima bean shares similarities in crop canopy.
566 However, some lima bean cultivars flower indeterminately and all cultivars have a longer
567 growing season, approximately 20 days longer, than snap bean (Johnson 2014; Maynard and
568 Hochmuth 2007). Endura 70WG (a.i. boscalid, BASF, Research Triangle Park, NC), Switch (a.i.
569 fludioxonil and cyprodinil, Syngenta Crop Protection, Greensboro, NC), Topsin 4.5 FL (a.i.
570 thiophanate-methyl, United Phosphorus, Inc., King of Prussia, PA), and Omega 500 F (a.i.
571 fluazinam, Syngenta Crop Protection, Greensboro, NC) are currently registered in the United
572 States for control of white mold caused by *S. sclerotiorum* on baby lima bean. When applied at
573 labeled rates and at the proper time, all these fungicides significantly reduced white mold
574 incidence on canola, potato and dry bean, compared with the non-treated control (Bradley et al.
575 2006; Johnson and Atallah 2006; Mahoney et al. 2014). Boscalid is a site-specific fungicide from
576 the succinate dehydrogenase inhibitors (SDHI) class (FRAC group 7) and is registered on lima
577 bean. It was among the most efficacious fungicides for white mold control in many crops (Lehner
578 et al. 2017; Mahoney et al. 2014). In a preliminary study by Everts et al. (2003), boscalid,
579 cyprodinil plus fludioxonil and *Bacillus subtillis* strain QST 713 applied at 10 days after first
580 flower significantly reduced white mold compared to non-treated plants. This timing was later
581 than guidelines developed for snap bean, and suggested that fungicides applied later in the season
582 may be efficacious including improving the yield of lima bean by up to 12% (Everts et al. 2002).

Using fungicide application guidelines developed for snap beans may lead to substantial yield loss in lima bean because those guidelines indicate sprays are not efficacious when applied at later growth stages (Lehner et al. 2017). In addition, information on application timing for managing white mold in lima bean is necessary to study fungicide efficacy, and the integration of fungicide management and cultural tactics, and biological controls such as *Bacillus amyloliquefaciens* D747 (Pethybridge et al. 2019a), *Coniothyrium minitans* (Everts 2006), and *Bacillus subtillis* strain QST 713 (Everts et al. 2003). The objective of this study was to determine the optimum time of application of boscalid (Endura) fungicide for white mold control in lima bean.

2.2. Materials and Methods

To determine the optimum time to apply fungicides for management of white mold in lima bean, trials with Endura 70WG fungicide were conducted during the summers of 2014, 2015, 2016, and 2017 at four locations in eastern Sussex County, Delaware. In each year the trials were conducted at two different locations, except for 2014, where the experiment was conducted only in one location, for a total of seven trials. The trials were conducted in commercial lima bean production fields, where all the locations had a history of white mold and the environmental conditions were typically favorable for disease development. Endura was applied at a rate of 0.76 kg ha^{-1} at six different timings with a carbon dioxide pressurized backpack sprayer (R&D Sprayers, BellSpray Inc., Opelousas, LA) equipped with hand-held boom with 4 nozzles spaced 46 cm apart, which are designed to cover the inner two rows and calibrated to deliver 187 liters ha^{-1} at 276 kPa. The application timings were: (i) at 20% flowering [approx. 30 days after planting (DAP)] when at least 20% of the plants had opened flowers; (ii) one week later (approx. 37 DAP) where 100% of the plants had opened flowers; (iii) two weeks later (approx. 44 DAP); (iv) three weeks later (51 -55 DAP); (v) at 20% flowering plus one week later [double treated (DT) or 30 and 37 DAP]; and (vi) non-sprayed [non-treated control (NTC)]. A summary of locations, cultivars, time of application of Endura and growth stages of lima bean is shown in Table 2.1. The variations shown in the application dates (in Table 2.1) is because the applications were conducted on different days due to weather conditions and because weather affected when plants achieved the target growth stage during the trial period. Treatments were arranged in a randomized complete block design (RCBD) with four replications. Plots were 3.0

m wide by 9.1 m long, each having four rows of lima beans spaced 76 cm apart. The growers irrigated, managed weeds and insects, and fertilized the crop according to standard commercial practices. Fungicides were applied to the field around the experiment by ground rig tractor and we made sure that there was no drift to our plots when the grower sprayed fungicides for control of white mold to the section of the field containing the experimental plots.

Immediately before harvest, three one-meter sections from inner two rows were selected in each plot for visual ratings. White mold disease incidence (DI), as the number of plants with visible signs or symptoms/total number of plants per 1 m section of a row; and disease severity (DS), rated as number of stalks or tillers with visible signs or symptoms of disease/total number of tillers present per plant in 1 m section of a row. Lima bean yield was recorded by removing all plants from three 1 m sections of a row (a total of 2.3 m^2 subsection of row area) per plot. To minimize a potential edge effect, during both ratings and at harvest, only plants from the two interior rows of the plots were rated and harvested. The harvested plants were threshed using a stationary thresher available in University of Delaware Carvel Research & Education Center (UD-REC) in Georgetown. Before weighing seed samples, the threshed seeds were cleaned using a 0.5 cm x 2 cm screen and handpicked for discolored or misshaped seeds or for any foreign materials present. In both ratings and harvest the average of the three randomly selected subsamples per plot was used for statistical analysis.

Table 2.1. Years, locations, cultivars, timings of Endura (a.i. boscalid) application used to evaluate the effect of timing of fungicide application to control white mold in lima bean from 2014 to 2017.

Year	Locations	Location code	Cultivars	Time of Endura application, days after planting (DAP)[a]
2017	Mulberry Knoll Rd. Lewes, DE	MK-17	Meadow	37[b], 45[c], 52[d], 60[e], and 37 + 45[f]
	Lynn Rd., Lewes, DE	LR-17	Cypress	31[b], 39[c], 46[d], 53[e], and 31 + 39[f]
2016	Lynn Rd., Lewes, DE	LR-16	Cypress	30[b], 37[c], 44[d], 50[e], and 27 + 37[f]
	Gill's Neck Rd. Lewes, DE	GN-16	Cypress	31[b], 41[c], 48[d], 54[e], and 31 + 41[f]
2015	Mulberry Knoll Rd. Lewes, DE	MK-15	Meadow	30[b], 37[c], 44[d], 51[e], and 30 + 37[f]
	John J Williams Hwy, Lewes, DE	JJW-15	Meadow	30[b], 37[c], 44[d], 51[e], and 30 + 37[f]
2014	Gill's Neck Rd. Lewes, DE	GN-14	Cypress	30[b], 37[c], 44[d], 51[e], and 30 + 37[f]

[a] Changes in DAP in different years was due to unfavorable weather conditions to spray or to variation in flowering onset due to weather.

[b] The fungicide was applied when approximately 20% of plants had one or more open blossom.

[c] The fungicide was applied when approximately 100% of plants had one or more open blossom.

[d] The fungicide was applied when approximately one week after 100% of plants had one or more open blossom.

[e] The fungicide was applied at approximately 10% of pods were at the pin-pod stage of development.

[f] Fungicides were applied when approximately 20% of plants had one or more open blossom and when approximately 100% of plants had one or more open blossom.

2.3. Statistical analysis

The effect of fungicide application timing on disease incidence, disease severity, and yield of lima bean was modeled using generalized linear mixed model (GLIMMIX) because the response variables were not normally distributed (SAS University Edition version 9.4 and JMP® Pro 14.1.0, SAS Institute Inc., Cary, NC, USA). GLIMMIX also recognizes the binomial

distribution of both the disease incidence and disease severity variables and the continuous distribution of the yield variable. Fungicide application timing and year were the fixed effects and location and block were random effects. Mean separation was conducted using Tukey-Kramer least squares means adjustment for multiple comparisons. Fitted model using simple linear regression analyses were used to predict the relationship between yield and disease incidence and disease severity.

2.4. Results

2.4.1. Disease incidence

Disease incidence was significantly affected by application timing by year (F=7.97, *P* <0.0001; Table 2.2) and therefore each year was analyzed separately. In 2014, where the experiment was conducted only once and disease incidence was low, there were no differences among fungicide application timing and the NTC (Table 2.2). In 2015, where disease incidence was also low, fungicides applied at 30, 37 or the double application significantly reduced disease incidence compared to the NTC. The highest disease incidence was observed in 2016 and fungicides applied at 30, 44, 51-55 DAP and the double treatment reduced disease incidence by 70%. 50%, 54%, and 69%, respectively compared to the NTC. In 2017, disease incidence was only significantly reduced by the double treatment in comparison to the NTC. However, fungicides applied 30, 37, and 44 DAP, reduced disease incidence by 60%, 82%, and 50% compared to application at 51-55 DAP.

2.4.2. Disease severity

The interaction between fungicide application timing and year was also significant for disease severity (F= 4.81, *P*<0.0001; Table 2). In 2014 when disease severity was low there were no significant differences in disease severity among fungicide application timings. In 2015 disease severity was moderate and all fungicide timings, including the late spray at 51-55 DAP, reduced disease severity compared to the NTC. Disease severity was also moderate in 2016 and was significantly reduced by fungicide applications at 30, 44, 51-55 DAP and the double treatment, by 62%, 67%, 63%, and 55%, compared to the NTC. The highest disease severity occurred in 2017. In that year, disease severity was highly variable among plots, and the fungicide application at 37 DAP reduced disease severity by 94% compared to the NTC.

Although other fungicide timings did not significantly reduce disease severity compared to the NTC, disease severity in plots sprayed 51-55 DAP was significantly higher, between 96% and 64%, than any other fungicide timings, (Table 2.2).

2.4.3. Lima bean yield

Lima bean yield was not significantly affected (F=1.29; *P*=0.2731; Table 2.2) by application timing. However, the yield was significantly affected by year (F=91.64; *P*<0.0001). For example, kg ha-1 in 2016 were significantly lower (*P*<0.0001) by 1,000 and 800 kg ha^{-1} compared to 2014 and 2017, respectively. Similarly, yields in 2015 were significantly lower (*P*<0.0001) by 1,000 kg ha^{-1} compared to 2014 and 2017, respectively (Figure 2.1).

2.4.4. Relationship between disease incidence, disease severity, and yield of lima bean

Yield of lima bean was significantly negatively correlated (t=-2.46; *P*= 0.0150) with disease incidence. The relationship was $kg\ ha^{-1} = 2.1 - 0.01*disease\ incidence + 0.6$. However, this relationship explained only 4% (or R^2=0.0351) of the variation in yield of lima bean. In addition, yield was not significantly correlated with disease severity, (t=-0.65; *P*= 0.5148) (Figure 2.2).

Table 2.2. Analysis of variance (Type III tests of fixed effects) of the fungicide[z] (Endura) application timing [Days after planting (DAP)] on disease incidence (%), disease severity (%), and yield (kg/ha) of lima bean at four locations from 2014 to 2017 in Lewis, DE and the means of disease incidence and disease severity of lima bean when fungicides were applied at different timings.

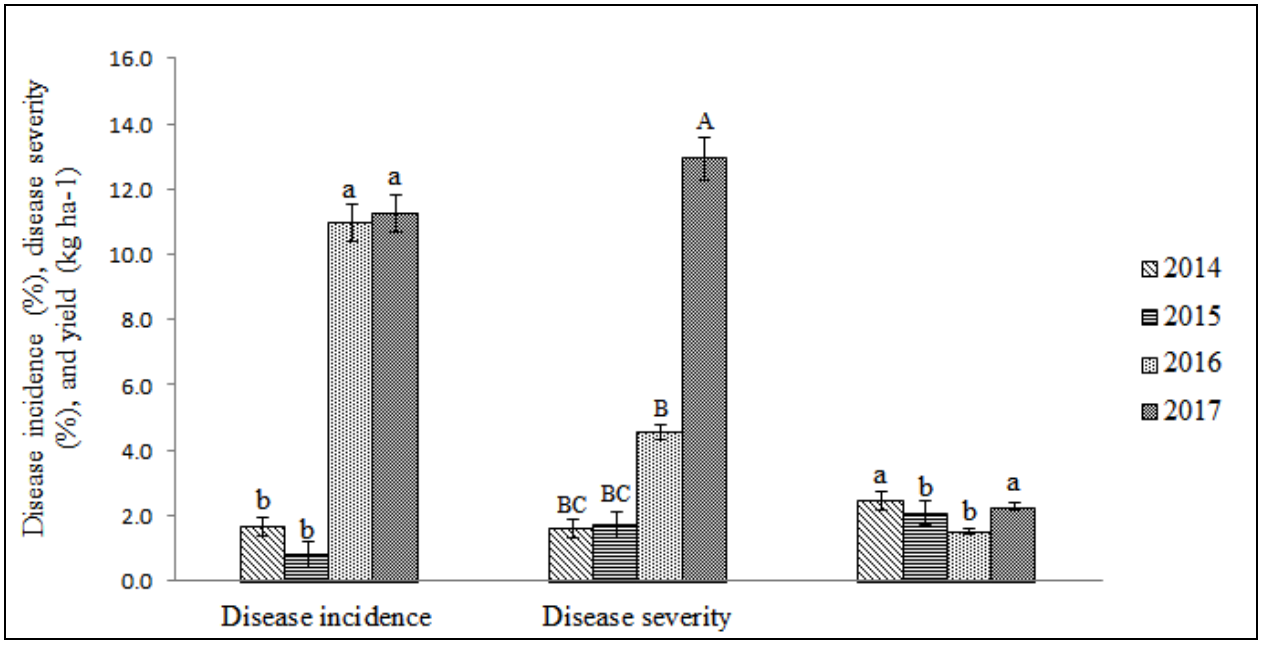
	Disease incidence				Disease severity				Yield (kg ha^{-1})			
Source of variance	DF	Mean Sq.	F	P > F	DF	Mean Sq.	F	P > F	DF	Mean Sq.	F	P > F
Endura timing	5	255.89	7.97	<0.0001	5	412.07	7.43	<0.0001	5	0.16	1.29	0.2731
Year	3	1469.69	59.81	<0.0001	3	1242.52	28.79	<0.0001	3	6.82	91.64	<0.0001
Endura timing*Year	15	1737.60	4.71	<0.0001	15	207.66	4.81	<0.0001	15	0.18	1.61	0.0773

Solutions for fixed effects

	Disease incidence[y]				Disease severity[y]			
Fungicide application timing	2014	2015	2016	2017	2014	2015	2016	2017
30+37 DAP	0.00 a	0.08 b	6.50 b	4.49 c	0.00 a	0.25 b	4.00 b	6.37 bc
30 DAP	0.83 a	0.15 b	6.27 b	8.27 bc	0.83 a	0.22 b	3.33 b	11.14 bc
37 DAP	0.83 a	0.20 b	12.45 ab	5.63 bc	0.83 a	0.61 b	4.67 ab	1.20 c
44 DAP	3.33 a	0.35 ab	10.37 b	11.35 bc	1.25 a	1.71 b	3.00 b	8.49 bc
53 DAP	3.33 a	0.35 ab	9.55 b	22.70 a	5.33 a	1.34 b	3.33 b	31.51 a
Non-treated control	1.67 a	0.96 a	20.64 a	15.06 ab	1.25 a	6.44 a	9.00 a	18.92 ab
Pr > F|	0.4342	0.0034	<0.0001	<0.0001	0.5887	<0.0001	0.0015	<0.0001

[z] Endura 70WG (a.i. boscalid, BASF, Research Triangle Park, NC) was applied at a rate of 0.76 kg ha^{-1} at five different timings or not applied.

[y]Means followed by the same letter within a column are not significantly different ($P \leq 0.05$) according to, Tukey-Kramer means comparison test).



701 Figure 2.2. Variations in disease incidence (%), disease severity (%), and yield (kg ha-1*1000) of
702 lima bean across years in Lewes, Delaware. Presence of the same letter above the bars indicates
703 no significant difference between years within each variable.

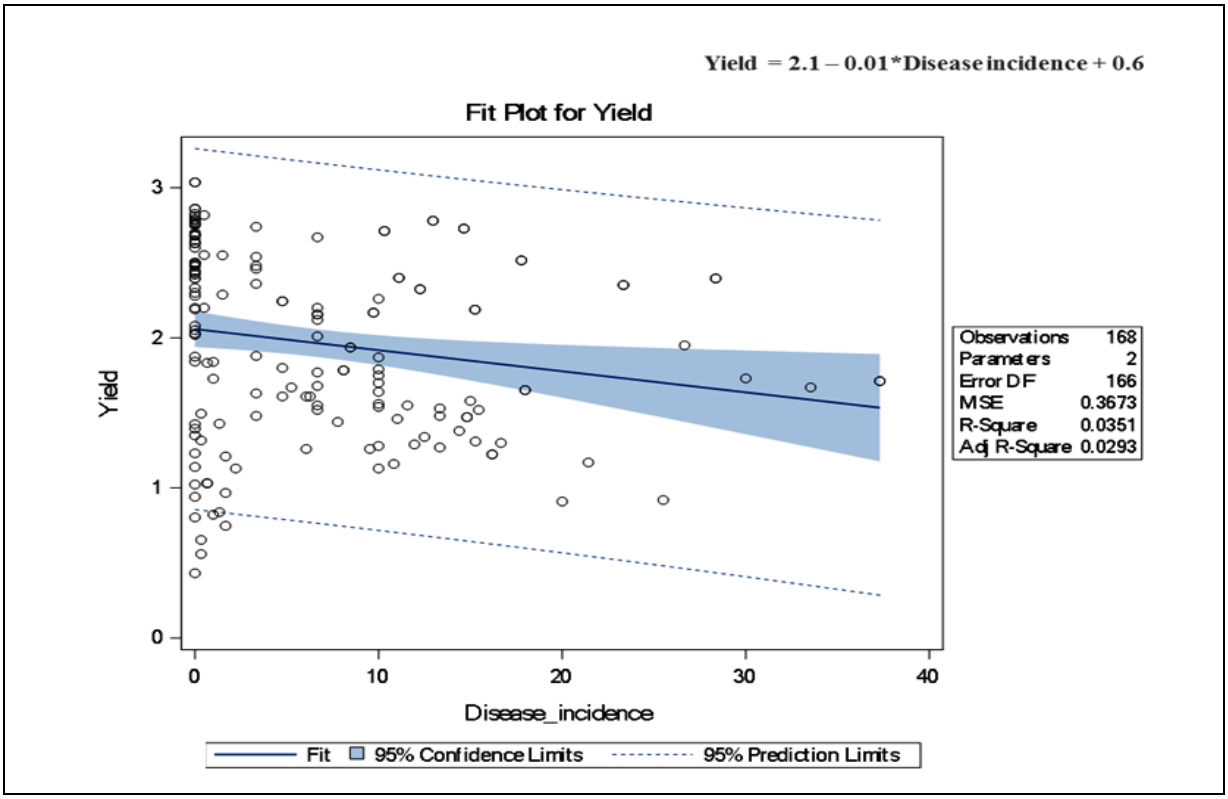


Figure 2.3. Fit plot of the relationship between yield and white mold disease incidence caused by *Sclerotinia sclerotiorum*. The plot consists of a scatter plot of the disease incidence and yield data overlaid with the regression line, and 95% confidence and prediction limits.

2.5. Discussion

The development and implementation of appropriate fungicide application timing and frequency guidelines is important to reduce substantial disease and yield loss in lima bean. However, the appropriate timing of a fungicide application for disease management can vary based crop type, growth stage, flowering stages and flower phenology, disease onset, soil moisture, environmental conditions, and specific pathogen or disease characteristics such as how initial infection occurs (Jeger 2004; Heffer Link and Johnson 2007; Matheron and Porchas 2004). For example, fungicide spray at 10 to 100 % of flowering stage was found to be effective to manage white mold in different crops (Dueck et al. 1983; Johnson and Atallah 2006; Schwartz et al. 2004; Willbur et al. 2019). In our study, earlier fungicide application timing(s) or spray at 20 to 100% flowering stage provided significant reduction in white mold incidence and severity

when disease pressure was moderate or high, compared to the NTC or the late application at 51-55 DAP. In addition, in 2016 the late applications performed well although in 2017, the latest application time (51-55 DAP) was the highest. In many crops with similar flower phenology and days-to-harvest as lima bean (McCreary et al. 2016), earlier application timing was most efficacious application to suppress white mold and improve yield. The importance of precise timing of fungicide applications has also been demonstrated in other pathosystems. The optimum application timing for boscalid on common bean was when plants reached 50 to 100% flowering (Schwartz et al. 2004). Johnson and Atallah (2006) also reported that disease incidence in potato was significantly reduced when thiophanate-methyl and fluazinam were applied when 100% of plants had one or more open blossoms. However, earlier applications were also effective where boscalid provided significant reduction of incidence when applied at 10% of primary inflorescences in potato plants. White mold incidence and/or severity increased when fungicide application occurred after 100% of snap bean plants had open flowers (Pethybridge et al. 2019b). They also found that late applications of thiophanate-methyl, a commonly used fungicide in New York for management of white mold in snap bean, were less effective than late application of boscalid and fluazinam. In contrast, we observed significant reductions in disease incidence and disease severity even when plants were sprayed one week after 100% flower, and in some years when sprayed two weeks after 100% flower. This indicates that sprays applied later than current guidelines could be used by growers, but also that they are not optimum and should be evaluated in combination with cultural or biological practices to further reduce disease. It likely also indicates that scheduling fungicide application based on both flowering, and environmental favorability may be more effective.

In this study, lima bean yield was not significantly affected by the fungicide application timing. However, the yield was significantly weakly correlated to DI. The lack of statistically significant direct effect of fungicide application on yield could have resulted from the relatively low disease incidence and disease severity observed, particularly in the years from 2014 to 2016, which might have been insufficient to cause significant yield difference. Alternatively, a yield sample larger than 2.3-m^2 row area harvested may have captured differences. Lima bean has large yield variations among plants (Ziska et al. 1985).

In the current experiment, we only evaluated one fungicide, boscalid. Boscalid, a site-specific fungicide from the succinate dehydrogenase inhibitors (SDHIs) class (Avenot and Michailides 2010; FRAC 2015; Matsson and Hederstedt 2001; McKaey et al. 2011), is registered on lima bean and might be more active when used preventively or sprayed at early flowering stages of lima bean than other fungicides. Lehner et al. (2017) and Mahoney et al. (2014) reported that boscalid was among the most efficacious fungicides for white mold control in dry bean compared to other fungicides. Other research has evaluated fungicides for white mold on lima bean and other crops. For example, a preliminary study by Everts et al. (2003) evaluated boscalid and cyprodinil plus fludioxonil applied at 10 days after first flower and both showed significantly reduced white mold compared to non-treated plants.

Several factors may favor late season disease development and impact efficacy of a fungicide application. For example, late season fungicide applications coincide with cool fall temperatures that are highly favorable to white mold development (Byrne et al. 2014; Huzar-Novakowiski et al. 2017; Mueller et al. 2004, 2002). Row closure within a lima bean field may also play a role in white mold development. Dense canopy and row closure provide an ideal microenvironment for white mold development, particularly apothecial emergence. Boscalid may not penetrate the canopy to reach senescent plant tissue lower in the canopy.

Resistance development to boscalid or other SDHI class of fungicides was reported on *S. sclerotiorum* in China (Wang et al. 2015), in Germany (Glättli et al. 2009; Stammler et al. 2010), and in France (Penaud et al. 2003). Although fungicide resistant populations of *S. sclerotiorum* have not yet been reported in mid-Atlantic region, this research will help differentiate white mold management failure due to improper timing from failures due to resistance development.

Developing disease management practices based on field history, diversity of the population of *S. sclerotiorum*, and application of fungicides at the optimum time will provide better management of white mold in lima bean. Our work will guide research on integrated disease management that combines fungicide applications with non-chemical practices such as avoiding excessive irrigation preceding and during flowering, rotation to non-hosts, use of resistant cultivars, use of cereal (such as rye) residues, or harvesting infested fields later than non-

infested fields (Arahana et al. 2001; Peltier et al. 2012; Pethybridge et al. 2019c; Yadav et al. 2015).

Although optimum timing for fungicides on lima bean was between 20 and 100% flowering, our study showed that later fungicide applications also provide some disease reduction. The double (30+37 DAP) boscalid application timing provided the most effective disease reduction. However, growers apply only one application for economic reasons. This information will assist in developing research on integrated methods to manage white mold in lima bean, and to research efficacy of additional fungicides. As part of integrated disease management practices, an application timing of boscalid within 3 weeks of 20% of plants with open blossom can significantly reduce disease severity.

Chapter 3: Variation in aggressiveness and oxalic acid production of *Sclerotinia sclerotiorum* isolates on lima bean, soybean, and common bean cultivars in the mid-Atlantic region.

ABSTRACT

Oxalic acid production by *Sclerotinia sclerotiorum* isolates has been associated with aggressiveness, which often expressed as lesion size or length. High variability of oxalic acid within *S. sclerotiorum* populations may influence performance of cultivar resistance and the effectiveness of fungicides across pathogen populations. The aggressiveness of twenty-five *S. sclerotiorum* isolates collected from the mid-Atlantic and other regions of the US was tested on five lima bean, two soybean (checks), and two common bean (checks) cultivars using the straw inoculation method. To determine, if oxalic acid (OA) was involved in the aggressiveness of the isolates, OA production was also quantified from infected plant tissue using spectrophotometry. *S. sclerotiorum* isolates significantly differed in their aggressiveness on the crops and cultivars inoculated. Lesion length on lima bean was the greatest (7.1 cm) compared to soybean (6.7 cm), and common bean (5.5 cm). Jackson Wonder lima bean developed the shortest lesion length of the cultivars, 1.7 cm less than the lesion length on NKS1990, which was the resistant soybean. Oxalic acid was greatest in Williams82, which was the susceptible soybean cultivar, and in 184-85, which was the susceptible lima bean cultivar. Isolates 13, which was obtained from soybean from NJ significantly caused the longest lesion length compared to all isolates. Isolate 13 also resulted in the highest oxalic acid accumulation compared to all, except isolate 4. There was slightly a weak but significant correlation (r=0.37; $P<0.0001$) between lesion length and oxalic acid accumulation by the isolates and the correlation ranged from 0.03 for isolate 7 to moderate, r=0.55 for isolate 25.

3.1. Introduction

Host resistance for diseases caused by *Sclerotinia sclerotiorum* is an effective management strategy in several crops such as soybeans (Huzar-Novakowiski et al. 2017; Vuong et al., 2004). However, no resistant or tolerant cultivars have been reported in lima bean. Likewise, there are few resistant or tolerant snap bean and dry bean cultivars (Lehner et al. 2017; Schwartz and Singh 2013; Bolton et al. 2006). Variability of pathogenicity factors within the *S. sclerotiorum* population is important in the development of tolerant or resistant cultivars. Lima bean cultivars should be screened against diverse population of *S. sclerotiorum* isolates, preferably genetically distinct and collected from different regions. Diversity of *S. sclerotiorum* has been identified by measuring isolate aggressiveness (or severity measured as lesion length /length) and by quantifying the production of virulence/pathogenicity factors such as oxalic and other organic acids (Taylor et al. 2015; Durman et al. 2005; Kull et al. 2004). Cessna et al. (2000) hypothesized that aggressiveness of *S. sclerotiorum* was related to variation in isolate oxalic acid production. Oxalic acid increases pathogenesis through acidification of host tissues and sequestration of calcium from host cell walls (Chen et al. 2013; Durman et al. 2005; Cessna et al. 2000).

Aggressiveness of *S. sclerotiorum* isolates on different hosts has been the subject of several studies and was reported to significantly vary among different isolates (Willbur et al. 2017; Zancan et al. 2015; Attanayake et al. 2013; Otto-Hanson et al. 2011; Ekins et al. 2007; Kull et al. 2004). Ekins et al. (2007) reported that isolates of *S. sclerotiorum*, collected from head and basal stem rots of sunflower from different locations in Australia differed significantly in aggressiveness (measured as stem lesion length) on the same crop. However the aggressiveness or lesion length was not related to the locations of collection or to the plant from which they were derived. A similar result was reported by Li et al. (2008) where *S. sclerotiorum* isolates collected from sunflower from China, Canada, and England varied significantly in aggressiveness, but the variation was not related to the geographic location of the isolates. There were significant differences in aggressiveness on dry bean among *S. sclerotiorum* isolates that originated from dry bean fields from different locations in Brazil (Zancan et al. 2015). In contrary, other studies reported that aggressiveness did not vary among isolates (Lehner et al. 2015; Attalah et al. 2004; Auclair et al. 2004; Sexton and Howlett 2004).

Aggressiveness of *S. sclerotiorum* also largely depends on the pathogen's physical and biochemical characteristics. *S. sclerotiorum* produces different virulence factors such as oxalic, succinic, malic, fumarie and glycolic acids (Rogelio et al. 1970), which may be pathogenicity determinants. Several research reports showed that some of these acids, particularly oxalic acid contributed to virulence of this pathogen in several crop plants. For example, Stephen et al. (2000) showed that pathogenesis of *S. sclerotiorum* requires the secretion of oxalic acid to suppresses the oxidative burst capacity of the host plants to cause infection in tobacco and soybean. Noyes and Hancock, (1981) showed that wilted sunflower leaves from infected plants contained over 15 times the oxalic acid of leaves of healthy plants. They speculated that oxalic acid was involved in the pathogenicity of *S. sclerotiorum*. Additional studies confirmed that oxalic acid is a pathogenicity determinant for *S. sclerotiorum* in common bean (Godoy et al. 1990; Tu 1985), the model plant tobacco, where it induces a programmed cell death (PCD) (Kyoung et al. 2008), tomato where it also induces PCD (Williams et al. 2011; Magro et al. 1984), sunflower (Magro et al. 1984), and Arabidopsis (Xiaoting et al. 2013; Rollins 2003). However, there are also reports that oxalic acid is not pathogenicity factor for *S. sclerotiorum*. For example, Liangsheng et al. (2015) reported that even though wild-type *S. sclerotiorum* produced up to 50 mM more of oxalic acid than UV-induced mutants that had lost oxalate production, the oxalate-minus mutants retained pathogenicity on plants. This result suggest that it is low pH, not oxalic acid itself, that establishes the optimum conditions for growth, reproduction, pathogenicity and virulence of *S. sclerotiorum*.

Significant variations in disease severity caused by *S. sclerotiorum* infections also differed among cultivars, lines, or genotypes of the same host. For example, Auclair et al. (2004) and Vear et al. (2004) reported that significant differences in aggressiveness or disease severity were detected among cultivars of soybean and sunflower, respectively. Kull et al. (2003) reported that isolates significantly varied in aggressiveness between susceptible (Williams82) and resistant (NKS1990) soybean cultivars. Significant differences were observed between the different *Brassica* genotypes in their responses to different isolates of *S. sclerotiorum* and there was also a significant host-pathogen interaction (Garg et al. 2010). Variations in aggressiveness of *S. sclerotiorum* isolates in agricultural populations may impact cultivar performance (Kull et al. 2003). Variability in isolate aggressiveness has been associated with problems in evaluating and

breeding for resistance in host crops (Kull et al. 2004, 2001; Carpenter et al. 1999). However, there were no available reports on aggressiveness variations among different hosts. Our isolates collected from different locations provide opportunities to study pathotype aggressiveness among different screening locations and therefore, the result in the current work will be the first report showing variations of *S. sclerotiorum* aggressiveness variations on different host plants.

It is hypothesized that aggressiveness or pathogenicity on the lima bean plants is directly related to the quantity of oxalic acid produced by isolates of *S. sclerotiorum*. However, there is no information on the range of oxalic acid production or pathogenicity of *S. sclerotiorum* populations in mid-Atlantic region. Nor is there information on how populations of *S. sclerotiorum* on lima bean in Delaware and Maryland and other production regions such as New York compare in diversity and aggressiveness. Isolates obtained from same crop or location may behave similarly in aggressiveness and result in similar oxalic acid accumulation. The objective of this project was to determine how variable isolates of *S. sclerotiorum* from the mid-Atlantic region were in their ability to cause disease on lima bean, soybean, and common bean and to determine if aggressiveness correlates to oxalic acid produced by these isolates. We screened lima bean cultivars for their susceptibility to isolates of *S. sclerotiorum* and included NKS1990 and Williams82 soybean cultivars as resistant and susceptible reference genotypes, respectively.

3.2. Materials and Methods

3.2.1. Fungal isolates

Diseased lima beans as well as other *S. sclerotiorum* host crops listed in table 4 were collected from different fields in the mid-Atlantic region of the US. Additional isolates were obtained from other groups working on *S. sclerotiorum* in US (Table 3.1). Infected plants were washed in running tap water for 3 to 5 minutes, small pieces of plant tissue were excised from the advancing lesion area and immersed in 20% Tween20 solution (Sigma-Aldrich, Westport Center Dr, St. Louis, MO), surface disinfested in a 5% NaOCl solution for 3 to 5 minutes, immersed into 95 % EtOH for 1 minute, and finally re-immersed into ddH_2O for 3 to 5 minutes to remove the disinfectants. The disinfected plant pieces were placed on PDA and incubated at 25 - 30ºC in the dark for approximately a week. The isolates were identified based on as the presence of buff to white mycelium and subsequent sclerotia formation. The cultures were transferred into quarter

strength PDA by single hyphal tip method as described in Mandal and Dubey (2012) and maintained in slant test tubes at 4 or 5 $^{\circ}$C in refrigerator. A total of 25 isolates from infected plants and a control (non-inoculated) were used in the aggressiveness study.

Table 3.1. List of isolates of *Sclerotinia sclerotiorum* collected from different states/locations and crops.

Isolate number	Isolate names[a]	Year of origin or received	Crop	Locations[b]	Isolates provided by:
1	SAS 15-100-2	2015	Tomato	St. Mary's Co., MD	Benjamin Beale
2	SAS 15-100-3b	2015	Tomato	St. Mary's Co., MD	Benjamin Beale
3	SAS 15-100-4	2015	Tomato	St. Mary's Co., MD	Benjamin Beale
4	SAS 15-100-6	2015	Snap bean	Sussex Co., DE	Own
5	SAS 15-100-7	2015	Lima bean	Sussex Co., DE	Own
6	SS8-Pe-DE	2017	Peas	Sussex Co., DE	Nancy F. Gregory
7	SS9-Pe-DE	2017	Peas	Sussex Co., DE	Nancy F. Gregory
8	SAS 15-100-12	2015	Snap bean	Sussex Co., DE	Dr. Nathan Kleczewski and Andy Kness
9	SAS 15-100-13	2015	Snap bean	Sussex Co., DE	Own
10	SAS JRS#723	2016	Soybean	ND[c]	Dr. James Steadman
11	SAS JRS#587	2016	Sunflower	OR[d]	Dr. James Steadman
12	SAS JRS#160	2016	Lima bean	Westley, CA	Dr. James Steadman
13	SS 1-NJ-Sb	2016	Soybean	NJ[e]	Dr. VanGessel and Dr. Nathan Kleczewski
14	SAS 15-100-1	2015	Sunflower	Madison, GA	Songbird blend Ultra, Madison, GA
15	SAS 15-100-3a	2015	Lima bean	Sussex Co., DE	Own
16	SS2-DE-Lb-i	2016	Lima bean	Sussex Co., DE	Own
17	SS2-DE-Lb-ii	2016	Lima bean	Sussex Co., DE	Own
18	SS3-DE-Lb-i	2016	Lima bean	Sussex Co., DE	Own
19	SS3-DE-Lb-ii	2016	Lima bean	Sussex Co., DE	Own
20	14207-1(A)	2016	Lima bean	NY[e]	Dr. Sarah Pethybridge
21	14207-1(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge
22	14207-2(A)	2016	Lima bean	NY	Dr. Sarah Pethybridge
23	14207-2(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge
24	14207-3(A)	2016	Lima bean	NY	Dr. Sarah Pethybridge
25	14207-3(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge

[a] Original name of the isolates;
[b] County and state from which isolates were obtained;
[c,d,e] represents unknown county or city.

3.2.2. Plant growth

Lima bean, soybean, and common bean seeds were planted during spring of 2017 and 2018 in the research greenhouse of the Department of Plant Science and Landscape Architecture at the University of Maryland. Seeds were directly planted to a substrate mix that approximately containing 15% perlite and 85% Canadian Sphagnum peat moss potting mix (Sun Gro Horticulture®, Silver St. Agawam, MA 01001-2907, US) in sterile 15 cm pots and placed on a greenhouse bench. Five lima bean cultivars (Cypress, C-elite, Jackson Wonder, 184-85, and Dixie Butterpea), two soybean cultivars [William82 (susceptible), NKS1990 (moderately resistant)], and two common bean cultivars (Beryl and 6122) were planted. After germination, plants were fertilized with a solution of 15N-5P-15K 100 ppm fertilizer at 250 ppm three times per week. The greenhouse temperatures were 20 ± 1°C (night, 12 h) and 26 ± 1°C (day, 12 h). The greenhouse had supplemental lighting of 12 hours each day. The cultivars were planted at 4 day intervals. After one month or when plants started developing the 5^{th} node one or two cultivars were inoculated each day with 25 isolates of *S. sclerotiorum* (the first 25 isolates listed in ascending order in Table 3.1). The time of inoculation was considered as block and the experimental design was RCBD with three replicates of each cultivar for each of the 25 isolates. The experiment was conducted twice (experimental run 1 in 2017 and experimental run 2 in 2018).

3.2.3. Aggressiveness test and lesion length

Lesion length was determined using the straw test procedure as described by Otto-Hanson et al. (2011) and modified by Zancan et al. (2015). For inoculation, sterile plastic drinking straws of approximately 5 mm in diameter and 2 cm long were used. One end of the straw was heat sealed. The open end of the straw was used to bore into the reverse side of a seven day old *S. sclerotiorum* culture on PDA at the advancing edge of the mycelia. The stem of each plant was cut 2 cm above the fourth node (i.e. the internode between the fourth and fifth node) and the straw containing agar and fungal mycelium was placed over the cut stem so that the stem was in contact with the mycelium. During and after inoculations, the greenhouse was maintained at 20°C nighttime and 26°C daytime. During the first 48 hours the plants were misted to keep the leaves and stems wet. After 8 days the inoculated plants were incubated for 8 days. The lesion length was measured in cm using a ruler.

3.2.4. Oxalic acid quantification

Oxalic acid [oxalate] from infected plant tissues collected from the experiment conducted in the spring of 2017 and 2019 was quantified using a diagnosis kit (Trinity Biotech) following the method of Davidson et al. (2016). Approximately 15 mg of infected stem (2 mm in size) tissue was excised and frozen under liquid nitrogen, ground in a pre-cooled mortar with pestle to break the cell walls in to a paste. Then 150 µl ice-cold oxalate assay buffer was added to the tissue paste and homogenized using a FastPrep instrument (MP Biomedicals) for 20 s. The homogenate was incubated for 10 min on ice and centrifuged at 10,000 x g for 5 min. The supernatant was collected in separate tubes and 1 - 50 µl of the supernatant was transferred into a 96-well flat-bottomed plate, mixed thoroughly, the volume standardized to 50 µl with oxalate assay buffer, and incubated at room temperature for 5 min.

Standard curve preparation: Oxalate standard was diluted to 1 mM (1nmol/µl) by adding 10 µl of 100 mM oxalate standard to 990 µl dH_2O, mixed well and 0, 2, 4, 6, 8, and 10 µl of the 1 mM the oxalate standard was added into a serious of wells in a 96-well plate and the volume was adjusted to 50 µl/well with oxalate assay buffer to generate 0, 2, 4, 6, 8, and 10 nmol/well of oxalate standard. Conversion: 2 µl of oxalate converter was added to each standard and sample well and mixed thoroughly and incubated at 30 ^{O}C for 1 hr.

Preparation of reaction mix: Reagents were mixed for the number of assays (standard and samples) to be performed. For each well, 50 µl of reaction mix containing: oxalate development buffer (46 µl), oxalate enzyme mix (2 µl), oxalate probe (2 µl) which had been prepared in advance was added and mixed well. The reaction mixes were then incubated at 37 ^{0}C for 60 min and the absorbance (450 nm) was measured using spectrophotometry (Trinity Biotech) (according to Durman, 2005). Within the enzymatic reaction oxalate is oxidized to carbon dioxide and hydrogen peroxide by oxalate oxidase and the hydrogen peroxide reacts with 3-methyl-2- benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB) in the presence of peroxidase to yield an indamine dye, which has an absorbance maximum at 590 nm. The intensity of the color produced is directly proportional to the concentration of oxalate in the sample.

Calculations: To calculate the oxalate amount in the 15 mg sample, the 0 oxalate standard reading was subtracted from all readings and an oxalate standard curve was plotted to apply the corrected sample reading to standard curve to get B nmol of oxalate amount in the sample wells. Sample oxalate concentration (C) = B/VxD = nmol/g = μM; where: B is the amount of oxalate in the sample well from Standard Curve (nmol), V is the sample volume used in the reaction well (ml), and D is the sample dilution factor.

3.3. Statistical analysis

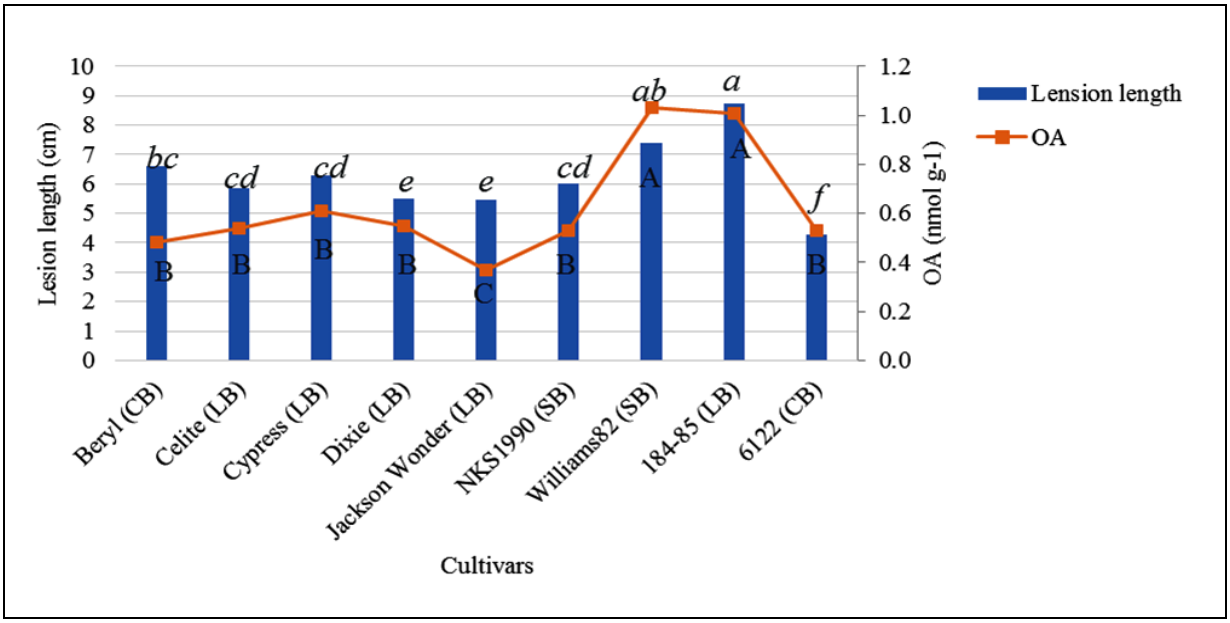
Lesion length (F = 1.84; *P*=0.1748) and oxalic acid data (F = 2.74; *P*=0.0978) from the two experiments were pooled for statistical analysis because there was no significant differences between the two experiments. Analysis of variance of mean straw test lesion length and oxalic acid quantification were performed using PROC REG models using SAS University Edition version 9.4 (SAS Institute Inc., Cary, NC, US). Type I error or α value was set at 0.05. For both lesion length and oxalic acid comparisons with overall average or analysis of mean (ANOM) procedure was used in jmp 14.1.0.0 (SAS Institute Inc., Cary, NC, US). An isolate was considered less or more aggressive when the average lesion length was shorter or longer, or the oxalic acid produced was higher or lower respectively, than the value of the overall mean of lesion length or oxalic acid of all isolates plus or minus one standard deviation. To identify resistant and susceptible lima bean cultivars, we compared the mean of each lima bean cultivar with the previously known resistant and susceptible cultivars NKS1990 and Williams82 soybean, and 6122 and Beryl common bean, respectively. No lima bean cultivars had been tested and therefore, no resistant or susceptible lima beans were used as checks. To compare the mean between the three crops, an average of one resistant (Jackson Wonder) and one susceptible (185-84) lima bean cultivar was used to compare with previously known resistant and susceptible soybean and common bean cultivars, respectively. Pearson correlation coefficients were calculated and simple linear regression using the equation "oxalic acid = $\beta_0+\beta_1$(lesion length)+Ɛ" tests were conducted to assess the relationship between disease development and oxalic acid accumulation in plants; where β_0 is the intercept, β_1 is the slope of linear relationship line, and Ɛ is error term.

Data of both lesion length and oxalic acid from the two years were analyzed together because there was no significant difference between the two years (P=0.54) nor was there a significant difference due to blocking (i.e. the inoculation timings; P=0.16).

3.4. Results

3.4.1. Assessment of variation in cultivars response to Sclerotinia sclerotiorum isolates infection (lesion length) using the straw test

The cultivars tested were significantly different in the mean lesion length caused by *S. sclerotiorum* isolates (F=45.64, P<0.0001). Mean comparisons between the cultivars were conducted in two different ways. Firstly, Tukey's all pair-wise comparison was done to screen all the cultivars based on their lesion length. Lima bean cultivar 184-85 had the longest lesions, which were significantly longer from all other cultivars except the susceptible soybean Williams82. The resistant common bean cultivar 6122 had the shortest lesion length, which was significantly shorter than all other cultivars compared (Figure 3.1). Dixie (lima bean), 6122 (common bean), and Jackson Wonder (lima bean) had significantly shorter lesion length by 0.5, 1.7, and 0.5 cm compared to the resistant NKS1990 soybean cultivar (Figure 3.1). However, the lesion lengths were significantly longer by 2.7, 0.5, and 1.4 cm for 184-85 (lima bean), Beryl (lima bean), and Williams82 (susceptible soybean) cultivars, respectively compared to NKS1990 (Table 3.2). Secondly, means were also compared with overall average mean decision chart - ANOM procedure to evaluate the response to *S. sclerotiorum* infection of five lima bean cultivar compared with NKS1990. There were significant differences between NKS1990 and the five cultivars in lesion length. Only one lima bean cultivar 184-85 had significantly longer lesions compared to NKS1990. Similarly and expectedly, a previously susceptible soybean cultivar had significantly longer lesions than NKS1990. Only 6122, a resistant common bean cultivar had significantly shorter lesion length than NKS1990 (Figure 3.2).



1019 Figure 3.1. The response of five lima bean (LB), two soybean (SB), and two common bean (CB)
1020 cultivars to infection of *Sclerotinia sclerotiorum* isolates and oxalic acid accumulation in the
1021 infected plant samples of these cultivars. Each blue bar indicates the mean of lesion length (cm)
1022 of the cultivar from 25 *S. sclerotiorum* isolates. The pick point in the orange line shows the
1023 average oxalic acid (nmol g^{-1}) accumulation in each cultivar quantified after infection by the 25
1024 *S. sclerotiorum* isolates. The small and italicized, and capital letters on the top of the bar and
1025 line graphs indicate significant differences among the cultivars in their lesion length and oxalic
1026 acid content, respectively.

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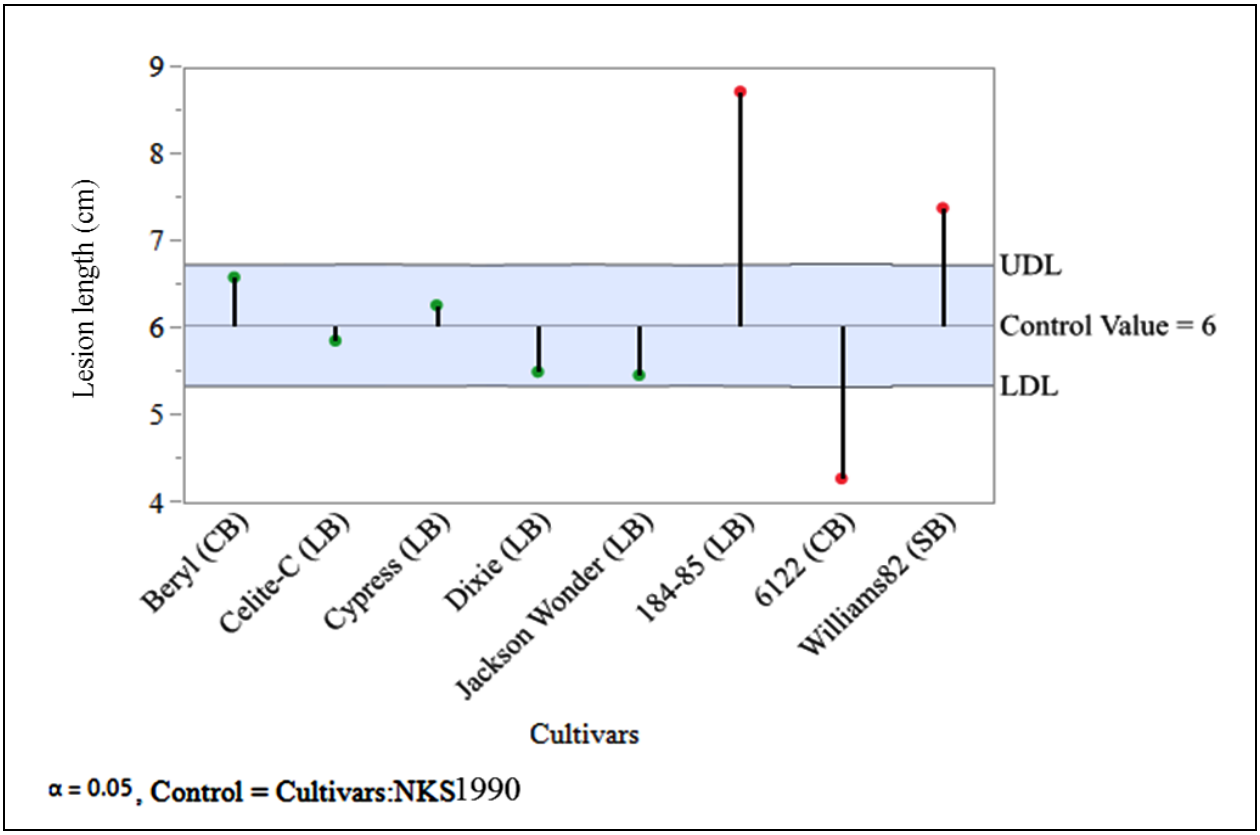

Figure 3.2. Comparisons of cultivars of lima bean, soybean, and common bean for lesion length with control decision chart. NKS1990, a resistant soybean cultivar, with a mean lesion length of 6 cm is the reference cultivar for comparison.

The blue shaded region is represents values that are not different from the average value. The cultivars with lesion length values that exceeded the upper decision limit (UDL) and lower decision limit (LDL) also exceeded the expectation criterion (i.e. 6 cm of the lesion length of NKS1990) based on $\alpha = 0.05$ and are represented by red dots.

Table 3.2. Analysis of variance and parameter estimates for lesion length of five lima bean, two soybean, and two common beans cultivars to *Sclerotinia sclerotiorum* inoculation resulted from regression analysis in SAS.

Analysis of Variance					
Source	DF	Type III SS	Mean square	F Value	Pr > F
Crops	2	433.17	216.58	52.01	<0.0001
Isolates	25	3062.05	122.48	69.64	<0.0001
Cultivars	8	1900.51	237.57	135.07	<0.0001
Solutions for fixed effects					
Variables	DF	Parameter estimate[a]	Standard error	t Value	Pr > |t|[b]
Intercept	1	-0.1695	0.2896	-0.59	0.5585
Cypress	1	0.2689	0.1991	1.35	0.1770
Dixie	1	-0.4963	0.2011	-2.47	0.0137
6122	1	-1.7060	0.2032	-8.40	<0.0001
184-85	1	2.7258	0.2000	13.63	<0.0001
Celite-C	1	-0.1016	0.2010	-0.51	0.6135
Beryl	1	0.5502	0.2004	2.75	0.0061
Jackson Wonder	1	-0.5350	0.1991	-2.69	0.0073
Williams82	1	1.3856	0.1991	6.96	<0.0001

[a] and [b] are the parameter estimates and P-value, respectively computed based on comparison of each cultivar with a reference resistance soybean cultivar, NKS1990.

All lima bean cultivars except 184-85 lima bean had equivalent lesion length as the resistant soybean cultivar, NKS1990. Common bean cultivar 6122 however, which had previously been identified as resistant, had the shortest lesions (were more resistant to *S. sclerotiorum* infection) compared to NKS1990. Common bean cultivar Beryl had significantly longer lesions (was less resistant to *S. sclerotiorum* infection) compared to its 6122. Williams82 is a susceptible soybean cultivar, and was similarly susceptible as common bean cultivar, Beryl. Lima bean cultivar 184-85 was the least resistant compared to all other cultivars in this experiment (Figure 3.1). Therefore, all lima bean cultivars except 184-85 have a similar resistance to *S. sclerotiorum* as NKS1990, but less resistance compared to 6122. In contrast, 184-

85 was the most susceptible lima bean cultivar compared to the susceptible soybean (Williams82) and susceptible common bean (Beryl) cultivar.

3.4.2. Assessment of variation in oxalic acid accumulation in infected plant parts among cultivars after inoculation with 25 Sclerotinia sclerotiorum isolates using the straw test

Oxalic acid accumulation differed significantly among the cultivars tested ($F=47.99$; $P<0.0001$). Susceptible lima bean 184-85 (i.e. 1.01 nmol g^{-1}) and susceptible soybean cultivar Williams82 (i.e. 1.03 nmol g^{-1}) accumulated significantly more oxalic acid compared to all the other cultivars tested (Figure 3.1). Williams82 had the highest oxalic acid accumulation (0.63 nmol g^{-1}) compared to the overall average mean value and was selected as a reference cultivar to compare the rest of the cultivars for their oxalic acid accumulation (ANOM analysis) . Beryl, the susceptible common bean, 0.48 nmol g^{-1}, and Jackson Wonder had 0.37 nmol g^{-1}, had significantly lower oxalic acid content compared to the overall average mean decision value. Based on the reference or control cultivar Williams82, all cultivars, except 184-85 accumulated significantly lower oxalic acid.

3.4.3. Assessment of variation in lesion length among lima bean, soybean, and common bean to Sclerotinia sclerotiorum isolates inoculation using the straw test

The three crops tested varied significantly ($F = 52.01$; $P<0.0001$) in lesion length following inoculation with 25 *S. sclerotiorum* isolates (Table 3.3). The average mean lesion length for lima bean, soybean, and common bean were 7.1 cm, 6.7 cm, 5.5 cm, respectively (Figure 3.4). The average lesion length on lima bean was significantly higher by 0.4 cm than on soybean. The common bean lesion length was significantly lower by 1.2 cm compared to soybean. There was also a significant interaction between isolates and crops (Table 3.3). The mean separation of Tukey's on the significant interaction effect between isolates and crops shown in (Table 3.3) was due to the existence of substantial differences between inoculated and non-inoculated control plants and also because there was only significant interaction between isolate 13 and lima bean (the Tukey's mean separation data not shown).

Table 3.3. Analysis of variance and parameter estimates for lesion length of lima bean, soybean, and common bean resulted from regression analysis in SAS.

Analysis of Variance					
Source	DF	Type III SS	Mean square	F Value	Pr > F
Crops	2	433.17	216.58	52.01	<0.0001
Isolates	25	3062.05	122.48	69.64	<0.0001
Cultivars	8	1900.51	237.57	135.07	<0.0001
Experiment	1	5.08	5.08	2.89	0.0894
Isolates*crops	50	549.93	10.99	2.64	<0.0001
Solutions for fixed effects					
Variables	DF	Parameter estimate[b]	Standard error	t Value	Pr > |t|[a]
Intercept	1	0.39	0.40	0.97	0.3314
Lima bean	1	0.38	0.17	2.22	0.0267
Common bean	1	-1.24	0.17	-7.17	<0.0001

[a] and [b] are the parameter estimates and P-value computed based on the average mean lesion length from the inoculation of 25 *S. sclerotiorum* isolates of lima bean and common bean in comparison to reference soybean.

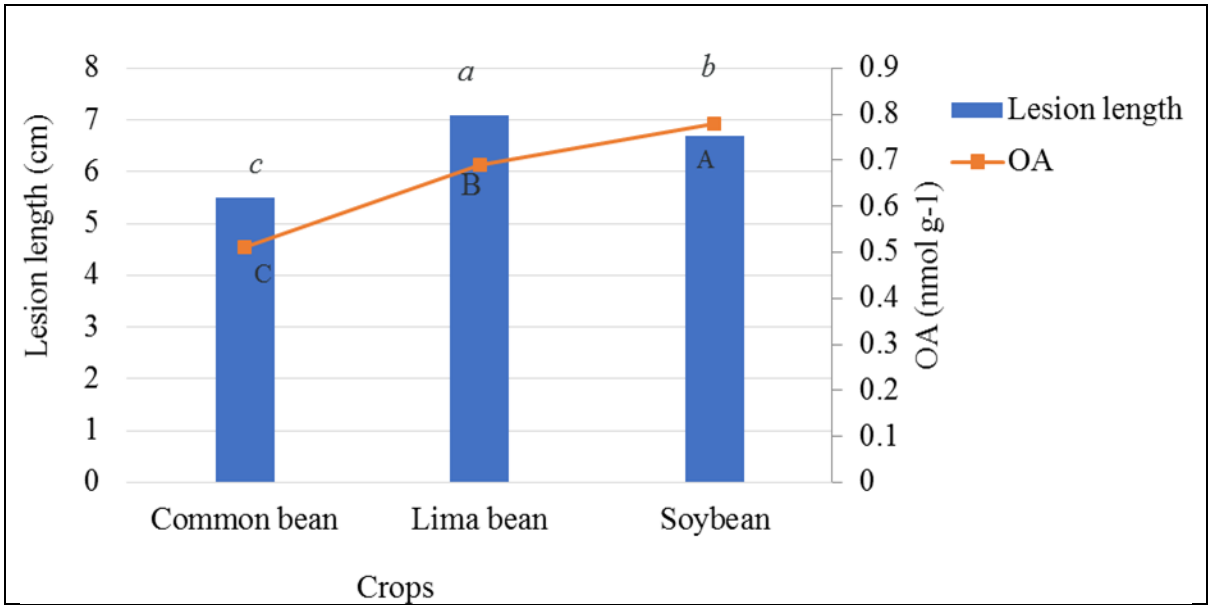

Figure 3.3. Lesion length and oxalic acid accumulation on common bean, lima bean, and soybean. The small and italicized, and capital letters on the top of the bar and line graphs indicate

significant differences in lesion length and oxalic acid accumulation among the crops, respectively.

3.4.4. Assessment of variation in oxalic acid accumulation between lima bean, soybean, and common bean crops following inoculation with 25 Sclerotinia sclerotiorum isolates

There was significant variation among the three crops tested in oxalic acid (nmol g^{-1}) accumulations after inoculation (F=21.16; P<0.0001). The average oxalic acid in the three crops was 0.51, 0.69, and 0.78 nmol g^{-1} in common bean, lima bean, and soybean, respectively (Figure 3.3). The average oxalic acid in lima bean and common bean were significantly lowered by 0.1 and 0.3 nmol g^{-1}, respectively compared to soybean (Figure 3.3). Based on comparison with overall average mean decision chart - ANOM, soybean and common bean has the highest and lowest OA compared to the overall average mean which is 0.662 nmol g^{-1} value (data not shown).

3.4.5. Assessment of variation in lesion length among Sclerotinia sclerotiorum isolates using the straw test

Using the pooled data of the two experiments, lesion length as measured by the straw test varied significantly among isolates (F = 15.8, P<0.0001). All isolates caused significantly longer lesions compared to the non-inoculated control (Figure 3.4). Based on the lesion length averaged over all the nine cultivars, isolate 13 produced the longest lesions with a mean value of 9.5 cm and isolate 6 produced the shortest lesions with a mean of 4.5 cm. Isolate 13 was selected as a reference isolate using ANOM procedure to compare aggressiveness among isolates. Pairwise comparisons of the isolates were made using Tukey's multiple mean comparison test to evaluate aggressiveness among the isolates.

Based on overall mean lesion length comparison averaged over five lima bean, two soybean, and two common bean cultivars, almost all the isolates resulted in significantly shorter lesions compared to isolate 13 (Figure 3.4; P<0.0001). Isolate 6 caused the shortest lesion length which was 5 cm less than the lesion length of isolate 13. Similarly, comparison with the ANOM decision chart (Figure 3.5), isolate 6 and 13 have the smallest and largest lesion length mean values, respectively. The lesion length ranged from 4.5 (isolate 6) to 9.5 cm (isolate 13). Isolate 8 had the second largest average values of lesion length, 7.2 cm (Figure 3.4) and also differed from

the overall mean. Therefore, isolate 6 was the least aggressive isolate and isolate 8 and 13 were the most aggressive isolates. We obtained isolate 6 from a snap bean collected from Sussex Co. DE, isolate 8 was obtained from a lima bean plant from ND and isolate 13 was obtained from a soybean plant in NJ.

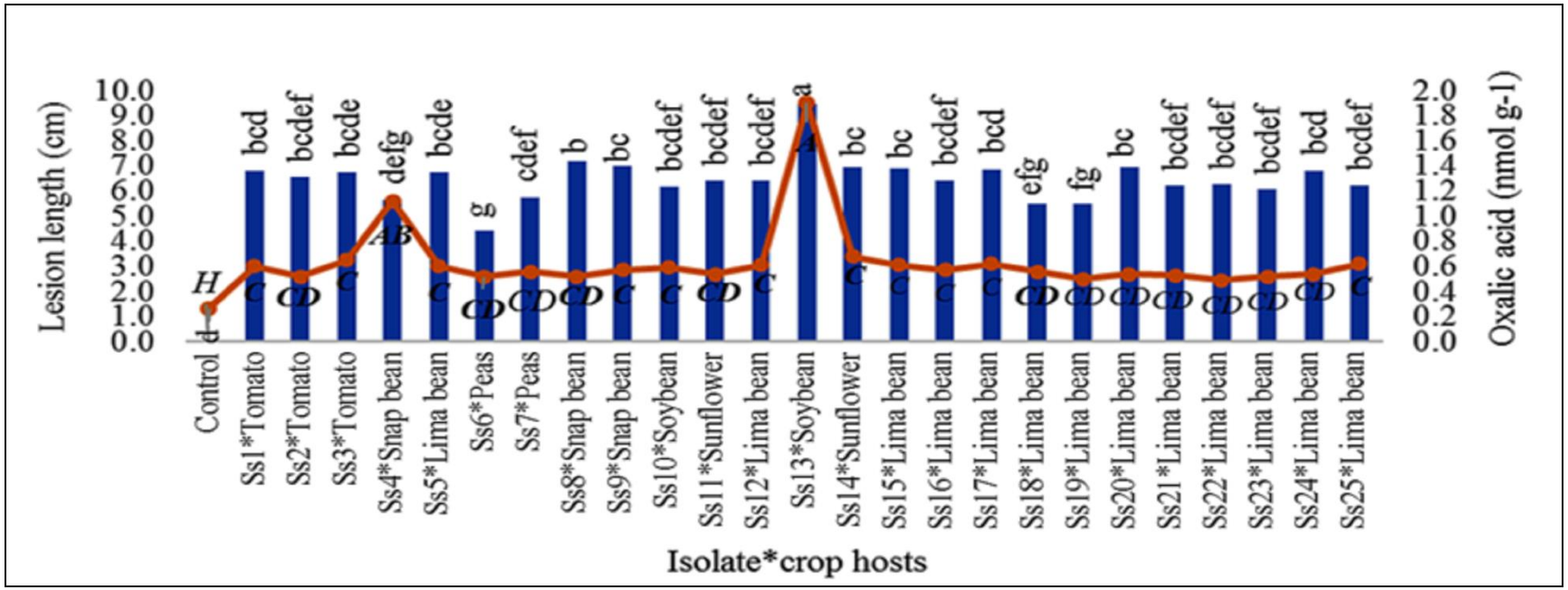

Figure 3.4. Mean stem lesion length (blue bar) and oxalic acid accumulation (orange line) from twenty-five *Sclerotinia sclerotiorum* isolates inoculated on nine cultivars following the straw test. Each bar length indicates the overall mean of each isolate from the nine cultivars with three replications (n=54) each used in the two experiments. Crop hosts indicated in the horizontal axis refer to the crop hosts where the isolates were originally obtained. Bars that have the same letter are not significantly different according to Tukey's test. The small, and capital and italicized letters on the top of the bar and line graphs indicate significant differences among the isolates in their lesion length and oxalic acid productions, respectively.

3.4.6. Assessment of variation among Sclerotinia sclerotiorum isolates in oxalic acid production following inoculation of five lima bean, two soybean, and two common bean cultivars

Oxalic acid accumulation (nmol g^{-1}) in the infected stem tissue following inoculation of nine cultivars by *S. sclerotiorum* isolates varied significantly (F = 27.4, *P*<0.0001). All the isolates accumulated higher oxalic acid than the non-inoculated control plant. In the inoculated plant tissue with isolate 13, there was significantly higher oxalic acid accumulation (1.6 nmol g^{-1}) than tissues inoculated with other isolates or the control plant. Based on the average oxalic acid accumulation measured from all the cultivars, tissues inoculated with isolate 13 and isolate 4 had the highest oxalic acid accumulations with a mean value of 1.9 and 1.1 nmol g^{-1}, respectively. The non-inoculated control plants had the least oxalic acid with a mean of 1.1 nmol g^{-1} (Figure 3.5, 3.6). However, only isolate 13, which was obtained from a soybean plant from NJ, accumulated a long lesion and high oxalic acid.

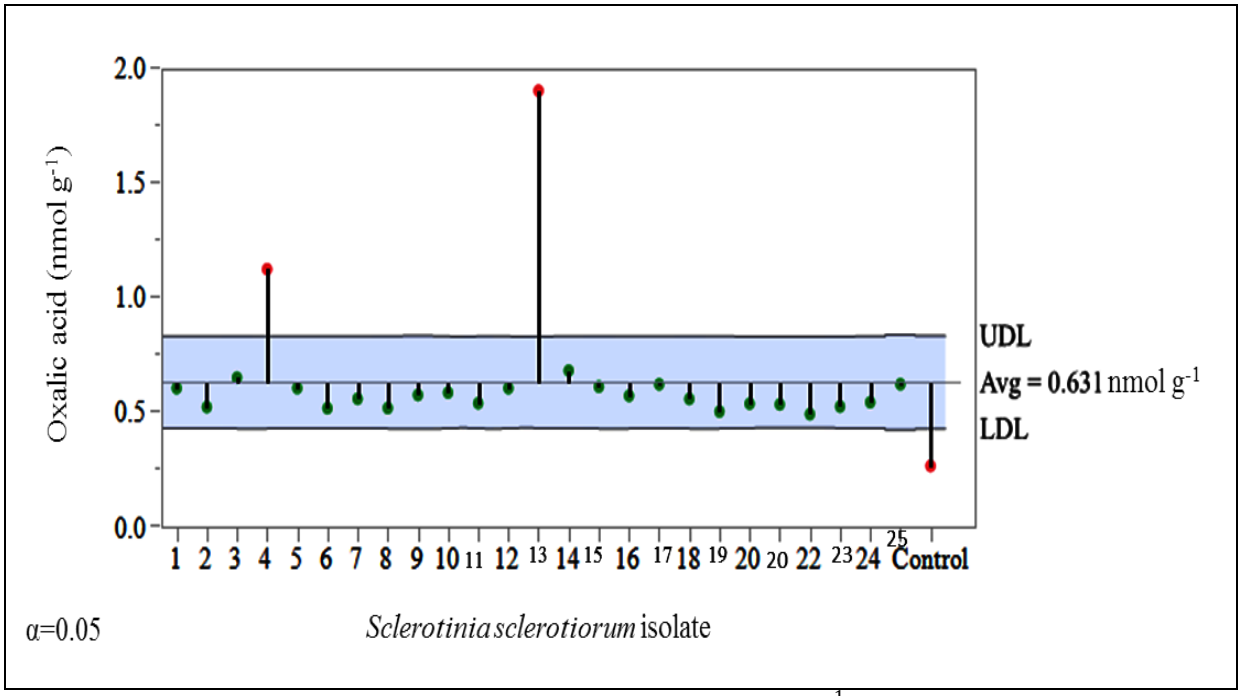

Figure 3.5. ANOM decision chart comparing oxalic acid (nmol g^{-1}) accumulation following inoculation with *S. sclerotiorum* isolates with the overall average value. The blue shaded region is the variances that are not different from the average value (which in this case is 0.631 nmol g^{-}

[1]). Isolates that exceed the upper decision limit are those that have a mean value that exceeds the expectation criterion based on $\alpha = 0.05$.

3.4.7. Assessment of correlation between lesion length caused by Sclerotinia sclerotiorum and oxalic acid production in common bean, soybean, and lima bean

Pearson's correlation coefficient test showed that there was a weak (r=0.37), but statistically significant correlation (*P<0.*0001) between lesion length and oxalic acid production by *S. sclerotiorum* isolates on the three crops tested in this experiment. Within the individual species, the correlations between lesion length and oxalic acid accumulation ranged from moderate in lima bean (r=0.54, $P < 0.0001$) to weak in common bean (r=0.26, $P < 0.0001$) and soybean (r=0.30, $P < 0.0001$) (Figure 3.6). In the scatterplot matrix, the narrow ellipse (in Figure 3.6b) reflects a moderate degree of correlation between the lesion length and oxalic acid accumulation in lima bean. Whereas the rounded ellipse, which is not diagonally oriented in soybean (in Figure 3.6c) indicates the lesion length and oxalic acid accumulation are less correlated. The correlation (r=0.36) between the lesion length and oxalic acid accumulation in common bean (Figure 3.6a) is in medium range between that of lima bean and soybean.

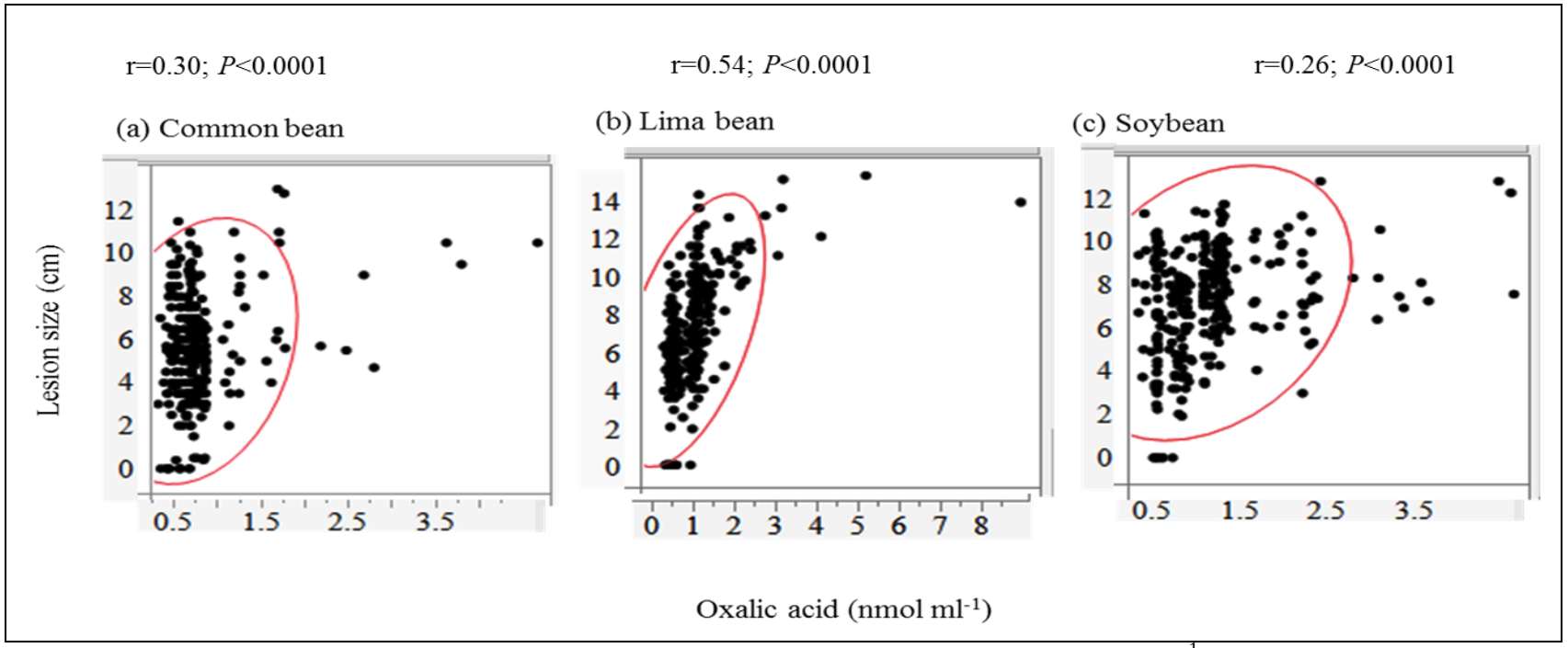

Figure 3.6. Scatterplot matrix of the correlations between lesion length (cm) and oxalic acid (nmol g^{-1}) accumulation by 25 *Sclerotinia sclerotiorum* isolates on common bean (a), lima bean (b), and soybean (c). The correlations are estimated by-row-wise method in jmp statistical software. The red ellipse region indicates a 95% bivariate normal density considering the assumption that each pair of variables has a bivariate normal distribution (two independent random variables),and the two variables in a bivariate normal are both are normally distributed, and have a normal distribution when both are added together). The ellipse encloses approximately 95% of the points. The narrowness of the ellipse (in b) reflects moderate degree of correlation between the variables. In c, the ellipse is rounded and is not diagonally oriented, indicating the variables are less correlated.

When the lesion length caused by the *S. sclerotiorum* infection by isolate and oxalic acid accumulation in the three crops was evaluated with fitted analysis, there was also a significant relationship (t=3.25; *P*=0.0034). The fitted model indicated that the oxalic acid level increased at a rate of 0.1 nmol g^{-1} as lesion length increases by one cm. The variance in oxalic acid, however, was predicted only by 31% (or R^2=0.305) from the lesion length (Figure 3.7).

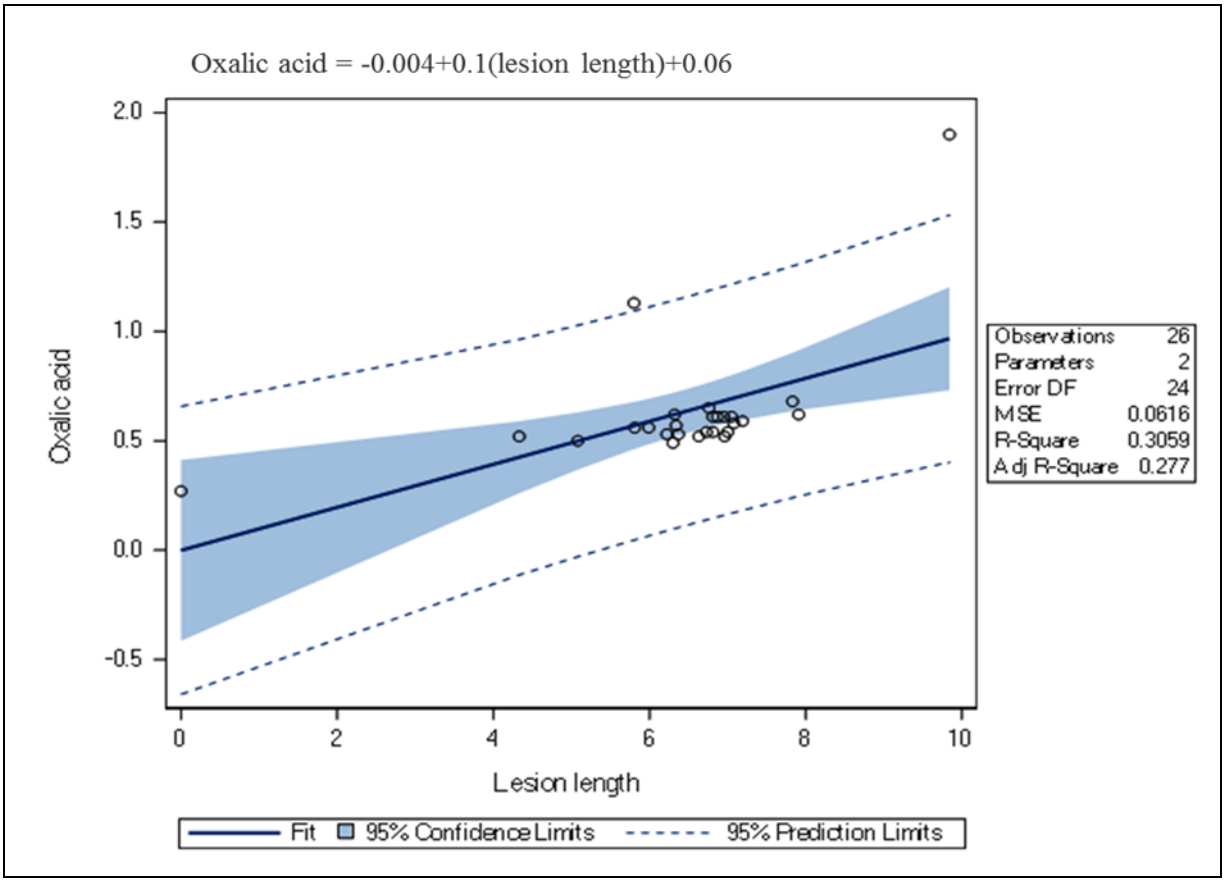

Figure 3.7. Scatter plot of the mean oxalic acid accumulation and lesion lengths overlaid with the regression line, and 95% confidence and prediction limits showing the relationship between lesion length and oxalic acid accumulation following inoculation with *Sclerotinia sclerotiorum* isolates.

3.4.8. Assessment of correlations between lesion length caused by Sclerotinia sclerotiorum and oxalic acid production in two common bean, two soybean, and five lima bean cultivars

The correlation coefficients between lesion length and oxalic acid production by 25 *S. sclerotiorum* isolates on the nine cultivars ranges from r=0.20 for Williams 82, which is a susceptible soybean cultivar, to r=0.47 for 184-85, which is a susceptible lima bean cultivar.

Correlations ranges from weak to moderate and were significant for all cultivars except Dixie a lima bean cultivar (Table 3.4).

Table 3.4. Correlations between lesion length (cm) and oxalic acid (nmol g^{-1}) accumulation following inoculation by 25 *Sclerotinia sclerotiorum* isolates on two common beans (CB), five lima bean (LB), and two soybean (SB) cultivars.

Cultivars (crops)	Correlation[a]	Count	Lower 95%	Upper 95%	Sig. Prob
Beryl (CB)	0.25	152	0.09	0.39	0.0021*
Celite-C (LB)	0.24	150	0.08	0.38	0.0032*
Cypress (LB)	0.21	156	0.06	0.36	0.0083*
Dixie (LB)	0.07	150	-0.10	0.23	0.3674
Jackson Wonder (LB)	0.20	156	0.03	0.33	0.0205*
NKS1990 (SB)	0.26	153	0.11	0.41	0.0010*
184-85 (LB)	0.47	153	0.34	0.60	<0.0001*
6122 (CB)	0.43	144	0.29	0.56	<0.0001*
Williams82 (SB)	0.20	156	0.03	0.33	0.0206*

[a]The correlation is based on the average of lesion length and oxalic acid accumulation of 25 *S. sclerotiorum* isolates on each of the nine cultivars used in this experiment (n=75). *indicates that the correlation between lesion length and oxalic acid accumulation was significant.

3.4.9. Assessment of correlations between lesion length and oxalic acid accumulations by Sclerotinia sclerotiorum

The correlation between the lesion length and the accumulation of oxalic acid within each isolate was also evaluated using the Pearson correlation coefficient. The correlation ranged from weak, r=0.03 (isolate 7) to moderate, r=0.55 (isolate 25). Out of the 25 isolates, there were 17 significant positive correlations between the length of the lesion and the production of oxalic acid (Table 3.5). There was no significant correlation between 8 of the 25 isolates. Correlation of the most aggressive isolate, isolate 13, was r=0.47 (P=0.0004) and was considered to be a moderate correlation (Table 3.5).

Table 3.5. Pearson's correlations between lesion length (cm) and oxalic acid (nmol g^{-1}) production of the 25 *Sclerotinia sclerotiorum* (Ss) isolates.

Isolate	Correlation[a]	Count	Lower 95%	Upper 95%	Signif. Prob.
Ss1	0.41	53	0.16	0.62	0.0021*
Ss2	0.16	53	-0.11	0.42	0.2424
Ss3	0.20	51	-0.08	0.45	0.1519
Ss4	0.43	53	0.18	0.62	0.0015*
Ss5	0.36	53	0.10	0.58	0.0075*
Ss6	-0.17	53	-0.42	0.11	0.2295
Ss7	0.28	52	0.01	0.51	0.0445*
Ss8	0.39	52	0.13	0.60	0.0048*
Ss9	0.54	52	0.31	0.71	<0.0001*
Ss10	0.29	54	0.02	0.51	0.0361*
Ss11	0.36	52	0.09	0.57	0.0093*
Ss12	-0.03	54	-0.30	0.24	0.8169
Ss13	0.47	53	0.23	0.66	0.0004*
Ss14	0.39	53	0.13	0.60	0.0038*
Ss15	0.45	52	0.21	0.65	0.0007*
Ss16	0.30	53	0.07	0.56	0.0137*
Ss17	0.30	53	0.08	0.56	0.0110*
Ss18	0.07	52	-0.21	0.34	0.6197
Ss19	0.33	53	0.06	0.55	0.0169*
Ss20	0.42	54	0.17	0.62	0.0017*
Ss21	0.16	54	-0.12	0.41	0.2606
Ss22	0.13	54	-0.14	0.38	0.3469
Ss23	0.41	53	0.16	0.62	0.0020*
Ss24	0.17	53	-0.11	0.42	0.2346
Ss25	0.55	49	0.32	0.72	<0.0001*

[a]The correlation is based on the average of lesion length and oxalic acid accumulation following inoculation of each isolate on the nine cultivars used in this experiment.

*indicates that the correlation between lesion length and oxalic acid accumulation was significant.

3.5. Discussions

S. sclerotiorum is an aggressive soil borne pathogen on soybean (Auclair et al. 2004; Mueller et al. 1999), common bean (Miklas et al. 2013; Del Río et al. 2004), and lima bean (Kee et al., 2004; Everts, 2016; unpublished and self-observation). Understanding how aggressiveness varies among *S. sclerotiorum* isolates may be useful tool for breeding programs aimed at

developing lima bean cultivars resistance to white mold. For example, Kull et al. (2003) suggested that the range in aggressiveness of *S. sclerotiorum* isolates in agricultural populations may impact cultivar performance.

In the current study, isolates significantly varied in their aggressiveness to five lima bean, two soybean, and two common bean cultivars. We confirmed significant differences among cultivars where lima bean cultivar 184-85 had significantly longer lesions than any other lima bean tested. There were also significant variations between the nine cultivars and on average, a common bean cultivar 6122 was the most resistant cultivar to *S. sclerotiorum*, whereas a lima bean cultivar 185-84 was the least tolerant cultivar compared to resistant soybean cultivar, NKS1990.

Lehner et al. (2015) also found that aggressiveness of *S. sclerotiorum* varied among different common bean cultivars. It ranged from susceptible in cultivars such as Beryl, NE1-06-12, Stampede, Eclipse, to partial resistance in cultivars 195, 11A-39, Cornell 603, G122, and Cornell 605. Similarly, Kabbage et al. (2014) showed that *S. sclerotiorum* isolates were moderate to strongly aggressive on soybean cultivars. Auclair et al. (2004) also reported that *S. sclerotiorum* aggressiveness significantly different among common bean cultivars. They proposed that the variation within the cultivars may have resulted from differences in plant architecture, cultivar height, maturity, or propensity to lodge. Boland and Hall (1987, 1986) also demonstrated that the response of soybean cultivars to *S. sclerotiorum* infection in field environments was positively correlated with plant height, maturity and lodging, suggesting that these variables may be related to mechanisms of disease escape.

We also observed differences in isolate aggressiveness as measured by the lesion lengths produced on the three plant species and in the oxalic acid accumulation in plant tissue. Lesion length and oxalic acid accumulation was highest in lima bean and soybean followed by common bean. There was no prior information on differences in lesion length and accumulation of oxalic acid from *S. sclerotiorum* in different crop species. Therefore, our finding may be the first to demonstrate these variations between different species of plants.

One of the objectives of this study was also to identify variability in aggressiveness among *S. sclerotiorum* isolates obtained from different hosts and different geographical locations and to identify representative isolates for use in cultivar testing and development of host resistance. Although there was little difference in aggressiveness among our isolates, we did identify isolate 13 as highly aggressive and propose that it can be used to evaluate lima bean cultivars. Additional isolates that differ in aggression should also be used.

Moreover, in the current experiment, there was a significant but weak correlation between lesion length and oxalic acid production by *S. sclerotiorum* isolates on the three crops tested in this experiment. The correlations ranged were moderate in lima bean and weak in both common bean and soybean. The results of the present study corroborate with the result reported by Willbur et al. 2017, where they showed that aggressiveness was only weakly correlated ($r = 0.26$, $P < 0.0001$) to isolate oxalate production by *S. sclerotiorum* in soybean.

Most previous studies on the aggressiveness of *S. sclerotiorum* reported that aggressiveness is associated with either the production of virulence factor such as oxalic acid or related compounds by the pathogen. These studies have documented the aggressiveness of this pathogen from a single crop or model plant study after inoculation with a single *S. sclerotiorum* isolate. The current experiment, however, examined the aggressiveness of *S. sclerotiorum* on three major hosts and used 25 isolates of *S. sclerotiorum*. We found that aggressiveness among the 25 *S. sclerotiorum* isolates collected from several crops and regions in US varied on three distinct but related crop species. Although the number of isolates analyzed in the current experiment was small, the variability of oxalic acid accumulation on lima bean, common bean, and soybean was high. Moreover, we demonstrated that there was significant variation between several isolates in terms of oxalic acid accumulation and that accumulation was related to aggressiveness or pathogenicity. The results can be used to make inferences about the population, especially from the mid-Atlantic region in the US. In general, this information can be used to evaluate lima bean lines for host resistance, which is needed because there are no commercially available resistant lima bean cultivars.

Here, our result showed that, there was little difference in aggressiveness within the mid-Atlantic *S. sclerotiorum* isolates which could facilitate the development of resistant lima bean

cultivars. Most lima bean cultivars screened were highly susceptible to *S. sclerotiorum*, so any inherent differences in white mold disease observed in the field may be a result of plant architecture. To separate the effect of plant architecture from aggressiveness factors such as oxalic acid, we suggested that performing a separate experiment by either directly spray the plants with OA after inoculation with *S. sclerotiorum* or developing an OA knockout *S. sclerotiorum* mutant and inoculate directly to the plant. In addition to studies on variation in aggressiveness of *S. sclerotiorum* population, more studies should be conducted to assess the genetic variability using microsatellite markers and mycelial compatibility groups (MCGs) of *S. sclerotiorum* isolates from lima bean fields in the region. More studies should also address the relationship between aggressiveness and genetic variability.

Chapter 4: Mycelial compatibility, molecular identification and comparison of *Sclerotinia sclerotiorum* isolates from different crops and regions in the United States

ABSTRACT

Sclerotinia sclerotiorum isolates collected from the mid-Atlantic and other regions of the US were evaluated for mycelial compatibility groups (MCGs) and genetic variability. *S. sclerotiorum* isolates from different crops and regions are thought to be more variable than isolates from the same crops and regions. Twenty-five (in 2017, set 1) and forty (in 2018, set 2) isolates, obtained from nine crops and eight states, were tested for MCGs. MCG was determined by pairing 7 day old *S. sclerotiorum* isolates on Diana Sermons' medium (DSM). Twelve MCGs were obtained in both set 1 and set 2. However, 82% and 64% of the isolates showed incompatible interactions on DSM, which was the largest group, including 24 and 37 isolates in set 1 and set 2, respectively. Genetic variability of isolates was also evaluated. DNA was extracted from 10 day old cultures. The ITS region was amplified using ITS1/ITS4 primer pairs and the amplicons were sequenced by Macrogene USA. Alignments were done using MUSCLE and sequence (including reference sequences of five *S. sclerotiorum*, three other sclerotinia species, *Botrytis cinerea*, and *Dumontinia tuberosa* from the NCBI) comparison and dendrogram construction was carried out using MEGA7 software. The Kimura model was applied, and bootstrap analysis was conducted with 1,000 replications, to assess group support. Sequence comparisons were not informative as ITS rDNA of most of the *S. sclerotiorum* isolates shared 100% sequence identity with those of *S. sclerotiorum* in NCBI DNA databases. Slight, 1.2% overall intraspecies sequence variability was observed between the Sclerotinia isolates from the current experiment and reference isolates from NCBI DNA database. There was also slight, 0.3% intraspecies variation within the *S. sclerotiorum* isolates collected from different crops and regions in the US, which may result from the homothallic (clonality) of *S. sclerotiorum*.

4.1. Introduction

One method to measure diversity of *S. sclerotiorum* is by evaluating mycelial compatibility groups (MCGs). Mycelial compatibility is defined as the ability of two strains of filamentous fungi to cross or fuse and form a continuous colony and is synonymous with vegetative compatibility (Schafer and Kohn, 2006). When paired in Diana Sermons ' medium (DSM) culture, some MCG members anastomose to form a compatible reaction line, and some do not. MCGs have been used as a tool to determine the genetic variation among *S. sclerotiorum* isolates collected from different crops and geographical locations (Willbut et al. 2019; Lehner et al. 2016; Koga et al. 2014; Otto-Hansen et al. 2011; Kull et al. 2004; Kohn et al. 1991). MCG is an assay of self and nonself recognition controlled by multiple loci (Bolton et al. 2006; Carbone et al. 1999) and is a good test for intraspecific heterogeneity in *S. sclerotiorum* (Otto-Hansen et al. 2011). MCGs may be genotypically unique (i.e. represent genetically different individuals) (Otto-Hansen et al. 2011; Li et al. 2008; Durman et al. 2003; Kohn et al. 1999). Each MCG is genotypically unique.

These specific genes involved in the incompatible MCG might be involved in programmed cell death (PCD) in the incompatible zones that the isolate carry out to avoid molecular parasites including cytoplasmic transmission of *S. sclerotiorum* mycoviruses (SsMYRV4) between the confronted incompatible isolates (Wu et al. 2017; Zhang and Nuss 2016). *S. sclerotiorum* genome contains a large repertoire of genes putatively involved in vegetative incompatibility, which is likely responsible for the high diversity of MCGs observed in the field. These genes that involved in these incompatible MCG interactions are called vegetative incompatible or *vic* genes (Marzano et al. 2015; Amselem et al. 2011). Melzer et al. (2002) reported that *S. sclerotiorum* isolates that have the same one nucleotide change in their *vic* loci are compatible to each other. Genetic analyses identified six diallelic *vic* genetic loci (termed as *vic1, vic2, vic3, vic4, vic6,* and *vic7*) involved in incompatible interactions in MCGs in filamentous fungi such as *Cryphonectria parasitica* (Zhang and Nuss 2016; Cortesi and Milgroom 1998). Vegetative incompatibility, which involves heterotrimeric guanine nucleotide-binding proteins (G proteins) signaling pathway, is controlled by specific loci termed *het* (heterokaryon incompatibility) loci. Reactive oxygen species (ROS) plays a key role in vegetative incompatibility-mediated PCD. The expression of G protein subunit genes, *het* genes, and ROS-

related genes were significantly down-regulated, and cellular production of ROS was suppressed in the presence of SsMYRV4 (Wu et al. 2017).

S. *sclerotiorum*, via oxalic acid also hijacks host pathways and induces cell death in host plant tissue resulted in distinctive apoptotic (pathogen's resistance mechanism) features (Kabbage et al. 2013). However, we need to implement a combination of microscopy (electron/florescence), chemical effectors and reverse genetics. PCD is also due to autophagy (host's mechanism). Both apoptosis and autophagy mediate resistance and susceptible host-microbes interactions. MCGs have also been found to be stable through successive sexual generations and after serial culturing, and the correlation between an MCG and a DNA fingerprint supports the synonymous relationships between MCGs and clones of *S. sclerotiorum*. MCGs of *S. sclerotiorum* also persist from year to year, covering wide geographic areas (Otto-Hansen et al. 2011; Kull et al. 2004; Hambelton et al. 2002; Kohli et al. 1992). Populations of *S. sclerotiorum* are clonal and several clones may infest each field (Durman 2005, 2003; Hambleton et al. 2002; Anderson and Kohn 1995). MCGs detect clonality among isolates of *S. sclerotiorum.*

MCGs also indicate isolate homogeneity and may be used to detect variation within a fungal population (Lehner et al. 2015; Kohn et al. 1990). Under laboratory conditions, MCG measures vegetative compatibility and intraspecific strain variation among isolates of *S. sclerotiorum*.

Diversity of *S. sclerotiorum* can also be studied using molecular approaches. Genetic variation is caused by mutation, selection, gene flow between populations, or genetic recombination within populations (Petrofeza et al. 2012). Genetic isolation of populations and drift can lead to genetic divergence and speciation (Petrofeza et al. 2012). Identifying *S. sclerotiorum* genetic structure and population dynamics is important to understanding how the underlying mechanisms are involved in the history of the pathogen and its distribution across various hosts and different geographical locations (Petrofeza et al. 2012). A number of studies reported on genetic diversity of *S. sclerotiorum* isolated from different hosts and from different geographical locations. Some of these studies found that populations of *S. sclerotiorum* vary genetically, however some studies found little or no genetic variation within and among *S. sclerotiorum* populations from the same geographic region or crop or from different locations and

crops. These results may reflect that the ability to detect genetic variation among *S. sclerotiorum* isolates varies with the region assessed in each experiment.

Manjunatha et al. (2014) found that the genomic DNA of *S. sclerotiorum* isolates from different geographical locations and various hosts in India had no variability in the ITS region and more homogeneity was observed with ITS-RFLP genetic study. However, the authors also found that the RAPD and URP (universal rice primer)-PCR banding pattern of isolates from different geographical locations were diverse. Use of the RAPD technique found differences among *S. sclerotiorum* isolates collected from canola from different geographical locations in Iran (Colagar et al. 2010). Sun et al. (2005), by using RAPD, found the genetic similarity of *S. sclerotiorum* isolates collected from different regions and hosts in China was high. SSR polymorphism and ITS sequencing has shown the existence of high genetic diversity among *S. sclerotiorum* isolates collected from oilseed plants from different geographical regions in India and sixty-five isolates were categorized into three evolutionary lineages (Sharma et al. 2018). Based on the results of RAPD and SSR analyses, 60 *S. sclerotiorum* isolates from eggplant from different geographic regions in Turkey represented 22 MCGs. Among these MCGs, 36% were represented by a single isolate and all isolates showed significant variability for virulence regardless of MCG and geographic origin. The 60 isolates were also grouped in two and three distinct clusters based on RAPD and SSR, respectively. The same authors reported that the genetic distance or clusters were not distinct relative to the MCG, geographical origin, and virulence, they suggested that diversity was related to the occurrence of clonal and sexual reproduction of *S. sclerotiorum* on eggplant in the areas surveyed (Tok et al. 2016).

Until this study, there was no information on MCGs and genetic diversity of populations of *S. sclerotiorum* on lima bean in Delaware, Maryland and other production regions such as New York. Increasing our knowledge of the genetic diversity and MCGs of *S. sclerotiorum* coupled with information on environmental factors that can influence white mold epidemics, can help us develop lima bean specific management guidelines or can be used for the development and utilization of host resistance. MCG or genetic diversity might be related to virulence or aggressiveness of *S. sclerotiorum* and high genetic diversity may lead to the development fungicide resistance. Therefore, MCG and genetic diversity could impact fungicide use and management of white mold caused by *S. sclerotiorum*.

Identifying an association between MCGs and genetic variation, and understanding their association with virulence factor such as oxalic acid is also important for developing strategies to combat the spread of *S. sclerotiorum* diseases and the mechanism of resistance of this pathogen. MCGs differ in their ability to persist from year to year as resistant sclerotia in soil (Durman et al. 2003). In the current experiment compatibility groupings and genetic diversity were used to study isolates of *S. sclerotiorum* collected from diverse crops and locations in the region. We anticipated that the knowledge generated on MCG and genetic diversity could be a valuable tool for management of *Sclerotinia* diseases, including maintaining efficacy of fungicides for lima bean in the mid-Atlantic region. The objective of the current research was to determine the mycelial compatibility groupings and genetic diversity among isolates of *S. sclerotiorum* collected from different fields of lima bean and other crops in the mid-Atlantic region.

4.2. Materials and Methods

4.2.1. Fungal cultures

Diseased lima beans and other host crops infected with *S. sclerotiorum*, such as soybean, common bean, tomato, sunflower, and four others were collected from different fields of the mid-Atlantic region of the US (Table 4.1). More isolates were obtained from Dr. James Steadman (University of Nebraska-Lincoln, NE), Dr. Sarah Pethybridge (Cornell University, NY), Drs. Mark VanGessel, Nathan Kleczewski, and Nancy Gregory (University of Delaware, DE), Dr. Karen Rane and Benjamin Beale (University of Maryland). Infested plants were washed in tap water for 3 to 5 minutes, small pieces excised from the advancing lesion area, immersed in 20% Tween20 solution (Sigma-Aldrich, Westport Center Dr, St. Louis, MO), surface disinfested in a 5% NaOCl solution in for 3 to 5 minutes, immersed into 95% EtOH for 1 minute, and re-immersed into ddH_2O for 3 to 5 minutes to remove the NaOCl. The disinfested plant pieces were placed on PDA and incubated at 25 - 30ºC in the dark for approximately a week. The isolates were identified based on as the presence of buff to white, mycelium and subsequent sclerotia formation. The isolates were transferred onto quarter strength PDA by single hyphal tip method as described in Mandal and Dubey (2012) and Moral et al. (1972) and maintained in slant test tubes at 4 or 5°C in refrigerator. Forty two isolates and a control (non-inoculated) were used in the aggressiveness study. Forty isolates were used for MCGs and e 43 isolates used for genetic diversity studies.

1416 Table 4.1. Isolates of *Sclerotinia sclerotiorum* collected from different states, counties and crops in the US.

Isolate number	Isolate names[a]	Year of origin or received	Crop	Locations[b]	Isolates provided by:
Ss1	SAS 15-100-2	2015	Tomato	St. Mary's Co., MD	Benjamin Beale
Ss2	SAS 15-100-3b	2015	Tomato	St. Mary's Co., MD	Benjamin Beale
Ss3	SAS 15-100-4	2015	Tomato	St. Mary's Co., MD	Benjamin Beale
Ss4	SAS 15-100-6	2015	Snap bean	Sussex Co., DE	Own
Ss5	SAS 15-100-7	2015	Lima bean	Sussex Co., DE	Own
Ss6	SS8-Pe-DE	2017	Peas	Sussex Co., DE	Nancy F. Gregory
Ss7	SS9-Pe-DE	2017	Peas	Sussex Co., DE	Nancy F. Gregory
Ss8	SAS 15-100-12	2015	Snap bean	Sussex Co., DE	Dr. Nathan Kleczewski and Andy Kness
Ss9	SAS 15-100-13	2015	Snap bean	Sussex Co., DE	Own
Ss10	SAS JRS#723	2016	Soybean	ND[c]	Dr. James Steadman
Ss11	SAS JRS#587	2016	Sunflower	OR[d]	Dr. James Steadman
Ss12	SAS JRS#160	2016	Lima bean	Westley, CA	Dr. James Steadman
Ss13	SS 1-NJ-Sb	2016	Soybean	NJ[e]	Dr. Van Gessel and Dr. Nathan Kleczewski
Ss14	SAS 15-100-1	2015	Sunflower	Madison, GA	Songbird blend Ultra Madison, GA
Ss15	SAS 15-100-3a	2015	Lima bean	Sussex Co., DE	Own
Ss16	SS2-DE-Lb-i	2016	Lima bean	Sussex Co., DE	Own
Ss17	SS2-DE-Lb-ii	2016	Lima bean	Sussex Co., DE	Own
Ss18	SS3-DE-Lb-i	2016	Lima bean	Sussex Co., DE	Own
Ss19	SS3-DE-Lb-ii	2016	Lima bean	Sussex Co., DE	Own
Ss20	14207-1(A)	2016	Lima bean	NY[e]	Dr. Sarah Pethybridge
Ss21	14207-1(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss22	14207-2(A)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss23	14207-2(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss24	14207-3(A)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss25	14207-3(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss26	14206-8(A)	2016	Lima bean	NY	Dr. Sarah Pethybridge

Ss28	14206-8(B)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss29	14207-7(A)	2016	Lima bean	NY	Dr. Sarah Pethybridge
Ss30	SS4-MD-Tm	2017	Tomato	St. Mary's Co., MD	Benjamin Beale
Ss31	SS5-MD-Sb	2017	Soybean	Sussex Co. DE	Own
Ss32	SS6-MBK-DE-Lb	2017	Lima bean	Fulton Co., MD	Own
Ss33	SS7-Milton-MD-Lb	2017	Lima bean	Sussex Co., DE	Own
Ss34	SS8-LR-DE-Lb	2017	Lima bean	Sussex Co., DE	Own
Ss36	SS9-MD-Lb	2017	Lima bean	Talbot Co., MD	Own
Ss37	SS10-MD-Lb	2017	Lima bean	Fulton Co., MD	Own
Ss38	SS11-MD-Gk	2018	Green Kale	Wicomico Co., MD	Dr. Karen Rane
Ss39	SS12-MD-Co	2018	Collards	Wicomico Co., MD	Dr. Karen Rane
Ss40	SS13-MD-Ka	2018	Kale	St. Mary's Co., MD	Benjamin Beale
Ss41	SS14-MD-Br	2018	Broccoli	St. Mary's Co., MD	Benjamin Beale
Ss42	SS15-MD-Tm	2018	Tomato	St. Mary's Co., MD	Benjamin Beale
Ss43	SS16-MD-Tm	2018	Tomato	St. Mary's Co., MD	Benjamin Beale
Ss44	SS18-MD-Tm	2019	Tomato	St. Mary's Co., MD	Benjamin Beale

[a] Original name of the isolates;

[b] County and state from which isolates were obtained;

[c,d,e] Represents unknown county. Note: In table 4.1, there are a total 42 *S. sclerotiorum* isolates. Isolate 27 and 35 were not included in the experiment due to lack of growth on PDA medium.

4.2.2. Mycelial compatibility groups (MCGs)

The MCG test was conducted in 2017 (set I) and 2018 (set II). During the first experimental period 25 isolates were used, and in the second experimental period additional isolates were included, bringing the total to 40 (Table 4.1). The MCG was determined by pairing the isolates in all possible combinations on Diana Simmons (DS) medium (Cubeta et al., 2001) as described in Mandal and Dubey, 2012 and Zancan et al, 2015. DS media consists of malt extract agar at 40 g/liter (Sigma-Aldrich, St. Louis), NaCl at 20 g/liter, Bacto peptone at 5 g/liter (BD Diagnostic Systems, Sparks, MD), Bacto agar at 15 g/liter (BD Diagnostic Systems), McCormick's red food dye (80 µl/liter), and McCormick's yellow food dye (80 µl/liter). A total of 300 and 780 combinations were obtained for set I and set II, respectively. In addition, each isolate was paired with itself and control (i.e. pure PDA plug). Before the MCG test, the isolates were grown on regular PDA medium and incubated at 24 ± 1 ^{O}C for one week. Mycelial discs (5 mm diameter) were taken from approximately 1 mm behind the advancing edge of the actively growing mycelial colony, and placed upside down 2.5 cm apart, on a plate of DS medium in 90 mm diameter Petri dish. Mycelial reactions were recorded after 7 days as incompatible when an apparent line of demarcation, a barrage zone, or a mycelia free zone is observed between the confronting paired isolates, and as compatible if there is no line of demarcation observed between the isolates. Radial growth of each isolate was also recorded to determine the growth or expansion performance of each isolate in the presence of the other isolate. The experiment was a completely randomized design with 2 replications.

4.2.3. Assessment and comparison of MCG diversity

The mycelial interactions were scored as 0 for compatible and 1 for incompatible, and on growth characteristics such as color, formation and type of barrage zone, and production of sclerotia. The isolates were then categorized into different MCGs. In the first set only 25 isolates were categorized (the first 25 isolates in ascending order in Table 4.1). In the second set, 15 additional isolates (numbered from 26 to 42 in Table 4.1), which were obtained from different locations and hosts, were added for 40 isolates. We first categorized the compatibility of *S. sclerotiorum* isolates into two groups: incompatible when an apparent line of demarcation, a barrage zone, was observed between the confronting paired isolates (Figure 4.1a) and compatible when there was no such zone between the isolates (Figure 4.1b). The incompatible groups were

further divided into sub-groups based on growth characteristics such as colony color, formation of sclerotia, and shape of the barrage zone (Table 4.2).

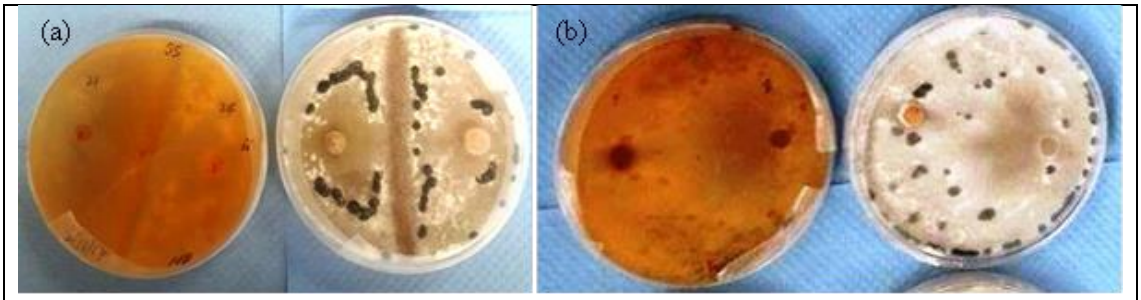

Figure 4.1. Incompatible (a) and compatible (b) reaction of isolates of *Sclerotinia sclerotiorum* 7 days after inoculation, respectively. The plates to the left of each pairing are the reverse side of the colony and the right the colony surface.

Table 4.2. Examples of growth characteristics of the incompatible Sclerotinia sclerotiorum groups grown on Dianna Sermon's medium.

Nature of separation	Color of MCG	Formation of sclerotia	General MCG group name[a]
Curvy grooved	White to brown	No	CGWTBN
Straight grooved	White to slight brown	Yes	CGWTBY
Curvy grooved	White to yellow	Yes	CGWTYY
Curvy grooved	White	Yes	CGWY
Curved intermediate	White	Half	CIWH
Curved intermediate	White	Yes	CIWY
Curvy mound	White	No	CMWN
Curvy mound	White to gray	Half	CMWTGY
Curvy mound	White	yes	CMWY
Intermediate grooved	White to yellow	Yes	IGWTYY
Intermediate grooved	White	Yes	IGWY
Intermediate intermediate	White to yellow	Yes	IIWTYY
Straight grooved	White	1/2th	SGWH
Straight grooved	White	No	SGWN
Straight grooved	White to yellow	Yes	SGWTYY
Straight grooved	White	Yes	SGWY
Straight intermediate	White to yellow	Yes	SIWTYY
Straight intermediate	White	Yes	SIWY
Straight mound	White to yellow	Yes	SMWTY
Straight mound	White	Yes	SMWY

[a]Arbitrary naming code or abbreviations given to MCG in this experiment only.

Chi-square analysis was used to determine the frequency of occurrence of MCGs within crops, and the locationwhere the isolates were obtained, and categorized into index groups based on Shannon index model. The Shannon index, *h*o, was computed for each MCG as follows:

$$ho = -\sum pi[\log(pi)];$$

where p*i* is the frequency of the ith MCG. Frequency is defined as the ratio between the number of isolates belonging to the ith MCG and the number of isolates in the sample. To correct for differences in sample sizes between populations, MCG diversity values were normalized by the maximum diversity in each population so that

$$Ho = ho/\ln k,$$

where *k* was the sample size. The total MCG diversity (H_{tot}) was partitioned into within- and among-population components (Goodwin et al. 1992 modified in Durman et al. 2003 and Vleugels et al. 2012).

4.3. Molecular characterization of *Sclerotinia sclerotiorum*

4.3.1. DNA extraction

DNA from 42 *S. sclerotiorum* isolates (Table 4.1) was extracted following a protocol described in Lehner et al., (2017). Isolates were sub-cultured onto PDA and incubated at 20 to 22°C. After 3 days, four agar plugs from the colony margin were transferred to a flask containing 150 ml PDB (potato dextrose broth at 24 g/liter of ddH_2O) and were incubated for 3 to 4 days at 20 to 22 °C. The resultant mycelial mat was removed, rinsed with ddH_2O in a Buchner funnel, blotted dry and lyophilized. DNA was extracted from approximately 0.015g lyophilized mycelium using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) following the manufacturer's instructions. DNA integrity was analyzed on an agarose gel (1% wt/vol agarose in Tris-acetate EDTA) amended with 0.5× (v/v) nucleic acid stain GelRed (Biotium Inc., Fremont, CA) and the concentrations were estimated in a spectrophotometer (Nanodrop 2000; Thermo Scientific, Waltham, MA).

4.3.2. PCR primers and Sequence analysis and Dendrogram construction

Identities of the isolates were confirmed by sequencing the ITS region, the *β*-tubulin, calmodulin, and aspartyl protease genes using standard PCR protocols (Baturo-Ciesniewska et al. 2017; White et al. 1990; Glass and Donaldson 1995). To amplify the rDNA of these regions, primer pairs obtained from different sources and own designed primers were used (Table 4.3). PCRs were performed using GoTaq Green Mix (Promega Corp., Madison, WI). The mix contains GoTaq DNA polymerase in 1x Green GoTaq reaction buffer (pH 8.5), 200 µM dNTP, 1.5 mM MgCl2, 1 µM each primer, 2.5 µl of DNA in a total volume of 25 µl/reaction. Amplifications for ITS and 18s rDNA were performed at initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 15 seconds, primer annealing at 52°C for 1 min, and elongation at 72°C for 1 min, with a 10-min final extension step at 72°C. Amplifications for *β*-tubulin gene were performed at initial denaturation temperature of 95°C for 2 minutes followed by 35 cycles of 15 seconds denaturation at 95°C, primer annealing at 55 °C for 1 minute, and elongation at

72°C for 1 min, with a 5 min final elongation step at 72°C. Amplifications for calmodulin and aspartyl protease genes were performed at initial denaturation temperature of 95°C for 2 minutes, followed by 35 cycles of 15 seconds denaturation at 95°C; primer annealing at 61^{O}C for 20 seconds; and elongation at 72^{O}C for 40 seconds, with final elongation temperature of 72^{O}C for 3 minutes. Water as blank template was used in all PCR run.

To confirm the amplification of the of gene or region, the PCR products were visualized following gel electrophoresis (100V) for about 35 minutes in 1x Tris-acetate-EDTA on 1.2 to 1.5 % agarose gel containing SYBR safe DNA gel stain using 1 kb ladder DNA size markers to estimate the size of amplified fragments. The PCR product from ITS and β-tubulin regions were sent to Macrogen USA and purification of the product and sequencing was conducted by the company.

Table 4.3. Primer codes, primer sequence, targets, expected size, and sources of primers of the polymerase chain reaction products amplified in this study.

Primer codes	Primer sequence (5' - 3')	Target (i.e. ITS or Gene)	Expected size (bp)	Sources
ITS1/ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	ITS region	540	D. Smith et al, 2017
HBITS1/HBITS2	CCTGATCCGAGGTCAACCAT TCATTACAGAGTTCATGCCC	ITS region	524	Own design
NS5/NS6	AACTTAAAGGAATTGACGGAAG GCATCACAGACCTGTTATTGCCTC	18S	250	D. Smith et al, 2017
HBNS1/HBNS2	ACGAATCGCATGGCCTTGTG CAGGTTAAGGTCTCGTTCGTT	18S	1,627	Own design
TU1/TU2	CCTGAAAAGCACCCCACTAT ACGGCACGAGGAACATACTT	Gene (*β-tubulin*)	494	D. Smith et al, 2017
HBTU1/HBTU2	TGGAACTGGTGCCGGTATGG GACGAAGGTGGAGGACATCT	Gene (*β-tubulin*)	874	Own design
HBTU3/HBTU4	TGTGACTGCCTTCAAGGTTTC CCGACGAAGGTGGAGGACAT	Gene *(β-tubulin*)	924	Own design
SsCadF1/SscadR1	TGTCCCAGTTCGACTCTCCTCT TGTTATTGCCCCCTTTGTTGGT	Gene (Calmodulin)	100	D. Smith et al, 2017
HBCadF/HBCadR	ATCTCAGCCCTATGGACACTTG GCTTTGCCGTACCTGGGAAAT	Gene (Calmodulin)	455	Own design
SSasprF/SSasprR	CATTGGAAGTCTCGTCGTCA TCAAACGCCAAAGCTGTATG	Gene (Aspartyl protease)	171	D. Smith et al, 2017

4.4. Molecular data analysis

The sequence data of the ITS and *β–tubulin* gene regions was edited using Bencling website (https://benchling.com). Sequence comparison was carried out using BLASTn on GenBank. Alignments were done using ClustalW through the latest version of Molecular Evolutionary Genetics Analysis (i.e. MEGA7) software package. A dendrogram was constructed from ITS rDNA sequences using maximum-likelihood algorithm (MEGA7 software). The Kimura two-parameter model was applied and bootstrap analysis with 1,000 replications was done to assess group support. The rDNA sequence that was obtained from the software was analyzed and compared with reference sequence from the National Center for Biotechnology Information (NCBI) to construct the dendrogram.

4.5. Results

4.5.1. Mycelial Compatibility Groupings (MCGs)

In the first set, a total of 82% of the mycelial reactions were incompatible whereas 18% were compatible. There was a significant difference ($X^2 = 131$, $P<0.0001$) between the frequency of the two reactions. All the isolates were compatible with themselves. Isolates that formed compatible reactions were placed into one MCG and isolates that formed incompatible reactions were assigned to different sub-MCG based on growth characteristics on DS medium. There were 48 MCG incompatible interactions of which most of these groups had ≤ 5 members. We selected 12 MCG that had greater than 5 members (Table 4.4).

1531 Table 4.4. Mycelial compatibility groups (MCGs), isolates, locations and crop host of isolate, number of MCG, number of isolates in
1532 each MCG, and Shannon index of *Sclerotinia sclerotiorum* (set 1 in 2017). Abbreviation was arbitrarily assigned to each MCGs.
1533 *Ho* is the normalized MCGs diversity.

Obs.	General MCGs[a]	Isolates[b]	Locations	Crop hosts	No. in each MCG	No. of isolates in MCG	*Ho*
1	SMWY	1,3,5,6,7,8,9,14,15, 21,24,25	St. Mary's Co., MD, Sussex Co., DE, Morgan Co., GA, NY	Lima bean, snap bean, and peas, Tomato, Kale, Broccoli, Sunflower	10	12	0.013
2	CMWY	1,2,3,5,6,7,8,9,11,13, 14,16,18,19,20,22,24,25	St. Mary's Co., MD, Wicomico, MD, Sussex Co., DE, Morgan Co., GA, NY, OR, Westley, CA	Lima bean, snap bean, and peas, Tomato, Kale, Broccoli, Sunflower	15	18	0.006
3	CMWN	3,4,5,7,9,15,16	St. Mary's Co., MD, Sussex Co. DE, NJ	Lima bean, snap bean, peas, Soybean	5	7	0.022
4	SIWY	3,6,7,8,12,14,16,18,19, 22,23,24,25	Leonardtown, MD, Sussex Co. DE, OR, Morgan Co., GA, NY	Tomato, lima bean, snap bean, peas, soybean, sunflower	10	13	0.011
5	SGWH	1,2,3,6,7,8,10,12,14,15, 18,19,20	St. Mary's Co., MD, Sussex Co. DE, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, Kale, Broccoli, sunflower, soybean	15	13	0.011
6	SGWY	1,2,3,4,5,6,7,8,9,10,11, 12,13,14,15,16,18,19,20, 21,22,23,24,25	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, Kale, Broccoli, sunflower, soybean	61	24	0.001
7	SGWN	1,2,3,4,8,9,11,12,13,14, 16,18,19,20,22,23	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA,	Lima bean, snap bean, peas, tomato, Kale, Broccoli, sunflower,	22	16	0.008

			Morgan Co., GA, NJ, NY	soybean			
8	SGWTGY	2,3,4,8,10,11,21,22,23,24	St. Mary's Co., MD, Sussex Co., DE, ND, NY	Lima bean, snap bean, peas, tomato, Kale, Broccoli	7	10	0.016
9	CGWTGN	2,4,10,12,18,19	St. Mary's Co., MD, Sussex Co., DE, ND, Oregon, NY	Lima bean, snap bean, peas, tomato, kale, broccoli, soybean	5	6	0.025
10	CGWY	1,2,3,4,5,6,9,10,11,13,14, 15,16,18,19,20,21,22,24	St. Mary's Co., MD, Sussex Co., DE, Stanislaus Co., CA, Morgan Co.,GA, NJ, ND, OR, NY	Lima bean, snap bean, peas, tomato, kale, broccoli, sunflower, soybean	24	19	0.005
11	SGWTYY	2,4,8,9,11,12,13,16,18,21, 22,23,24,25	St. Mary's Co., MD, Sussex Co., DE, Stanislaus Co., CA, ND, OR, NY	Lima bean, snap bean, peas, tomato, kale, broccoli, sunflower, soybean	10	14	0.010
12	CGWTYY	4,5,20,22,23,24,25	Sussex Co., NY	Lima bean, snap bean, peas	5	7	0.022

1534 [a] Arbitrary abbreviations or code given to MCG and the details is shown in Table 4.3; [b] isolate code given only for this research.

In the second set, the majority (66 %) of the reactions were incompatible and only 34% were compatible. There was significant variations (X^2 = 180, P<0.0001) between the frequency of occurrence of compatible and incompatible MCGs. All the isolates were compatible with themselves. Isolates that formed compatible reactions were placed into one MCG and isolates that formed incompatible reactions were assigned to different sub-MCGs based on growth characteristics on DS medium. There were 49 MCG incompatible interactions and most had ≤ 5 numbers. Therefore, we selected 12 MCG that had greater than five numbers (Table 4.5).

1543 Table 4.5. Mycelial compatibility groups (MCGs), isolates, locations, crop hosts, number of MCG, number of isolates in each MCG,
1544 and Shannon index of Sclerotinia sclerotiorum (set 2 in 2018). Abbreviation was arbitrarily assigned to each MCGs. H0 is the
1545 normalized MCGs diversity.

Obs.	General MCGs[a]	Isolates codes[b]	Location/states	Crop type	No. in each MCG	No. of isolates in MCG	H_0
1	SGWTYY	1,2,3,4,5,8,10,11,12, 13,14,15,16,17,18,1 9,20,21,22,23,24,26, 27,28,29,30,31,32,3 3,34,36,37,38,39,40, 41,42	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, broccoli, sunflower, soybean	132	37	0.00131
2	CGWTYY	1,2,3,4,5,8,10,11,12, 13,24,27-32,36,37,38-42	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, green kale, collards, broccoli, sunflower, soybean	76	26	0.00496
3	SGWY	1,2,3,4,5,8,10,11,12, 13,14,15,16,17,19,2 0,21,22,23,24,28,29, 30,31,32,33,34,36,3 7,38,39,40	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, green kale, collards, broccoli, sunflower, soybean	64	33	0.00249
4	IGWTYY	1,2,3,4,5,8,10,11,12, 14,16,17,18,19,20,2 1,22,23,24,26,27,28, 29,30,31,32,33,34,3 6,37,38,39,40,41,42	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, green kale, collards, broccoli, sunflower, soybean	46	37	0.00131
5	CGWY	3,4,5,8,10,12,13,14, 15,16,19,20,22,23,2 8,30,31,34,35,36,37, 38,39,40,41	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, green kale, collards, broccoli,	22	25	0.00536

				sunflower, soybean			
6	SIWTYY	1,2,3,6,8,10,12,14,15,16,17,18,20,22,23,24,29,30,31,33,34,37,10,41	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, broccoli, sunflower, soybean	22	26	0.00496
7	IGWY	3,4,5,8,12,13,14,16,19,20,21,22,26,24,28,31,34,36,37,38,39,40	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, green kale, collards, broccoli, sunflower, soybean	19	22	0.00669
8	IIWTYY	1,3,4,5,9,12,13,17,19,21, 22,28,29,31,34,41,42	St. Mary's Co., MD, Sussex Co., DE, ND, OR, Stanislaus Co., CA, NJ, NY	Lima bean, snap bean, peas, tomato, kale, broccoli, soybean	11	17	0.00935
9	SMWTY	3,12,17,18,19,20,23,24, 29,32,36,38,39,42	St. Mary's Co., MD, Wicomico Co. MD, Sussex Co., DE, ND, OR, NJ, NY	Lima bean, snap bean, peas, tomato, kale, green kale, collards, broccoli, soybean	9	14	0.01136
10	CIWH	5,6,7,9,14,15,20,38	St. Mary's Co., MD, Sussex Co., DE, Stanislaus Co., CA, Morgan Co., GA, NJ, NY	Lima bean, snap bean, peas, sunflower, soybean, tomato, kale, broccoli	7	9	0.01593
11	CIWY	5,6,8,10,12,16,21,22,23	Sussex Co., DE, OR, Stanislaus Co., CA, Morgan Co., GA, NY	Lima bean, snap bean, peas, sunflower, soybean	7	10	0.01484
12	CMWTGY	6,8,9,10,12,16,22,23	Sussex Co., DE, ND, OR, NY	Lima bean, snap bean, peas, sunflower, soybean	7	8	0.01715

1546 [a] Arbitrary abbreviations or code given to MCG and the details is shown in Table 11; [b] isolate code given only for this research.

4.5.2. Genetic diversity of Sclerotinia sclerotiorum

PCR amplification of the ITS region of all isolates using ITS1/ITS4, ITS4/ITS5, and HBITS1/ITS2 primer pair produced DNA fragments between 520 to 550 bp .

4.5.2.1. Identification of Sclerotinia sclerotiorum, the casual agents of white mold in lima bean and other plants by polymerase chain reaction (PCR) technique

Amplicons of all *S. sclerotiorum* isolates obtained with HBITS1/ITS2 and ITS1/ITS4 primers resulted in a 524-bp and 540-bp product, respectively (Figure 4.2).

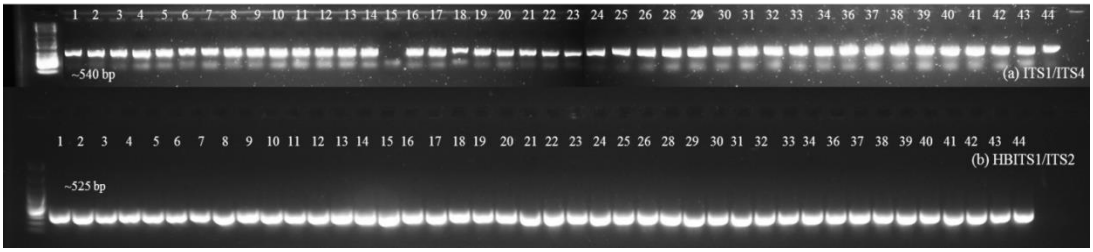

Figure 4.2. Products of polymerase chain reaction amplification of the ITS region of *Sclerotinia sclerotiorum* isolates originating from different regions and crops in US using primer pairs ITS1/ITS4 (row a) and HBITS1/ITS2 (row b).

4.5.2.2. Genetic comparison using a dendrogram based on rDNA sequences of Sclerotinia sclerotiorum isolates

DNA sequences obtained from the current experiment from fragments of the ITS rDNA (amplicons from primers ITS1 and ITS4) from 44 *S. sclerotiorum* isolates were used to construct a dendrogram using the maximum-likelihood algorithm. Sequences of five *S. sclerotiorum* (NCBI) isolates were used as references (Figure 4.3). In addition, sequences from *S glacialis*, *S. tetraspora*, *S. homeocarpa*, *Botrytis cinera*, and *Dumontinia tuberosa* were included for additional comparison (Figure 4.3). The dendrogram based on rDNA ITS1 sequences demonstrated that no or very little intraspecific variation was found in the ITS1-ITS4 regions of the 42 *S. sclerotiorum* isolates, which all were in a single clade (Figures 4.3).

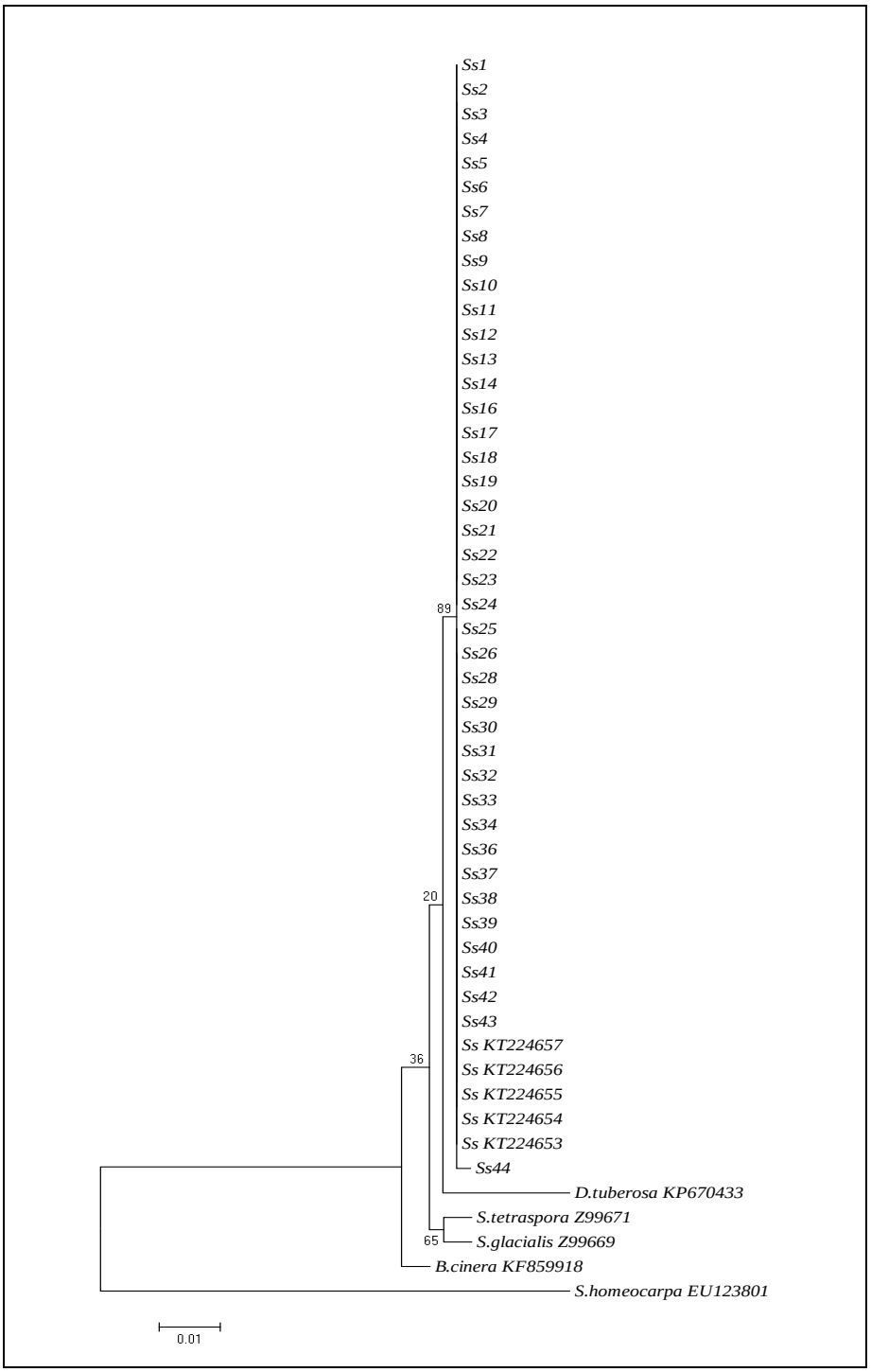

Figure 4.3. Dendrogram of variation among DNA sequences of ITS1 region of *S. sclerotiorum* collected from different crops and different geographical locations in the US. Note: The percentage of trees in which the associated taxa clustered together is shown next to the branches. There were a total of 441 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

4.5.2.4. PCR method for the detection of thiophanate-methyl-resistant Sclerotinia sclerotiorum using amplification of β-tubulin gene

PCR with primers TU1/TU2, HBTU1/TU2, and HBTU3/TU4 amplified a single DNA fragment of *β-tubulin* gene of ~495bp and ~874 bp sizes, respectively with all *S. sclerotiorum* isolates used in this experiment (Figure 4.4; Table 4.6).

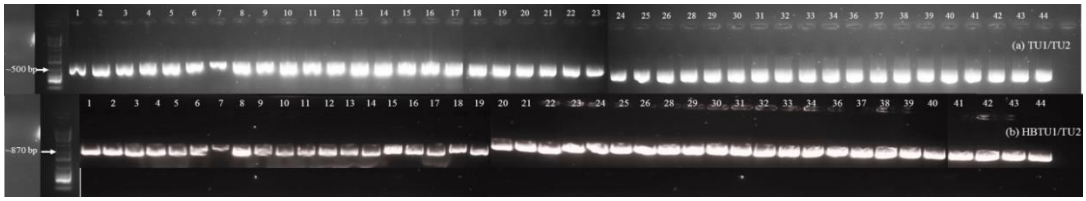

Figure 4.4. Products of polymerase chain reaction amplification of *Sclerotinia sclerotiorum* isolates originating from different regions and crops in USA based on the β-tubulin gene. Products of appropriate size were identified using both primer sets of TU1/TU2 (row a) 494 bp and HBTU1/TU2 (row b) 870 bp, respectively.

4.5.2.3. Most Sclerotinia sclerotiorum isolates cannot be differentiated on the basis of partial DNA sequences of the β-tubulin gene.

Based on the *β-tubulin* gene sequences; (i) all the isolates except isolate 1 (Ss1), isolate 4 (Ss4), isolate 10 (Ss10), isolate 29 (Ss29), isolate 40 (Ss40), and isolate 11 (Ss11), were identical to each other and the reference isolate (Figure 4.6); (ii) isolate 1 (Ss1), isolate 4 (Ss4), isolate 10 (Ss10), isolate 29 (Ss29), and isolate 40 (Ss40) were identical to each other; (iii) isolate 11 (Ss11), which originated from a sunflower plant in OR, is different from all other isolates. Isolate 4, which originated from a snap bean plant in DE, had high oxalic acid production when inoculated to lima bean, soybean, and common bean (Figure 3.6 in chapter 3), which was significantly different from the rest of the *S. sclerotiorum* isolates.

Isolate 1, isolate 2, isolate 11, and Ss_*β-tubulin*_MH796667, a reference isolate from NCBI were selected from the three clades of identical partial sequences of the *β-tubulin* genes (Figure 4.5). The sequence variations were compared using

Clustal Omega algorism in Benchling software. The few bp differences are highlighted in red in Figure 4.6. Thirty-five isolates, represented by isolate Ss2, were identical to the reference isolate Ss_*β-tubulin*_MH796667. Five isolates, represented by isolate Ss1, differed from the reference isolate by one bp change from G to A at position 376 (Figure 4.6). Isolate Ss11 differed from the reference isolate by two changes (T to A and C to T at position 265 and 337, respectively) (Figure 4.6).

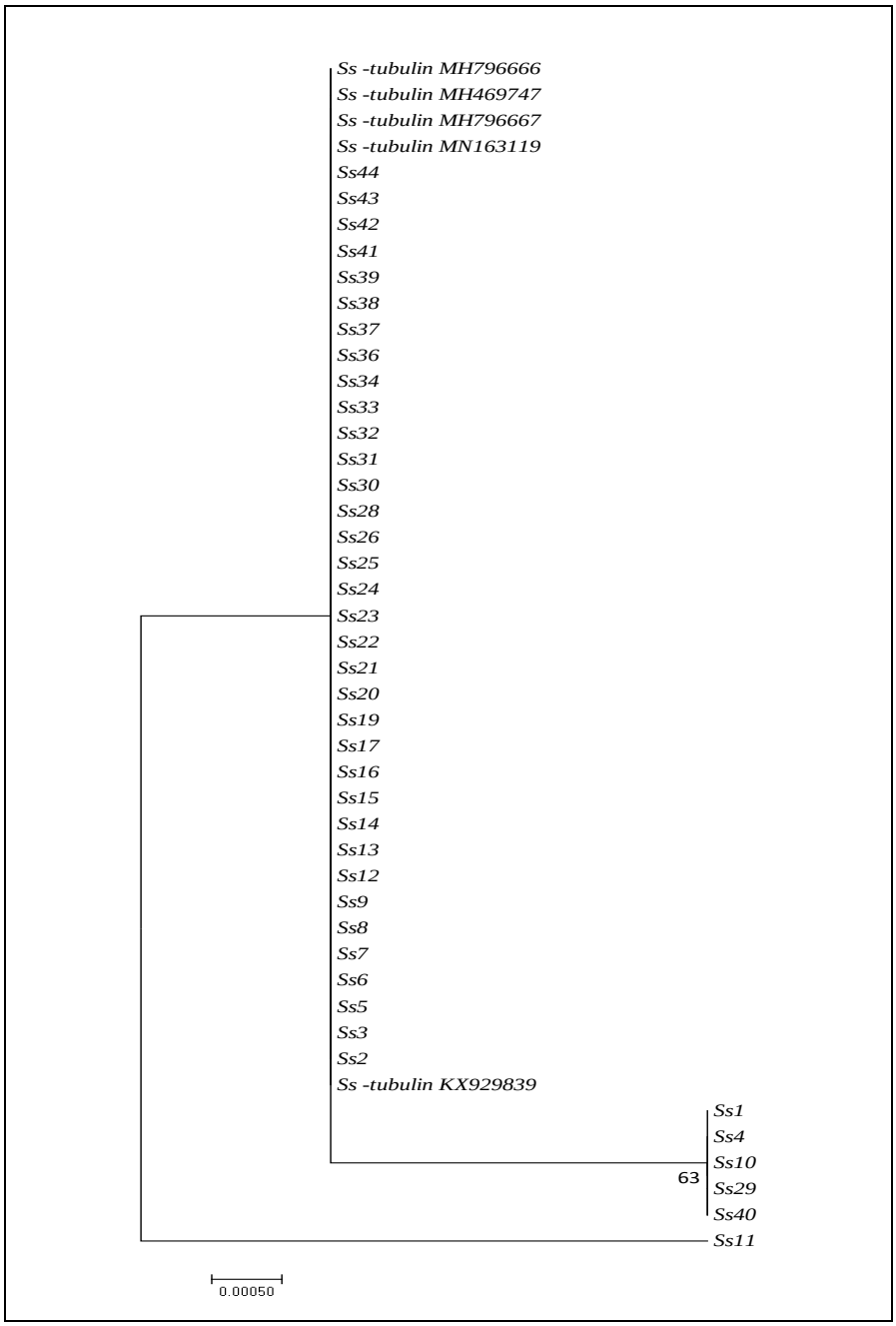

Figure 4.5. Dendrogram of variation among DNA sequences of *β-tubulin* gene of *S. sclerotiorum* collected from different crops and different geographical locations in the US.

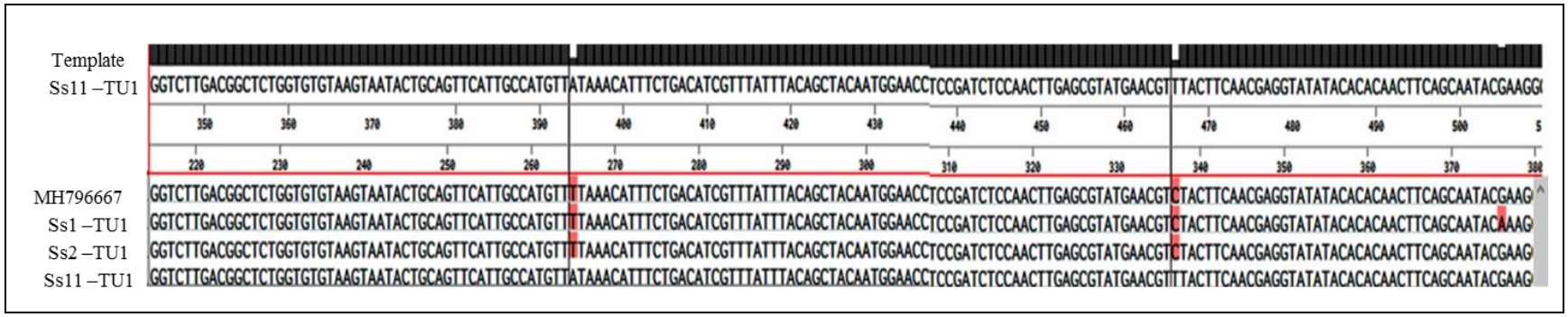

Figure 4.6. Alignment of the partial DNA sequence of the *β-tubulin (TU) gene* of selected *Sclerotinia sclerotiorum* isolates using Clustal Omega in Benchling software package.

4.5.2.5. Amplification of calmodulin gene of Sclerotinia sclerotiorum

PCR with primers SsCadF/STCadR and HBSsCadF/STCadR resulted in amplification of all *S. sclerotiorum* isolates examined in this experiment. The primer pair SsCadF/SsCadR amplified a single fragment of ~100 bp and primer pair HBSsCadF/CadR amplified ~450 bp, respectively in all of the *S. sclerotiorum* isolates (Figure 4.7; Table 4.6).

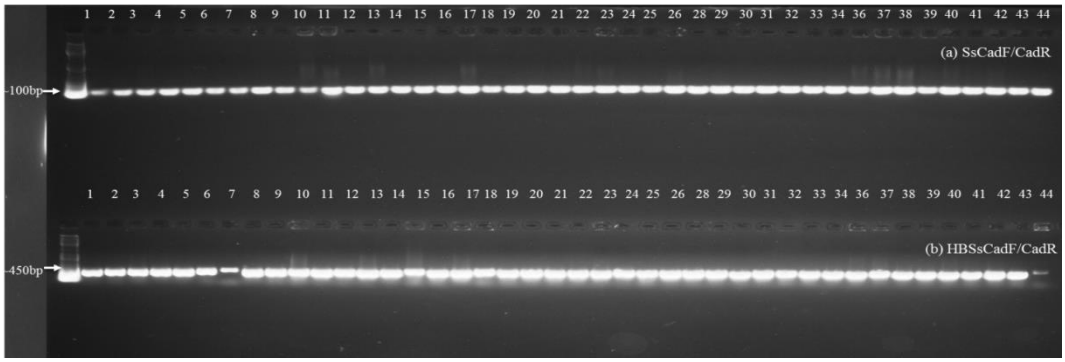

Figure 4.7. Products of polymerase chain reaction amplification of *Sclerotinia sclerotiorum* isolates originating from different regions and crops in US using (row a) primers SsCadF_CadR (row a) with ~100 bp and HBCadF_CadR (row b) with ~450 bp, respectively.

4.5.2.6. Amplification of aspartyl protease (aspr) genes of Sclerotinia sclerotiorum

Amplification of all *S. sclerotiorum* isolates tested in this experiment with SSasprF/SSasprR primers resulted in a ~100 bp product. The intensity of the band visualized on the gel with this primer pair was weak for isolate 4, 5, 16, 30, and 34 and no band was observed for isolate number 6 and 7, respectively (Figure 4.8; Table 4.6).

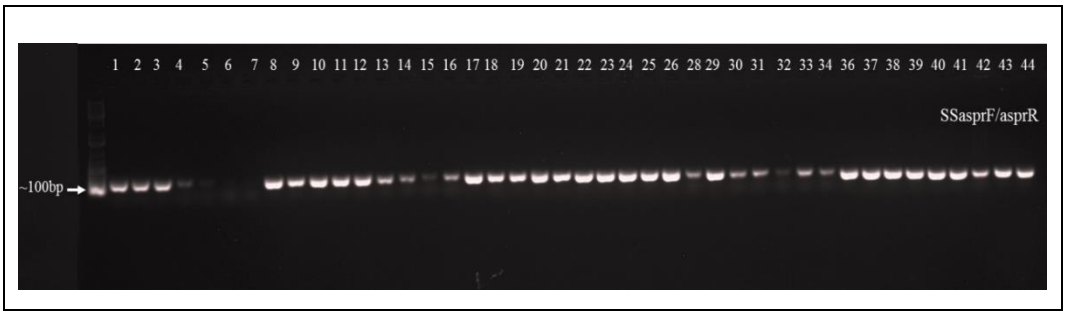

Figure 4.8. Products of polymerase chain reaction amplification of Sclerotinia sclerotiorum isolates originating from different regions and crops in US using primer pairs, SSasprF/SSasprR resulted in a ~100 bp product.

4.5.2.7. Presence of 18S or introns in rDNA in Sclerotinia sclerotiorum

Primer pairs HBNS1/NS2 and NS5/NS6 were used to check the presence of introns or 18S rDNA in *S. sclerotiorum* isolates. The amplification with these primer pairs, HBNS1/NS2 and NS5/NS6, produced a single fragment of ~1,600 bp and 250 bp, respectively. The gel product of the PCR product from NS5/NS6 primer pair is shown in Figure 4.9.

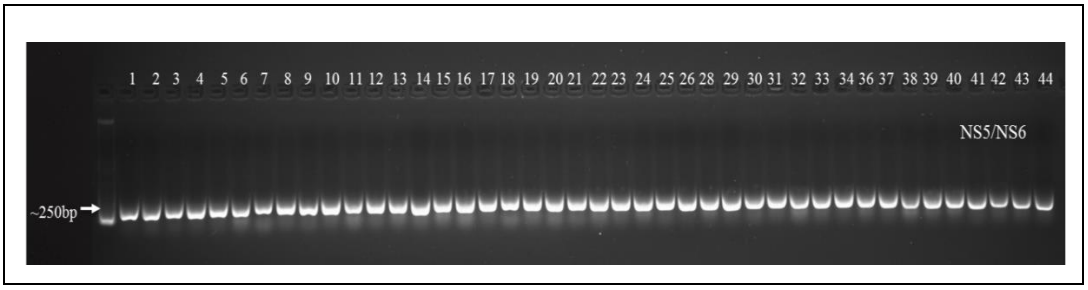

Figure 4.9. Products of the polymerase chain reaction amplification from *Sclerotinia sclerotiorum* isolates originating from different regions and crops in US using primers NS5/NS6 with ~250 bp.

Table 4.6. Origin of *Sclerotinia sclerotiorum* isolates and results of polymerase chain reaction (PCR) assays and analyses on ribosomal DNA (rDNA) for species differentiation.

Code[a]	Host	Year	Locations	SSU[b]					Product	
S. sclerotiorum				ITS1	HBNS1/ NS2	NS5/ NS6	NS1/ NS8	*β-tubulin*	Ssaspr	Sscad
Ss1	Tomato	2015	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss2	Tomato	2015	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss3	Tomato	2015	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss4	Snap bean	2015	Sussex Co., DE	+	+	+	-	+	+	+
Ss5	Lima bean	2015	Sussex Co., DE	+	+	+	-	+	-	+
Ss6	Peas	2017	Sussex Co., DE	+	+	+	-	+	-	+
Ss7	Peas	2017	Sussex Co., DE	+	+	+	-	+	-	+
Ss8	Snap bean	2015	Sussex Co., DE	+	+	+	-	+	+	+
Ss9	Snap bean	2015	Sussex Co., DE	+	+	+	-	+	+	+
Ss10	Soybean	2016	ND	+	+	+	-	+	+	+
Ss11	Sunflower	2016	OR	+	+	+	-	+	+	+
Ss12	Lima bean	2016	Westley, CA	+	-	+	-	+	+	+
Ss13	Soybean	2016	NJ	+	+	+	-	+	+	+
Ss14	Sunflower	2015	Madison, GA	+	+	+	-	+	+	+
Ss15	Lima bean	2015	Sussex Co., DE	-	+	+	-	+	+	+
Ss16	Lima bean	2016	Sussex Co., DE	+	+	+	-	+	+	+
Ss17	Lima bean	2016	Sussex Co., DE	+	+	+	-	+	+	+
Ss18	Lima bean	2016	Sussex Co., DE	+	+	+	-	-	+	+
Ss19	Lima bean	2016	Sussex Co., DE	+	+	+	-	+	+	+
Ss20	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss21	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss22	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss23	Lima bean	2016	NY	+	+	+	-	+	+	+

Ss24	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss25	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss26	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss28	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss29	Lima bean	2016	NY	+	+	+	-	+	+	+
Ss30	Tomato	2017	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss31	Soybean	2017	Sussex Co. DE	+	+	+	-	+	+	+
Ss32	Lima bean	2017	Fulton Co., MD	+	+	+	-	+	+	+
Ss33	Lima bean	2017	Sussex Co., DE	+	+	+	-	+	+	+
Ss34	Lima bean	2017	Sussex Co., DE	+	+	+	-	+	+	+
Ss36	Lima bean	2017	Talbot Co., MD	+	+	+	-	+	+	+
Ss37	Lima bean	2017	Fulton Co., MD	+	+	+	-	+	+	+
Ss38	Green Kale	2018	Wicomico Co., MD	+	+	+	-	+	+	+
Ss39	Collards	2018	Wicomico Co., MD	+	-	+	-	+	+	+
Ss40	Kale	2018	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss41	Broccoli	2018	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss42	Tomato	2018	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss43	Tomato	2018	St. Mary's Co., MD	+	+	+	-	+	+	+
Ss44	Tomato	2018	St. Mary's Co., MD	+	+	+	-	+	+	+

[a] code is given to the current experiment.

[b] Presence of introns in small subunit (SSU) reaction with HBNS1/NS2, NS5/NS6 and NS1/8.

‘+’ or ‘-' indicate the presence or absence, respectively, of the PCR product.

4.6. Discussion

In the current experiment, isolates that originated in each location had a heterogeneous mix of MCGs. Similarly, most isolates belonged to numerous MCGs. None of the isolates were found either compatible or incompatible with all other isolates. Each MCG contained isolates from different locations and hosts. MCGs on cultivated hosts reported to be more complex, indicating that agricultural practices had influence on MCG frequencies and patterns (Kull et al. 2004). Out of 42 isolates collected from different geographical locations and hosts in the US, 12 MCGs were identified. Mandal and Dubey, (2012), observed a similar pattern where within 24 isolates from six different crops and 10 regions in India, there was heterogeneity of MCG and none of the isolates were found compatible or incompatible with all other isolates and each MCG had isolates that originated from different locations and hosts. Both our result and the result from the Mandal and Dubey, (2012) indicated the existence of high variability within *S. sclerotiorum* populations from different crops and geographical locations.

The existence of high variability within the *S. sclerotiorum* isolate in terms of MCGs found between different geographic locations and different hosts may have an effect on the disease phenotype within cultivars of lima bean, soybean, and common bean tested in this experiment because each MCG is genotypically unique. The unique genes that control MCG may be associated to programmed cell death (PCD) that *S. sclerotiorum* uses to protect itself from other antagonistic including from plant resistance. There could be an association between MCG (and PCD) and oxalic acid production by *S. sclerotiorum*. It was previously demonstrated that during plant-microbe interactions PCD can mediate both resistance and susceptible interactions. It was also reported that PCD in multicellular organisms occur by apoptosis (a mechanism by which pathogen uses hosts specialized cellular machinery to kill itself) and autophagy (hosts' mechanism of cleaning out of damaged cells and re-generation of new cells) which both are regulated biological processes that play a central role in tissue homeostasis, development, and disease (Pattingre et al. 2005). Kabbage et al. (2013) reported that transgenic plants expressing anti-apoptotic protein (Bcl-2) family

members inhibited wild type *S. sclerotiorum* induced programmed cell death and disease development. *S. sclerotiorum*, via oxalic acid hijacks host pathways and induces cell death in host plant tissue resulting in distinctive apoptotic features in a time and dose dependent manner. This suggests that autophagy is a defense response in plant-microbes interactions also called suppression of autophagy. Thus, the control of cell death, dictated by the plant (autophagy) or the fungus (apoptosis), can lead to opposing outcomes. The type of cell death (not PCD itself) is decisive to the outcomes of certain plant-microbes interactions (Kabbage et al. 2013).

The ITS region sequence, is highly conserved within *S. sclerotiorum*, and was not informative in identifying heterogeneity within the species. All *S. sclerotiorum* isolates from the current experiment and reference sources are closely related and all grouped into a single clade, which differed from other *Sclerotinia species* and other fungal species. The neighbor-joining analysis further demonstrates the lack of intraspecies variability within the rDNA of the *S. sclerotiorum* isolates used in the current study.

There was sequence similarity between all the isolates, except for isolates 20, 28, and 44 which have a single mutation. ‘In addition, all of *S. Sclerotiorum* isolates sequenced had thymine at the right end position, except for isolate number 30, which has no known sequence at that end (data not shown). Baturo-Ciesniewska et al. (2017) and Njambere et al. (2008) reported similar findings on similarity within the species. One-hundred percent sequence similarity with a quality score of $Q \geq 20$ of most isolates also occurred with previously published sequences in NCBI (Baturo-Ciesniewska et al. 2019 unpublished; Shahoveisi et al. 2019 unpublished; Dubey et al. 2018 unpublished) (Figure 4.3).

The lack of variability among the isolates is because the selected genetic markers amplified highly conserved regions of the same species. Our work demonstrate the lack of intraspecies variation or presence of strong clonality in agreement of *S. sclerotiorum* populations from Canada or US from different crops

(Baturo-Ciesniewska et al. 2017; Jeon et al. 2006; Cubeta et al. 1997; Kohli and Kohn 1996; Kohn 1995; Kohli et al. 1992; Kohn et al. 1991).

When other methods, or more appropriate and informative genetic sequences, were used, *S. sclerotiorum* population have been shown to have high genetic diversity and provided evidence of both clonal and sexual reproduction. The presence of high genetic diversity of *S. sclerotiorum* existed in 36 isolates obtained from oilseed rape (Mert- Türk et al. 2007), from Turkey, ; from 79 isolates from dry bean (Gomes et al. 2011), in isolates from Brazil, ; from canola in Australia (Sexton and Howlett, 2004); and from different crops in New China and Zealand (Sun et al. 2005; Carpenter et al. 1999).

The *S. sclerotiorum β-tubulin* DNA sequences were 494 bp in length and had few differences, which were not adequate to differentiate isolates collected from different crops and different geographical locations in the US. Isolates obtained from lima bean are represented in the first two clades indicating that there exist small variations within isolates obtained from the same crop. The lima bean isolates were from different geographical locations, isolate 20 to isolate 28 are from NY, isolate 5 is from DE, isolate 12 is from CA, and all are in the first clade. However isolate 29 is from NY and is in the second clade. In general, we were unable to clearly separate *S. sclerotiorum* isolates, and there were no distinct correlations among location and crop source. *S. sclerotiorum* is homothallic, and selfing is presumed to be common, which may contribute to this low genotypic diversity.

The 18S rRNA variation was used to differentiate species of Sclerotinia (Baturo-Ciesniewska et al. 2017) and can be used to confirm *Sclerotinia species* identity. We used NS primer pairs and confirmed that the isolates used were S. *sclerotiorum.* In addition, similarity in these bands indicates that there were no intraspecific variations within the isolates. Studies showed that NS1/NS8 primer pair was used to differentiate *S. trifolium* isolates (Baturo-Ciesniewska et al. 2017; White et al. 1994).

Both the genotypic (MCGs and molecular markers) and phenotypic (lesion length) characteristics of the *S. sclerotiorum* isolates tested in the current project may enhance the development of improved screening of lima bean cultivars for white mold resistance. In conclusion, although the morphological characters demonstrated that the *S. sclerotiorum* population is genetically diverse, the molecular data showed no or few polymorphisms at both the ITS and *β-tubulin* genes among these isolates. More research on the *S. sclerotiorum* isolates using whole genome sequencing or molecular markers from different genetic regions is important to understand the population structure of *S. sclerotiorum*. Therefore, we proposed to sequence the entire genome (or additional selected genomic regions) where greater DNA polymorphisms exist, to better understand these isolates.

Chapter 5: Sensitivity of *Sclerotinia sclerotiorum* collected from the mid-Atlantic region of the United States to six fungicide active ingredients

ABSTRACT

Sclerotinia sclerotiorum causes white mold that can lead to high yield loss on lima bean in the mid-Atlantic region. Control of the disease largely relies on use of fungicides. However, no studies have examined the regional population of *S. sclerotiorum* for sensitivity to labeled active ingredients (a.i.s). *In-vitro* bioassays were conducted twice in 2017 and 2019 to determine the sensitivity of 40 *S. sclerotiorum* isolates to boscalid, fludioxonil, cyprodinil, thiophanate-methyl (TM), prothioconazole, and fluazinam a.i.s. Each fungicide was diluted in Dimethyl Sulfoxide (DMSO) and amended to potato dextrose agar (PDA) at two concentrations. Three, 5-mm diameter plugs of *S. sclerotiorum* were transferred to 9-cm diameter plates amended with a fungicide or non-amended, DMSO. The diameter of the mycelial growth was measured daily for 3 days after the first day of transfer and the mean mycelial growth value obtained after the third day was used to calculate percent reduction in mycelial growth using the following formula:$\%\ reduction\ in\ mycelial\ \ growth = \left(\frac{IsgDSMO - Isgal}{IsgDSMO}\right) * 100$; Where; *IsgDMSO* is the mean mycelial growth of the isolate after the third day grown on potato dextrose agar amended with DMSO (control) and *Isgai* is the mean mycelial growth of the isolate after the third day grown on potato dextrose agar amended with DMSO and the fungicide active ingredient (a.i.), boscalid. In addition, correlation between isolates sensitivity to each a.i. and isolate's capability to produce lesion length and oxalic acid (from chapter 3) was analyzed. Data from the two years were analyzed together because there was no significant variation between the two years (P=0.0754). Data from each assay was also analyzed separately. Isolates were significantly different in percent reduction in mycelial growth in all a.i.s. There were significant differences between the two concentrations for all, except cyprodinil and fludioxonil a.i.s. There were also significant interactions between the concentrations

and isolates for all, except boscalid and TM a.i.s. There was only significant correlation between the isolates' sensitivity to boscalid and LS and OA.

5.1. Introduction

White mold is difficult to manage due to its existence as sclerotia in the soil and because of its wide range of hosts. Despite the difficulty, however, a number of approaches have been used to control *S. sclerotiorum* in different crops. For example, prophylactic protectant fungicides such as fludioxonil (Mueller et al. 2002), boscalid, picoxystrobin, pyraclostrobin, and thiophanate-methyl (Huzar-Novakowiski et al. 2017) in soybean, Thiophanate-methyl (TM), fluazinam, and procymidone (Lehner et al. 2015) in common bean, fluopyram, benomyl, tebuconazole, and vinclozolin (Mahoney et al. 2014; Morton et al. 1989; Steadman 1979; Hunter et al. 1978) in dry and white beans. Biofumigant volatiles, from six different crop plants including *Brassica juncea* 'Vittasso' (Warmington and Clarkson 2015) have been used to determine effects on carpogenic germination of sclerotia and mycelial growth of *S. sclerotiorum* and the result showed that all biofumigant plants significantly reduced both germination of sclerotia and mycelial growth of *S. sclerotiorum*. Efficacy of the biopesticide Double Nickel LC (*Bacillus amyloliquefaciens* D747 strain) for the management of white mold in snap and red kidney beans were conducted (Pethybridge et al. 2019). However, their result showed that no significant variations between this biopesticide and conventional fungicide in disease incidence reduction and also there was no significant difference in white mold incidence between 2.34 and 4.68 liters/ha of Double Nickel LC in either crop.

Successful prevention of fungicides resistance development of fungal pathogens requires identifying factors related to the sources, production, and spread of resistance. One of those key factors is variation in sensitivity of fungal pathogens to specific fungicide active ingredients (a.i.s) (Avenot and Michailides 2010). Sensitivity of *S. sclerotiorum* isolates to fungicide a.i.s can be studied *in-vitro* by determining the mycelial growth of the isolates on PDA amended with different fungicide a.i.s. In addition, determination of *in-vitro* fungicide-sensitivity of *S. sclerotiorum* isolates provides useful information in understanding the potential for resistance development in field situations and can also be used to in developing effective fungicide-resistance strategies (Ma et al. 2009). The efficiency of chemical

fungicides depends on the mode of action of the molecule at the physiological level on one or more components of the life cycle of the pathogen (Matheron et al. 2004). In addition, reduction in fungicide sensitivity by *S. sclerotiorum* isolates might develop due to mutation in the isolate's partial sequence of target genes that are affected by specific a.i.s such as the *β-tubulin* gene that encodes specific protein (Lehner et al. 2017). However, laboratory tests are not always reliable predictors and detection of less sensitive isolates under laboratory conditions may not relate to the performance of the a.i.s under field condition (Stevenson et al. 2019).

To date, there is no commercial lima bean cultivar(s) with high levels of resistance to white mold, and growers rely mainly on fungicide applications. Spray of fungicides year after year, especially of site-specific (or fungicides that primarily act on a single target sites) products, can select for resistant isolates and, consequently, may lead to control failures (Brent and Hollomon 2007). Evaluating the sensitivity of *S. sclerotiorum* to the registered fungicides is important for white mold and resistance management. In US, several fungicides are registered for white mold control in several crop plants. Some of the common fungicides used to control white mold in lima bean and their mode of actions are shown in Table 1.1.

5.2. Mode of action of fungicides used to control *Sclerotinia sclerotiorum*

5.2.1. Succinate dehydrogenase inhibitor (SDHI) fungicides

Succinate dehydrogenase inhibitor (SDHI) fungicides include active ingredients such as boscalid, fluopyram, thifluzamide, bixafen, carboxin, fluaxapyroxad, isopyrazam, penthiopyrad and sedaxane (FRAC 2015; McKaey et al. 2011). They play an important role in controlling diseases caused by a broad range of plant pathogenic fungi including white mold (caused by *S. sclerotiorum*, Liu et al. 2018; Stammler et al. 2007), gray mold (caused by *botrytis cinerea*, Zhang et al. 2007; Leroux et al. 2003), Alternaria late blight (caused by *Alternaria alternata*, Avenot et al. 2008a). SDHI fungicides are locally systemic, meaning that they specifically bind to the ubiquinone-binding site (Q-site), which is a functional part of the tricarboxylic cycle and of the mitochondrial complex II, thereby inhibiting fungal mitochondrial respiration chain (Klappach and Stammler 2019; Avenot and

Michailides 2010; Matsson and Hederstedt 2001). By binding to the ubiquinone-binding site of this enzyme, the fungicide block SDH-mediated electron transfer from succinate to ubiquinone (Klappach and Stammler 2019). SDHI, outside of disease control also have beneficial effects on plant physiology the effect of which often called as Plant Health or Plant Performance (Gullickson 2016). In general, these fungicides are considered as excellent candidates for managing fungicide resistance development and optimizing diseases control (Avenot and Michailides 2010; Avenot et al. 2008a; Zhang et al. 2007; Stammler et al. 2007a).

Boscalid, a group 7 fungicide (Varner and Terpstra, 2007) is effective against different stages of fungal development, mainly against spore germination, germ tube elongation but also inhibits other stages such as appressoria formation or mycelial growth (Stammler 2008). Boscalid controls a broad range of fungal pathogens in a wide crops such as dry bean, canola, soybean, oilseed (Mahoney et al. 2014' Bradely et al. 2006; Huzar-Novakowiski et al. 2017; Spitzer et al. 2017; McCreary et al. 2016; Atallah et al 2006; Lehner et al. 2017; Kee et al. 2004; Mahoney et al. 2014). Boscalid has a medium resistance risk that may be results either from the application strategy, the fungus under control, or mode of actions for specific fungi and provided effective control against white mold disease in several crops. Nonetheless, there are no boscalid-resistant field isolates of *S. sclerotiorum* target pathogen has been reported in the US so far.

5.2.2. Anilino-pyrimidine and phenylpyrrole group of fungicides

Anilinopyrimidine (AP) group of fungicides include cyprodinil and pyrimethanil, and others active ingredients, whereas phenylpyrrole (PP) group of fungicides include fludioxonil, are one of the most widely used fungicides in many crops to control white mold and other fungal diseases including *B. cinerea* (Muller et al. 2013). AP fungicides inhibit the sysnthesis of amino acids and PP fungicides interfere with the osmotic signal transduction pathway, affecting the germination of spores abd growth of mycelia. Cyprodinil (AP) is a systemic fungicide and move upwar in the plant, and translocated within the plant system, thus protecting the plant from the attack of pathogenic fungi, or limiting an already established infection;

whereas fludioxonil (PP) is non-systemic that has long residual activity and mainly inhibits the germination of fungal conidia (Cremlyn 2009). Fludioxonil can cause accumulation of glycerol in *S. sclerotiorum* (Duan et al. 2013). Switch, a combination of Scholar SC (50% fludioxonil) and Vangard (75% cyprodinil) are the fungicides to supply fludioxonil and cyprodinil active ingredients, respectively.

5.2.3. Methyl benzimidazole carbamates (MBCs) group of fungicides

The methyl benzimidazole carbamates (MBCs) are broad-spectrum fungicides that have been used worldwide for the control of many plant-pathogenic fungi and they are classified by FRAC as code 1, include with several other groups within a broad class of fungicides that inhibit mitosis and cell division (Olaya and Geddens 2019; FRAC 2014). They are single-site inhibitors that are effective at relatively low doses and their main biological activity against fungi is inhibition of mycleial growth and malfunction of the germ tube (Olaya and Geddens 2019; Muller et al. 2013).

MBC fungicides have both preventive (protective) and early infection (or curative) activity on target fungicide (Olaya and Geddens 2019; Muller et al. 2013; Varner and Terpstra 2007; del Rio et al. 2004). Protective mode of action is when the active ingredient of a given fungicide forms a barrier inside the plant system and prevent fungal infection by stopping spores from germinating, whereas curative fungicides have activity against early fungal infection when applied 24 to 48 hours after infection (Gullickson 2016). MBC fungicides have systemic properties, but they cannot move downward in the plant. They are effective when complete coverage of the plant achieved (Muller et al. 2013). Topsin-M (thiophanate-methyl or TM a.i.), one of a benzimidazole group of fungicides, has an acropetal penetrant properties and is absorbed by the roots and leaves of the treated plants (Olaya and Geddens 2019). TM disrupts fungal mitosis by interfering with *β-tubulin* assembly (Iyer and Makris 2010). TM was first registered as a pesticide in the U.S. in 1973 for use as a fungicide. Other groups of fungicides in the MBCs include benomyl, carbendazim, thiobendazole, and fuberidazole (Olaya and Geddens 2019; Quaranta 2012).

5.2.4. Demethylation Inhibitor (DMI) group of fungicides

DMI fungicides which were introduced in the mid-1970s include the triazoles (Mehl et al. 2019; Muller et al. 2013). DMI fungicides inhibit one specific enzyme, C14-demethylase, which has a role in sterol production in fungi and cause abnormal growth to the fungus that results to death (Muller etl al. 2013). These fungicides may be applied preventatively or early in the fungal infection processes. Most DMI fungicides are locally syetemic and more mobile in plant tissue than QoI fungicides (Muller et al. 2013). However, proline (41 % Prothioconazole) which is one of a DMI (Triazoles) group of fungicides has a systemic demethylation inhibitor activity. It acts against susceptible fungi through the inhibition of demethylation at position 14 of lanosterol or 24-methylene dihydroano-sterol, both of which are precursors of sterols in fungi; i.e., it works through disruption of ergosterol biosynthesis (Ergosterol, a precursor to Vitamin D2, is an important component of fungal cell walls) (USEPA 2007).

5.2.5. Pyridinamine group of fungicides

Omega 500 F (40 % fluazinam) is a multi-site contact fungicide that belongs to the pyridinamine family group of fungicides and has systemic mode of action. Through multi-site modes of action, fluazinam attacks pathogens to provide disease control for legume vegetables, beans, onions, peanuts and potatoes without encouraging resistance development. It also features low use rates and flexible application methods. Fluazinam (Group 29 fungicide) offers consistent white mold control with respect to reduced disease incidence and severity, yield response, and economic return (Mahoney et al. 2014; Varner and Terpstra 2007, Vieira et al. 2010, Pynenburg et al. 2011).

5.3. Rationale and objective

In the mid-Atlantic region of the US, there are no fungicide application guidelines developed for white mold on lima beans. Growers currently use guidelines developed for snap beans or soybean (Everts 2016; Steadman 1979; Hunter et al. 1978) presuming that lima bean shares some similarities with snap bean and soybean

in terms of crop canopy. In addition, there is no information on the sensitivity of *S. sclerotiorum* isolates collected from the region to the common fungicide active ingredients registered in US and used in different crops. Sensitivity and development of resistance of *S. sclerotiorum* isolates to different fungicide active ingredients have been reported in other regions.

For example, SDHI resistant *S. sclerotiorum* were reported under field conditions on oilseed rape in China (Wang et al. 2015), in Germany (Stammler et al. 2010; Glättli et al. 2009). Resistance-mutants to PhenylPyrroles such as fludioxonil have been reported in both field and laboratory populations of *S. sclerotiorum*. (Kuang et al. 2011). In their result, they reported that the laboratory fludioxonil mutants were less fit than their parental isolates in terms of mycelial radial growth, pathogenicity and sclerotial production. Field populations of *S. sclerotiorum* resistance to benomyl, which is very closely related to thiophanate-methyl, have also been reported in Michigan, USA (Koenraadt et al. 1992). Resistance to thiophanate-methyl has been reported in Illinois field population of *S. sclerotiorum* (Mueller et al. 2002) and associated with point mutations in the *β-tubulin* gene, which alter amino acid sequences at the benzimidazole-binding site (Lehner et al. 2015; Ma and Michailides 2005; Koenraadt et al. 1992). In *S. sclerotiorum*, these mutations result in the replacement of glutamine (GAG) by alanine (GCG) at codon 198 (E198A) or of phenylalanine (TTC) by tyrosine (TAC) at codon 200 (F200Y) in the *β-tubulin* gene (Yang et al. 2004). Resistance to dicarboxamide in field isolates of *S. sclerotiorum* in China was also identified (Zhou et al. 2014).

Although boscalid and other active ingredients have been used for many years to manage *S. sclerotiorum*, sensitivity to this active ingredient has not been reported in populations of field isolates of *S. sclerotiorum* from lima bean in the mid-Atlantic regions of US. Therefore, assessing the sensitivity of *S. sclerotiorum* to the registered fungicides is important to identify if there exists development of resistance by *S. sclerotiorum*. Thus, the objective of this *in-vitro* bioassay was to determine the sensitivity of 40 *S. sclerotiorum* isolates to the a.i.'s boscalid, fludioxonil, cyprodinil, thiophanate-methyl, prothioconazole, and fluazinam.

5.4. Materials and Methods

5.4.1. In vitro growth of S. sclerotiorum on fungicide-amended potato dextrose agar

An *in vitro* bioassay was done to determine the efficiency of six registered fungicides on the growth of *S. sclerotiorum* isolates collected from different regions of the US. This experiment was conducted following a protocol according to the protocol of Lehner et al, 2017 and Muller et al, 2002. Six fungicides, Endura 70 WG (70 % boscalid, BASF Corp., Florham Park, NJ), Scholar SC (50 % fludioxonil, Syngenta Crop Protection, Greensboro, NC), Vangard (75 % cyprodinil, Syngenta Crop Protection, Greensboro, NC), Topsin 4.5 FL (45 % thiophanate-methyl, United Phosphorus, Inc., King of Prussia, PA), Proline (41 % Prothioconazole, Syngenta Crop Protection, Greensboro, NC), Omega 500 F (40 % fluazinam, Syngenta Crop Protection, Greensboro, NC), and Scholar SC (50% fludioxonil, Syngenta, Sudlersville, MD) were used for this bioassay.

The fungicides were diluted in dimethyl sulfoxide (DMSO) to obtain 100 mg of a.i. ml^{-1} stock solution. To obtain the desired concentrations, serial dilutions were made from the stock solution in distilled water. The concentrations were 0 (PDA + DMSO), 1.0, and 5 µg of boscalid ml^{-1}; 0, 0.5, and 1.0 µg of fludioxonil and cyprodinil ml-1; 0, 5.0, and 10 µg of thiophanate-methyl ml-1; 0, 1.0, and 5 µg prothioconazole ml^{-1}; and 0, 0.05, and 0.1 µg of fluazinam ml^{-1}. The fungicides were added to cool (42 to 50^0C) PDA and mixed thoroughly. The selection of these concentrations was made from different related research work based on better reduction of the mycelial growth of *S. sclerotiorum* in each of the a.i., mainly based on the EC_{50} values. For example, boscalid significantly reduced the mycelial growth of *S. sclerotiorum* from 77 to 100% at 1.0 and 56 to 83% at 5.0 µg ml^{-1}, respectively (Lehner et al. 2017; Matheron and Porchas, 2004). Similarly fludioxonil was reported reduced the mycelial growth from 95 to 99% at 0.1 µg ml^{-1} and from 77 to 100% at 1.0 µg ml^{-1}, respectively (Matheron and Porchas 2004); fluazinam reduced from 95 to 99% at 0.1 and from 77 to 100% at 1.0 µg ml^{-1}, respectively (Matheron and Porchas, 2004); thiophanate-methyl from 53 to 93% reduction at a rate ranges from 5.0 to 10 µg ml^{-1} (Lehner et al. 2017; Mueller et al. 2002); and Hou et al. (2018), based on EC_{50}

value, showed cyprodinil significantly reduced the radial growth of *S. sclerotiorum* at 0.5 and 1.0 µg ml^{-1}.

Twenty milliliters of amended and non-amended (control) PDA containing each of the above concentrations were poured into 9 cm diameter petri dishes. Initial cultures of *S. sclerotiorum* isolates were grown on regular PDA for approximately one week. Plugs (5 mm diameter) were taken from actively growing margins of the colony, and one plug was transferred to the center of each of four replicated plates of each treatment concentration. The plates were placed in a growth chamber at 25°C under constant fluorescent light at 100-µE·m^{-2}·s^{-1}. Each plate with a mycelial plug was considered as an experimental unit. The diameter of the radial growth was measured daily for 3 days after the first day of transfer and the mean radial growth value obtained after the third day was used for statistical analysis.

5.4.2. Statistical analysis

The experiment was conducted as a completely randomized design (CRD). Each sampling unit, which was replicated three times, consisted of a petri dish with three sub-samples (plugs of *S. sclerotiorum* culture). The experiment was conducted twice, once in 2017 and once in 2018. The diameter of the *S. sclerotiorum* radial growth on each petri dish was measured daily for 3 days after the first day of transfer and the mean radial growth measured after the third day was used to calculate percent reduction in radial growth due to fungicide using the following formula:

$$Percent\ reduction\ in\ mycelial\ \ growth\ (PRMG) = \left(\frac{MgDSMO - Mgai}{MgDSMO}\right) * 100$$

Where; *MgDMSO* is the mean mycelial growth of the isolate after the third day grown on potato dextrose agar amended with DMSO (control) and *Mgai* is the mean mycelial growth of the isolate after the third day grown on potato dextrose agar amended with DMSO and the fungicide. Data from the two years were analyzed together because there was no significant difference between the two years (*P*=0.0754).

Analysis of variance (ANOVA) was conducted using Proc GLIMMIX in SAS (SAS Institute, Inc., Cary, NC) to determine if there were significant differences in percent reduction in mycelial growth among the isolates or between the two fungicide concentrations. The isolates and fungicide concentrations were considered fixed variables and the replications and years as random factors. When there was a significant interaction between the isolate and fungicide concentration, the percent reduction in growth of isolates at each fungicide concentration was analyzed separately. Means comparisons were conducted using Tukey-Kramer multiple mean comparison analysis method at $\alpha = 0.05$. In addition, proc corr or Pearson's correlation analysis was conducted in SAS (SAS Institute, Inc., Cary, NC) to determine if there were relationships between sensitivity of the isolates to each fungicide a.i. at the higher concentration and 1) lesion length (chapter 3), 2) oxalic acid production (chapter 3), and 3) the reduction in growth due to the other fungicides. An analysis of the effect of each fungicide was conducted separately because different concentrations of each fungicide were tested and fungicides were tested in separate experiments.

5.5. Results

5.5.1. Sensitivity of Sclerotinia sclerotiorum isolates to boscalid

There was a significant difference (F=8.36; $P<0.0001$) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates to boscalid relative to the DMSO control. The average percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates from the two concentrations on PDA amended with boscalid ranged from 35% for isolate 13 to 74% for isolate 12, respectively (Table 5.2). There was a significant difference (F=563.51; $P<0.0001$) between the two concentrations of boscalid in the percent reduction in mycelial growth of *S. sclerotiorum* isolates. There was no significant interactions (F=0.88; $P=0.6775$) between the isolates' sensitivity to boscalid and the two concentrations of boscalid (Table 5.1). Therefore, the percent reductions in mycelial growth of isolates on the two concentrations were combined for the means separation test. Based on the average percent reduction in mycelial growth from the two concentrations of boscalid, isolate 12 significantly had the

greatest percent reduction (74%) compared to all other isolates, except isolate 6 (70%), 10 (70%), 14 (72%), 22 (67%), and 25 (67%). Isolate 13 significantly had the least (35%) percent reduction compared to all isolates, except isolate 2 (37%), 11 (40%), 26 (44%), and 39 (36%). Isolates 12 and 13 were the most and the least sensitive isolates, respectively (Table 5.2).

There was significant correlation between percent reduction in mycelial growth of the isolates (grown in boscalid at 5 μg ml^{-1}), and 1) lesion length (r=-0.28; *P*=0.0004), and 2) oxalic acid production by each isolate (r=-0.23; *P*=0.0040), respectively (Table 5.13).

Table 5.1. Analysis of variance of percent reduction in mycelial growth between *Sclerotinia sclerotiorum* isolates grown on potato dextrose agar amended with DMSO (control) and boscalid at two concentrations.

Source	DF	Anova SS	Mean square	F Value	Pr > F
Isolate	39	47747.81	1447.83	8.36	<0.0001
Concentration	1	89041.79	80041.79	563.51	<0.0001
Isolate*Concentration	39	4412.99	183.87	0.88	0.6775

Table 5.2. Estimate of the percent reduction in mycelial growth of *Sclerotinia sclerotiorum* isolates grown for three days on potato dextrose agar amended with DMSO and boscalid.

Isolate number	Estimate of the percent reduction in mycelial growth	
12	74	a*
14	72	ab
6	70	ab
10	70	abc
25	67	abcd
22	67	abcde
4	64	bcdef
32	64	bcdefg
19	63	bcdefg
33	63	bcdefg
7	63	bcdefg
34	61	cdefgh
24	59	defghi
1	58	defghi
29	58	defghij
31	58	defghij
5	57	efghijk
38	56	fghijkl
28	55	ghijklm
23	54	ghijklm
20	54	ghijklm
16	52	hijklmn
37	52	hijklmn
18	52	hijklmn
9	51	ijklmn
40	50	ijklmn
15	49	jklmno
3	48	klmno
8	48	klmno
21	47	lmno
36	47	lmno
30	47	lmno
42	46	mnop

17	45	nopq
41	45	nopq
26	44	nopqr
11	40	opqr
2	37	pqr
39	36	qr
13	35	r

*Numbers followed by the same letter are not significantly different according to Tukey-Kramer at $\alpha = 0.05$ level.

5.5.2. Sensitivity of Sclerotinia sclerotiorum isolates to cyprodinil

There was a significant difference (F=9.97; *P*<0.0001) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates grown in cyprodinil relative to the DMSO control. There was no significant difference in the percent reduction in mycelial growth of *S. sclerotiorum* isolates (F=0.97; *P*<0.3248) between the two concentrations of cyprodinil. However, there was a significant interactions (F=36; *P*<0.0001) between the isolates' sensitivity to cyprodinil and the two concentrations of cyprodinil (Table 5.3). Therefore the percent reductions in mycelial growth of isolates on the two concentrations were analyzed separately for the means separation test.

Cyprodinil at 0.5 μg ml^{-1} caused a significant difference (F=4.76; *P*<0.0001) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates grown in relative to the DMSO control. The percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates ranged from -14% for isolate 17 to 31% for isolate 18, respectively (Table 5.4). This indicates that some isolates grew better in the presence of the low concentration of cyprodinil than in its absence. *S. sclerotiorum* isolate sensitivity to cyprodinil significantly related with the concentrations of this a.i. At 0.5 μg ml^{-1} cyprodinil, isolate 18 significantly had the greatest reduction (31%) in mycelial growth compared to all, except isolates 1 (19%), 4 (9%), 6 (10%), 12 (6%), 20 (10%), 20 (10%), 21 (14%), 24 (7%), 25 (27%), 39 (7%), and 42 (31%) and isolate 17 significantly had the least (-14%) percent reduction compared to all, except isolates 1 (19%), 18 (31%), 21 (14%), 25 (27%), and 42 (3%). Isolates 18 and 17

were the most and the least sensitive isolates to cyprodinil at 0.5 μg ml^{-1}, respectively. Similarly, there was a significant difference (F=35.34; *P*<0.0001) in percent reduction of mycelial growth within the 40 *S. sclerotiorum* isolates grown in cyprodinil at 1 μg ml^{-1} relative to the DMSO control, which ranged from 0% for isolate 40 to 29% for isolate 1, respectively (Table 5.4). Similarly, at 1 μg ml^{-1} of cyprodinil, isolate 1 significantly had the greatest reduction in mycelial growth (29%) than any other isolate, except isolate 42 (23%) and isolate 40 significantly had the least (0%) percent reduction compared to all, except isolates 1 (29%), 3 (11%), 6 (7%), 7 (10%), 8 (7%), 12 (11%), 16 (11%), 18 (11%), 20 (16), 21 (11%), 24 (7%), and 42 (27%) (Table 5.4). The growth of five of the six isolates (18, 42, 1, 21, and 20) that were most inhibited at 0.5 μg ml^{-1} were also among the most inhibited at 1 μg ml^{-1}. However the mycelial growth of isolate 25 was reduced by 27% at 0.5 but not at 1.0 μg ml^{-1}.

There was no significant correlation between the percent reduction in mycelial growth of isolates grown in cyprodinil at 1 μg ml^{-1} and 1) lesion length (r=-0.13; *P*=0.1214), and 2) oxalic acid production by each isolate (r=-0.16; *P*=0.0548) (Table 5.13).

Table 5.3. Analysis of variance of percent reduction in mycelial growth between *Sclerotinia sclerotiorum* isolates grown on potato dextrose agar amended with DMSO (control) and cyprodinil at 0.5 μg ml^{-1} and 1 μg ml^{-1}.

Source	DF	Anova SS	Mean square	F Value	Pr > F
Isolate	39	26794.07	687.03	9.97	<0.0001
Concentration	1	66.96	66.96	0.97	0.3260
Isolate*Concentration	39	7003.50	179.58	3.61	<0.0001

Table 5.4. Estimate of the percent reduction in mycelial growth of *Sclerotinia sclerotiorum* isolates grown for three days on potato dextrose agar amended with DMSO and cyprodinil at two concentrations.

Concentration 1 (0.5 μg ml^{-1})			Concentration 2 (1 μg ml^{-1})		
Isolate number	Estimate of the percent reduction in mycelial growth		Isolate number	Estimate of the percent reduction in mycelial growth	
18	31	a*	1	29	a*
42	31	a	42	23	a
25	27	ab	20	16	b
1	19	abc	18	11	bc
21	14	abcd	21	11	bc
20	10	abcde	16	11	bc
6	10	abcde	12	11	bc
4	9	abcde	3	11	bc
39	7	abcde	7	10	cd
24	7	abcde	24	7	cde
12	6	abcde	8	7	cde
16	5	bcde	6	7	cde
3	5	bcde	4	4	def
29	5	bcde	2	3	ef
23	4	bcde	11	2	ef
7	2	bcde	30	1	ef
11	1	cde	22	0	f
26	1	cde	19	0	f
9	1	cde	26	0	f
28	0	cde	25	0	f
15	0	cde	23	0	f
34	0	cde	17	0	f
13	0	cde	28	0	f
14	0	cde	15	0	f
10	0	cde	33	0	f
36	0	cde	34	0	f
38	0	cde	14	0	f
5	0	cde	31	0	f
40	0	cde	29	0	f
41	0	cde	32	0	f
37	0	cde	13	0	f
33	-1	cde	37	0	f
30	-2	cde	10	0	f
8	-2	cde	39	0	f
32	-6	cde	36	0	f
31	-7	de	38	0	f

2	-8	de	5	0	f
22	-10	de	41	0	f
19	-12	e	9	0	f
17	-14	e	40	0	f

*Numbers followed by the same letter are not significantly different according to Tukey-Kramer at $\alpha = 0.05$ level.

5.5.3. Sensitivity of Sclerotinia sclerotiorum isolates to fludioxonil

There was a significant difference (F=1.83; *P*=0.0017) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates grown on fludioxonil relative to the DMSO control. There was no significant difference (F=1.32; *P*<0.2513) between the two concentrations of fludioxonil in the percent reduction in mycelial growth of *S. sclerotiorum* isolates. However, there was a significant interaction (F=2.13; *P*=0.0002) between the isolates' sensitivity to fludioxonil and the two concentrations of fludioxonil (Table 5.5). Therefore, the percent reductions in mycelial growth of isolates on the two concentrations were analyzed separately for the means separation test (Table 5.6).

There was a significant difference (F=1.61; *P*=0.0208) in percent reduction of mycelial growth within the 40 *S. sclerotiorum* isolates grown in fludioxonil at 0.5 μg ml^{-1} relative to the DMSO control. The percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates at 0.5 μg ml^{-1} concentration of fludioxonil ranges from 90% for isolate 20 to 100% for isolate 29, respectively (Table 5.6). Similarly, there was a significant difference (F=1.66; *P*=0.0091) in percent reduction of mycelial growth within the 40 *S. sclerotiorum* isolates grown in fludioxonil at 1 μg ml^{-1} relative to the DMSO control and the percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates at 1 μg ml^{-1} concentration of fludioxonil ranges from 88% for isolate 2 to 100% for isolate 29, respectively (Table 5.10).

There was no significant correlation between the reduction in mycelial growth of isolates on fludioxonil at 0.5 μg ml^{-1}, and 1) lesion length (r=0.15; *P*=0.0597), and 2) oxalic acid production by each isolate (r=0.11; *P*=0.1905) (data not shown). Similarly, there was no correlation between the isolates (grown in fludioxonil at 1 μg

ml^{-1}) percent reduction in mycelial growth, and 1) lesion length (r=0.01; *P*=0.9489), and 2) oxalic acid production (Chapter 3) by each isolate (r=0.03; *P*=0.6344) (Table 5.13).

Table 5.5. Analysis of variance of percent reduction in mycelial growth between *Sclerotinia sclerotiorum* isolates grown on potato dextrose agar amended with DMSO (control) and fludioxonil at 0.5 µg ml-1 and 1 µg ml-1.

Source	DF	Anova SS	Mean square	F Value	Pr > F
Isolate	39	2095.11	53.72	1.83	0.0017
Concentration	1	37.95	37.95	1.32	0.2513
Isolate*Concentration	39	2389.36	61.27	2.13	0.0002

Table 5.6. Estimate of the percent reduction in mycelial growth of *Sclerotinia sclerotiorum* isolates grown for three days on potato dextrose agar amended with DMSO and fludioxonil at two concentrations.

Concentration 1 (0.5 µg ml^{-1})			Concentration 2 (1 µg ml^{-1})		
Isolate number	Estimate of the percent reduction in mycelial growth		Isolate number	Estimate of the percent reduction in mycelial growth	
29	100	a*	19	100	a*
41	100	a	23	100	a
39	100	a	25	100	a
3	100	a	16	100	a
37	100	a	26	100	a
25	100	a	29	100	a
26	100	a	8	100	a
36	100	a	3	100	a
10	100	a	41	100	a
2	99	a	5	100	a
5	99	a	4	100	a
4	99	a	11	100	a
40	99	a	9	99	a
34	99	a	20	99	a
21	99	a	32	99	a
15	99	a	10	99	a
33	99	a	24	99	a
1	99	a	40	99	a
22	99	a	39	99	a

13	99	a	42	99	a
42	99	a	17	99	a
17	98	a	33	99	a
31	98	a	12	98	a
18	98	a	30	98	a
9	98	a	31	98	a
14	97	ab	22	98	a
30	97	ab	15	98	a
24	97	ab	36	98	a
12	97	ab	18	98	a
32	97	ab	13	98	a
11	96	ab	34	98	a
19	96	ab	37	98	a
8	96	ab	14	97	a
38	95	ab	38	97	a
16	94	ab	28	97	a
28	94	ab	6	97	a
7	94	ab	21	96	a
23	92	ab	1	96	a
6	92	ab	7	95	a
20	90	b	2	77	b

*Numbers followed by the same letter are not significantly different according to Tukey-Kramer at $\alpha = 0.05$ level.

5.5.4. Sensitivity of Sclerotinia sclerotiorum isolates to fluazinam

There was a significant difference (F=7.90; *P*=0.0046) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates grown on fluazinam relative to the DMSO control. There was also a significant difference in the percent reduction in mycelial growth of *S. sclerotiorum* isolates (F=105.21; *P*<0.0001) between the two concentrations of fluazinam. There was also a significant interaction (F=10.45; *P*=0.0435) between the isolates' sensitivity to fluazinam and the two concentrations of fluazinam (Table 5.7) Therefore the percent reductions in mycelial growth of isolates on the two concentrations were combined for the means separation test.

At the lower concentration of fluazinam at 0.05 μg l^{-1}, there was a significant difference (F=3.38; *P*<0.0001) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* relative to the DMSO control. The percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates ranged from 46% for isolate 41 to 98% for

isolate 28, respectively (Table 5.8). *S. sclerotiorum* isolate sensitivity to fluazinam significantly related with the concentrations of this a.i. At 0.05 μg ml^{-1}, isolate 28 significantly had the greatest reduction (98%) in mycelial growth compared to isolates 42 (50%) and 41 (46%) and isolate 41 had the least (46%) percent reduction compared to all except isolates 4 (75%), 10 (75%), 12 (70%, and 42 (50%). Isolates 28 and 41 were the most and the least sensitive isolates to fluazinam at 0.05 μg ml^{-1}, respectively. Similarly, there was a significant difference (F=4.92; *P*=0.0056) in percent reduction of mycelial growth within the 40 *S. sclerotiorum* isolates at 0.1 μg ml^{-1} The percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates at 0.1 μg ml^{-1} fluazinam ranged from 44% for isolate 12 to 98% for isolate 29, respectively (Table 5.8). At 0.1 μg ml^{-1} of fluazinam, isolate 29 significantly had the greatest reduction (96%) in mycelial growth compared to all except isolates 1 (96%), 2 (68%), 4 (93%), 10 (61%), 36 (82%), 37 (80%), 38 (67%), and 41 (93%) and isolate 12 had the least (44%) percent reduction (Table 5.8). Isolate 41, which had significantly less growth at the higher concentration of 0.1 μg ml^{-1} was only reduced by 50% at the lower concentration.

There was no significant correlation between the reduction in mycelial growth of the isolates grown in fluazinam at 0.05 μg ml^{-1}, and lesion length (r=-0.10; *P*= 0.2321), however, there was a significant negative correlation between the reduction in mycelial growth of the isolates grown in fluazinam at 0.05 μg $ml^{-1}$and oxalic acid production by each isolate (r=-0.16; *P*= 0.0471). Similarly, there was no correlation in reduction in mycelial growth of isolates grown in fluazinam at 0.1 μg ml^{-1} and 1) lesion length (r=-0.02; *P*= 0.7682), and 2) oxalic acid production by each isolate (r=0.06; *P*= 0.4780) (Table 5.13).

Table 5.7. Analysis of variance of percent reduction in mycelial growth between *Sclerotinia sclerotiorum* isolates grown on potato dextrose agar amended with DMSO (control) and fluazinam at two concentrations, crop and location sources of the isolates.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate	39	30656.31	786.06	7.90	0.0046
Concentration	1	92142.66	92142.6	105.21	<0.0001
Isolate*Concentration	39	49552.09	1270.5	10.45	0.0435

Table 5.8. Estimate of the percent reduction in mycelial growth of *Sclerotinia sclerotiorum* isolates grown for three days on potato dextrose agar amended with DMSO and fluazinam.

Concentration 1 (0.05 μg ml^{-1})			Concentration 2 (0.1 μg ml^{-1})		
Isolate number	Estimate of the percent reduction in mycelial growth		Isolate number	Estimate of the percent reduction in mycelial growth	
28	98	a*	29	96	a*
36	95	a	1	96	a
3	95	a	4	93	ab
25	95	a	41	93	ab
33	94	a	36	82	abc
34	93	a	37	80	abcd
24	93	a	2	68	abcd
16	92	a	38	67	abcd
30	92	a	10	61	abcd
40	91	a	19	58	bcd
1	91	a	39	55	cd
37	91	a	21	55	cd
23	91	a	17	55	cd
15	91	a	8	53	cd
8	91	a	26	53	cd
26	90	a	32	52	cd
20	89	a	31	52	cd
39	89	a	3	52	cd
9	88	a	14	52	cd
7	88	a	25	52	cd
5	88	a	23	50	cd
32	88	a	15	50	cd
38	86	a	13	50	cd
2	86	a	30	50	cd

18	86	a	11	50	cd
6	85	a	34	50	cd
21	84	a	24	50	cd
19	82	a	40	49	cd
31	81	a	9	49	cd
11	81	a	5	48	cd
17	80	a	6	48	cd
13	79	a	16	48	cd
22	79	ab	28	47	cd
14	78	ab	22	47	cd
29	76	ab	33	47	cd
10	75	abc	7	47	cd
4	75	abc	42	46	cd
12	70	abc	18	46	cd
42	50	bc	20	44	d
41	46	c	12	44	d

*Numbers followed by the same letter are not significantly different according to Tukey-Kramer at $\alpha = 0.05$ level.

5.5.5. Sensitivity of Sclerotinia sclerotiorum isolates to prothioconazole

There was a significant difference (F=18.28; *P*<0.0001) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates grown in prothioconazole relative to the DMSO control. There was a significant difference in reduction in mycelial growth of *S. sclerotiorum* isolates (F=50.94; *P*<0.0001) between the two concentrations of prothioconazole. There was also significant interaction (F=8.39; *P*<0.0001) between the isolates' sensitivity to prothioconazole and the two concentrations of prothioconazole (Table 5.9). Therefore, the percent reductions in mycelial growth of isolates on the two concentrations were analyzed separately for the means separation test.

At the lower concentration, there was a significant difference (F=17.66; *P*<0.0001) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates grown in prothioconazole at 1 μg ml^{-1} relative to the DMSO control. The percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates at 1 μg ml^{-1} concentration of prothioconazole ranges from 0% for isolate 42 to 82% for isolate 9

(Table 5.10). At 1 μg ml^{-1}, isolate 9 significantly had the greatest reduction in radial growth of any isolate (82% reduction) compared to all, except isolates 4 (74%) and 29 (77%) and isolate 42 significantly had the least (0% reduction) percent reduction compared to all, except isolates 2 (44%), 4 (74%), 9 (82%), 29 (77%), 36 (42%), 37 (49%), and 41 (32%). Isolates 9 and 42 were the most and the least sensitive isolates at 1 μg ml^{-1} of prothioconazole, respectively. Similarly, at 5 μg ml^{-1} prothioconazole there was a significant difference (F=10.51; $P<0.0001$) in percent reduction of mycelial growth among the 40 *S. sclerotiorum* isolates and the percent reduction in mycelial growth ranged from -1% for isolate 5 to 96% for isolate 36, respectively (Table 5.10). At 5 μg ml^{-1}, isolate 36 significantly had the greatest reduction (96%) in radial growth compared to all, except isolates 1 (79%), 2 (59%), 4 (92%), 9 (51%), 10 (63%), 28 (56%), 29 (49%), 34 (46%), 37 (93%), 38 (60%), 39 (69%), 40 (58%), and 41 (93%) and isolate 5 significantly had the least (-1%) percent reduction compared to all, except isolates 1 (79%), 2 (59%), 4 (92%), 10 (63%), 28 (56%), 36 (96%), 37, (97%), 38 (60%), 39 (67%), 40 (58%), and 41 (93%) (Table 5.10).

There was no significant correlation between the percent reduction in mycelial growth of isolates grown in prothioconazole at 1 μg ml^{-1} and 1) lesion length (r=-0.07; P= 0.4118), and 2) oxalic acid production by each isolate (r=0.08; P=0. 0.3043). Similarly, there was no correlation between the reduction in mycelial growth of isolates grown in prothioconazole at 5 μg ml^{-1} and 1) lesion length (r=-0.04; P= 0.6007), and 2) oxalic acid production by each isolate (r=0.08; P= 0.3405) (Table 5.13).

Table 5.9. Analysis of variance of percent reduction in mycelial growth between *Sclerotinia sclerotiorum* isolates grown on potato dextrose agar amended with DMSO (control) and prothioconazole at two concentrations, crop and location sources of the isolates.

Source	DF	Anova SS	Mean square	F Value	Pr > F
Isolate	39	251468.23	6447.90	18.28	<0.0001
Concentration	1	17969.81	17969.81	50.94	<0.0001
Isolate*Concentration	39	74204.87	1902.69	8.39	<0.0001

Table 5.10. Estimate of the percent reduction in mycelial growth of *Sclerotinia sclerotiorum* isolates grown for three days on potato dextrose agar amended with DMSO and prothioconazole at two concentrations.

Concentration 1 (1 μg ml^{-1})			Concentration 2 (5 μg ml^{-1})		
Isolate number	Estimate of the percent reduction in mycelial growth		Isolate number	Estimate of the percent reduction in mycelial growth	
9	82	a*	36	96	a*
29	77	a	37	93	ab
4	74	ab	41	93	ab
37	49	bc	4	92	ab
2	44	cd	1	79	abc
36	42	cde	39	67	abcd
41	32	cdef	10	63	abcde
38	27	cdefg	38	60	abcdef
25	27	cdefg	2	59	abcdef
1	22	cdefg	40	58	abcdef
10	22	cdefg	28	56	abcdefgh
26	20	defg	9	51	abcdefgi
8	18	defg	29	49	abcdefgi
17	16	efg	34	46	abcdefgi
7	14	fg	42	40	bcdefgi
6	13	fg	25	34	cdefghi
15	13	fg	24	27	cdefghi
34	13	fg	33	27	cdefghi
3	12	fg	26	18	defghi
40	12	fg	30	16	defghi
14	10	fg	32	15	defghi

24	10	fg	22	12	efghi
31	9	fg	21	10	efghi
18	9	fg	23	9	fghi
16	8	fg	14	8	fghi
20	8	fg	17	8	fghi
19	8	fg	8	8	fghi
12	7	fg	19	7	fghi
22	7	fg	31	7	fghi
5	7	fg	11	7	fghi
32	7	fg	7	6	fghi
21	7	fg	18	6	ghi
11	6	fg	12	4	hi
33	5	fg	6	4	hi
13	4	g	15	2	i
23	4	g	3	2	i
30	4	g	13	2	i
28	2	g	16	1	i
39	1	g	20	1	i
42	0	g	5	-1	i

*Numbers followed by the same letter are not significantly different according to Tukey-Kramer at $\alpha = 0.05$ level.

5.5.6. Sensitivity of Sclerotinia sclerotiorum isolates to thiophanate-methyl

There was a significant difference in percent reduction of mycelial growth (F=5.89; *P*<0.0001) among the 40 *S. sclerotiorum* isolates grown in thiophanate-methyl. The average percent reduction in mycelial growth of 40 *S. sclerotiorum* isolates from the two concentrations on PDA amended with thiophanate-methyl ranged from 75% for isolate 7 to 100% for isolate 25, respectively (Table 5.12). There was a significant difference (F=15.61; *P*<0.0001) between the two concentrations of thiophanate-methyl in the percent reduction in mycelial growth of *S. sclerotiorum* isolates. However, there was no significant interactions (F=1.08; *P*=0.3434) between the isolates' sensitivity to thiophanate-methyl and the two concentrations of thiophanate-methyl (Table 5.11). Therefore the percent reductions in mycelial growth of isolates on the two concentrations were combined for the means separation test. Isolate 25 significantly had the greatest percent reduction (100%) in mycelial growth in thiophanate-methyl compared to all, except isolates 5 (81%), 6 (76%), 7 (75%), 13 (85%), 15 (76%), 28 (79%), and 25 (79%) and isolate 7

significantly had the least (75%) percent reductions in mycelial growth compared to all, exept isolates 2 (87%), 5 (81%), 6 (76%), 13 (85%), 15 (76%), 28 (79%), 25 (79%), and 31 (86%) (Table 5.12).

There was no correlation of percent reduction in mycelial growth between the isolates grown in thiophanate-methyl at 10 μg ml^{-1} and 1) lesion length (r=0.14; *P*= 0.0933), and 2) oxalic acid production by each isolate (r=0.05; *P*= 0.5454) (Table 5.13).

Table 5.11. Analysis of variance of percent reduction in mycelial growth between *Sclerotinia sclerotiorum* isolates grown on potato dextrose agar amended with DMSO (control) and thiophanate-methyl at two concentrations.

Source	DF	Anova SS	Mean square	F Value	Pr > F
Isolate	39	23365.27	599.11	5.89	<0.0001
Concentration	1	1586.23	1586.23	15.61	<0.0001
Isolate*Concentration	39	4297.73	110.20	1.08	0.3434

Table 5.12. Estimate of the percent reduction in mycelial growth of *Sclerotinia sclerotiorum* isolates grown for three days on potato dextrose agar amended with DMSO and thiophanate-methyl compared to isolates grown on potato dextrose agar amended with DMSO.

Isolate number	Estimate of the percent reduction in mycelial growth	
25	100	a*
36	98	ab
38	98	ab
29	98	ab
42	98	ab
9	98	ab
40	97	ab
1	97	ab
17	97	ab
41	97	ab
39	97	ab

4	97	ab
24	97	ab
37	96	ab
34	96	ab
28	96	ab
16	96	ab
3	95	abc
32	95	abc
8	94	abc
33	94	abc
21	94	abc
10	94	abc
22	94	abcd
26	94	abcde
23	94	abcde
14	93	abcde
12	93	abcde
19	93	abcde
11	93	abcde
20	92	abcde
2	87	abcdef
31	86	abcdef
13	85	bcdef
5	81	cdef
30	79	def
18	79	ef
15	76	f
6	76	f
7	75	f

*Numbers followed by the same letter are not significantly different according to Tukey-Kramer at $\alpha = 0.05$ level.

Table 5.13. Pearson correlation coefficients of the relationship between lesion length, oxalic acid produced by 25 *Sclerotinia sclerotiorum* isolates, and percent reduction in mycelial growth of these isolates grown on boscalid, cyprodinil, fludioxonil, fluazinam, prothioconazole, and thiophanate-methyl active ingredients. The r and Prob > |r| were based on N = 300 under H0: Rho=0.

		Lesion length	Oxalic acid	Boscalid	Cyprodinil	Fludioxonil	Fluazinam	Prothioconazole
Oxalic acid	r p	0.6013 0.0001						
Boscalid (5 µg ml^{-1})	r p	-0.2839 0.0004	-0.2337 0.0040					
Cyprodinil (1 µg ml^{-1})	r p	-0.1270 0.1214	-0.1571 0.0548	0.0420 0.6097				
Fludioxonil (1 µg ml^{-1})	r p	-0.0063 0.9489	0.0391 0.6344	0.1931 0.0179	-0.0556 0.4995			
Fluazinam (0.1 µg ml^{-1})	r p	-0.0222 -0.7682	0.0574 0.4854	-0.2012 0.0136	0.0948 0.2486	-0.0153 0.8522		
Prothioconazole (5 µg ml^{-1})	r p	0.0431 0.6007	0.0784 0.3405	0.0416 0.6131	0.1027 0.2113	0.0066 0.9365	0.3570 <0.0001	
Thiophanate-methyl (10 µg ml^{-1})	r p	0.1376 0.0933	0.0498 0.5454	0.0439 0.5940	0.0586 0.4760	0.2876 0.0004	0.4625 <0.0001	0.3118 0.0001

5.6. Discussions

In the current research, sensitivity of *S. sclerotiorum* isolates, collected from different crops and various geographical locations in the mid-Atlantic and other regions of US, to six fungicides, boscalid, fluazinam, prothioconazole, fludioxonil, thiophanate-methyl, and cyprodinil were evaluated. Isolates significantly varied in their sensitivity to all fungicides a.i's, indicating that variation in sensitivity to all six a.i.s may exist within the *S. sclerotiorum* populations. The sensitivity of the isolates to cyprodinil, fluazinam, fludioxonil, and prothioconazole significantly correlated with the concentrations of the a.i.'s used. However, the isolates' sensitivity to boscalid (1 and 5 µg ml^{-1}) and thiophanate-methyl (5 and 10 µg ml^{-1}) were not significantly correlated with the two concentrations, respectively.

Differences in isolate sensitivity to boscalid may result from historical fungicide use patterns on different crops at different geographical locations. For example, isolate 12 originated from a soybean plant from GA and was highly sensitive to boscalid. Isolate 13, which originated from soybean plant from NJ, was much less sensitive to boscolid. This result is similar to previous studies where *S. sclerotiorum* isolates obtained from different geographical locations and various crops showed significant differences in mycelial growth under *in-vitro* conditions (Hu et al. 2018; Wang et al. 2009; Zhang et al. 2007). Similar findings were also reported by Hu et al. (2018) and Yi-lou et al. (2012) that showed there were significant differences in boscalid sensitivity between *S. sclerotiorum* isolates in the Anhui Province of East China and the province of SW China, respectively.

Variations in percent reduction in mycelial growth in boscalid within isolates might be related with characteristics of an isolate that are also related to infection (measured as lesion length) or the potential of the isolate to produce strong virulence factor such as oxalic acid. For example, in the current experiment, there was a significant negative correlation between the isolates reduction in mycelial growth in boscalid, and lesion length on lima bean, soybean, and common bean, and oxalic acid production. For example, isolate 13 caused higher lesion length and produced significantly higher oxalic acid than isolate 12 (Figure 3.5, 3.6 in Chapter 3). As an

aggressive isolate, interestingly it was the least sensitive isolate to boscalid, which is registered for use on lima bean, common bean, and soybean. Similar result was previously reported by Wang et al. (2015) stated that boscalid resistant mutants of *S. sclerotiorum* had slower radial growth, no ability to produce sclerotia, lower virulence and lower oxalic acid production.

Isolates 25 and 7 were the most and the least sensitive isolates to thiophanate-methyl, respectively. Differences in the sensitivity of the isolates to thiophanate-methyl may be correlated either with the source crops or location of the isolates. Isolate 25 which had the greatest percent reduction in mycelial growth was obtained from lima bean from NY and isolate 7 which had the least reduction was obtained from peas from DE. However fungicide use patterns in these two fields is not known.

Isolates 1 and 40 were the most and the least sensitive isolates to cyprodinil at 1 μg ml^{-1}, respectively. Isolates 29 and 20 were the most and the least sensitive isolates to fludioxonil at 0.5 μg ml^{-1}, respectively. We found significant differences in the sensitivity of isolates to fludioxonil. Matheron and Porchas (2004) found that the mycelial growth of *S. sclerotiorum* collected from Yuma Co., Arizona, USA were significantly different at 1.0 μg ml^{-1} fludioxonil. We did not see a significant correlation between the isolates sensitivity to fludioxonil and lesion length or oxalic acid production. A contrary result was reported by Duan et al. (2013) which showed that fludioxonil may present good opportunities to control *S. sclerotiorum* by increasing glycerol biosynthesis and decreasing contents of oxalic acid.

Isolates 29 and 12 were the most and the least sensitive isolates to fluazinam at 0.1 μg ml^{-1}, respectively. Isolate 28 which had the greatest percent reduction in mycelial growth at 0.05 μg ml^{-1} of fluazinam was obtained from lima bean from NY and isolate 41 which had the least percent reduction was obtained from broccoli from St. Mary's Co., MD. Similarly, isolate 29 which had the greatest percent reduction in mycelial growth at 0.1 μg ml^{-1} of fluazinam was obtained from lima bean from NY and isolate 12 which had the least percent reduction was obtained from soybean from GA.

Isolates 36 and 5 were the most and the least sensitive isolates at 5 μg ml^{-1} of prothioconazole, respectively. Isolate 9 which had the greatest percent reduction in mycelial growth at 1 μg ml^{-1} of prothioconazole was obtained from snap bean from DE and isolate 42 which had the least percent reduction was obtained from tomato from St. Mary's Co., MD. Similarly, isolate 36 which had the greatest percent reduction in mycelial growth at 5 μg ml^{-1} of prothioconazole was obtained from lima bean from Tablot Co., MD and isolate 5 which had the least percent reduction was obtained from lima bean from DE.

In conclusion, sensitivity of *S. sclerotiorum* isolates collected from different crops and geographical locations in the mid-Atlantic and other regions of the US to six fungicide's a.i.s including boscalid, fludioxonil, cyprodinil, thiophanate-methyl, prothioconazole, and fluazinam was established. Based on percent reduction in mycelial growth all isolates had a range of sensitivity to all a.i.s. This isolates sensitivity depend on the concentrations of the a.i.s of all, except boscalid and thiophanate-methyl where there were no interactions between the sensitivity and concentrations in the later two a.i.s.

For example, the sensitivity of the isolates in boscalid (at the two concentrations) ranged from 35% for isolate 13 to 74% for isolate 12. Therefore, isolates 13 and 12 were the least and the most sensitive isolates to boscalid, respectively. Similarly, in thiophanate-methyl, the sensitivity ranged from 75% for isolate 7 to 100% for isolate 25 and isolate 7 and 25 were the least and the most sensitive isolates to thiophanate-methyl, respectively. At 0.5 μg ml^{-1} cyprodinil, isolate 17 (-14%) and isolate 18 (31%) were the least and the most sensitive isolates, respectively. At μg ml^{-1} of cyprodinil, isolate 40 (0%) and isolate 1 (29%) were the least and the most sensitive isolates, respectively. At 0.5 μg ml^{-1} of fludioxonil, isolate 20 (90%) and isolate 29 (100%) were the least and the most sensitive isolates, respectively. At 1 μg ml^{-1} of fludioxonil, isolate 2 (88%) and isolate 19 (100%) were the least and the most sensitive isolates, respectively. At 0.05 μg ml^{-1} fluazinam, isolate 41 (46%) and isolate 28 (98%) were the least and the most sensitive isolates, respectively. At 0.1 μg ml^{-1} of fluazinam, isolate 12 (44%) and isolate 29 (96%) were

the least and the most sensitive isolates, respectively. At 1 μg ml^{-1} prothioconazole, isolate 42 (0%) and isolate 9 (82%) were the least and the most sensitive isolates, respectively. At 5 μg ml^{-1} of prothioconazole, isolate (-1%) and isolate 36 (96%) were the least and the most sensitive isolates, respectively. Furthermore, isolates' sensitivity to boscalid was negatively correlated to lesion length (r=-0.28397; *P*=0.0004) and oxalic acid production (r=-0.23370; *P*=0.0040). Fungicide sensitivity to fluazinam was positively correlated to fungicide sensitivity to prothioconazole (r=0.35695; *P*<.0001) and thiophanate methyl (r=0.46247; *P*=<.0001). Likewise, fungicide sensitivity to fludioxonil was positively correlated to fungicide sensitivity to boscalid (r=0.19309; *P*=0.0179) and thiophanate methyl (0.28760; *P*=0.0004). However, fluazinam sensitivity was negatively correlated to boscalid sensitivity (r= -0.20119; *P*=0.0136).

Overall, our results showed the existence of variations, within *S. sclerotiorum* isolates obtained from different crops and geographic locations, in sensitivity to different a.i.s and will advance our understanding of the sensitivity level of each of the a.i.'s and might provide a reference point for future fungicide resistance monitoring programs.

Chapter 6: Conclusions

Understanding the diversity, or whether *S. sclerotiorum* in a particular geographic region is a homogenous population or a collection of different populations, as well as the prevalence of recombination has important implications for selecting representative isolates to be used in developing and testing effective and durable disease management practices, particularly development of appropriate fungicide(s) for that region. Effective management of white mold and fungicide application guidelines are important considerations in an integrated management system. Therefore, the diversity of fourty-two *S. sclerotiorum* isolates collected from eight states and ten different crops in the US was studied by 1) evaluating the lesion length and oxalic acid production on nine (five from lima bean, two soybean, and two common bean) cultivars, 2) mycelial compatibility grouping (MCGs) and molecular/genetic characterization, and 3) determining sensitivity (*in-vitro* bioassay) to six fungicides active ingredients (a.i.s. The effect of application timing of Endura (a.i. boscalid) on disease incidence, disease severity, and yield of lima bean was also evaluated.

In the aggressiveness study, isolate 13 (obtained from Soybean, NJ) and isolate 6 (Snap bean, DE) were the most and least aggressive isolates, respectively. Isolates were also significantly different in oxalic acid production, and isolate 13 and isolate 4 were the highest oxalic acid producers. The crop of origin or location of origin of the isolates may result in the differences in lesion size and oxalic acid production.

In the study of MCGs, majorities (~75%) of the interactions were incompatible and molecular/genetic variability within populations ranged from 1 to 2%, and among population was 98 to 99%. These results and the small, 0 to 0.35 Shannon index (*Ho*) values of the MCGs, showed the existence of high diversity within the *S. sclerotiorum* isolate populations and might indicate that our isolate collection would reproduce sexually rather than through vegetative (asexual) reproduction. The molecular characterization demonstrated high sequence similarities

and identical sequences among our isolates based on ITS region and *β*-tubulin gene. However, a single mutation occurred in the ITS region for isolates 20 (C than A), 28(C than A), and 44 (A than T), respectively in position 16, 20, and 437 bp, respectively.

In *in-vitro* bioassays, there were significantly differences among isolates in percent reduction in mycelial growth for all six a.i.s. There were significant differences among isolates between the two concentrations for all fungicides, except cyprodinil and fludioxonil. There were also significant interactions between the concentrations and isolates for all fungicides, except boscalid and TM. Based on the percent reduction in mycelial growth, the most and least sensitive isolates were selected for each a.i.s. Significant correlation existed only between the isolates' sensitivity to boscalid and lesion length and oxalic acid production. The isolates variability in sensitivity to almost all a.i.s might have resulted from the sources (either crops or geographical locations) of the isolates or the isolates' aggressiveness. However, further genetic marker analysis is needed for conformation.

Disease incidence was reduced by 6.4%, 5.4%, 3.9%, and 7.6% compared to NTC when fungicides were applied 30 DAP ($P<0.0001$), 37 DAP ($P<0.0001$), 44 DAP ($P<0.0128$), and 30+37 DAP ($P<0.0001$, respectively. These application timings also reduced disease severity by 5.7%, 8.0%, 6.0%, and 7.0% compared to NTC, respectively. Earlier (i.e. 30 to 44 DAP) or within 2 weeks of 20% flowering and double treatment of boscalid reduced disease incidence and disease severity and improved yield of lima bean.

In the current study all the diversity measures, except molecular characterization, demonstrated significant and high varibility among *S. sclerotinia* isolates collected from different crops and geographical locations. However, the molecular/genetic or sequence results indicated that the isolates have high similarity. This genetic result might be due to the homothallic nature of *S. sclerotiorum*.

In conclusion, even though the morphological characters such as MCGs, pathogenicity test, and oxalic acid productions demonstrated that the *S. sclerotiorum*

population is diverse, the molecular data showed a low level of diversity among the isolates. Further analysis on the *S. sclerotiorum* isolates using other molecular techniques or examination of different genetic regions/genes is necessary to understand the population structure of the isolates. This research improves our understanding of the diversity of the mid-Atlantic *Sclerotinia sclerotiorum* population and suggests that, during selection of resistant lima bean cultivars, plants should be challenged by an array of *S. sclerotiorum* isolates, not just one putatively aggressive or susceptible isolate. My research also establishes guidelines for timing of fungicide management of white mold and developed baseline data on isolate sensitivity to fungicides.

Recommendations

Although research continues on *S. sclerotiorum*, comprehensive preventive or curative measures for management of disease caused by this pathogen are not available yet. With knowledge of the signs and symptoms of the fungus, determination of effective fungicides and proper application timing along with awareness of the favorable environmental conditions and the host species, we can manage white mold. Moreover, knowledge of field history and continuous scouting and sampling techniques, should be included in an integrated management program that includes cultural, chemical, and biological control methods. To date, management of *S. sclerotiorum* using chemical fungicides is considered as the most effective way to manage white mold in lima bean and other crops. Furthermore, whole genome sequence analysis approach should be used to precisely identify specific gene(s), if any; that are responsible for variability among our *S. sclerotiorum* isolates in lesion length produced on host plants, oxalic acid production, MCGs, and sensitivity to fungicide a.i.s.

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