ASSOCIATION MAPPING TO IDENTIFY SCLEROTINIA SCLEROTIORUM GENOMIC REGIONS AFFECTING AGGRESSIVENESS OF LESION FORMATION ON SUNFLOWER STEMS

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

MASTER OF SCIENCE

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ABSTRACT

Sclerotinia sclerotiorum is one of the most destructive pathogens of sunflower in the United States and worldwide. Distinctive symptoms include mid-stalk rot, basal stalk rot and head rot from subsequent infection of the sunflower head. This fungal pathogen has a remarkably broad host range of over 400 dicot plants. However, little is currently known about the virulence strategies that allow *S. sclerotiorum* to successfully infect a wide range of plant hosts. The goal of our project was to identify *S. sclerotiorum* virulence determinants and effectors that contribute to disease development on sunflower. We evaluated a diverse collection of 232 *S. sclerotiorum* isolates for aggressiveness of mid-stalk lesion formation on two sunflower inbred lines. In addition, we performed genotyping-by-sequencing on 191 isolates to identify genetic markers for genome-wide association mapping to identify candidate genes associated with aggressiveness. A total of eight loci associated with variation in aggressiveness were identified.

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LIST OF ABBREVIATIONS

AM .....Association Mapping

Avr ……Avirulence

Bp .....Base pair

EDTA .....Ethylene-di-amine tetra-acetic acid

End-PD ……..…endo-polygalacturonase proteins

ETI …….Effector-triggered immunity

Exo-PG …….....exo-polygalacturonase proteins

FAO .....Food and agriculture organization

GQ ……..Genotype quality

HR ……..Strong hypersensitive

LD .....Linkage Disequilibrium

MDS ……..Multi-dimensional scaling

PAMPs ……...Pathogen-associated molecular patterns

PCA ……Principal component analysis

PCD ……Response result in programmed cell death

PCR .....Polymerase Chain Reaction

PDA ……...Potato dextrose agar media

PME ……..Pectin methyl esterase

PTI …….Pathogen -triggered immunity

RNA ……..Ribonucleic Acid

SNP .....Single Nucleotide Polymorphism

USDA ……United States Department of Agriculture

USDA-ARS …….....USDA, Agricultural research service

1. INTRODUCTION

Sunflower (*Helianthus annuus L.)* is one of the most important oil crops in the world as well as in the US. It ranks fifth after soybean (*Glycine max* (L.) Merr.), rapeseed (*Brassica napus* L.), cottonseed (*Gossypium hirsutum* L.), and groundnut (*Arachis hypogaea* Fabr) in total production (Harter *et al*. 2004; Muller *et al*. 2011) and it is the second largest hybrid crop, second only to maize (*Zea mays* L.) (Zulauf *et al*. 2017). Sunflower is a member of the Asteraceae family, which includes lettuce as well as ornamental plants such as chrysanthemum. More than 65 different species included in the genus *Helianthus,* Sunflower is one of the most important oil crops in the genus (Andrew *et al.* 2013). For the last 25 years, global sunflower production gradually increasing (FAO, 2012). The United States contributes around 5% of the total world sunflower production, it thrives in all fifty states, but commercial production is concentrated primarily in the northern great plains. Despite the considerable effort to improve the productivity of sunflower, growers still face huge challenges that cause loss of productivity. The loss of productivity of sunflower is attributed by several biotic and abiotic factors. Diseases are among the most important biotic factors that limit sunflower production in the US. Sclerotinia diseases – notably basal stalk rot, mid-stalk rot and head rot have periodically caused damaging losses (Weber and Toit, 2017). Sclerotinia diseases caused by a necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary are among the most significant diseases of sunflower and other crops in the US and globally. The yield loss due to these diseases could be as high as 30% depending on the susceptibility of the host and the environmental condition (Willets and Wong, 1980; Prudy, 1979).

Sclerotinia diseases caused by *S. sclerotiorum* is one of the most economically important diseases for sunflower production (Willets and Wong, 1980; Boland and Hall, 1994). Due to the

increase of sunflower and other susceptible crops production throughout the world and in the US, the incidence and severity of this disease also dramatically increased (Boland and Hall, 1994). The incidence and severity of sclerotinia mid-stalk rot are highly variable from year to year and field to field due to the difference in the amount of inoculum (sclerotia in the soil and airborne ascospore) and environmental conditions for successful host penetration, infection and colonization by the fungus (Turkington *et al*. 1991; Parker *et al.* 2014). The management of sclerotinia mid-stalk rot disease is very difficult through the persistence of sclerotia viable for a long time in the soil and the inability of controlling airborne ascospores produced from the long stored sclerotia in the soil under conducive environmental conditions (Maserevic and Gulya, 1992; Gulya *et al*. 1997; Leite, 2014). Controlling the ascospores that cause sclerotinia mid-stalk rot and head rot is impossible as ascospores can travel long distance through wind. Thus, the lack of single exclusive control measure to reduce the effect of this disease on sunflower production, integrated control measures of cultural, biological, chemical and genetic resistance should be employed. The cultural method uses quarantine methods including tillage to bury the sclerotia (Cook *et al*. 1975), minimum seeding rate to reduce lodging and to increase intra and inter row spacing (Jurke and Fernando, 2008) and exclusion measures to prevent the introduction of *Sclerotinia sclerotiorum* isolates with seed to new areas. But, the most important tools for controlling this disease in sunflower are planting in non-infested soil and preventing sclerotia build up in soils (Prudy, 1979; Sharma *et al*. 2015). For the last five decades, the control of sclerotinia stem rot using biological agent has been investigated. More than 35 species of bacteria and fungi that are antagonistic or mycoparasite to Sclerotinia spp. (Adams and Ayers, 1979; Smolinska and Kowalska, 2018). Some of the organisms has been investigated as a biological agent for *Sclerotinia sclerotiorum* in sunflower (Burgess and Hepworth, 1996;

Abdollahzadeh *et al*. 2006). Seed treatment and pre-plant soil fumigation are also practiced in sunflower farming but currently, there is no registered chemical fungicide to control sclerotinia mid-stalk rot in the United States. As far as there is no completely immune sunflower inbred line available, genetic resistance will be the most important control method to this disease in the near future (Leite, 2014). Thus, phenotypic and genotypic characterization of large and diverse collection of *S. sclerotiorum* isolates for the identification of pathogen virulence factors and characterization of their functions and host targets has led to improve the overall understanding of how this fungus cause disease and this will help to develop a new way to control the sclerotinia disease in the future.

The development of numerous molecular marker technologies including single nucleotide polymorphism (SNP) has been used in genetic mapping in a range of crops including sunflower. The power of SNP in genome mapping can be extended to the construction of haplotypes, which increase the information content. Sequencing methods are particularly suitable for the construction of molecular markers, since they facilitate identification of several SNP alleles (Bhattramakki and Rafalski, 2001; Qin *et al*.2017). It is now common to phenotype and genotype large and diverse collection of isolates to identify molecular markers to facilitate the identification of molecular/virulence factors that cause disease in plants. Now, association mapping is increasingly being used to discover the associations between genotype and phenotype. Association studies can be carried out by testing for association of phenotypic traits with markers spread across the genome (genome scan) or with markers in the region of candidate genes (candidate gene approach). Association mapping offers the unique opportunity of linking diversity analysis, identification of marker-trait associations and resistance development.

2. LITERATURE REVIEW

2.1. Sunflower Production and Distribution

Sunflower (*Helianthus annuus L.)* belongs to the Asteraceae family (Andrew *et al*. 2013). It is an annual oil crop that is grown across wide range of environments in temperate and subtropical region. It was first domesticated in about 3000 BC by native Americans in the eastern part of the United States. The native Americans mainly used sunflower as food and they extracted oil (Seiler *et al*., 2017). There are two major types of sunflower grown: oilseed type – for vegetable oil production and Confectioners type – for human and bird consumption (Berglund, 2007). It is mainly grown in temperate and subtropics, but now it flourishes in different agro-climatic zones. The optimum temperature for sunflower growth is within the range of 20-25°C. Low temperature or frost can damage it at any growth stage, but also higher temperatures decrease yield and oil content of the seeds (Thomaz *et al*., 2012).

While sunflower is native to North America, its development as an oilseed crop first took place in Russia. Currently, the United States ranks tenth in global sunflower production. Ukraine, the Russian Federation, Argentina, China and France are the top five producers of sunflower respectively (FAOSTAT, 2015/16). In the United States, the commercial production of sunflower started in the 1960s (Berglund, 2007). Around 1.35 million acres of sunflower was grown in the 2016/17 growing season. The primary production zones of sunflower in the United States are the northern great plains (North Dakota, South Dakota, Minnesota), central great plains (Kansas, Nebraska, and Colorado) and California. The average yield was around 1339 LBS/acre (http://www.sunflowernsa.com/).

2.2. *Sclerotinia sclerotiorum* and its Taxonomy

S. sclerotiorum is a necrotrophic generalist fungal pathogen that causes diseases on more than 400 plant species across the world (Boland and Hall, 1994; Willets and Wong, 1980). The majority of crops susceptible to this fungus belong to the subclass dycotyledonae but around 25 potential host belongs to the subclass monocotyledonae (Boland and Hall, 1994). *S. sclerotiorum* is widely distributed globally and more than 60 names are used to refer to diseases caused by this fungal pathogen including cottony rot, watery soft rot, crown rot, stem rot, blossom blight and white mold (Bolton *et al*. 2006, Prudy, 1979). It is often called "sclerotinia disease". It was first designated in 1837 as *Peziza sclerotiorum* (Purdy 1979) and recognized as a sunflower pathogen in 1861 (Prudy, 1979; Kote, 1985). After the description of the genus sclerotinia, its name changed to *Sclerotinia libertiana* (Purdy 1979). Though, due to conflict with the rules of the International Code of Botanical Nomenclature, the name *Sclerotinia libertiana* later changed to *S. sclerotiorum* de Bary in 1884 and it has been taxonomically categorized as being part of the Sclerotiniaceae family, in the order Helotiales, in the class Discomycetes and in the Ascomycota phylum (Bolton *et al*. 2006). The Sclerotiniaceae family is well known for the formation of black melanised hyphal aggregates called sclerotia. The sclerotia are a survival structure for harsh environmental conditions and serve as the main source of inoculum for disease infection.

S. sclerotiorum de Bary reproduce both sexually and asexually (Willets and Wong, 1980). The sexual reproduction results from the germination of apothecia from sclerotia. During sexual reproduction, ascospores developed and released from apothecia through self-fertilization. Due to its homothallic characteristics, a single ascospore can complete its life cycle (Willets and Wong, 1980; Saharan and Mehta, 2008; Clarkson *et al*. 2014). The asexual reproduction of this fungus is considered as the most common form of reproduction and it happens through

myceliogenic germination resulting new sclerotia or mycelium that finally results from the germination of sclerotia (Willets and Wong, 1980, Kohli *et al*. 1992). Thus, both sexual and asexual reproduction results in a mainly clonal population structure (Kohli *et al*. 1992).

2.3. Distribution and Economic Importance of *S. sclerotiorum*

S. sclerotiorum has been recognized for more than 150 years as a serious pathogen for numerous crops both in the field and during transit to the market (Willets and Wong, 1980; Bolland and Hall, 1994). This pathogen is distributed all over the world but mostly common in the temperate regions (Willets and Wong, 1980). Historically, *S. sclerotiorum* is the most important fungal disease of sunflower in major sunflower producing countries including Argentina, Brazil, Canada, China, Chile, Tanzania, Turkey and the United States (Bolland and Hall, 1994; Gulya, 1997; Masirevic and Gulya, 1992).

The amount of yield loss on sunflower duet to *S. sclerotiorum* diseases mainly depends on the part of the plant infected by the pathogen. The basal stalk rot caused by the germination of sclerotia inoculum in the soil to form mycelia and it results in the rotting of the basal stalk, root system and wilting of the whole plant. The severity of basal stalk rot and the yield loss depends on the amount of sclerotia inoculum in the soil and the age of the plant at the beginning of infection. *Sclerotinia sclerotiorum* infection at the seedling stage kills rapidly and causes stand failure and up to 98% yield loss whereas infection at later stage of the plant cause around 12% yields loss (Masirevic and Gulya, 1992). Sclerotinia head rot, initiated by air born ascospores, can cause a total yield loss, it causes the falling of plant head, reduce the number and weight of seeds as well oil content (Masirevic and Gulya, 1992; Leite, 2014; Zimmer and Hoes, 1978). Sclerotinia mid-stalk rot, initiated by air-borne ascospores, is one of the most significant sclerotinia disease in sunflower it has been an increasingly important yield limiting disease of

sunflower (Kurle *et al*. 2001; Leite, 2014; Saharan and Mehta, 2008). Yield losses due to sclerotinia mid-stalk rot range from 0-100% (Purdy, 1979) and are dependent on susceptibility of the varieties and environmental conditions conducive for pathogen development (Purdy, 1979; Bell *et al*. 1990). Disease severity is positively correlated with the amount of sclerotia inoculum in the soil resulting in ascospores released (Boland and Hall, 1988). Optimum RH, temperature and extended periods of foliar wetness required for successful infection during and after crop flowering (Boland and Hall, 1988). In North Dakota, the cost of annual yield loss caused by *Sclerotinia sclerotiorum* have not been fully investigated, but it has been estimated that yield loss and cost of fungicide applications can exceed $30 million annually (Grafton *et al.*, 2002).

2.4. Life Cycle, Infection Strategy and Symptoms of *S. sclerotiorum*

2.4.1. Life Cycle

Sclerotinia sclerotiorum is a wide host range, necrotrophic pathogen; it gets its nutrients from dead or decaying plant cells. The life cycle of *Sclerotinia sclerotiorum* is initiated by the infection of host plant either through carpogenic or myceliogenic germination (Dueck 1977; Willets and Wang 1980). It has four stages in its life cycle: sclerotia, apothecium, ascospore and mycelium (Prudy, 1979), but *Sclerotinia sclerotiorum* spends about 90% of its lifecycle as sclerotia, which act as the dormant stage of the fungus (Adam and Ayers 1979; Willets and Wong 1980). The viability and survival of sclerotia in the soil are affected by many factors including the soil type, previous crop planted and environmental factors. It has been reported that the maximum and minimum number of apothecia production were recorded in sandy loam soil and sandy soil respectively (Saharan and Mehta, 2008). The combined effect of high temperature and high soil moisture are probably the two most deleterious environmental factors for sclerotia survival. Under conducive environmental condition, the overwintered sclerotia can germinate

myceliogenically or carpogenically. The carpogenic germination of sclerotia which results in the production of a small mushroom-like structure called apothecium. Ascospores develop from apotheca and are ejected to the surrounding environment. Then, ascospores become airborne and most ascospores fall in the immediate locality but, some ascospores travel long distance by wind current (Bolton et al. 2006; Willets and Wong, 1980; Prudy, 1979). After ejection, ascospores fall on living or nonliving plant parts. Ascospore may directly penetrate healthy host tissues and establish infection (Bolton et al. 2006; Willets and Wong, 1980; Prudy, 1979; Saharan and Mehta, 2008). High moisture condition and cool temperature under the plant canopy helps the carpogenic germination, successful infection and growth of the pathogen. In myceliogenic germination, the sclerotia produce mycelium. The infection of susceptible host plants by mycelium often occurs at or below the soil-line. Sclerotia germinate in the presence of exogenous nutrients and produce hyphae which cause infection by first invading nonliving organic matter and forming a mycelium, which is a transitional necessity for mycelial infection of host plant (Bolton et al. 2006; Saharan and Mehta, 2008; Willets and Wong, 1980).

2.4.2. Infection Strategy and Symptoms

S. sclerotiorum is the most economically important pathogen for sunflower production in the United States and globally (del Rio *et al*. 2007; Gulya, 1997; Nelson and Lamey, 2000). It has three principal modes of infection in sunflower (Bolton *et al.* 2006; Masirevic and Gulya, 1992). Infection may result at the stem base (basal stalk rot) from mycelia germinated from sclerotia in the soil (Purdy, 1979; Bolton *et al.* 2006; Willets and Wong, 1980), Mid-stalk rot, from the germination and penetration of ascospores at wound sites in the stalk (Mclean, 1958; Mancal *et al* 19882; Abawi and Grogan, 1975; Willets and Wong, 1980), and head rot from the germination of ascospores on senescent flowers or leaves (Abawi and Grogan, 1975; Bolton *et*

al. 2006; Willets and Wong, 1980). Sunflower is the only crop that *S. sclerotiorum* consistently infects through the roots (Lu, 2003). Though, it is the germination of ascospores on senescent tissues and flowers/heads which is epidemiologically important as the common of epidemics occur after flowering (Sutton and Deverall, 1983; Willets and Wong, 1980). Ascospores discharged from the apothecia at the base of the plants in soil constitute an important source of primary infection. On germination, ascospore gives rise to infection hyphae and initial penetration of the host tissue takes place either directly by mechanical pressure through the cuticle or through the natural openings; hyphae may also penetrate already wounded or injured tissues (Bolton et al. 2006; Willets and Wong, 1980). Cellulolytic and Pectiolytic enzymes are responsible for cell and membrane degradation resulting in subsequent death of the cells (Morrall *et al*. 1982). In addition, the oxalic acid creates an acidic environment in and around the site where many degradative enzymes are most active (Godoy *et al*. 1990; Morrall and Thomson. 1991; Cessna *et al*. 2000). Myceliogenic germination of sclerotia results in direct mycelial infection of the host plant (Le Tourneau, 1979; Willets and Wong, 1980).

The economic damage of *S. sclerotiorum* in sunflower production is depends on the type of disease and symptoms detected. This pathogen mainly affects three parts of the sunflower plant, which can infect the base and root system (basal stalk rot), the middle-stem/stalk (mid-stalk rot) and the head of the plant (head rot) (Masirevic and Gulya, 1992; Harvenson, 2011; Leite, 2014). The basal stalk rot can occur at any growth stage of the plant (seedling to maturity), but most infection occurred near the flowering stage of the plant. It is initiated through root infection from the germination of sclerotia existed in the soil. If there is infection at the seedling stage, it may cause stand failure but there is no spread of disease to other plants (Zimmer and Hoes, 1978; Masirevic and Gulya, 1992). The main symptom for basal stalk rot is sudden wilting

of the entire above ground plant without any foliar lesion. A light brown, water-soaked lesions appears at the base of the plant and surrounds the rod. Under conducive moisture condition, the lesion may be covered by a white, cottony mycelium. Then, the fungus develops internally and destroys the internal tissue of the stalk. The rotten stalk filled with black sclerotia and the diseased plant can lodge easily. Head rot may occur before flowering (i.e. bud rot) or at the end of flowering or later. The infection may start at any part of the spectacle. The initial symptoms are described dark, soft water-soaked lesions on the back side of the heads, covered by white mycelial growth covering portions of the developing seeds. As the disease advances, the fungus rots the interior of the head, leaving only the vascular elements intact. The interior of the head is filled by large numbers of irregular-shaped sclerotia. Lastly, there is a complete disintegration of the head with exposed fibrous vascular elements. A mass of sclerotia falls in to the base of the plant and will be the source of inoculum for the next season (Bolton *et al.* 2006; Harvenson, 2011; Willets and Wong, 1980). The mid-stalk rot is usually occurring between the late vegetative stage and maturity via ascospores discharged from mushroom-like apothecia. Ascospores land on leave wounds, initiate infection and colonize leaf tissue and proceed towards the petiole, finally the stalk of the plant (Masirevic and Gulya, 1992; Leite, 2014). The lesion appeared from infection in mid-stalk is similar to the lesion appeared in basal stalk rot infection (Willets and Wong, 1980). The only difference is the mode of infection. Under favorable moisture condition, white mycelium covers lesion and sclerotia formed inside the stalk. Then, it causes wilting above the site of infection, the plant rots at the site of infection and will result in breakage of stems at the point of infection, resulting in death and complete yield loss in affected plants.

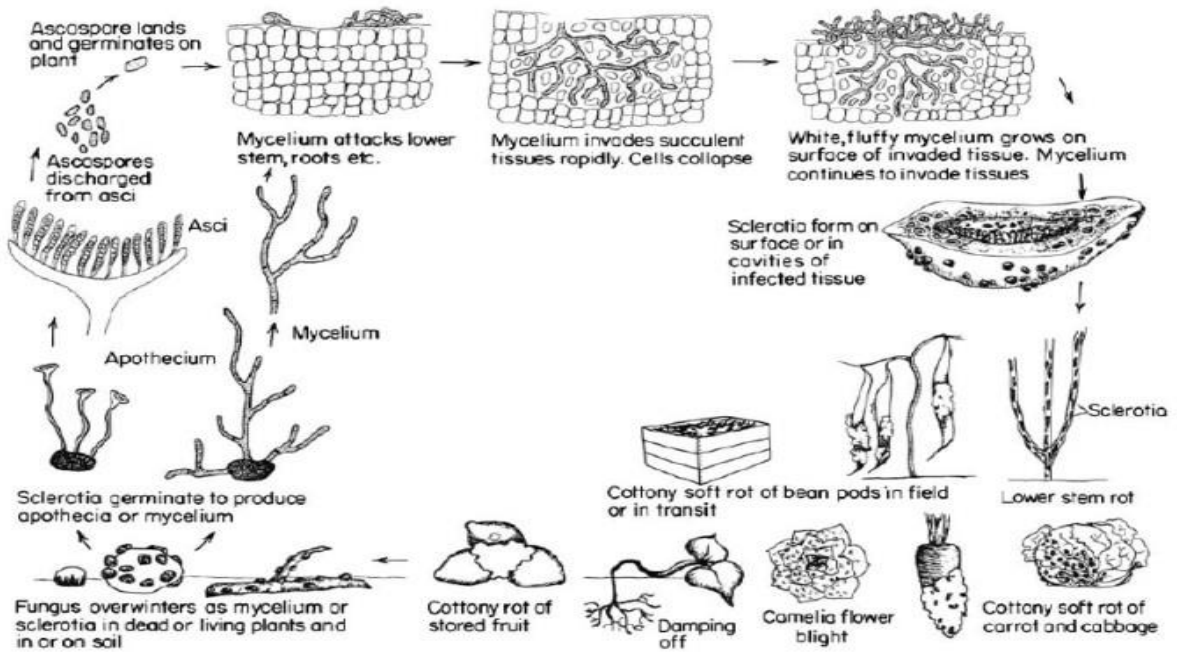


Figure 2.1. *S. sclerotiorum* disease cycle (Bolton et al., 2006)

2.4.3. *S. sclerotiorum* Disease Control in Sunflower

Sclerotia mid-stalk rot disease of sunflower is one of the most devastating disease that affect sunflower production in the US and globally. Controlling this disease is very difficult due to the persistence of viable sclerotia in the soil for long period of time, the lack of immunity in both cultivated sunflower genotypes, the travel of ascospores for long distance and the lack of effective chemical control (Maserevic and Gulya, 1992; Gulya *et al.* 1997; Bolton et al. 2006). Therefore, the most effective method to control the effect of this disease is based on an integrated control measure.

Cultural methods are the most practicable but unsuccessful method to control this disease including quarantine during seed importation, spacial isolation, avoidance of excessive nitrogen fertilization, moisture regulation, sanitation, mulching of the soil, timely sowing, crop rotation and tillage operation (Masirevic and Gulya, 1992; Bazzalo *et al*. 1985; Duncan *et al*. 2006; Saharan and Mehta, 2008). Planting in non-infested soil and preventing the buildup of sclerotia

in the soil are the most effective tools for managing sclerotinia diseases of sunflower. Currently, there is no any chemical registered to control sclerotinia diseases on sunflower either in the US or in North Dakota. Thus, the most effective control of this disease on sunflower is mostly based on an integrated program of measures, which include several cultural practices.

Biological control using fungal and bacterial mycoparasite which are associated with the sclerotinia species is becoming an important method of disease control. More than 35 species of fungi and bacteria are implicated to be antagonistic or mycoparasites of *Sclerotinia* spp. (Adams and Ayers 1979; Kamal *et al*. 2015; Zeng *et al*. 2012). The most well examined antagonistic fungi used as a biological control includes *Aspergillus ustus, Bacillus cereus, Bacillus subtilis, Coniothyrium minitans, Trichoderma harzianum, Trichoderma atroviride, Trichoderma asperellum, streptomyces lydicus (Adams and Ayers 1979,* Geralldine *et al. 2013;* Jones *et al. 2015, Prudy, 1979).The genus trichoderma* are the most well studied antagonistic microorganisms against the sclerotia of *S. sclerotiorum* which can neutralize the sclerotia structures in the soil and are used as a biological control agent (Aleandri *et al.* 2015*).*The basic mechanism used *Trichoderma* to degrade and disintegrate the sclerotia and to control *S. sclerotiorum* disease includes*; mycoparasitism (Geraldine et al. 2013),* antibiosis *(Vinale et al. 2008) a*nd systemically induced resistance *(Nawrocka and Małolepsza, 2013).* Though, the effectiveness of these biological agents is short term, in the long term, many of these agents are compromised either by the changes in the virulence of *S. sclerotiorum* or environmental condition (Whipps and Budge, 1990). Genetic resistance is the only economical and sustainable means of controlling this disease (Dennis *et al*. 2008; Neik *et al*. 2017). The genetic resistance of sunflower is partial and controlled by multiple genes. Many wild species of *Helianthus annus* have high resistance genes (Davet *et al*. 1991, Takulder

et al. 2016). The incorporation of these genetic resources using integrative approaches combining omic technologies (genomics, transcriptomics, proteomics, metabolomics and phenomics) using bioinformatic tools in developing commercial varieties that have effective resistance level suitable for sunflower cultivation in areas where this disease is endemic.

2.5. Host Range

S. sclerotiorum is the most successful necrotrophic fungal pathogen which has a broad host range worldwide, predominantly dicotyledonous plants, although a number of agriculturally important monocotyledonous plants are also susceptible. Records of susceptible hosts of this pathogen are scattered throughout the published scientific literature, but the most comprehensive host index of this pathogen is identified by Boland and Hall 1994 includes 408 species of plants 42 subspecies in 278 genera and 75 families that are susceptible to infection. Most of these host plants are economically important crops including, Sunflower (*Helianthus annus*), Canola/Oilseed rape (*Brassica napus*), Soybean *(Glycine max*), Sugar beet (*Beta vulgaris*), Peanut (*Arachis hypogaea*), mustard (*Brassica juncea*), and garden lettuce (*Lactuca sativa*) (Boland and Hall 1994; Heffer, 2007; Chitrampalam *et al*. 2008; Saharan and Mehta, 2008; Peltier *et al*. 2012). The nonspecific and omnivorous nature of this pathogen makes control of disease in crop production very challenging, because it restricts the number of non-host crops that can be included in crop rotations and in the development of either chemical or host resistance management options.

Table 2.1. A list of some of agronomic and vegetable crops reported as highly susceptible to *S. sclerotiorum* (Boland and Hall, 1994).

Crop	Scientific Name
Sunflower	*Helianthus annuus*
Lettuce	*Lactuca sativa*
Scarlet runner bean	*Phaseolus coccineus*
Lima bean	*P. limensis*
Green and dry bean	*P. vulgaris*
Pea	*Pisum sativum*
Field pea	*P. sativum*
Clovers (red, white etc.)	*Trifolium spp.*
Cowpea	*Vigna sinensis*
Table beet	*Beta vulgaris*
Rapeseed	*Brassica napus*
Cole crops	*Brassica oleracea*
Watermelon	*Citrullus vulgaris*
Cucumber	*C. sativus*
Winter squash	*C. maxima*
Pumpkin	*C. pepo*
Summer squash	*C. pepo var. melopepo*
Peppermint	*Mentha piperita*
Crownvetch	*Coronilla varia*
Lentil	*Lens culinaris*
Alfalfa	*Medicago sativa*
Sweet clovers	*Melilotus spp.*
Onion	*Allium cepa*
Flax	*Linum flavum*
Cotton	*Gossypium hirsutum*
Peanut	*Arachis hypogea*
Buckwheat	*Fagopyrum esculenthum*
Tomato	*Lycopersicum esculentum*
Tobacco	*Nicotiana tabacum*
Potato	*Solanum tuberosum*
Carrot	*Daucus carota var. sativa*

2.6. The Biology of *S. sclerotiorum*

S. *sclerotiorum* causes infection in sunflower which occurs at any growth stage from seedling to maturity either by means of ascospores which are discharged from apothecia into the air or by mycelium germinated from sclerotia residing in the soil or from neighboring infected plants (Willets and Wong, 1980; Prudy, 1979). The biology of S. *sclerotiorum* depends on four developmental stages: Sclerotia, mycelium, apothecium (ascospore) and microconidia (Bolton *et al*. 2006; Willets and Wong, 1980). Better understanding of the biology of S. sclerotiorum may provide deep insight into its pathogenicity, genetic variation, modes of reproduction, distribution and population structure of the pathogen and severity of disease on economically important crops.

S.sclerotiorum can propagate through both carpogenic germination and myceliogenic germination. The carpogenic germination of sclerotia results in the production of apothecium, which produces ascospores (Masirevic and Gulya, 1992). The myceliogenic form of reproduction is regarded as the most common (Kohli *et al*. 1992; Kohli 1995; Cubeta *et al*. 1997). It occurs through the germination of sclerotia to form mycelium, that ultimately in production of additional sclerotia (Bolton *et al.* 2006; Willets and Wong 1980). In addition to these, S. *sclerotiorum* can form microconidia in culture, which is the rare feature of the pathogen (Willets and Wong 1980; Masirevic and Gulya, 1992). Both carpogenic germination and myceliogenic germination mostly results in clonal populations (Kohli *et al*. 1992). The genetic diversity of S. *sclerotiorum* is highly likely affected by the type of reproduction (Aldrich-Wolfe *et al*. 2015). Recent research studies clearly demonstrate that recombination and out crossing through sexual reproduction results in genetic variation within *S. sclerotiorum* populations, which may increase the genetic diversity of the pathogen

(Carbone *et al* 1999; Atallah *et al* 2004; Hemmati *et al*. 2009; Attanayake *et al*. 2013). The genetic variation ultimately results in phenotypic variation and affects the adaptability of the pathogen in a specific agro-climatic zone (Carbone *et al* 1999; Atallah *et al* 2004; Hemmati *et al*. 2009; Attanayake *et al*. 2013).

Mycelial compatibility groups (MCGs) and DNA profiling are used to differentiate the clonal lineages of *S. sclerotiorum* from each other. MCGs are determined through an assay of phenotypes for a self-recognition system controlled by multiple loci (Carbone *et al*. 1999). Mycelial incompatibility, recognized as a reaction line among paired mycelia in culture, and molecular marker assays show a clonal population structure with a high level of intraspecific heterogeneity (Kohn *et al*. 1990; Kohn *et al*. 1991; Kohli *et al.* 1992; Kohli *et al*. 1995).

In addition to recombination and outcrossing, genetic diversity in *S. sclerotiorum* is known to occur between geographic regions, as well as within agricultural fields (Purdy 1979; Carpenter *et al.* 1999; Kohn *et al*. 1990; Kohn *et al.* 1991; Karimi *et al.* 2011; Attanayake *et al.* 2013). Geographically isolated *S. sclerotiorum* populations from Australia, Norway, China and the United States were genetically differentiated and found to share no mycelial compatibility groups, and also were differentiated phenotypically based on mycelial pigmentation, growth rate, sclerotia production, oxalic acid levels and sensitivity to some fungicides (Attanayake *et al*. 2013; Kamvar *et al*. 2017; Clarkson *et al*. 2017). However, these populations could not be distinguished based on their virulence on Sunflower, canola and dry bean (Attananayake *et al*. 2013; Clarkson *et al*. 2017; Kamvar *et al*. 2017). There is no confirmation of host preference in different isolates, since there was no greater genetic similarity between isolates infecting similar hosts than between isolates from different hosts (Carpenter *et al*. 1999). An understanding of the level of genetic diversity within fields and between regions is

important for the development of management strategies that are effective against all isolates within a pathogen population (Aldrich-Wolfe *et al*. 2015; Sharma et al. 2015).

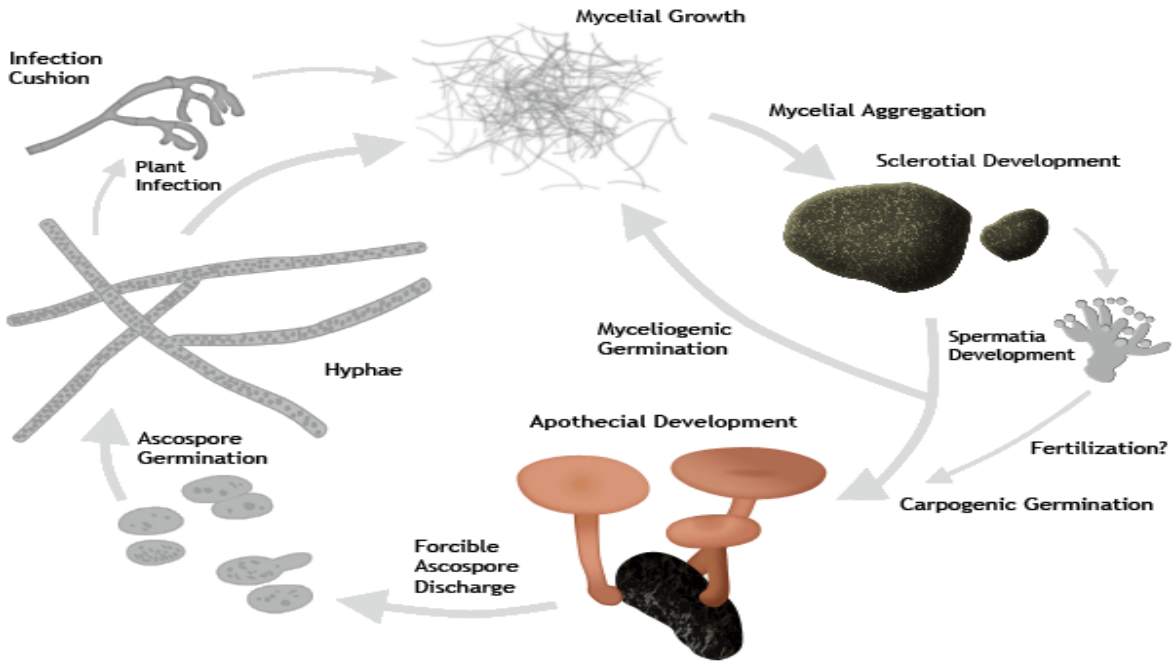


Figure 2.2. The developmental life cycle of *S. scleriotiorum* (Rollins, 2007).

2.6.1. Sclerotia

The sclerotia is a compact mass of hardened mycelium that contains food reserves and used as a survival structure for harsh environmental conditions, being dormant until favorable environmental conditions return. It has been described as round and cushion-like, spherical, irregular or flatten shaped and up to several cm in length (Prudy, 1979; Willets and Wong, 1980).

The biology of Sclerotia is very important in the understanding of the life cycle and reproduction of *S. sclerotiorum* and for disease management. A fully developed sclerotia has three developmental stages: initiation, growth, and maturation (Townsend, 1975; Willets and Wong, 1980). The Initiation phase starts through the formation of small discrete sclerotial primordia. Factors that initiate primordia include; mechanical barrier, shortage and nutrients

imbalance, chemical substances produced by the fungus itself, light and temperature (Willets, 1972; Christias and Lockwood, 1973; Willets and Wong, 1980). Then, the primordium rapidly develops into a white compact hyphal mass and results young sclerotia within three days (Willets and Wong, 1980; Coly-Smith and Cooke, 1971). During growth phase nutrients are transported to the growing young sclerotia through a few translocator hyphae (Coly-Smith and Cooke, 1971). At the maturation phase, the sclerotia stops growing in size, and is characterized by internal changes, deposition of structural and storage nutrients, dehydration and pigmentation. When the sclerotia become mature enough, the white mycelium aggregates turn black and capable of resisting adverse environmental conditions to serves as a resting structure in the life cycle of the fungus (Bolton *et al*. 2006; Coly-Smith and Cooke, 1971; Willets and Wong, 1980). The black color at maturity is due to the presence of melanin, which is thought to play a role in protection against adverse environmental conditions in S. *sclerotiorum* (Bell and Wheeler 1986; Henson *et al*. 1999; Bolton *et al*. 2006).

The sclerotia of *S. sclerotiorum* can sustain its viability in the soil for approximately 4 1/2 years and serve as a primary survival structure (Bolton et al. 2006). Survival in the soil for a long period depends on the environmental conditions and the soil microbial community (Abawi and Grogan 1979; Adams and Ayers 1979; Willets and Wong, 1971). The environmental conditions affecting longevity of sclerotia includes: soil pH, temperature, soil moisture, soil texture and the depth of the sclerotia buried (Schmidt, 1970; Willets and Wong 1980; prudy, 1979). Soil pH and soil temperatures have minimum impact on the viability and survival of sclerotia, but extreme soil temperatures of >35˚C for 3 weeks or more continuously can be deleterious (Philips, 1986; Adams and Ayers 1979; Sharma *et al*. 2015). Excessive soil moisture increase the activities of the soil microbial community and it has been found that it adversely affects the survival of

sclerotia and results a 100% loss of viability (Adams and Ayers 1979; del Rio, 1999; Smolinka and Kowalinska, 2018).

Sclerotia mostly germinate in the spring either myceliogenically or carpogenically (Willets and Wong, 1980; Prudy, 1979; Wang *et al*. 2012). The myceliogenic germination rate of sclerotia depends on the environmental conditions and the degree of melanization of the rind, completely melanized rind prevented the sclerotia from germinating in the absence of an energy source (Huang and Erikson, 2008; Abawi and Grogan, 1979). The presence of enough nutrient substrate initiate myceliogenic germination whereas enough soil moisture initiates carpogenic germination (Boland and Hall 1987; Abawi and Grogan 1979; Wu and Subbarao 2008; Kader *et al*. 2018). The types of disease in sunflower caused by s. *sclerotiorum* depends on the types of sclerotia germination. Myceliogenic germination occurs after the rind was ruptured and cause basal stalk rot. Carpogenic germination results in airborne ascospores and causes both mid-stalk rot and head rot. Temperature is important for carpogenic germination and it has been reported that germination can occur between 0.5-25 ºC, but the optimum temperature range between 10 and 20ºC, with the highest germination rate at 15ºC whereas myceliogenic germination can occur up to extreme low temperature of -20 ºC (Abawi and Grogan 1979; Hao *et al* 2003; Clarkson *et al*. 2004; Clarkson *et al*. 2007).

2.6.2. Apothecia (Ascospore)

Apothecia are sexual fruiting bodies of S. *sclerotiorum* and are comprised of a stalk (stipe) and a head (disc) (Abawi and Grogan 1979; Bolton *et al*. 2006). The head is cup-shaped or saucer-like structure, yellowish-brown in color and up to 10 mm in diameter. The stalk (stipe) is cylindrical, smooth and light-brown in color (Willets and Wong 1980). Ascospores are produced within the asci in the hymenium layer on the upper surface of the head of apothecium

(Bolton *et al.* 2006). Ascospores are the only infectious spores produced by *S. sclerotiorum* which is significant to the epidemiology of the diseases caused by this fungus. Apothecia production occurs after certain period of sclerotia dormancy and requires preconditioning for at least 2 to 3 weeks at 10-15^{0}C in moist soil within the top 2 cm of the soil surface (Abawi and Grogan, 1979; Sharma *et al*. 2015; Masirevic and Gulya, 1992).

The stipes results from the actively growing fungal cells in the region of sclerotia cortex. After the stipes emerge from the soil, they continue to grow upward to a height of about 1 cm and if they are exposed to ultraviolet light (<390 nm), they differentiate into apothecia. The tip of the stipe expands to form a top surface made up of the hymenium, a number of asci and sterile supportive hair like structures are born on the surface of hymenium. In the asci, sexual recombination occurs, and the products are eight ascospores, neatly lined-up near the tip of each ascus. After the maturation of asci, ascospores are forcibly released in response to environmental change; light, temperature, relative humidity, slight moisture tension, and are discharged to heights that will introduce the spores to air currents (Abawi and Grogan 1979; Bolton *et al*. 2006, Sharma *et al*. 2015). The release of ascospores takes an average 1-2 weeks but it may take up to 3 weeks. Ascospore survival and viability after release are the most significant factor in the life cycle of *S. sclerotiorum* and disease development (Macdonald and Boland, 2004; Sharma *et al*. 2015; Clarkson et al. 2004). The release and survival of ascospores are affected by environmental factors. Optimum temperature is one of the important factors for the release of ascospore. Ascospores are released at a temperature range of 5-30ºC, but most intensive discharge occurs between 19 to 22ºC (Clarkson et al 2003; Maserevic and Gulya, 1992). Relative Humidity and precipitation also have a greater effect on ascospore release, survival and germination of ascospores, but the effect of relative humidity is higher than either precipitation

or temperature. High RH reduces ascospore survival (Abawi and Grogan, 1975; Bardin and Huang, 2001; Qandah *et al*. 2011). After release, ascospores can be carried by air currents long distance. The distances of airborne ascospores travelled can differ depending on the environmental condition and other factors, with estimates ranging from 25 meters up to several kilometers (Suzui and Koayashi 1972; Williams and Stelfox 1979; Saharan and Mehta, 2008).

2.6.3. Variability of *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum isolates variability has been well studied by different criteria including morphological characters (Willets and Wong, 1980), Geographic variation (Prudy, 1979), isoenzymes and DNA restriction fragment length polymorphism (Kohn et al. 1988), mycelial compatibility grouping (Kohli et al. 1992) and Pathogenicity (Ghasolia and Shivpuri, 2007; Sharma *et al*. 2015). Understanding the genetic, morphological and pathogenic variability of *Sclerotinia sclerotiorum* isolates helps to develop better breeding program and identify host resistance in affected crops including sunflower.

Extensive research has been conducted on the phenotypic variability on *S.sclerotiorum* isolates. The morphological variability includes the number, size and shape of sclerotia, in mycelia growth rate, pigmentation, color of the colony and intraspecific compatibility between isolates (Kohn *et al.* 1991; Morrall *et al*. 1982; Hemmati *et al*. 2009, Garg *et al*. 2010). The intraspecific compatibility or incompatibility between isolates has been used for morphological characterization and it has been used in investigation of *S. sclerotiorum* to differentiate isolates into different groups (Kohli et al. 1992; Bolton et al. 2006; Aldrich-Wolfe et al. 2015). When *S.sclerotiorum* isolates are grown together in an agar culture medium, an incompatible interaction between mycelia has been observed as a zone of hyphal cell lysis and reduced growth (Kohn *et al.* 1991; Kohn et al. 1995). This clearly indicates that there is a genetic variation in

S. sclerotiorum isolates field population and this suggests that the distinct genotypes that create the population are each conserved (Kohn *et al.* 1991; Ge *et al*, 2012; Roger *et al*. 2008; Aldrich-Wolfe *et al*. 2015). A number of molecular techniques including SSR markers, DNA fingerprinting and genome sequencing have been used to identify the genetic diversity among isolates (Kohn *et al.* 1991; Kohli and Kohn, 1998; Aldrich-Wolfe *et al*. 2015; Tok *et al*. 2016; Sharma *et al*. 2018). Compatibility grouping and DNA fingerprinting methods were used by several researchers to classify populations of *S. sclerotiroum* into different groups (Kohn *et al.* 1991; Kohli et al. 1995; Carbone and Kohn, 2001; Saharan and Mehta, 2008, Riou *et al*. 1991). In addition to Morphological and molecular techniques, Pathogenic variability has been used in many research studies to distinguish the various isolates of this pathogen. Pathogenic variability has been associated with the production of oxalic acid, hemicellulose and other pectalytic enzymes. These factors appear to be the primary determinants of pathogenicity (Noyes and Hancock, 1981; Saharan and Mehta, 2008; Sharma *et al.* 2015).
S. sclerotiorum evolutionary history and pathogenic variability is important for the identification development of host resistance. Variation between isolates on the basis of morphology and pathogenicity depends on the level of toxins associated with their virulence (Willets and Wong, 1980).

2.7. Host-Pathogen Interaction and Resistance Development

After the publication of H.H Flor's waork on the genetics of the interaction between flax and its obligate rust pathogen, *Malamspora lini*, that the study of plant-pathogen interaction gained a substantial focus by plant pathologists and others to develop innate resistance in plants. Plant-pathogen interaction is a multiple process, mediated by molecules derived from both sides of the plant and the pathogen which mainly include proteins, small RNA, sugar and enzymes

(Boyd et al. 2013). Many studies on host-pathogen interactions revealed that plant-pathogenic microorganisms use molecular effectors to infect host plants and cause disease. These secreted molecular effectors are the key factors which determine their pathogenicity and allow their successful colonization inside the host plant. In response to these molecules, plant derived molecules are involved in the recognition of these pathogen effectors in order to elicit defense response. These microbial elicitors, also known as pathogen-associated molecular patterns (PAMPs), are recognized by the membrane-localized patterns (PRRs) of plants trigger PAMP-triggered immunity (PTI), which serves as the first line of defense for the plant (Bod et al. 2013; Zipfel et al. 2014). Most plat pathogen effectors can suppress this first line of defense and recognized by the host effectors or Avr proteins produced by R genes. The interaction between pathogen effectors and the Avr proteins initiate strong defense response called effector-triggered immunity (ETI). This process is referred to as 'gene-for-gene' resistance.

Many proteins and molecular effectors of *S. sclerotiorum* are identified in different plant hosts. These molecular effectors include, ethylene inducing enzymes, polygalacturonase proteins (end-PG and exo-PG), cysteine-rich protein, hemicellulose, phosphatidase, pectin methyl esterase (PME), protease, proteolytic enzyme, cellulose and oxalic acid. These proteins help hyphae inter cellular penetration of plant tissue during infection by degrading the cell wall of the host plant (Lumsden and Dow, 1973; Garg *et al*. 2010, Garg *et al*. 2011, Dallal Bashi *et al*. 2010, Davar *et al*. 2012, Lyu *et al*. 2015, Wang *et al*. 2015; Zuppini *et al*. 2005, Zhu *et al*. 2011).

Oxalic acid and Cellulolytic enzymes are the most important virulence factors produced by *S. sclerotiorum* hyphae and they play a key role in the penetration, infection and colonization of host plants (Lumsden and Dow, 1972; Noyes and Hancock, 1981, Saharan and Mehta, 2008; Williams *et al*. 2011). Oxalic acid decreases the pH level of the host cell which results reduced

host resistance (Marciano *et al.* 1983; Wang *et al*. 2009; Wei and Clough, 2016). In addition, it increases the function of cell-wall degrading enzymes by reducing the pH bellow the optimum and by reducing the acidic polygalacturonase inhibition by plant defense polygalacturonase inhibiting proteins (Lumsden, 1976; Favaron *et al*. 2004; Kubicek *et al*. 2014). Oxalic acid works in cooperation with other cell wall degrading enzymes to hydrolyse Ca^{2+}, stomatal opening and closure by abscisic acid and suppress oxidative burst, which is very important in early plant defense response (Cessna et al. 2000; Guimarese and Stotz, 2004; Laluk and Mengistie, 2010). cellulase enzymes degrade cellulose in infected tissue late in pathogenesis (Saharan and Mehta, 2008). Pectin methyl esterase is responsible for demethylation of pectin and endopolygalacturonase is responsible for the hydrolysis of the middle lamella. Both enzymes enhance the penetration of the host cell by the hyphae (Lumsden, 1976). In addition to playing in the penetration, colonization and infection, enzymes and other virulence factors that breakdown the cell wall and host plant tissue contents also contribute to pathogenesis by providing a supply of nutrients to sustain the intensive metabolic activity of *S. sclerotiorum* (Saharan and Mehta, 2008; Lyu *et al*. 2016).

Genetic resistance is the most effective solution to control the effect of this pathogen in sunflower production, but there are a number of limiting factors in the deployment of resistance including *S. sclerotiorum* genetic diversity and the lack of immunity in cultivated sunflower (Gulya et al. 1997; Hammati et al. 2009). The identification and characterization of *S. sclerotiorum* virulence factors and their functions in sunflower and other economically important crops improve our understanding how this pathogen cause disease. The better understanding of the biology of this pathogen will led a new way to develop new diseases control measures.

2.8. Genomic Advances in *S. sclerotiorum*

The first whole genome of *S. sclerotiorum* isolate 1980 UF-70 was sequenced by the Broad Institute. A comparative study of *S. sclerotiorum sequenced* genome and its closely related fungi *Botrytis cinerea* sequenced genome has been conducted (Amselem et al. 2011). The S. sclerotiorum genome is predicted to contain 10,637 genes and 16 chromosomes (Amselem et al. 2011). Derbyshire et al. 2017 re-sequenced the whole genome of *S. sclerotiorum* isolate 1980 UF-70 and they assembled 16 chromosomes, 11,130 gene models and also, they predicted 70 effector candidates. Having this resource available publicly has allowed further investigation into the penetration, infection and colonization strategies and the mechanisms of pathogenicity of *S. sclerotiorum* in many susceptible crops including sunflower.

2.9. Association Mapping

Many advances are being made in sunflower breeding that has been reflected by continual increased production globally. The conventional method, which involves in exploiting adapted and wild germplasm for crop improvement played a key role in sunflower improvement. This approach comprehends firstly the assemblage of accessions from different origin, evaluation of such accessions for the desirable trait, and identification of accession(s) with desirable traits. This is followed by crossing to adapted germplasm most often deficient in the trait of interest to develop inbred, DH or backcross (BC) populations. The developed populations are then phenotyped and genotyped to identify linked molecular markers to facilitate the incorporation of desirable traits in breeding programs via marker-assisted selection (Somers *et al*., 2007, Aktar *et al*. 2010). Association mapping involves the search for genotype-phenotype correlations in unrelated individuals and is often faster and more profitable than traditional biparental mapping (Myles *et al*. 2001). It was first successfully used for identification of alleles at loci contributing

to susceptibility to human diseases (Borton *et al*. 2009). AM has been suggested to be applicable to any set of genotypes and to detect QTL for as many traits that show variation (Flint. 2011). Thus, gene mapping efforts are shifting from conventional approach to a more accurate association mapping.

3. RATIONALE AND OBJECTIVES

Sclerotinia diseases are caused by a necrotrophic pathogen *Sclerotinia sclerotiorum*, which is one of the most destructive diseases of sunflower and more than 400 crop species. Due to its wide host range and lack of complete resistance in many affected crops including sunflower, it causes significant economic damage in the US and worldwide. As a necrotrophic pathogen, *S. sclerotiorum* is well known by its mode of nutrient acquisition from host cells killed by effector proteins, cell wall-degrading enzymes and toxins produced by this pathogen. Significant progress has been made in our understanding of this pathogen and some of its effectors including oxalic acid in disease development, the importance of cell wall-degrading enzymes and other secreted proteins. Unlike biotrophic pathogens, which resistance is mostly associated with a strong hypersensitive (HR) response result in programmed cell death (PCD) through the process of either pathogen-triggered immunity (PTI) or effector-triggered immunity (ETI) after pathogen effectors are recognized by plant immune receptors (Raffaele and Kamoun 2012; Bourras *et al*. 2016; Williams *et al*. 2016; McCaghey *et al.* 2018). Necrotrophic pathogens, such as *S. sclerotiorum* can hijack the hypersensitive machinery through the delivery of its toxins and cell wall degrading enzymes that target the plant cell in order to kill it (Lorang *et al*., 2012; Salguero-Linares *et al*., 2019). *S. sclerotiorum* deployment of toxins and cell wall degrading enzymes during infection and disease development may reduce the importance of effectors and therefore, it has been understudied area of *S. sclerotiorum* research.

With the absence of completely resistant commercially available sunflower inbred lines or chemicals and long-term viability of the fungus in the soil; cultural methods were used by farmers to manage *Sclerotinia* diseases resulted limited success. Thus, development of genetically resistant inbred lines is the best solution to overcome this problem. Better

understanding and identification of this pathogen's aggressiveness factors and determinants that are deployed during infection and disease development and their mechanism how these factors interact with the host plant help the scientific community to develop genetically resistant crops through either conventional or molecular breeding. Additional research is required to identify *S. sclerotiorum* aggressiveness factors and their mechanisms and also to increase our knowledge of *S. sclerotiorum* aggressiveness factors. Our proposed project aims to identify genetic factors and determinants that contribute to disease development and differences in *S. sclerotiorum* isolates aggressiveness on mid-stalk tissue of two sunflower inbred lines. Our approach of using the mid-stalk tissue rather than the basal stalk or the head tissue help as to avoid some technical challenges associated with quantifying isolate virulence for a diverse and large collection of isolates. Even if we didn't test the basal stalk tissue in our experiments, we considered that at least some virulence factors and determinants are involved in virulence regardless of mode of entry or tissue infected.

The phenotypic and genotypic data of our experiments were used to identify molecular markers to facilitate the identification of virulence factors and determinants that cause disease infection and development in plants. Association mapping study was used to discover the associations between genotype and phenotype. An association studies can be carried out by testing for association of phenotypic traits with markers spread across the genome or with markers in the region of candidate genes. it offers the unique opportunity of linking diversity analysis, identification of marker-trait associations and resistance development. Therefore, this study was conducted to address the following objectives:

1. Evaluate the virulence of a large and diverse collection of *S. sclerotiorum* isolates on mid-stalk tissues of two sunflower inbred lines.

2. Perform genotyping-by-sequencing to improve marker density for previously genotyped isolates and to genotype additional isolates to facilitate association mapping.
3. Conduct association mapping to identify candidate genetic factors in *S. sclerotiorum* that contribute to differences in isolate aggressiveness on sunflower.

4. MATERIALS AND METHODS

This study comprised three main components:

1. Phenotyping (screening) the aggressiveness of a large and diverse collection of *S. sclerotiorum* isolates on mid-stalk tissues of two sunflower inbred lines.
2. Perform genotyping-by-sequencing to improve marker density for previously genotyped isolates and to genotype additional isolates to facilitate association mapping.
3. Conduct association mapping to identify candidate genetic factors in *S. sclerotiorum* that contribute to differences in isolate virulence on sunflower.

4.1. Experimental Materials

In this experiment two USDA sunflower inbred lines and 234 S. *sclerotiorum* isolates were used. Inbred line HA207 is highly susceptible to S. *sclerotiorum* basal stalk and head rot diseases whereas inbred line HA441 is moderately resistant to these diseases, but the response to mid-stalk infection on both inbred lines was not known. The S. *sclerotiorum* isolates were collected from 24 U.S states, with a small number of isolates collected outside the U.S (133 of them provided by Dr. Berlin Nelson, North Dakota State University, 48 isolates from Dr. James Steadman, University of Nebraska, Lincoln and the rest 53 isolates from our lab)., but the majority of them were collected from sunflower, canola, dry bean and soybean fields in the North Central U.S (Appendix I and II).

4.2. Phenotyping

The main objective of this experiment was to collect phenotypic data for aggressiveness in causing stem lesions for our 234 S. *sclerotiorum* isolates on inbred line HA207 and 222 S. *sclerotiorum* isolates on inbred line HA441. The phenotypic data was used, in conjunction with

SNP marker data derived from genotyping-by-sequencing, for association mapping to identify genetic factors that cause variation in aggressiveness among isolates.

4.2.1. Experimental Location and Plant Growth

Plants for our phenotyping experiments were grown under greenhouse condition at the USDA-ARS, Northern crop science lab. greenhouses, which is located in Fargo, North Dakota. The seeds of both inbred lines were planted in 24-cell plastic flats (each cell 5.7 × 7.6 cm) filled with Sunshine SB 100B potting mixture from February 2016 to March 2018. During plant growth in greenhouse, plants were watered daily, fertilized once a week and the greenhouse adjusted to increase the light period from 8 to 16 h dark/light cycles, florescent lights used to increase the light period and during the winter season. After seven weeks of growth, plants were used for artificial inoculation.

4.2.2. Inoculum Preparation and Inoculation Technique

During inoculum preparation, a single sclerotium was plated on potato dextrose agar (PDA) media for each isolate and incubated for four days at room temperature. After germination and mycelial growth on PDA media plates, a 6 mm plug from the tip of growing mycelia was transferred to minimal media (1g NaOH, 3g DL-Malic Acid, 2g NH_4NO_3, 0.1g $MgSO_4$ x $7H_2O$, 39g Bacto agar, per liter of distilled water, Guo and Stotz, 2004) using a sterilized cork borer. The transferred mycelia were grown for approximately a day and twelve 7 mm sterile filter discs saturated with potato dextrose broth were placed around the growing mycelia such that the advancing colony would just grow over the discs within 24h. After a day the mycelia covered the filter disc and plants were inoculated by using Parafilm to affix the colonized filter discs on the sunflower stalk between the first and second set of true leaves.

4.2.3. Phenotypic Data Collection and Analysis

After 8 days post inoculation, the lesion lengths were measured using digital calipers. A series of experiments was conducted to evaluate all isolates for aggressiveness on both sunflower inbred lines. Each experiment included 22 isolates and 2 check isolates and an N of 10 plants per isolate, with inoculated plants arranged in a randomized complete block design. Experiments were conducted such that each isolate evaluation was replicated three times, resulting in evaluation of lesion lengths on a total of 30 plants of each inbred line per isolate. The two check isolates which exhibit consistent lesion sizes have been used to evaluate the consistency across experiments. The data was analyzed to determine statistically significant differences in isolate virulence and differences between experiments and replicates with SAS software (Version 9.4) using Proc Mixed Model. Additional comparisons of the relationship between genotypes response to isolate aggresiveness were assessed by Pearson correlation coefficients using the data analysis function in Microsoft Excel.

4.3. Genotyping

4.3.1. DNA Extraction

To produce mycelial tissue for DNA isolation, single sclerotia was grown on PDA media plates for approximately 4 days at room temperature for each isolate. After 4 days of mycelial growth, a sterile filter paper (Whatman No. 1) was placed in a new PDA plates and a 6 mm mycelium plug from the growing mycelium was transferred in to the center of the filter circle on the PDA plates. The transferred mycelium was grown for 3 days at room temperature. Then, a mycelial mat from the filter circle was collected, transferred in to a glass scintillation vial, and stored at -80°C. The tissue samples were subsequently lyophilized for 48 hrs. using a Labconco freeze dryer (FreeZone® 6, 12 and 18 Liter) and the lyophilized mycelium was stored at -20°C

prior to DNA isolation. For DNA isolation, 20 mg of lyophilized mycelium was transferred to a disruption tube containing 800μm zirconium beads, flash frozen in liquid nitrogen, and ball milled in a pre-cooled adapter using Mixer Mill MM301 with 2 cycles of 1.5 min duration, 30hz frequency. The sample tubes were re-frozen in liquid nitrogen between milling cycles. DNA was subsequently isolated using a GeneJet Plant DNA kit (Thermo Scientific) according to the manufacturer's instructions.

4.3.2 Assessment of Quality and Quantity of DNA Samples for GBS

Genomic DNA for each sample was quantified on a Qubit 3.0 fluorometer and then stored at -20^{o}C. DNA quality and size were evaluated by agarose gel electrophoresis on 1% TAE agarose gels. DNA samples of sufficient quantity and quality from a total of 227 isolates were submitted to LGC Genomics for genotyping-by-sequencing (GBS). GBS libraries were prepared from DNA samples digested with Ms1I and sequenced on an Illumina NextSeq 500 V2 sequencer to produce approximately 1 million 75bp reads per sample.

4.4. Genotyping-by-sequencing (GBS) Data Analysis

The raw sequencing reads were trimmed and filtered to remove low-quality reads or regions within the reads using publicly available software Trimmomatic (Bolger et al., 2014). The high-quality sequencing reads were mapped to a *S. sclerotiorum* isolate 1980 UF-70 reference genome using BWA-MEM (BurrowsWheeler aligner-Maximal exact matches) (Li, 2013). BWA is a hash-based aligner which uses hash tables to store the information of either the reference genome or short reads (Li, 2009; Ye *et al*., 2015). BWA outputs alignments in SAM format which contains alignment data in human readable tab-delimited text form. We used the 'view' command of SAMtools to convert mapped reads from SAM to BAM format, which is a preferred format for the downstream variant detection analyses due to its relatively smaller size.

The sort and index utilities of SAMtools were used to sort and index BAM files according to the chromosomal positions for downstream analysis. Sequence variants (INDELs/SNPs) were called from the processed BAM file using SAMtools: mpileup/BCFtools, to obtain the VCF (variant call format). VCFtools was used to remove variants/sample with read depth (DP) of 4 and genotype quality (GQ) of 10 (Danecek *et al*., 2011).

4.4.1 Assessment of Genotypic Data

The VCF file was assessed in multiple steps to obtain a genotypic file containing variants and isolates with minimal missing data. First, all the multi-allelic variants were removed followed by removal of variants with missing data > 90%. The remaining variants were used to select the isolates with genotypic data for more than 60% of the selected variants. A total of 203 isolates with 6045 variants were obtained. Since, *S. sclerotium* contains a haploid genome, any heterozygous calls were coded as a missing data and the missing data for variants and isolates were calculated. Any isolates with missing data > 60% were removed followed by the removal of variants with > 60% of missing genotype. A total of 190 isolates with 2014 markers were obtained for association mapping.

4.4.2. Association Analysis

Association analysis was done using genotypic variants and estimated lesion length in both inbred lines as a phenotypic data to identify significant marker-trait associations for virulence. A genomic data analysis software JMP® Genomics v 8.0 was used to perform the association analysis. Mixed models (MM) to control for type I errors (false positives) caused by genetic bottlenecks as a result of population structure (Q-matrix) and population structure as well as familial relatedness (Q-K matrix) was used for association analysis. Q matrix was computed using Principal component analysis (PCA) and multi-dimensional scaling (MDS). The

eigenvalue of 10 principal components (Pcs) were evaluated to select only those PCs that accounted for 50% of the variation and used as Q matrix to control for the population structure. The MDS technique grouped the isolates into multiple dimension based on variability within the isolates. A dimension after which the rate of decrease in badness-of-fit criterion are significantly reduced is selected as Q matrix in MDS technique. The Kinship matrix was obtained by calculating the probability of two individual to share an allele from same ancestral locus, also known as Identity by Descent (IBD) (Harris, 2011; Zhang *et al.,* 2016). Quantile-quantile (QQ) plots of Q-model (PCA) and Q-K model (PCA+IBD and PCA+MDS) were drawn using the negative log10-transformed P-values obtained in each model. QQ-plots can be used to infer the extent to which the observed significance is obtained by chance (Madel et al., 2013). The model that followed the expected line more closely are expected to produce a lesser false-positive, and thus, selected as best-fit model in this association analysis.

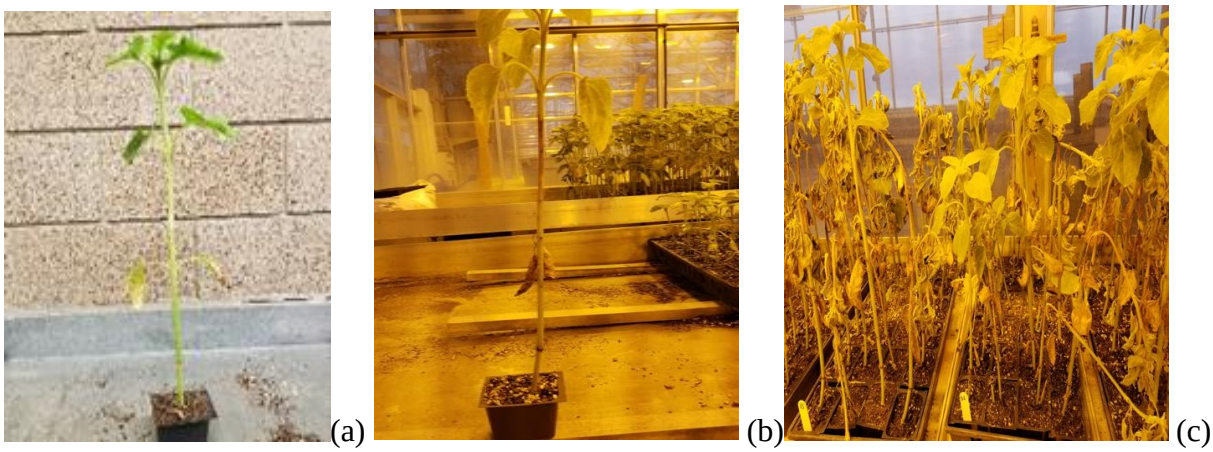
4.4.3. Linkage Disequilibrium

To calculate linkage disequilibrium decay, variants with 10% or fewer missing data were selected. Pairwise linkage disequilibrium was calculated between all SNPs residing within a single chromosome. The distance between the compared markers and their correlation coefficient was used to generate a graph and visualize the linkage decay.

5. RESULT

5.1. Greenhouse Testing

After an average three days of post inoculation, a lesion around the point of inoculation becomes clearly visible (Figure 5.1b). The lesion growth continues and covers most of the mid-stalk of the plant in the next five days. On most aggressive isolates at the eight days of post inoculation, mycelium was found inside and outside of the infected middle stalks, and the stalk above the point of inoculation wilted, collapsed and some inoculated stalks characteristically exhibited a white cottony mold inside the collapsed stalk (Figure 5.1c).



(a) (b) (c)

Figure 5.1. The apperance of plants at the date of artificial inoculation (a), at the four days of post inoculation (b) and mid-stalk lesion after 7 days post inoculation (c)

5.2. Statistical Analysis

The mean mid-stalk lesion length of 30 plants for each of 222 isolates in inbred line HA441 and for each of 232 isolates in inbred line HA207 was used for statistical analysis. For both inbred lines, the mean phenotypic data was ranked to determine the significant differences in virulence that *S. sclerotiorum* isolates exhibit and the potential for our approach to successfully identify genetic factors underlying these virulence differences. The ranked phenotypic data clearly shows that there is a significant difference in virulence /aggressiveness among isolates in both inbred lines (Figure 5.2, 5.3, 5.4, 5.5). The mean mid-stalk lesion length

data was analyzed using SAS software (Version 9.4) using Proc mixed model. The Analysis of variance showed significant difference among 222 isolates in inbred line HA441 and 232 isolates in inbred line HA207 in relation to mid-stalk lesion length ($P \leq 0.0001$) (Table 5.1 and Table 5. 2). Even though inbred line HA441 was considered more resistant to basal-stalk rot disease than inbred line HA207, in this experiment it was more susceptible to mid-stalk rot disease as compared to HA207. Larger lesions were observed for many isolates as compared to inbred line HA207. The overall mean lesion clearly indicates that inbred line HA441 was more susceptible than inbred line HA207 for mid-stalk disease (Appendix I and II). In some cases, isolates collected from the same geographical location had relatively similar levels of aggressiveness in terms of mean lesion length. For example, three isolates collected from Argentina on HA2017 and five isolates collected from North Dakota on HA441 showed relatively similar level of aggressiveness. But, in most cases, however, there were substantial difference in aggressiveness within and among the two inbred lines. The estimated mean calculated using SAS software (Version 9.4) Proc mixed model where block experiments were regarded as random variable and the calculated estimated mean was used in association mapping for each respective inbred line.

Table 5.1. The Analysis of variance of 222 isolate on inbred line HA441

Effect	DF	Den DF	F Value	Pr>F
Isolate	221	5954	2	<.0001

Table 5.2. The Analysis of variance of 232 isolate on inbred line HA207

Effect	DF	Den DF	F Value	Pr>F
Isolate	231	6300	4071	<.0001

5.3. Correlation of Genotype Responses Aggressiveness

A significant positive correlation (r^2=0.6478, P<0.001, Figure 5.2) was observed between inbred line HA441 and HA2017 for their response to 232 *S. sclerotiorum* isolates aggressiveness.

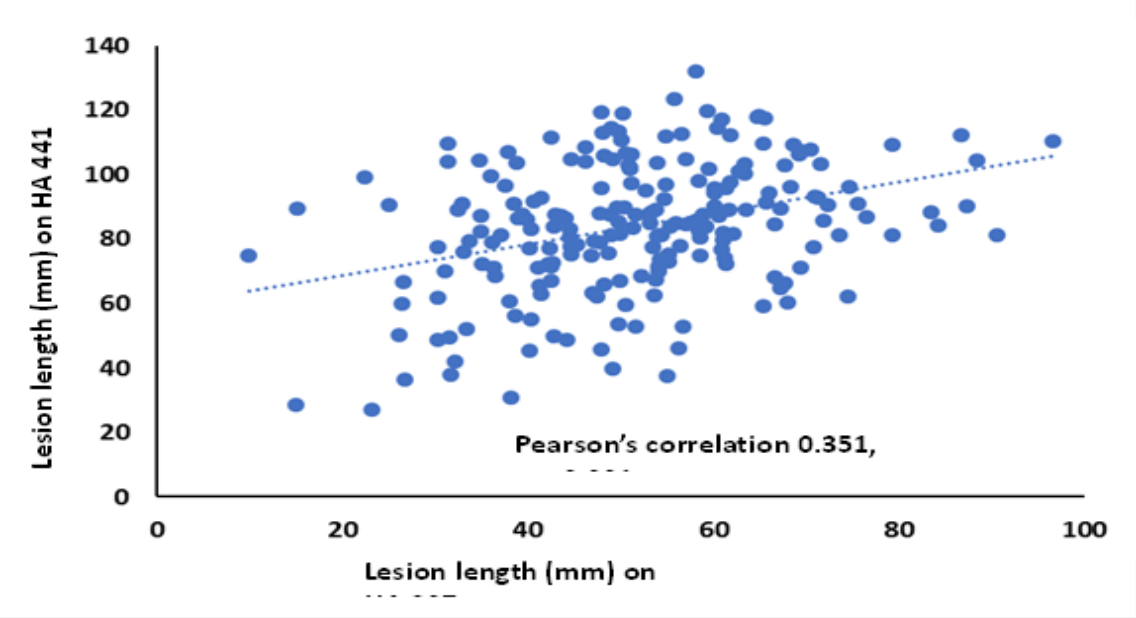


Figure 5.2. Correlation for mid-stalk lesion length in two sunflower inbred lines.

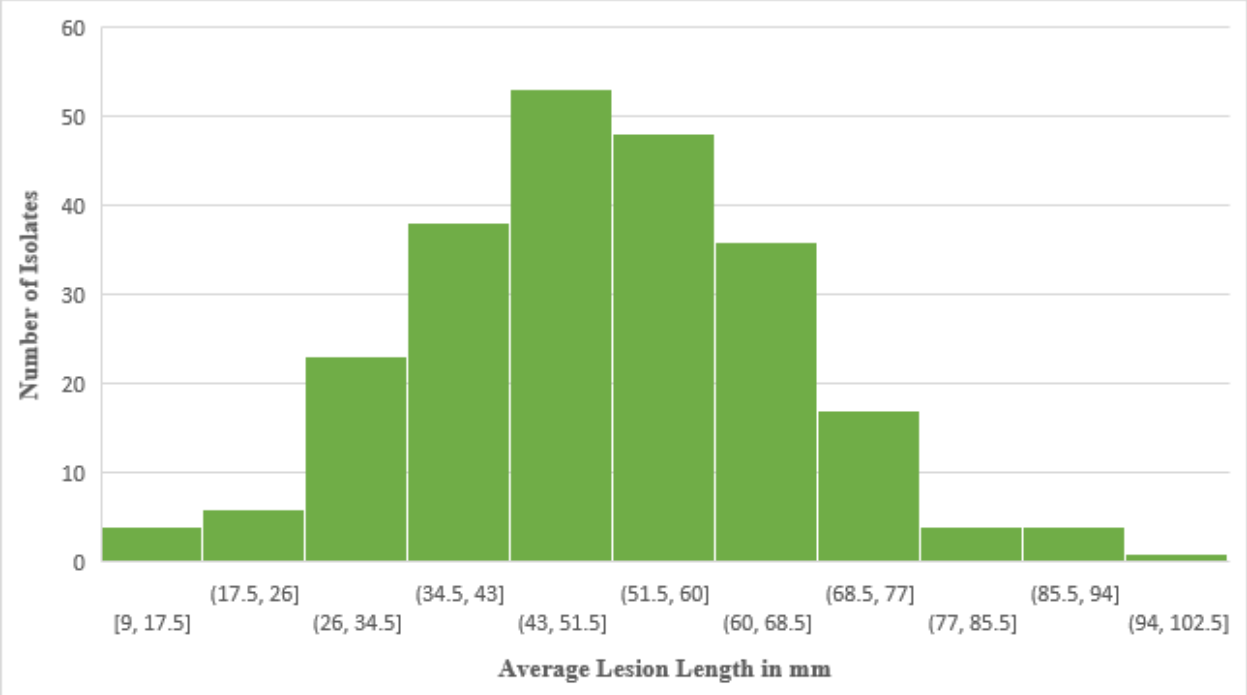


Figure 5.3. The distribution of isolates based on their aggressiveness on inbred line HA207

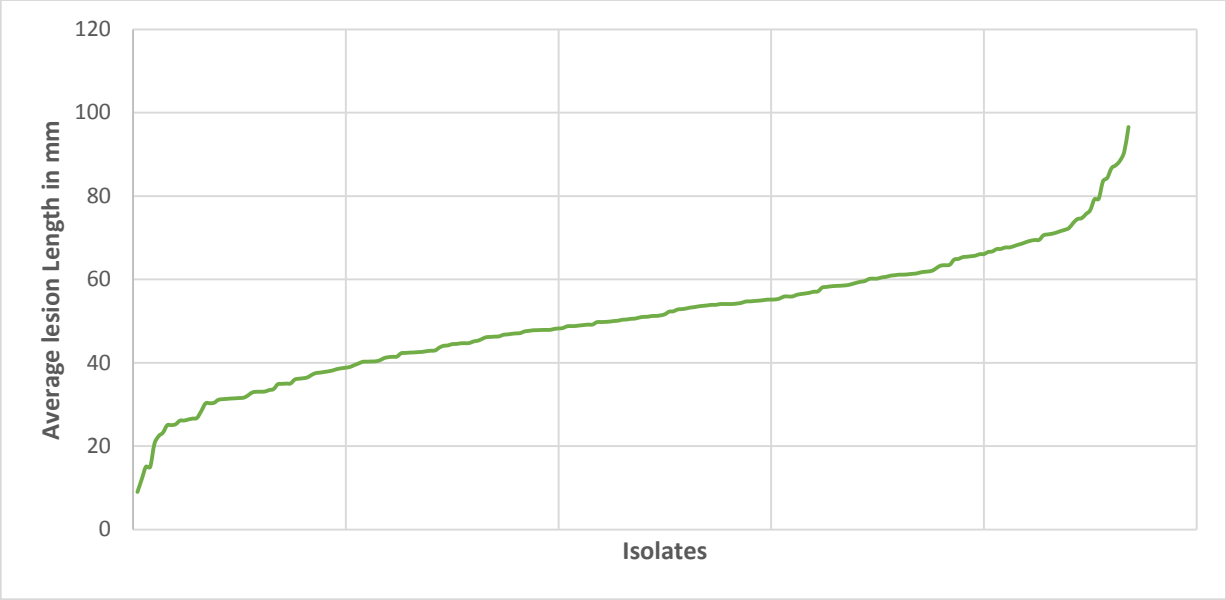


Figure 5.4. The lesion length of 232 S. *sclerotiorum* isolates on mid-stalk tissue of inbred line HA207

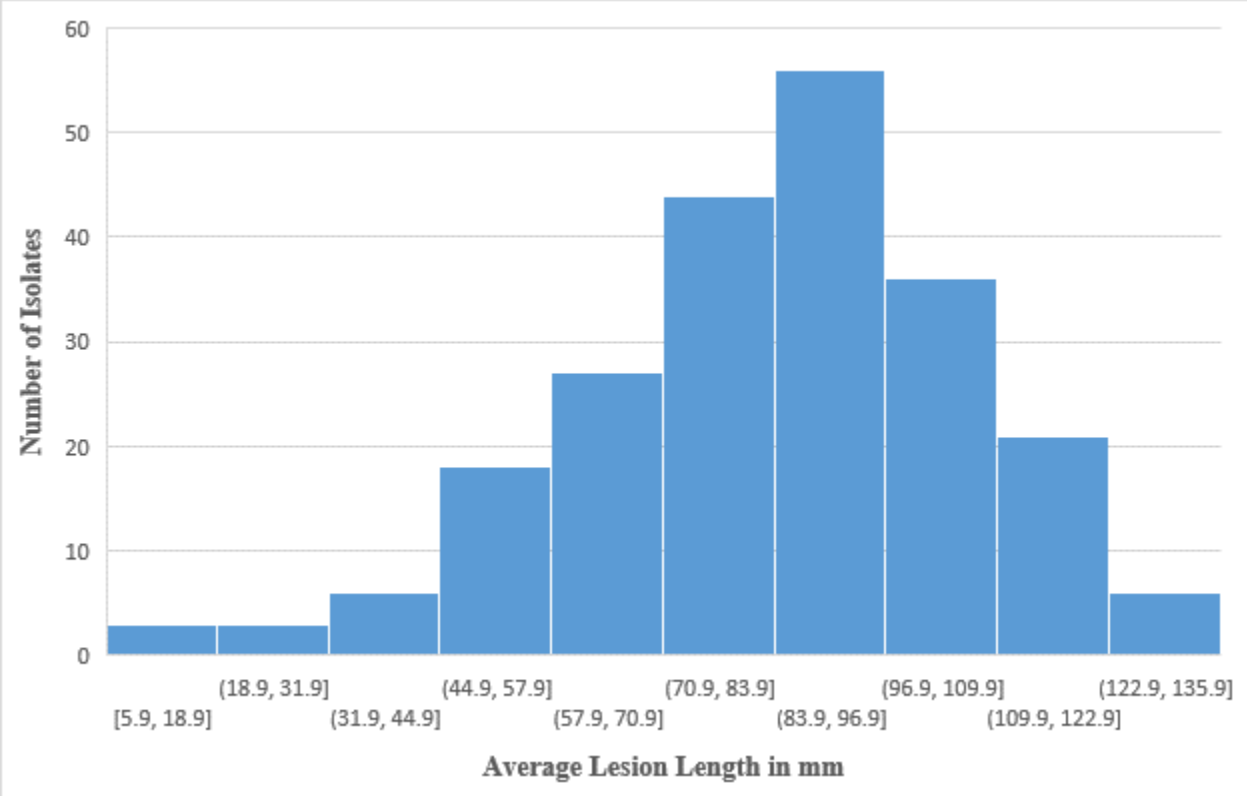


Figure 5.5. The distribution of isolates based on their aggressiveness on inbred line HA441

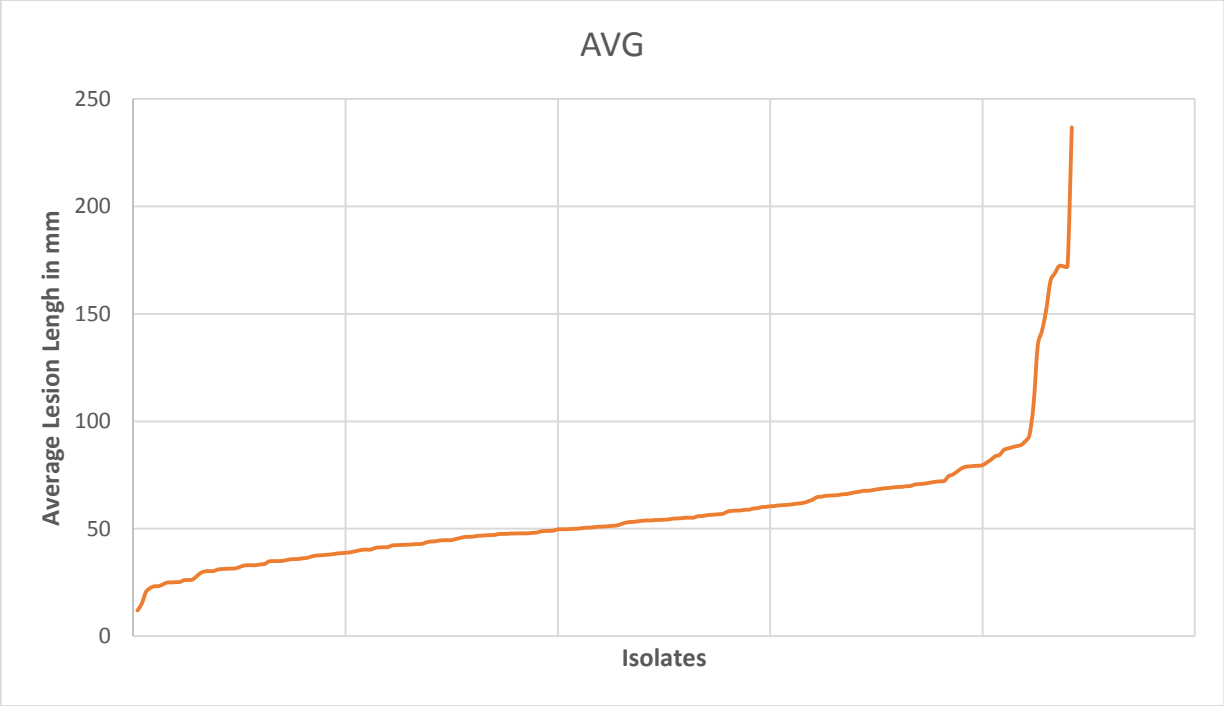


Figure 5.6. The lesion length of 222 S. *sclerotiorum* isolates on mid-stalk tissue of inbred line HA441

5.4. Population Structure and Relationship Matrix

PCA analysis resulted in 4 PCs that explained 50% variation within the data set and were selected as Q matrix to correct for the population structure in this association analysis using Q model and Q-K model (Figure 5.7). Likewise, screen plot obtained from MDS technique showed a reduction in rate of decrease in badness-of-fit criterion after fourth dimension, and thus 4 dimensions were used as Q matrix in Q-K model (Supplementary Figure 1). The heat-plot generated using IBD matrix (Figure 5.8) shows a presence of significant diversity between isolates as well as a few groups of isolates that are potentially clonal. The QQ plot showed that the Q-K model using PCA and IBD matrix as the best fit model compared to other model used in this study (Supplementary Figure 2).

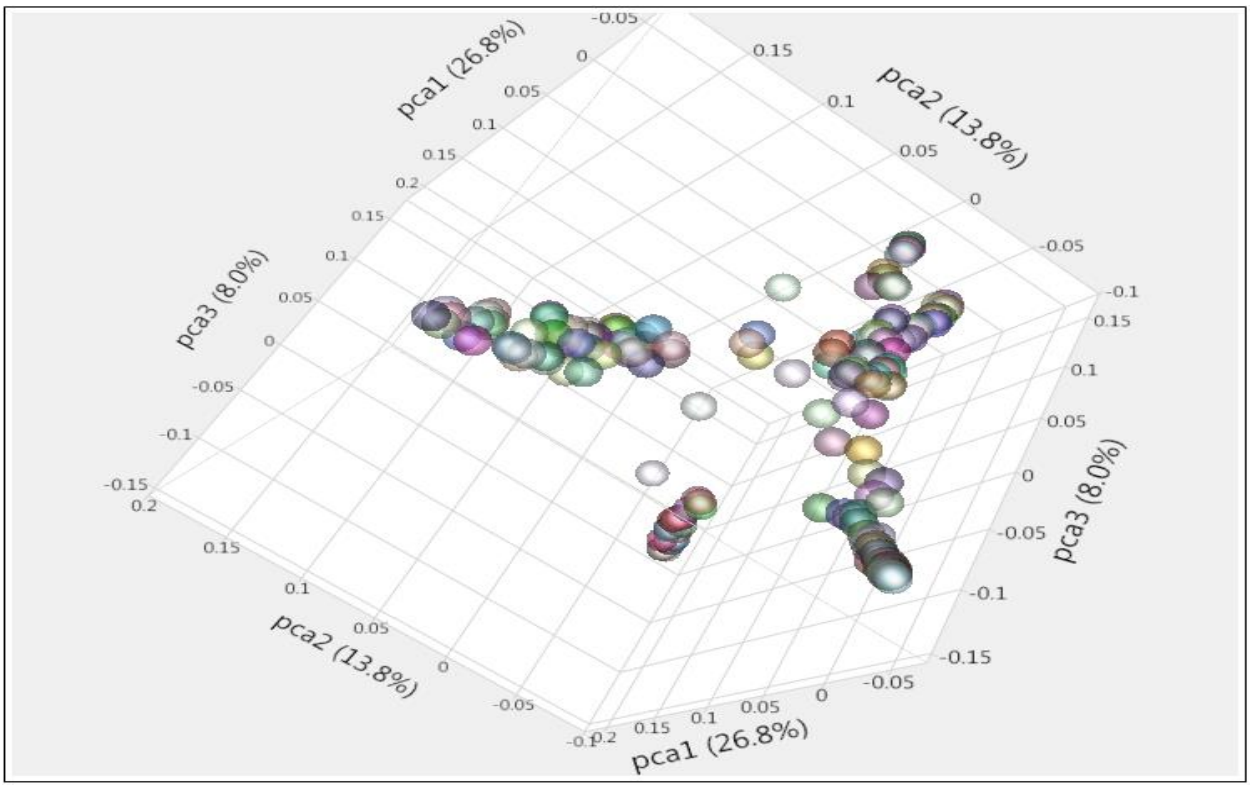


Figure 5.7. A 3D scatterplot generated using PCA showing clustering based on 3PCs.

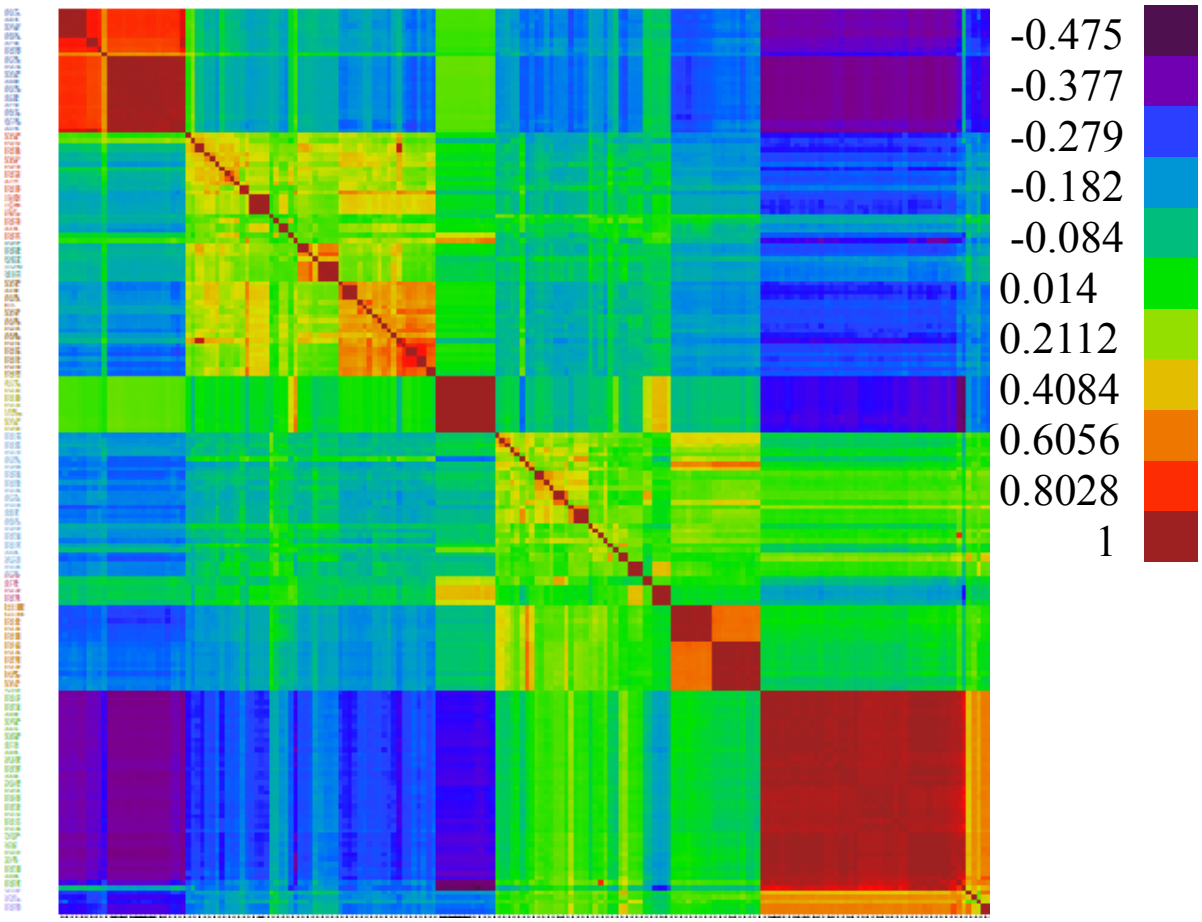


Figure 5.8. A heatmap of 190 isolates generated using Identity by descent (IBD) value used to infer the familial relatedness between the isolates. The color represents IBD (shown on top right corner) that explains the degree of relatedness between the isolates. IBD of 1 shows a perfect relationship and the degree of relatedness reduces with decrease in this value.

5.5 Association Mapping

In this study, markers associated with aggressiveness in S. *sclerotiorum* isolates against both sunflower inbred lines were identified by association mapping analysis using JMP genomics software. From the sequenced 227 isolates, only 190 isolates were used in the analysis. Eight markers were significantly associated (negative log10-transformed P-values >=3) with aggressiveness on HA441 but there was not any marker significantly associated with aggressiveness on inbred line HA207. The eight significantly associated markers linked to aggressiveness on HA441 were located on chromosomes; 2, 4, 6, 10, 13, 14, and 16.

The linkage disequilibrium analysis was done to identity the overall linkage decay within the *S. sclerotium* genome. Considering the overall genome, we observed correlation of 90% or above between markers that are 100 bp to 3.81 MB apart. On the contrary, we observed linkage decay between marker as close as 37 bp apart (Supplementary Table 1). The observation of such pattern of linkage disequilibrium has not been reported yet, and thus have no clear explanation on such behavior in this pathogen. However, the genes that are residing within 10 Kb of the associated markers have been listed as candidate genes associated with those markers (Table 5.3).

Table 5.3. List of SNP markers significantly associated with aggressiveness on inbred line HA441 and genes flanking 10KB of the significant markers

Marker	Chr	Pos	P-value	Genes	-10 KB	+10 KB	JGI ID	JGI annotation	NCBI blastp
CP017817_1_2688943	Chr04	2688943	3.79	sscle_04g039740	2678943	2698943	SS1G_14283T0	60S acidic ribosomal protein P0	60S acidic ribosomal protein P0 [Sclerotinia sclerotiorum 1980 UF-70]
				sscle_04g039750			SS1G_14282T0	WD repeat protein WDR4	
				sscle_04g039760			SS1G_14281T0		hypothetical protein
				sscle_04g039770			SS1G_14280T0	Uroporphyrinogen decarboxylase	
				sscle_04g039780			SS1G_14279T0	Cytosine deaminase FCY1 and related enzymes	
				sscle_04g039790			SS1G_14278T0	Molecular chaperone (DnaJ superfamily)	
				sscle_04g039800			SS1G_14277T0	Amino acid permease	
CP017827_1_347971	Chr14	347971	3.68	sscle_14g097990	337971	357971	SS1G_13627T0	Sedlin, N-terminal conserved region	similar to trafficking protein particle complex subunit 2 [Botrytis cinerea T4]
				sscle_14g098000			SS1G_13626T0	NA	NA
				sscle_14g098010			SS1G_13625T0	Core histone H2A/H2B/H3/H4	Histone H4, partial [Tolypocladium capitatum]
				sscle_14g098020			SS1G_13624T0	NA	NA
				sscle_14g098030			SS1G_13619T0	NA	NA

Table 5.3. List of SNP markers significantly associated with aggressiveness on inbred line HA441 and genes flanking 10KB of the significant markers (continued)

Marker	Chr	Pos	P-value	Genes	-10 KB	+10 KB	JGI ID	JGI annotation	NCBI blastp
CP017815_1_181142	Chr02	181142	3.30	sscle_02g012090	171142	191142	SS1G_12887T0	NA	putative riboflavin transporter mch5 protein [Botrytis cinerea BcDW1] (78% match on 63% query)
				sscle_02g012100			SS1G_12886T0	Glucose-6-phosphate 1-dehydrogenase	
				sscle_02g012110			SS1G_12885T0	60S ribosomal protein L35A/L37	
				sscle_02g012120			SS1G_12883T0	NA	
				sscle_02g012130			SS1G_12882T0	NA	similar to vacuolar ATPase assembly integral membrane protein VMA21 [Botrytis cinerea T4] (81% idenity on 83% query cover)
				sscle_02g012140			SS1G_12881T0	SNF2 family DNA-dependent ATPase	
CP017826_1_604521	Chr13	604521	3.27	sscle_13g093530	594521	614521	SS1G_06542T0	Serine/threonine protein kinase	
				sscle_13g093540			SS1G_06546T0	Zinc finger, C3HC4 type (RING finger)	
				sscle_13g093550			SS1G_03650T0	Glycolipid 2-alpha-mannosyltransferase (alpha-1,2-mannosyltransferase)	
				sscle_13g093560			SS1G_06547T0	GlycosylTransferase Family 31	
				sscle_13g093570			SS1G_06548T0	Galactokinase	
				sscle_13g093580			SS1G_06549T0	GPCR_Rhodpsn	putative tetratricopeptide repeat protein 15 protein [Botrytis cinerea BcDW1] (88% idenity on 99% query cover)

Table 5.3. List of SNP markers significantly associated with aggressiveness on inbred line HA441 and genes flanking 10KB of the significant markers (continued)

Marker	Chr	Pos	P-value	Genes	-10 KB	+10 KB	JGI ID	JGI annotation	NCBI blastp
CP017823_1_317107	Chr10	317107	3.12	sscle_10g075710	307107	327107	SS1G_08244T0	Domain found in Dishevelled, Egl-10, and Pleckstrin (DEP)	putative vacuolar membrane-associated protein iml-1 protein [Botrytis cinerea BcDW1] \
				sscle_10g075720			SS1G_08245T0	Uncharacterized conserved protein	putative ribosome biogenesis protein urb1 protein [Botrytis cinerea BcDW1]
				sscle_10g075730			SS1G_08246T0	Calcium-responsive transcription coactivator	
				sscle_10g075740			SS1G_08247T0	Vesicle trafficking protein Sec1	
CP017819_1_826493	Chr06	826493	3.10	sscle_06g050700	816493	836493	SS1G_07245T0	60S ribosomal protein L13a	
				sscle_06g050710			SS1G_07244T0	BCL2-associated athanogene-like proteins and related BAG family chaperone regulators	putative bag domain-containing protein [Botrytis cinerea BcDW1]
				sscle_06g050720			SS1G_07243T0	Glutaredoxin and related proteins	
				sscle_06g050730			SS1G_07242T0	Chalcone_isomerase	
				sscle_06g050740			SS1G_07241T0	NA	NA
				sscle_06g050750			SS1G_07240T0	Transposon-encoded proteins with TYA, reverse transcriptase, integrase domains in various combinations	
				sscle_06g050760			SS1G_08962T0	Chromo (CHRromatin Organisation MOdifier) domain	
				sscle_06g050770			SS1G_07238T0	Leucine rich repeat proteins, some proteins contain F-box	

Table 5.3. List of SNP markers significantly associated with aggressiveness on inbred line HA441 and genes flanking 10KB of the significant markers (continued)

Marker	Chr	Pos	P-value	Genes	-10 KB	+10 KB	JGI ID	JGI annotation	NCBI blastp
CP017829_1_433804	Chr16	433804	3.07	sscle_16g108520	423804	443804	SS1G_10216T0	CUE domain	putative cue domain-containing protein [Botrytis cinerea BcDW1]
				sscle_16g108530			SS1G_10218T0	Serine/threonine protein kinase	
				sscle_16g108540			SS1G_10220T0	SPRY domain-containing proteins	
				sscle_16g108550			SS1G_10222T0	NA	NA
				sscle_16g108560			SS1G_10224T0	Translocase of outer mitochondrial membrane complex, subunit TOM37/Metaxin 1	
CP017829_1_410763	Chr16	410763	3.00	sscle_16g108400	400763	420763	SS1G_10204T0	Mitochondrial carnitine-acylcarnitine carrier protein	
				sscle_16g108410			SS1G_10205T0	obsolete_pf08575	Mitochondrial carrier protein [Rutstroemia sp. NJR-2017a WRK4] (67% idenity in 98% query cover)
				sscle_16g108420			SS1G_10206T0	Zinc finger, C3HC4 type (RING finger)	
				sscle_16g108430			SS1G_10207T0	Glyco_hydro_1	mitotic-spindle organizing 1 protein [Rutstroemia sp. NJR-2017a BVV2] (83% identity in 93% query cover)
				sscle_16g108440			SS1G_10208T0	Uncharacterized conserved protein	putative kinetochore protein spc25 protein [Botrytis cinerea BcDW1]
				sscle_16g108450			SS1G_10209T0	NA	putative rna polymerase i-specific transcription initiation factor rrn6-like protein [Botrytis cinerea BcDW1] (81% identity in 100% query cover)
				sscle_16g108460			SS1G_10210T0	Thioredoxin-like protein	60S ribosomal L3 protein [Rutstroemia sp. NJR-2017a BVV2]
				sscle_16g108470			SS1G_10211T0	Predicted coiled-coil protein	coiled-coil domain-containing protein [Sclerotinia borealis F-4128] (85% idenity in 100% query cover)
				sscle_16g108480			SS1G_10212T0	Ribosomal protein L3	
				sscle_16g108490			SS1G_10213T0	Cytochrome oxidase complex assembly protein 1	putative cytochrome oxidase assembly protein [Botrytis cinerea BcDW1]
				sscle_16g108500			SS1G_10214T0	NA	NA

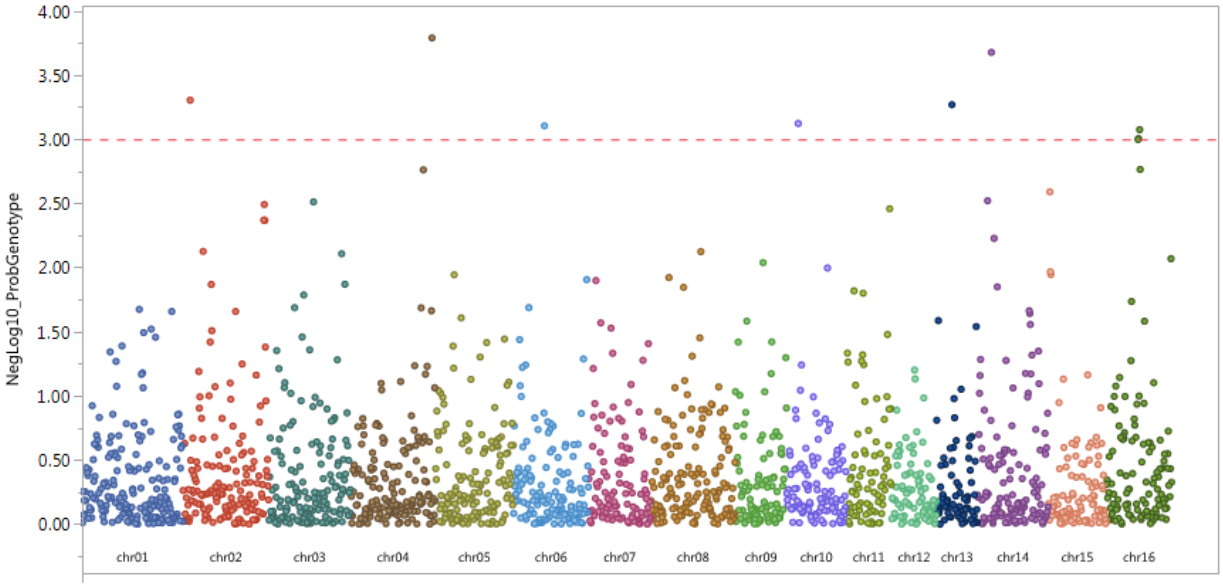


Figure 5.9. Manhattan Plot for **HA441** using Q model. A total of 2014 variants for 190 isolates were used to conduct the association mapping. Three PCA that explained > 50% of the variation were used to correct for the population structure (Q). The Identity by Descent (IBD) matrix was used to calculate the familial relatedness and correct for the kinship (K).

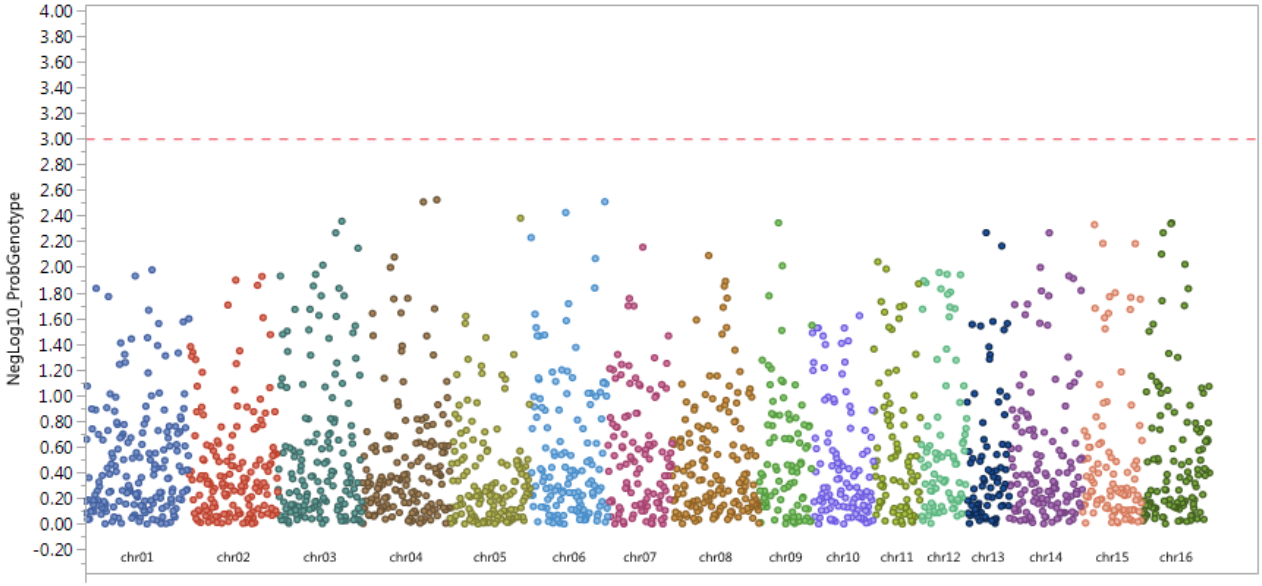


Figure 5.10. Manhattan Plot for **H207** using Q model. A total of 2014 variants for 190 isolates were used to conduct the association mapping. PCA that explained > 50% of the variation were used to correct for the population structure.

6. DISCUSSION

Since the start of sunflower research in the 1960s, several improved sunflower inbred lines have been released with the aim of improving production, oil and confection quality and disease resistance. But, the production of sunflower in the US still affected by many fungal pathogens including S. *sclerotiorum* diseases; head rot, basal stalk rot and mid-stalk rot. This is mainly due to the absence of effective agronomic control measures and the lack of complete genetic resistance on these released sunflower inbred lines to contain the effect of this pathogen. The better understanding of the biology and its interaction of this pathogen with plant hosts during infection and disease development will help the scientific community to develop a new strategy to control its effect on sunflower and other susceptible crops. Thus, the principal objective of this project was to identify genetic factors that contribute to differences in isolate virulence on two sunflower inbred lines. The identification, analysis and functional characterization of this pathogen virulence factors is now an essential tool to further our understanding of how S. *sclerotiorum* infection and disease development process is regulated and how it can evade host plant defense systems. Many plant pathogen virulence factors have already been identified and shown to act as effector proteins which manipulate host plant machinery for a range of fungal pathogens (Bolton *et al*. 2008b; Tyler 2009; Derbyshire et al. 2017; Sang *et al*. 2019). The identification, analysis and functional characterization of virulence genetic factors in S. *sclerotiorum* is a fundamental area of research and the scientific community anticipates that these candidate genetic factors and their function will ultimately lead to the discovery of new control measures to economically destructive pathogens including S. *sclerotiorum*.

In our study, a large and diverse collection of isolates collected from many host crops and different geographical regions were tested aimed at identifying genetic factors that cause variation in aggressiveness among 222 *S. sclerotiorum* isolates on inbred line HA441 and 232 S. *sclerotiorum* isolates on inbred line HA207. There was a significant variability in virulence was successfully demonstrated among S. *sclerotiorum* isolates in both inbred lines. However, in this phenotyping study, S. *sclerotiorum* isolates exhibited significant variation in terms of experiments and inbred lines. This may not be surprising as isolates collected from different host crops and different geographical regions. Analysis of variance results showed significant difference among S. *sclerotiorum* isolates on both sunflower inbred lines in relation to mid-stalk lesion length ($P \leq 0.0001$). The analyzed estimated phenotypic mean data and the filtered sequence variants (SNPs) from the genotyping-by-sequencing (GBS) data analysis were subsequently used for association mapping analysis using JMP genomics software. The PCA analysis resulted in 4 PCs that explained 50% variation within the data set and were selected as Q matrix to correct for the population structure in this association analysis using Q model and Q-K model. The genome wide association analysis was conducted using 2014 markers for both inbred lines. From this analysis, a total of eight candidate genes were identified for HA441 and but there was no any significant marker associated with aggressiveness on inbred line HA207. The results of the analysis that accounted for population structure was adopted after setting the cutoff value of statistic at significance level of 0.01. The power of association studies depends on the level of population structure and genetic variation (Hamblin and Jennink, 2011, Duncan and Brown, 2013). Identifying and taking into consideration, population structure (Q matrix) as a fixed effect and differences in genetic relatedness among S. *sclerotiorum* isolates within the subpopulations as random effects reduces the number of false positives (Fusari *et al*. 2013; Wei

et al. 2017). Our analysis result indicated that more than four clusters were appropriate in assigning the population structure within the S. *sclerotiorum* isolates used in this study.

Some of the notable results we identified during the analysis are; First, the sunflower inbred line HA441 is considered as moderately resistant to head rot and basal stalk rot diseases as compared to inbred line HA207. But the phenotypic experiment clearly indicates that inbred line HA441 is more susceptible as compared to inbred line HA207 for mid-stalk rot disease. Second, though the secretion of oxalic acid has been championed as the key infection and disease development strategy for *S. sclerotiorum*, an in-depth analysis and JGI and NCBI genome browser search reveal other candidate virulence determinants potentially important in the infection and disease development process were identified. Third, the linkage disequilibrium analysis results linkage decay as close as 37 bp apart. The observation of such pattern of linkage disequilibrium has not been reported yet, and thus have no clear explanation on such behavior in this pathogen. The identification of the *S. sclerotiorum* virulence determinants and functional characterization of these candidate virulence determinants will provide deep insights into how other virulence determinants alongside oxalic acid may contribute to the infection cycle and disease development in sunflower and other affected crops. Although we didn't make any direct associations yet, the identified candidate virulence determinants need further experimental investigation to explore whether they truly are virulence determinants with an effector role.

7. CONCLUSION

Sunflower (*Helianthus annuus L.)* is one of the most important oil crops for the US economy. But, the production of this crop is challenged by both biotic and abiotic factors, mainly diseases. Sclerotinia diseases are caused by a necrotrophic fungal pathogen *Sclerotinia sclerotiorum* causes a major yield loss in sunflower and other legume and oil crops. The lack of effective control measure against these diseases make their effect more problematic. Better knowledge about the biology of this fungus and its interaction with host plants helps the research community to develop durable resistance in host plants to contain its effect in crop production. Thus, the overarching goal of this project was to develop insight into the genetic factors that cause difference in aggressiveness among the large and diverse collection of *S. sclerotiorum* isolates on mid-stalk tissue of two sunflower inbred lines. This was accomplished through the identification of genetic factors that create virulence/aggressiveness difference among the large and diverse collection of *S. sclerotiorum* isolates. The genetic factors identified in this experiment should be further investigated and their virulence determinants such as protein, small molecule, and small RNA effectors that contribute to disease development on sunflower should be well characterized.

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APPENDIX A. THE AVERAGE LESION LENGTH, ESTIMATED MEANS AND OTHER STATISTICAL ANALYSIS RESULTS FROM PROC MIXED MODEL IN INBRED LINE HA207

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
1980	9.94866667	37.7503	6.3654	1980	Nebraska	Dry Bean
Arg1	83.5406667	53.8023	6.3184	2000	Argentina	Sunflower
Arg154	74.6903333	60.8921	6.51	NA	Argentina	Sunflower
Arg2	52.3353333	58.2297	6.3353	2000	Argentina	Sunflower
Arg213	52.7917241	67.0811	6.8833	NA	Argentina	Sunflower
Arg228	58.6326667	60.1123	6.4527	NA	Argentina	Sunflower
Bean03	66.7093103	37.9567	6.322	2003	NA	Dry Bean
BN102	59.1748276	53.287	6.3758	2008	North Dakota	Dry Bean
BN105	31.6193333	54.762	6.3706	2008	North Dakota	Dry Bean
BN109	48.3123333	59.0641	6.3231	2008	North Dakota	Dry Bean
BN110	61.1963333	49.4137	6.3586	2007	North Dakota	Canola
BN112	75.676	53.1819	6.3177	2007	North Dakota	Canola
BN113	58.4223333	44.6477	6.3586	2008	North Dakota	Soybean
BN114	58.191	51.2102	6.3462	2008	North Dakota	Soybean
BN117	53.6563333	37.4821	6.3703	2008	North Dakota	Dry Bean
BN118	54.9976667	51.7836	6.3646	2008	North Dakota	Dry Bean
BN119	15.186	46.2297	6.343	2008	North Dakota	Dry Bean
BN121	61.1266667	52.9526	6.3535	2008	North Dakota	Dry Bean
BN123	55.4146667	49.0751	6.3186	2008	North Dakota	Sunflower
BN126	67.3113333	42.443	6.3242	2008	North Dakota	Sunflower
BN132	53.0173333	31.755	6.331	2008	North Dakota	Soybean
BN133	51.222	56.3121	6.3272	2008	North Dakota	Dry Bean
BN134	42.774	47.3664	6.3122	2008	North Dakota	Soybean
BN135	48.226	52.6304	6.3919	2008	North Dakota	Soybean
BN138	46.2733333	46.98	6.3919	2008	Minnesota	Dry Bean
BN140	58.8883333	58.495	6.4175	2008	Minnesota	Soybean
BN143	53.238	45.0909	6.3285	2008	Minnesota	Sunflower
BN144	70.9433333	40.1545	6.3606	2008	Minnesota	Soybean
BN151	40.31	30.9378	6.3275	2008	North Dakota	Dry Bean
BN153	51.2303333	28.7674	6.3919	2008	Nebraska	Soybean
BN154	38.6733333	53.5709	6.3612	2008	Nebraska	Dry Bean
BN155	35.0253333	20.1881	6.4518	2008	North Dakota	Sunflower
BN156	56.4793333	46.5393	6.4005	2008	North Dakota	Dry Bean
BN157	36.2796667	71.5356	6.3434	2008	North Dakota	Dry Bean
BN158	38.814	44.7023	6.31	2008	North Dakota	Dry Bean

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
BN160	88.482	58.7315	6.3826	2008	North Dakota	Sunflower
BN161	34.839	55.7668	6.3491	2008	North Dakota	Sunflower
BN162	61.8163333	36.7435	6.3826	2008	North Dakota	Canola
BN163	60.1963333	64.9985	6.5338	2008	North Dakota	Sunflower
BN166	39.8283333	54.7534	6.375	2008	North Dakota	Sunflower
BN167	79.3356667	55.1823	6.3839	2008	North Dakota	Canola
BN169	54.1006667	64.9635	6.3659	2008	North Dakota	Dry Bean
BN172	72.305	25.3722	6.4319	2008	North Dakota	Dry Bean
BN173	79.244	47.5915	6.4319	2008	North Dakota	Canola
BN174	31.542	41.3383	6.3839	2008	North Dakota	Sunflower
BN175	53.7613333	61.4988	6.4319	2008	North Dakota	Canola
BN176	58.461	44.4242	6.4319	2008	North Dakota	Soybean
BN178	67.6686667	40.8585	6.3659	2008	North Dakota	Soybean
BN183	50.594	37.4768	6.4319	2008	North Dakota	Sunflower
BN186	55.139	64.4098	6.4319	2008	North Dakota	Canola
BN187	43.6466667	55.4825	6.4319	2008	North Dakota	Sunflower
BN191	70.5796667	40.1664	6.4225	2008	Nebraska	Soybean
BN193	61.6523333	54.2869	6.4618	2006	Colorado	Sunflower
BN195	40.3343333	46.6207	6.3655	2008	Nebraska	Dry Bean
BN196	53.544	43.3609	6.4618	1996	Colorado	Dry Bean
BN201	44.5066667	47.8476	6.4618	2008	South Dakota	Soybean
BN203	42.618	61.5462	6.5159	2006	Minnesota	Soybean
BN208	47.1046667	40.8729	6.5159	1997	Iowa	Soybean
BN209	62.1479667	26.8759	6.4038	1998	Illinois	Soybean
BN210	41.4746667	42.5632	6.3355	2002	Ohio	Soybean
BN212	26.1066667	38.3019	6.4038	2008	Iowa	Soybean
BN213	42.951	46.4866	6.3897	2008	Iowa	Soybean
BN215	37.5326667	46.176	6.3322	2008	Iowa	Soybean
BN216	50.0053333	54.5475	6.5159	2008	Iowa	Soybean
BN219	58.53	61.3983	6.3694	2008	Michigan	Soybean
BN220	55.1493333	56.5705	6.321	2000	Wisconsin	Soybean
BN221	67.6676667	23.9656	6.3143	2000	Wisconsin	Soybean
BN222	66.0486667	54.1548	6.3536	2000	Wisconsin	Soybean
BN227	26.1316667	42.6828	6.3736	2003	Wisconsin	Soybean
BN228	20.561	45.2809	6.4561	2003	Wisconsin	Soybean
BN231	33.102	58.5322	6.4561	2002	North Dakota	Canola
BN232	42.5523333	73.1492	6.4561	2003	Wisconsin	Tobacco
BN234	32.8743333	32.4435	6.303	2004	Missouri	Soybean
BN241	41.1116667	55.2937	6.3296	2008	North Dakota	Soybean
BN243	54.363	51.8066	6.4561	2005	Montana	Houndstongue
BN244	68.98	42.1259	6.4561	2007	Montana	Safflower

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
BN246	33.669	55.2987	6.3576	2008	Kansas	Sunflower
BN249	56.792	5.366	6.8111	NA	NA	NA
BN251	47.6373333	62.6309	7.4439	NA	Washington	Lentil
BN252	37.9566667	54.1012	7.4439	NA	Washington	Lentil
BN253	49.7583333	25.6742	8.2816	NA	Washington	Lentil
BN254	12.2024138	41.4119	7.4439	NA	Washington	Lentil
BN255	58.33	43.7412	7.4439	NA	Washington	Pea
BN257	49.8003448	34.5885	7.4439	NA	Washington	Pea
BN258	31.0935	46.5768	7.4439	NA	Washington	Pea
BN260	37.1110345	48.963	7.4439	NA	Washington	Gourd
BN261	39.4403448	27.5371	7.4439	NA	Washington	Gourd
BN262	30.2875862	25.7227	6.7359	NA	Washington	Gourd
BN265	42.2758621	56.277	6.2926	NA	Washington	Potato
BN267	44.662069	72.0772	6.3542	NA	Washington	Potato
BN268	23.2362069	57.9013	6.4232	NA	California	Lettuce
BN269	15.062	64.6665	6.3597	NA	California	Lettuce
BN270	54.0886667	58.3185	6.3761	NA	California	Lettuce
BN272	71.8843333	53.0003	6.4232	NA	California	Lettuce
BN273	55.9033333	32.3763	6.4232	NA	Oregon	Potato
BN274	67.956	43.7614	6.3763	NA	Oregon	Cauliflower
BN276	54.218	49.5143	6.4232	NA	Oregon	Cauliflower
BN279	51.0023333	35.4483	6.4232	NA	Oregon	Snap Bean
BN280	30.3783333	33.4626	6.4232	NA	South Carolina	Parsley
BN281	41.3403333	41.3881	6.3294	NA	South Carolina	Lettuce
BN291	47.5163333	65.5781	6.3058	NA	North Carolina	Cabbage
BN293	33.4503333	50.0102	6.401	NA	North Carolina	Cabbage
BN303	31.4646667	39.8828	6.3141	NA	North Carolina	Cabbage
BN304	42.3523333	22.3833	6.3373	NA	North Carolina	Cabbage
BN306	46.1816667	65.514	6.3425	NA	Idaho	Dry Bean
BN307	55.1966667	56.5909	6.3509	NA	Arkansas	Canola
BN308	30.2326667	59.2705	6.3502	NA	Georgia	Brassica olearacea
BN310	25.2496667	53.6289	6.3893	NA	Georgia	Broccoli
BN311	66.5673333	40.9497	6.4501	NA	Georgia	Cabbage
BN312	52.8626667	51.1365	6.4579	NA	Georgia	Carrot
BN313	42.4703333	55.3991	6.2912	NA	Georgia	Tomato
BN314	60.109	65.2646	6.2978	NA	Georgia	Cabbage
BN315	34.9256667	65.2625	6.4813	NA	Oklahoma	Lettuce
BN316	49.0456667	72.4093	6.3671	NA	Florida	Petunia
BN317	38.4993333	38.0082	6.388	NA	Florida	Cucumber
BN318	68.3076667	44.6592	6.388	NA	Florida	Tomato
BN319	53.891	27.2256	6.3448	NA	Florida	Potato

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
BN320	62.8276667	66.7252	6.3237	NA	Florida	Tomato
BN321	26.3946667	58.5336	6.3671	NA	Florida	Cabbage
BN322	33.0456667	56.0862	6.4813	NA	Florida	Tomato
BN323	31.2503333	66.0632	6.4813	NA	Arizona	Lettuce
BN324	56.2956667	87.9229	6.3393	NA	Arizona	Lettuce
BN325	48.952	47.3538	6.3229	NA	Arizona	Lettuce
BN327	44.7146667	50.8967	6.4469	NA	Arizona	Lettuce
BN328	54.6916667	55.6481	6.3405	NA	Arizona	Lettuce
BN329	87.371	55.1448	6.4552	NA	Arizona	Lettuce
BN330	33.0306667	58.077	6.3176	NA	Arizona	Lettuce
BN331	46.312	55.5698	6.4552	NA	Arizona	Lettuce
BN332	44.6926667	50.9764	6.3093	NA	Arizona	Lettuce
BN333	45.2793333	56.6781	6.343	NA	Connecticut	Tomato
BN334	51.0416667	40.4238	6.343	NA	Maine	Bush Bean
BN335	45.7043333	63.9248	6.343	NA	Massachussetts	Squash
BN336	44.0663333	60.3899	6.3177	NA	New York	Cabbage
BN337	53.89	40.4741	6.4663	NA	New York	Soybean
BN338	37.6356667	65.4549	6.3393	NA	New York	Cabbage
BN500	61.1366667	57.1293	6.439	2007	North Dakota	Canola
BN700	60.8853333	50.1637	6.3857	2007	North Dakota	Canola
BN900	31.417	50.5937	6.3226	2007	North Dakota	Canola
Breck1	64.903	34.8586	6.3776	2004	Minnesota	Sunflower
C1_231	49.8713333	62.5438	6.3115	NA	NA	NA
C2_264	44.1486667	61.3884	6.373	NA	NA	NA
C3_135	65.3396667	72.874	6.3455	NA	NA	NA
CA1	46.708	25.8471	6.3085	2005	California	Sunflower
CA2	54.81	51.1933	6.3571	2003	California	Sunflower
Carr1	60.992	48.9048	6.3115	2004	North Dakota	Sunflower
Carr2	69.2733333	38.6428	6.33	2004	North Dakota	Sunflower
Carr3	32.156	55.4457	6.3155	2004	North Dakota	Sunflower
Cockle04	42.8896667	40.6542	6.3333	2004	NA	Cocklebur
Fargo1	48.7876667	49.9906	6.328	2004	North Dakota	Sunflower
FM150	34.997	47.4389	6.3378	NA	North Dakota	Sunflower
FM170	61.3206667	48.6919	6.3378	NA	North Dakota	Sunflower
FM224	36.4946667	54.3123	6.3624	NA	North Dakota	Sunflower
FM227	52.2543333	53.8339	6.3425	NA	North Dakota	Sunflower
FM261	39.0016667	37.1082	6.3055	NA	North Dakota	Sunflower
Grandin1	40.2546667	39.7368	6.3309	2004	North Dakota	Sunflower
JS461	47.0473333	39.7898	6.3096	2003	Washington	Dry Bean
JS465	47.8613333	48.8274	7.5006	2003	Washington	Dry Bean
JS472	51.633	57.4943	6.4198	2003	Oregon	Dry Bean

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
JS474	47.8853333	64.7166	6.3391	2003	Oregon	Dry Bean
JS501	38.1913333	73.4036	6.3142	2004	Tasmania	Dry Bean
JS558	51.3765	63.6079	6.3768	2004	Minnesota	Dry Bean
JS561	50.376	61.7098	6.3569	2004	Minnesota	Dry Bean
JS577	65.6856667	64.1446	6.3548	2004	Nebraska	Dry Bean
JS578	90.611	56.2717	6.4198	2004	Nebraska	Dry Bean
JS591	60.6063333	58.7361	6.4042	2004	Oregon	Dry Bean
JS596	68.5886667	59.3935	6.3305	2004	California	Dry Bean
JS598	70.7846667	57.9717	6.4198	2004	California	Dry Bean
JS599	49.1533333	47.7059	6.329	2004	California	Dry Bean
JS601	63.529	53.0929	6.3768	2004	California	Dry Bean
JS611	65.4306667	46.6648	6.3716	2004	Michigan	Dry Bean
JS644	50.8533333	23.3339	6.3192	2005	Nebraska	Dry Bean
JS659	64.7466667	62.0835	6.351	2005	North Dakota	Dry Bean
JS665	50.0913333	67.6206	7.5375	2005	Michigan	Dry Bean
JS676	56.6263333	49.4661	6.3716	2005	California	Dry Bean
JS679	25.019	49.0078	6.3569	2005	California	Dry Bean
JS681	69.4826667	61.0442	6.3768	2005	California	Dry Bean
JS698	60.176	38.9766	6.3768	2007	North Dakota	Dry Bean
JS701	59.4276667	62.5859	6.3768	2007	North Dakota	Dry Bean
JS705	55.8866667	65.2438	6.3491	2007	North Dakota	Dry Bean
JS708	58.0426667	53.4854	6.3718	2007	North Dakota	Dry Bean
JS709	35.975	52.5749	6.3414	2007	North Dakota	Dry Bean
JS710	59.5843333	43.4087	6.3718	2007	North Dakota	Dry Bean
JS711	65.5636667	76.2611	6.4537	2007	North Dakota	Dry Bean
JS714	47.8803333	49.2469	6.396	2007	North Dakota	Dry Bean
JS715	57.0376667	61.3647	6.3468	2007	North Dakota	Dry Bean
JS717	37.8036667	40.76	6.3047	2007	North Dakota	Dry Bean
JS719	86.6793103	13.1653	6.319	2007	North Dakota	Dry Bean
JS728	50.5373333	69.9279	6.3541	2007	Colorado	Dry Bean
JS765	60.4813333	45.9673	6.3202	2007	Washington	Dry Bean
JS766	54.891	64.3451	6.3569	2007	Washington	Dry Bean
JS771	22.3883333	44.6042	6.3358	2007	Washington	Dry Bean
JS803	76.606	53.3964	6.3718	2008	Michigan	Dry Bean
JS806	46.7913333	47.6335	6.3244	2008	Michigan	Dry Bean
JS815	71.224	64.9346	6.3548	2008	Michigan	Dry Bean
JS840	41.4216667	40.4109	6.351	2009	Michigan	Dry Bean
JS841	47.7913333	38.1548	6.3716	2009	Michigan	Dry Bean
JS843	67.2633333	28.1027	6.3802	2009	Michigan	Dry Bean
JS845	71.5746667	67.5998	6.3569	2009	Michigan	Dry Bean
JS861	47.81	48.4848	6.3144	2009	Michigan	Dry Bean

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
JS887	48.1163333	62.8119	6.3548	2010	Nebraska	Dry Bean
JS890	46.9433333	24.0382	6.5252	2010	Colorado	Dry Bean
JS891	74.4786667	59.2425	6.3267	2010	North Dakota	Dry Bean
JS913	49.732	33.8267	6.3189	2010	North Dakota	Dry Bean
MarshE04	69.452	48.5787	6.301	2004	NA	Marsh Elder
MN1	24.9926667	31.8776	6.3166	2010	Minnesota	Sunflower
MN2	63.35	51.9719	6.375	2010	Minnesota	Sunflower
MN3	54.0923333	48.5501	6.4467	2010	Minnesota	Sunflower
MN4	45.112	49.6965	6.3911	2010	Minnesota	Sunflower
MN5	26.7546667	61.3447	6.3624	2010	Minnesota	Sunflower
MN6	46.133	38.2605	6.3297	2010	Minnesota	Sunflower
MN8	40.597	42.8036	6.4196	2010	Minnesota	Sunflower
ND44	31.328	52.3733	6.4196	2011	North Dakota	Canola
NE031	54.0796667	65.1403	6.6855	2010	Nebraska	Canola
NE274	28.392	58.355	6.3078	NA	Nebraska	Dry Bean
NE567	26.6006667	56.572	6.3401	NA	Nebraska	Dry Bean
NE574	66.073	46.054	6.5288	NA	Nebraska	Dry Bean
NE590	36.1703333	46.5028	6.3255	NA	Nebraska	Dry Bean
NE710	48.7518519	60.5523	6.401	NA	Nebraska	Dry Bean
NE743	55.9023333	71.0745	6.3293	NA	Nebraska	Dry Bean
NECanola	48.8206667	61.8243	6.302	2007	Nebraska	Canola
R140	53.3603571	45.3692	6.4501	NA	NA	NA
R224	44.4733333	52.1379	6.4606	NA	NA	NA
R260	42.437	62.2198	6.3474	NA	NA	NA
Ragweed	54.724	56.3727	7.5186	2004	NA	Ragweed
Soy01	49.157	74.2561	6.3185	2001	NA	Soybean
Sun87	50.319	84.4288	6.3533	1987	Canada	Sunflower
SunA146	61.9056667	43.6907	6.3382	2002	NA	Sunflower
SunA226	57.1205	62.5274	6.3317	2002	NA	Sunflower
SunA320	84.3306667	73.904	6.34	2002	NA	Sunflower
Wells1	63.3916667	53.8383	6.3382	2004	North Dakota	Sunflower
WM245	96.569	43.3987	6.3337	2003	Nebraska	Dry Bean
WM714	73.5393333	51.8292	6.3362	2004	Nebraska	Dry Bean

APPENDIX B. THE AVERAGE LESION LENGTH ESTIMATED MEANS AND OTHER STATISTICAL ANALYSIS RESULTS FROM PROC MIXED MODEL IN INBRED LINE HA441

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
1980	27.994	67.0196	19.0732	1980	Nebraska	Dry Bean
Arg1	82.149	78.201	19.0373	2000	Argentina	Sunflower
Arg154	53.1486207	74.1231	19.9237	NA	Argentina	Sunflower
Arg2	93.3648276	89.9521	19.6043	2000	Argentina	Sunflower
Arg213	236.752667	238.32	19.5123	NA	Argentina	Sunflower
Arg228	66.9723333	56.9428	19.1235	NA	Argentina	Sunflower
Bean03	80.8663333	70.8368	19.1235	2003	NA	Dry Bean
BN102	35.7713333	42.6881	19.2959	2008	North Dakota	Dry Bean
BN105	24.142	68.922	31.5298	2008	North Dakota	Dry Bean
BN109	72.0666667	78.9835	19.2959	2008	North Dakota	Dry Bean
BN110	69.872	88.5672	22.9042	2007	North Dakota	Canola
BN112	79.571	86.4878	19.2959	2007	North Dakota	Canola
BN113	165.5	99.3778	19.2684	2008	North Dakota	Soybean
BN114	58.8333333	66.6917	19.0047	2008	North Dakota	Soybean
BN117	172.166667	59.8972	22.9117	2008	North Dakota	Dry Bean
BN118	172.166667	111.89	19.1691	2008	North Dakota	Dry Bean
BN119	168.833333	94.1176	19.0687	2008	North Dakota	Dry Bean
BN121	172.166667	105.51	19.1691	2008	North Dakota	Dry Bean
BN123	152.166667	55.6603	19.6298	2008	North Dakota	Sunflower
BN126	142.166667	98.9488	19.2363	2008	North Dakota	Sunflower
BN132	135.5	96.4949	19.0228	2008	North Dakota	Soybean
BN133	42.774	61.0718	19.2363	2008	North Dakota	Dry Bean
BN134	48.226	121.26	19.3125	2008	North Dakota	Soybean
BN135	46.2733333	124.29	19.3125	2008	North Dakota	Soybean
BN138	58.8883333	98.9698	19.2363	2008	Minnesota	Dry Bean
BN140	53.238	99.7877	19.3125	2008	Minnesota	Soybean
BN143	70.9433333	104.41	19.2363	2008	Minnesota	Sunflower
BN144	40.31	89.2355	19.2383	2008	Minnesota	Soybean
BN151	51.2303333	94.493	19.2948	2008	North Dakota	Dry Bean
BN153	68.88	98.4754	19.2948	2008	Nebraska	Soybean
BN154	38.6733333	84.8287	19.2948	2008	Nebraska	Dry Bean
BN155	35.02533333	81.6812	31.6094	2008	North Dakota	Sunflower
BN156	56.4793333	47.3994	19.1662	2008	North Dakota	Dry Bean
BN157	36.2796667	106.53	19.1662	2008	North Dakota	Dry Bean
BN158	38.814	110.79	19.1662	2008	North Dakota	Dry Bean
BN159	88.482	26.1565	19.3705	2008	North Dakota	Canola

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
BN160	78.0682	110.76	19.1662	2008	North Dakota	Sunflower
BN161	34.839	118.64	19.1662	2008	North Dakota	Sunflower
BN162	61.8163333	112.52	19.3718	2008	North Dakota	Canola
BN163	60.1963333	101.49	31.7032	2008	North Dakota	Sunflower
BN166	39.8283333	126.99	19.6169	2008	North Dakota	Sunflower
BN167	79.3356667	102.09	31.7032	2008	North Dakota	Canola
BN169	54.1006667	103.11	19.2948	2008	North Dakota	Dry Bean
BN172	72.305	87.6266	19.1662	2008	North Dakota	Dry Bean
BN173	47.616	96.0838	19.125	2008	North Dakota	Canola
BN174	79.244	244.34	19.0807	2008	North Dakota	Sunflower
BN175	31.542	86.8646	19.1578	2008	North Dakota	Canola
BN176	53.7613333	112.04	19.4075	2008	North Dakota	Soybean
BN178	58.461	54.5388	19.1799	2008	North Dakota	Soybean
BN183	67.6686667	85.065	19.1961	2008	North Dakota	Sunflower
BN186	50.594	62.3323	18.7468	2008	North Dakota	Canola
BN187	25.183	77.2345	19.2361	2008	North Dakota	Sunflower
BN191	55.139	62.5721	19.1676	2008	Nebraska	Soybean
BN193	43.6466667	58.7631	19.3622	2006	Colorado	Sunflower
BN195	70.5796667	75.7828	19.2361	2008	Nebraska	Dry Bean
BN196	61.6523333	96.6578	19.2361	1996	Colorado	Dry Bean
BN201	40.3343333	88.6702	19.2361	2006	Minnesota	Soybean
BN203	53.544	94.2378	19.2361	2008	Indiana	Soybean
BN208	44.5066667	89.7178	19.2361	1997	Iowa	Soybean
BN209	35.401	57.0075	19.2465	1998	Illinois	Soybean
BN212	42.618	89.5258	19.1799	2008	Iowa	Soybean
BN215	47.1046667	70.6046	19.0891	2008	Iowa	Soybean
BN216	23.319	98.8522	19.2361	2008	Iowa	Soybean
BN219	62.1479667	83.0838	19.2361	2008	Michigan	Soybean
BN220	41.4746667	84.0813	19.0891	2000	Wisconsin	Soybean
BN222	26.1066667	48.9012	19.2185	2000	Wisconsin	Soybean
BN227	35.886	58.7539	19.1182	2003	Wisconsin	Soybean
BN228	42.951	90.2521	19.2955	2003	Wisconsin	Soybean
BN231	37.5326667	73.1705	19.1799	2002	North Dakota	Canola
BN232	50.0053333	69.8335	19.5814	2003	Wisconsin	Tobacco
BN234	58.53	77.1962	19.1182	2004	Missouri	Soybean
BN243	29.565	45.6249	19.1591	2005	Montana	Houndstongue
BN244	55.1493333	67.3965	19.5814	2007	Montana	Safflower
BN246	67.6676667	49.1781	19.5814	2008	Kansas	Sunflower
BN249	66.0486667	75.7661	19.024	NA	NA	NA
BN251	26.1316667	9.8056	23.4081	NA	Washington	Lentil
BN252	20.561	62.6988	19.5814	NA	Washington	Lentil

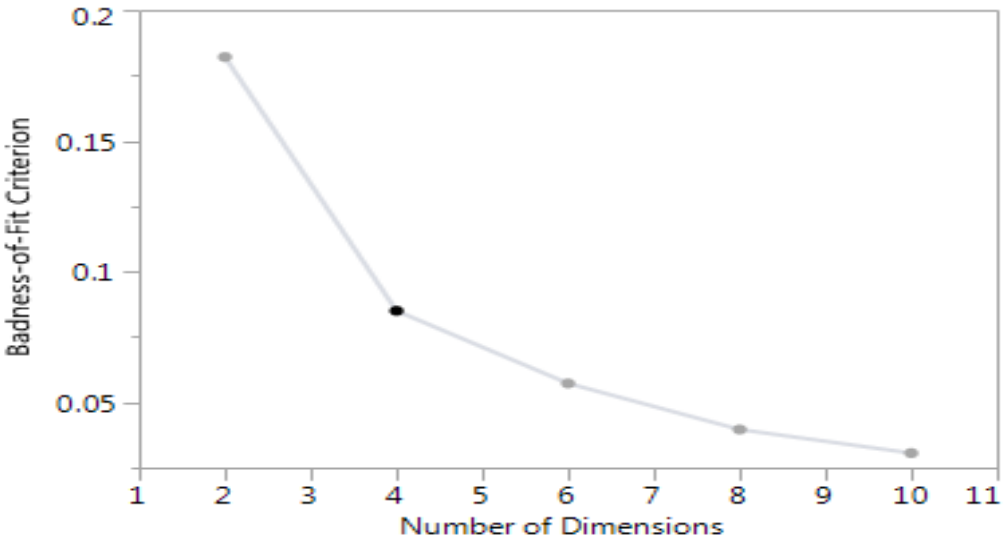
Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
BN253	33.102	42.1691	19.5814	NA	Washington	Lentil
BN254	42.5523333	58.4055	19.5814	NA	Washington	Lentil
BN255	32.8743333	83.7655	18.9116	NA	Washington	Pea
BN257	41.1116667	76.1035	19.5814	NA	Washington	Pea
BN258	54.363	48.4598	19.5814	NA	Washington	Pea
BN260	68.98	71.1333	20.1998	NA	Washington	Gourd
BN261	69.8167	66.3258	19.5814	NA	Washington	Gourd
BN262	33.669	9.5289	19.1037	NA	Washington	Gourd
BN265	56.792	24.11	19.2331	NA	Washington	Potato
BN267	47.6373333	74.9381	19.4854	NA	Washington	Potato
BN268	37.9566667	82.4021	19.4854	NA	California	Lettuce
BN270	49.7583333	55.9834	19.0327	NA	California	Lettuce
BN272	66.15333	67.3971	19.4854	NA	California	Lettuce
BN273	12.2024138	97.5178	19.4854	NA	Oregon	Potato
BN276	58.33	57.2022	19.04	NA	Oregon	Cauliflower
BN279	49.8003448	53.9592	19.3854	NA	Oregon	Snap Bean
BN280	31.0935	45.1982	19.3854	NA	South Carolina	Parsley
BN281	37.1110345	42.6572	19.3854	NA	South Carolina	Lettuce
BN291	39.4403448	68.0754	19.3551	NA	North Carolina	Cabbage
BN293	30.2875862	99.0221	19.632	NA	North Carolina	Cabbage
BN304	42.2758621	84.4851	19.0743	NA	North Carolina	Cabbage
BN307	44.662069	79.5751	19.632	NA	Arkansas	Canola
BN310	23.2362069	106.65	19.632	NA	Georgia	Broccoli
BN311	83.78533	90.7701	19.632	NA	Georgia	Cabbage
BN312	75.265	82.2468	19.632	NA	Georgia	Carrot
BN313	15.062	76.4155	19.632	NA	Georgia	Tomato
BN314	79.0265	86.0085	19.632	NA	Georgia	Cabbage
BN315	54.0886667	93.5338	19.632	NA	Oklahoma	Lettuce
BN316	71.8843333	98.6211	19.632	NA	Florida	Petunia
BN317	55.9033333	98.4925	19.3585	NA	Florida	Cucumber
BN318	67.956	54.9201	19.632	NA	Florida	Tomato
BN319	54.218	86.3098	19.3585	NA	Florida	Potato
BN321	51.0023333	43.8042	19.3585	NA	Florida	Cabbage
BN322	30.3783333	111.81	19.3585	NA	Florida	Tomato
BN323	41.3403333	103.77	19.3282	NA	Arizona	Lettuce
BN324	47.5163333	97.5878	19.3282	NA	Arizona	Lettuce
BN325	33.4503333	89.0178	19.3282	NA	Arizona	Lettuce
BN327	31.4646667	74.7395	19.3282	NA	Arizona	Lettuce
BN329	42.3523333	74.1651	19.3282	NA	Arizona	Lettuce
BN330	46.1816667	77.3031	19.3282	NA	Arizona	Lettuce
BN331	55.1966667	100.92	19.3282	NA	Arizona	Lettuce

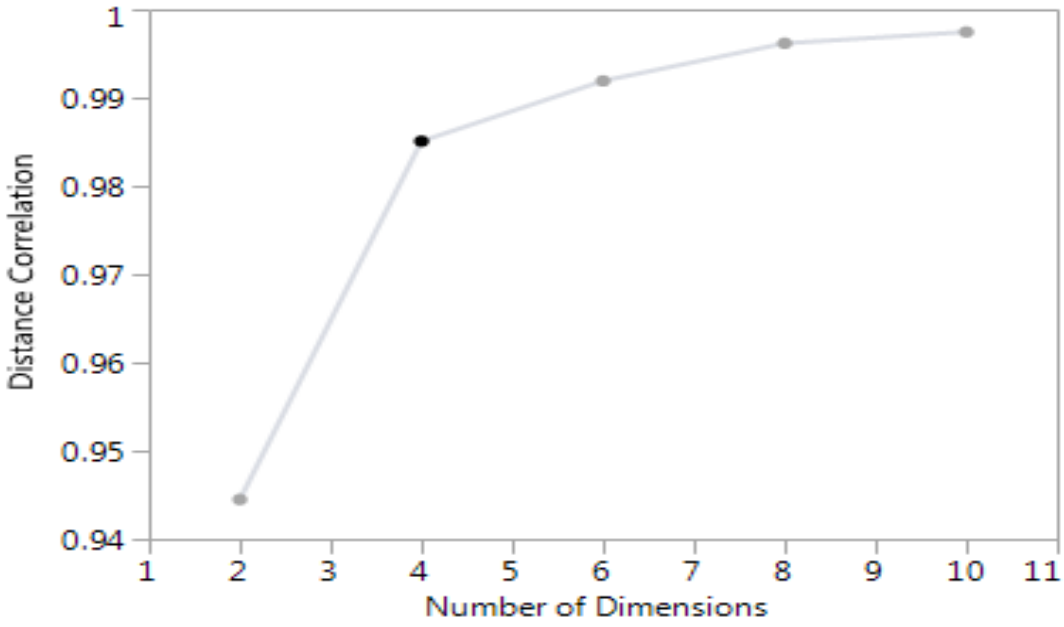
Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
BN332	30.2326667	90.7755	19.2045	NA	Arizona	Lettuce
BN333	25.2496667	87.1295	19.2045	NA	Connecticut	Tomato
BN334	66.5673333	79.5024	19.137	NA	Maine	Bush Bean
BN335	52.8626667	93.8452	18.924	NA	Massachussetts	Squash
BN336	42.4703333	80.4151	19.2045	NA	New York	Cabbage
BN337	60.109	117.52	19.2045	NA	New York	Soybean
BN338	34.9256667	110.16	19.2045	NA	New York	Cabbage
BN500	49.0456667	118.9	19.2045	2007	North Dakota	Canola
BN700	38.4993333	79.5636	19.0898	2007	North Dakota	Canola
BN900	68.3076667	54.3032	19.2412	2007	North Dakota	Canola
Breck1	53.891	59.0923	19.5627	2004	Minnesota	Sunflower
C1_231	62.8276667	83.2323	19.5123	NA	NA	NA
C2_264	26.3946667	83.1296	19.5123	NA	NA	NA
C3_135	33.0456667	93.1435	19.7683	NA	NA	NA
CA1	31.2503333	87.3585	24.1994	2005	California	Sunflower
CA2	56.2956667	73.03	19.2858	2003	California	Sunflower
Carr1	48.952	82.4466	19.5627	2004	North Dakota	Sunflower
Carr2	44.7146667	83.0147	22.9013	2004	North Dakota	Sunflower
Carr3	54.6916667	82.1913	19.5627	2004	North Dakota	Sunflower
Cockle04	87.371	93.8679	19.5123	2004	NA	Cocklebur
Fargo	78.833	135.75	20.2583	2004	North Dakota	Sunflower
FM150	33.0306667	69.6038	19.2247	NA	North Dakota	Sunflower
FM170	46.312	88.707	19.8199	NA	North Dakota	Sunflower
FM224	44.6926667	73.5846	19.5627	NA	North Dakota	Sunflower
FM227	45.2793333	89.53	19.8199	NA	North Dakota	Sunflower
FM261	51.0416667	95.942	19.9923	NA	North Dakota	Sunflower
Grandin1	45.7043333	51.1897	19.5772	2004	North Dakota	Sunflower
JS461	44.0663333	47.8214	19.7855	2003	Washington	Dry Bean
JS465	53.89	29.0016	19.2322	2003	Washington	Dry Bean
JS472	37.6356667	84.0831	19.7847	2003	Oregon	Dry Bean
JS474	61.1366667	86.2421	19.4024	2003	Oregon	Dry Bean
JS501	60.8853333	96.917	20.0993	2004	Tasmania	Dry Bean
JS558	31.417	62.188	20.0993	2004	Minnesota	Dry Bean
JS561	64.903	70.1621	20.0806	2004	Minnesota	Dry Bean
JS577	107.8592	108.42	20.0993	2004	Nebraska	Dry Bean
JS578	49.8713333	79.1428	19.7829	2004	Nebraska	Dry Bean
JS591	44.1486667	87.2283	20.0993	2004	Oregon	Dry Bean
JS596	65.3396667	90.0641	19.5823	2004	California	Dry Bean
JS598	46.708	106.08	20.0993	2004	California	Dry Bean
JS599	54.81	110.82	20.0993	2004	California	Dry Bean
JS601	60.992	117.01	19.7855	2004	California	Dry Bean

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
JS611	69.2733333	110.45	19.7847	2004	Michigan	Dry Bean
JS644	32.156	113.93	19.7829	2005	Nebraska	Dry Bean
JS659	42.8896667	43.554	19.7114	2005	North Dakota	Dry Bean
JS665	48.7876667	117.48	19.7855	2005	Michigan	Dry Bean
JS676	34.997	75.0023	20.0993	2005	California	Dry Bean
JS679	61.3206667	127.03	20.0993	2005	California	Dry Bean
JS681	36.4946667	124.46	19.5823	2005	California	Dry Bean
JS698	52.2543333	86.6356	19.6097	2007	North Dakota	Dry Bean
JS701	39.0016667	100.99	19.7829	2007	North Dakota	Dry Bean
JS705	40.2546667	97.1837	20.0993	2007	North Dakota	Dry Bean
JS708	47.0473333	116.98	19.5799	2007	North Dakota	Dry Bean
JS709	47.8613333	131.44	20.0806	2007	North Dakota	Dry Bean
JS710	51.633	100.7	19.2553	2007	North Dakota	Dry Bean
JS711	47.8853333	106.36	19.5799	2007	North Dakota	Dry Bean
JS714	38.1913333	113.46	19.6097	2007	North Dakota	Dry Bean
JS715	51.3765	104.8	19.2379	2007	North Dakota	Dry Bean
JS717	50.376	114.67	19.5799	2007	North Dakota	Dry Bean
JS719	65.6856667	112.82	19.6097	2007	North Dakota	Dry Bean
JS728	90.611	102.11	19.6097	2007	Colorado	Dry Bean
JS765	60.6063333	85.9371	19.2481	2007	Washington	Dry Bean
JS766	68.5886667	66.4747	20.0993	2007	Washington	Dry Bean
JS771	70.7846667	93.7726	19.6097	2007	Washington	Dry Bean
JS803	49.1533333	94.0659	19.6097	2008	Michigan	Dry Bean
JS806	63.529	76.2646	19.4761	2008	Michigan	Dry Bean
JS815	65.4306667	90.9158	19.7829	2008	Michigan	Dry Bean
JS840	50.8533333	109.72	20.0993	2009	Michigan	Dry Bean
JS841	64.7466667	104.78	19.4761	2009	Michigan	Dry Bean
JS843	50.0913333	107.3	20.0993	2009	Michigan	Dry Bean
JS845	56.6263333	64.7038	19.7829	2009	Michigan	Dry Bean
JS861	25.019	111.27	19.6097	2010	Nebraska	Dry Bean
JS887	69.4826667	75.8733	20.0993	2010	Colorado	Dry Bean
JS888	59.4276667	84.9404	20.0806	2010	Colorado	Dry Bean
JS890	55.8866667	93.5632	19.4888	2010	Colorado	Dry Bean
JS913	58.0426667	104.97	19.2014	2010	North Dakota	Dry Bean
MarshE04	35.975	61.352	19.2764	2004	NA	Marsh Elder
MN1	59.5843333	76.7755	19.2247	2010	Minnesota	Sunflower
MN2	65.5636667	75.4616	19.2011	2010	Minnesota	Sunflower
MN3	47.8803333	88.2733	19.5627	2010	Minnesota	Sunflower
MN4	57.0376667	89.3282	19.8268	2010	Minnesota	Sunflower
MN5	37.8036667	96.3109	19.0886	2010	Minnesota	Sunflower
MN6	86.6793103	91.3783	19.4221	2010	Minnesota	Sunflower

Isolate	Raw Mean	Estimate Mean	Standard Error	Collection Year	Collection Location	Plant Collected From (host)
MN8	50.5373333	53.0416	19.5627	2010	Minnesota	Sunflower
ND44	47.7913333	67.7442	19.4357	2011	North Dakota	Canola
NE031	60.4813333	79.8473	19.5627	2010	Nebraska	Canola
NE274	54.891	67.5296	18.9682	NA	Nebraska	Dry Bean
NE567	22.3883333	101.88	19.5627	NA	Nebraska	Dry Bean
NE574	76.606	75.622	19.9269	NA	Nebraska	Dry Bean
NE590	46.7913333	79.3099	19.3377	NA	Nebraska	Dry Bean
NE710	71.224	99.736	19.2947	NA	Nebraska	Dry Bean
NE743	41.4216667	75.576	19.5627	NA	Nebraska	Dry Bean
NECanola	67.2633333	92.6599	19.5123	2007	Nebraska	Canola
R140	71.5746667	84.9668	19.6956	NA	NA	NA
R224	47.81	32.9221	19.4449	NA	NA	NA
R260	48.1163333	83.3615	19.2905	NA	NA	NA
Ragweed	46.9433333	92.1764	19.7818	2004	NA	Ragweed
Soy01	87.9476667	82.6953	19.5123	2001	NA	Soybean
Sun87	84.36925	90.9206	19.5136	1987	Canada	Sunflower
SunA146	74.4786667	87.3827	19.2	2002	NA	Sunflower
SunA226	49.732	95.6908	19.3349	2002	NA	Sunflower
SunA320	88.9256	108.34	19.5123	2002	NA	Sunflower
Wells1	69.452	74.6954	18.9378	2004	North Dakota	Sunflower
WM245	24.9926667	65.3404	19.0019	2003	Nebraska	Dry Bean
WM714	24.9926667	86.0313	19.4221	2004	Nebraska	Dry Bean

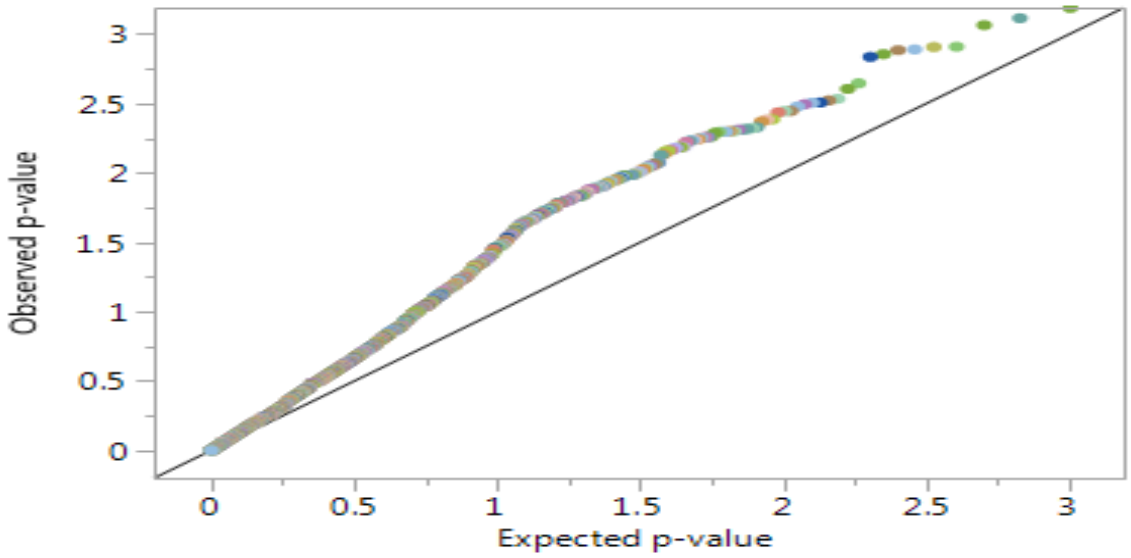
APPENDIX C. SUPPLEMENTARY FIGURE 1



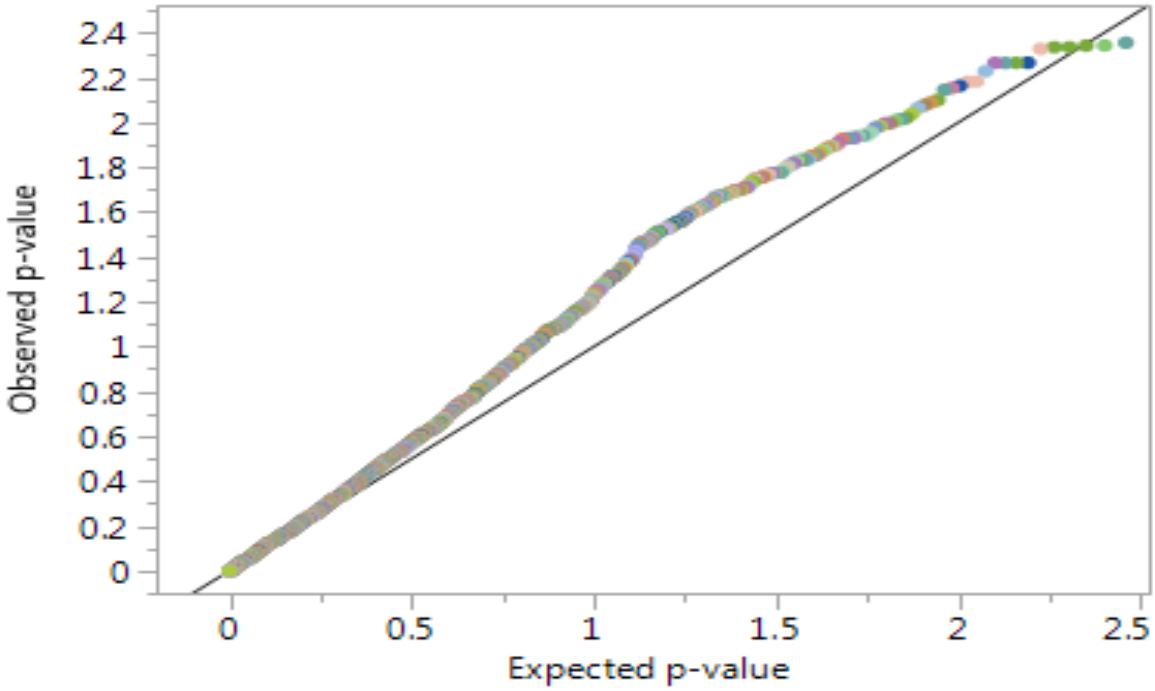


APPENDIX D. SUPPLEMENTARY FIGURE 2

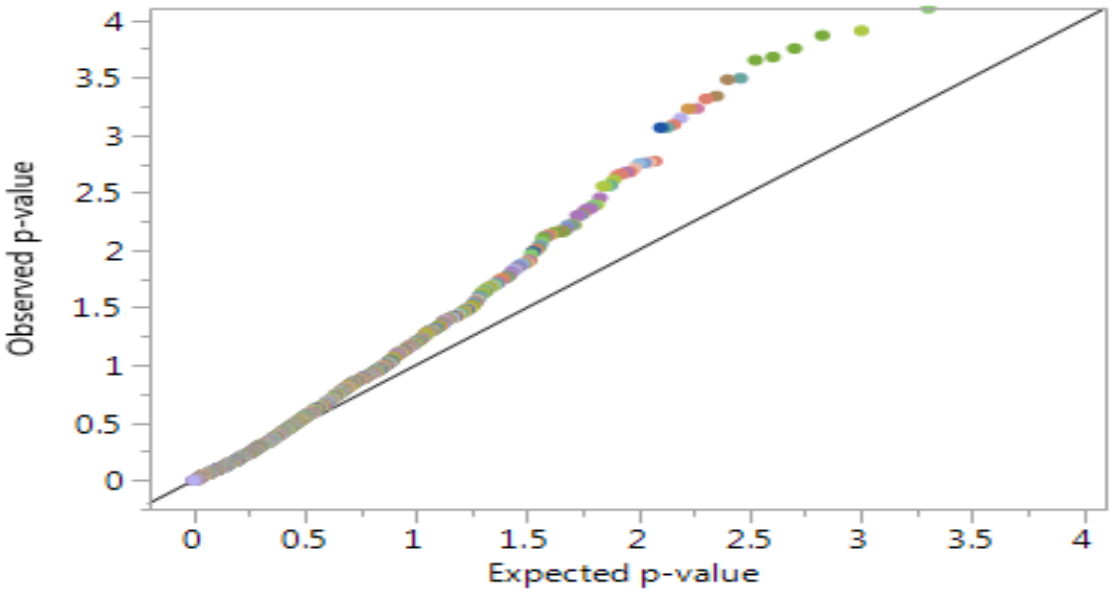
Q-50perc-207



Q K- 50perc PCA-207



Q-50perc-HA441



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