STUDIES ON DIVERSITY OF *ALTERNARIA ALTERNATA* ASSOCIATED WITH ALTERNARIA LEAF SPOT IN SUGAR BEET

By

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ABSTRACT

STUDIES ON DIVERSITY OF *ALTERNARIA ALTERNATA* ASSOCIATED WITH ALTERNARIA LEAF SPOT IN SUGAR BEET

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Alternaria leaf spot (ALS) is one of the foliar diseases that can impact sugar beet. Recently, ALS has been an increasing issue in fields from several states in the United States including Michigan. One of the most common causative agents of ALS is *Alternaria alternata*. This same organism has been reported as a pathogen not only in sugar beet, but also in a number of other crops. Michigan is the second most agriculturally diverse state in the United States and a wide range of crops are grown in the state. Since *A. alternata* is a pathogen on a number of crops currently grown and associated with sugar beet crop rotation in Michigan, it is important to learn about the interactions between *A. alternata* and its hosts. Therefore, the current projects were carried out to examine some of the host-pathogen interactions in *A. alternata* with a focus on ALS in sugar beet. The first project addressed developing effective inoculation methods for ALS in the laboratory, greenhouse or in the field. An effective inoculation method for ALS in sugar beet is important for assessing potential management tools for ALS such as screening for resistance and for fungicide efficacy. A detached leaf assay and a greenhouse inoculation with the inoculum in 0.2% malt extract as an outside nutrient source indicated a significant difference between susceptible and resistant varieties for the disease. Field results showed a significant effect of inoculation in three out of four years but showed issues with other pathogens and still need further testing. However, preliminary evidence for a consistent inoculation was observed in 2021, where the inoculum in an outside nutrient source, similar to what we used in the greenhouse, gave higher disease severity than a non-inoculated control or water-suspended

inoculum. The diversity of *A. alternata* was observed in the second and third projects. Aspects of the host range of *A. alternata* were examined by isolating the pathogen from four crops growing in Michigan and cross-inoculating each isolate across four hosts. All the *A. alternata* strains caused lesions on all four host crops tested regardless of the crop from which they originally were isolated. Therefore, the results supported a wide host range of *A. alternata*. Furthermore, genetic diversity of *A. alternata* was examined in the third project. A total of 48 *A. alternata* isolates, collected from four hosts in Michigan, and *in-silico* data for an additional 15 *A. alternata* isolates from different hosts in other geographic areas, were used in a genetic analysis using three loci. The phylogenetic tree in this study agreed with previous reports of a high level of genetic diversity for *A. alternata*. This was the first study to examine diversity on beet and indicated similar diversity to what has been observed on other crops like potato and apple. No clear separation was observed between *A. alternata* strains related to the host from which they were originally collected. This further supports a lack of host specialization of *A. alternata* among the diverse hosts examined. Therefore, care should be taken when including these crops in a crop rotation system. If these crops are grown in rotation, the potential for disease development needs to be considered for appropriate management. The pathogen diversity also has implications for screening for host resistance.

This dissertation is dedicated to my husband and parents who have been with me all the time by giving me the love, support, and encouragement to fulfill this achievement.

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KEY TO ABBREVIATIONS

µl	Micro liter
ABC	ATP binding cassette
ALS	Alternaria leaf spot
ANOVA	Analysis of variance
AUDPC	Area under the disease progress curve
BCV	*Beet chlorosis virus*
BWYV	*Beet western yellows virus*
C	Celsius
CLS	Cercospora leaf spot
CMA	Corn meal agar
Cyp51	Sterol 14-alpha demethylase
Cytb	Cytochrome b
DMI	Demethylase inhibitors
DNA	Deoxyribo nucleic acid
dNTP	Deoxyribonucleotide triphosphate
EC50	Half maximal effective concentration
EndoPG	Endoploygalacturonase
FRAC	Fungicide Resistance Action Committee
HCV8	Half-strength clarified V8
HST	Host specific toxin
Inc.	Incorporated

ITS	Internal transcribed spacer region
L	Liter
LSD	Least significant difference
MFS	Major facilitator superfamily
$MgCl_2$	Magnesium chloride
MI	Michigan
ml	Milliliter
mm	Millimeter
MN	Minnesota
MO	Montana
MSU	Michigan State University
NCBI	National center for biotechnology information
OMA	Oatmeal agar
PCR	Polymerase chain reaction
Pgs1	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase
Ppm	Parts per million
QoI	Quinone outside inhibitor
REACh	Research and Education Advisory Council
RH	Relative humidity
Sp.	Species (singular)
Spp.	Species (plural)
Syn.	Synonym
SVREC	Saginaw Valley Research and Extension Center

Tma22	Translation machinery associated protein
Tris-EDTA	Tris(hydroxymethyl)aminomethane - ethylenediaminetetraacetic acid
USDA	United States Department of Agriculture
UV	Ultraviolet
vol/vol	Volume to volume
wt/vol	Weight to volume
x	Multiplied by

CHAPTER 1

ALTERNARIA LEAF SPOT OF SUGAR BEET: HISTORY, SYMPTOMS, HOST RANGE, CAUSATIVE AGENT, EPIDEMIOLOGY, AND MANAGEMENT

History of sugar beet production

Sugar beet (*Beta vulgaris*) is an important crop for sugar extraction in many countries around the world (Smigocki et al. 2009). Before the nineteenth century, the primary source to obtain sucrose was sugar cane (*Saccharum* spp.) and sugar cane was the only commercial source of sucrose (Cooke and Scott 1993). The main disadvantage of sugar cane is its limited distribution in the world. Sugar cane production is restricted to tropical and subtropical areas of the world (Cooke and Scott 1993). In the nineteenth century, sugar beet (*Beta vulgaris* subsp. *vulgaris* L.) was introduced as a crop from which to extract sugar (sucrose) in Europe (Smigocki et al. 2009, Cooke and Scott 1993), a temperate region.

The origin of the genus *Beta* was in Middle Eastern and Eastern Mediterranean countries (Smigocki et al. 2009, Doney 1996). Beet was first domesticated between 2000 and 1500 B. C. and the first domesticated beet was a leafy vegetable (Harveson et al. 2009, Winner 1993, Smigocki et al. 2009, Doney 1996). In addition, some *Beta vulgaris* were used for medicinal purposes (Smigocki et al. 2009, Harveson et al. 2009). Since then, different types of *Beta vulgaris* were developed. These cultivated beets have been categorized into four main groups based on the external features of the crop. The four groups are leaf beet, garden beet, fodder beet and sugar beet (Lewellen et al. 2009). Spinach beet and Swiss chard are grouped under leaf beets where the leaves are used for salads or cooked greens. Garden beet such as red beet is used as a root vegetable for human consumption, although the leaves also may be eaten. They have succulent storage organs and mostly have very thin lignified tissues (Lewellen et al. 2009).

Fodder beets are mainly grown for animal feed. Sugar beets are used to extract sucrose and ethanol production. Typically, the sucrose concentration of a sugar beet root can range from 14-20% of the fresh weight (Wyse 1979, Campbell 2002). Fodder and garden beets also can be used for sugar extraction, but the sucrose concentration is lower (3-6% from garden beets and 6-12% from fodder beet) (Lewellen et al. 2009).

The German chemist, Andreas Margraff discovered that some beets (fodder beets, called mangolds) contain sucrose and he determined that extraction of sucrose from beets is possible. However, sucrose extraction was not a success until one of his students, Franz Karl Achard started extracting sucrose from beets. Achard started the first sugar factory at Cunern in lower Silesia (Poland) in 1801 (Harveson et al. 2009, Winner 1993). Then production and factories spread to other countries in Europe including, France, Germany, Austria, and Russia (Winner 1993). The first attempt to establish a sugar factory in the United States was in 1838 in Northampton, Massachusetts by two Americans, Edward Church and David Lee Child but it was shut down in 1840 (Harveson et al. 2009, Winner 1993). Several other attempts to establish sugar factories were made in Michigan, Wisconsin, and Illinois, but they all were unsuccessful (Harveson et al. 2009). The first successful sugar factory in the United States was built in central California in 1870 (Harveson et al. 2009, Winner 1993).

In Michigan, Dr. Robert C. Kedzie was the pioneer to establish the local sugar beet industry. Therefore, he was considered the “Father of the Michigan beet sugar industry” (Michigan Sugar Company 2015a). In 1897, what is now Michigan Sugar Company established its first sugar beet factory in Bay City, Michigan. In 1899, eight new sugar beet factories were built in different cities in Michigan including Kalamazoo, Rochester, Benton Harbor, Alma, West Bay City, Essexville (a second factory), and Caro. The number of factories increased to

sixteen by 1905 (Michigan Sugar Company 2015a). Currently, a single company, Michigan Sugar Company operates four factories in Bay City, Caro, Croswell, and Sebewaing in Michigan (Michigan Sugar Company 2015b).

Since the 1930s, sugar beet yield has been improved (Draycott 2006). For recent yields, in 2004 the average sugar yield from sugar beets in the world was 34 million tons (Draycott 2006). In 2004, Europe and the European Union produced 26 and 19 million tons of sugar beet with Africa (0.8 million tons), North and central America (4 million tons), South America (0.4 million tons) and Asia (2 million tons) accounting for the remainder of the production (Draycott 2006). Currently in the United States, sugar beet is grown in four regions which includes a total of 11 states. This includes the Great Lakes region (Michigan), the Upper Midwest (Minnesota and North Dakota), the Great Plains (Colorado, Montana, Nebraska and Wyoming) and the Far West region (California, Idaho, Oregon, and Washington) (USDA, Economic Research Service, 2021). In the United States, the total acres of sugar beet planted in 2021 was 1,161,500 acres and a total of 35, 675, 000 tons of sugar production was obtained (USDA-National Agricultural Statistical Services 2021). In the United States, an average of 55-60% of sugar was extracted from sugar beet and an average 40-55% of sugar was extracted from sugar cane domestically since 2000 (ERS 2021). This indicates the importance of sugar beet for domestic sugar production in the United States.

Alternaria leaf spot in sugar beet

The production and yield of sugar beet in the world is affected by a number of diseases and they can be characterized as foliar, root and post-harvest diseases (Franc 2009). One of the diseases that can affect sugar beet is Alternaria leaf spot (ALS) (Franc 2009, McFarlane et al. 1954). Several *Alternaria* species can cause ALS, of which the two most commonly reported are

Alternaria brassicae and *Alternaria alternata* (Franc 2009, Gray 1995). Other species also have been reported to cause ALS, including *Alternaria betae-kenyensis*, *Alternaria tenuissima* (now recognized as a synonym of *A. alternata*), *Alternaria chenopodii*, *Alternaria ashwinii* and *Alternaria dilkushana* (Misra et al. 2021, Grover and Gupta 1973, Woudenberg et al. 2015, Simmons 2007, Khan et al. 2020). However, the classification of some members of this genus has been somewhat uncertain due to the morphological plasticity of some *Alternaria* species and lack of genetic differentiation associated with certain characters (Woudenberg et al. 2013, Woudenberg et al. 2015). In addition, some of these potential species are not fully described.

ALS in sugar beet has a wide geographic distribution. It has been reported that ALS occurs in all regions where sugar beets are grown (Dunning and Byford 1982, Franc 2009), but the level of severity varies in different regions (Franc 2009, Dunning and Byford 1982). In some regions in Pakistan, ALS is considered a major foliar disease and research has been conducted to manage ALS (Abbas et al. 2014). In India, ALS can cause moderate to severe damage on sugar beet and was reported in a number of regions including Punjab, Delhi, Jammu and Kashmir (Srivastava 2004, Misra et al. 2021). In Russia, ALS in sugar beet also is considered an economically important disease (Lastochkina et al. 2018, Gannibal 2018, Стогниенко et al. 2019). ALS in sugar beet has been reported in several counties in Europe such as the Netherlands, Romania, Belarus, Poland, Germany, Slovakia and the United Kingdom, but is generally considered a minor issue in these countries (Hanse et al. 2015, Hudec and Rohačik 2002, Bălău 2009, Dunning and Byford 1982, Kolomiets et al. 2010, Pusz 2007, Dorn 1950, Russel 1965). In the United States, McFarlane et al. (1954) reported that a new form of Alternaria leaf spot was identified in California sugar beet fields, and the pathogen was identified

as *A. brassicae*. McFarlane et al. (1954) noted that *Alternaria tenuis* (older name of *A. alternata*) also had been identified causing ALS on sugar beet in the United States before 1950.

Historically ALS was considered a minor foliar disease of sugar beet in the United States (Franc 2009). But recently ALS has caused increasing issues and potential significant yield losses in Michigan sugar beet fields (Rosenzweig et al. 2017, Rosenzweig et al. 2019). Increased incidence also has been reported in other states (Khan et al. 2020). Due to its relative lack of importance in the US and Europe, little research has been done on ALS in sugar beet (Franc 2009). With the potential increasing issues from this disease, it is important to learn about this host-pathogen system and examine potential management strategies for this disease

Signs and symptoms of ALS

The primary symptom for ALS is necrotic lesions on the leaf (Figure 1.1A) (Franc 2009, McFarlane et al. 1954, Srivastava 2004, Rosenzweig et al. 2019). The symptoms usually appear on older leaves first (Franc 2009, McFarlane et al.1954). The lesions of ALS start as 2-5 mm circular to irregular shaped lesions on the leaves. As the disease progresses, these can coalesce to form large necrotic lesions with an average diameter of 10 mm for *A. alternata* (Khan et al. 2020, Srivastiva 2004, Franc 2009) whereas the lesion size can increase up to 15 mm with *A. brassicae* (Srivastava 2004). The lesions are usually dark brown in color with a lighter center (Khan et al. 2020, Srivastava 2004, Franc 2009). They can occur anywhere on the leaf but frequently are found at the margin of leaves (Srivastava 2004, Yu et al. 2016). Under conducive conditions, such as high humidity (>90% RH) and cool temperature (16˚- 22˚C), lesions can be observed with a velvety blackish appearance corresponding to heavy sporulation of the pathogen (Srivastava 2004, Franc 2009). Sometimes, shot holes can be observed at the center of the lesions (Srivastava 2004, McFarlane et al. 1954) when necrotic tissue breaks and falls out of the leaf. On

some sugar beet varieties, reddish colored margins may form around ALS lesions (McFarlane et al. 1954). When the lesions occur on the leaf margins, the leaves may curl upward as the lesions coalesce (Figure 1.1A) (Srivastava 2004). When the environmental conditions [humidity (>90%) and cool temperature (16°- 22°C)] are favorable for the disease, lesions can accelerate leaf senescence and a rapid necrosis on the leaves resulting in a loss of the entire leaf (Franc 2009, McFarlane et al. 1954). As well as the leaf, some *Alternaria* species (especially reported for *A. brassicae*) may infect the petioles and seed stalks (McFarlane et al. 1954, Srivastava 2004) causing long and narrow necrotic spots. However, infection on petioles occurs rarely (Srivastava 2004). In addition, *A. alternata* may cause seedling damage and storage rot of sugar beet (Abada 1994, Kolomiets et al. 2010, Larran et al. 2000, Misra et al. 2021, Srivastava 2004). As well as causing disease, *A. alternata* has been reported as a potential endophyte of beet (Larran et al. 2000), showing a diversity of interactions for this fungus.

ALS can be favored when plants have symptoms of beet yellows [caused by *Beet western yellow virus* (BWYV), *Beet chlorosis virus* (BCV), and *Beet mild yellowing virus* (BMYV)] or Fusarium yellows which promote yellowing in the plant (Russel 1965, Franc 2009). ALS also can be observed when the plants are under stress conditions such as nutrient deficiencies, which accelerate yellowing (Franc 2009).

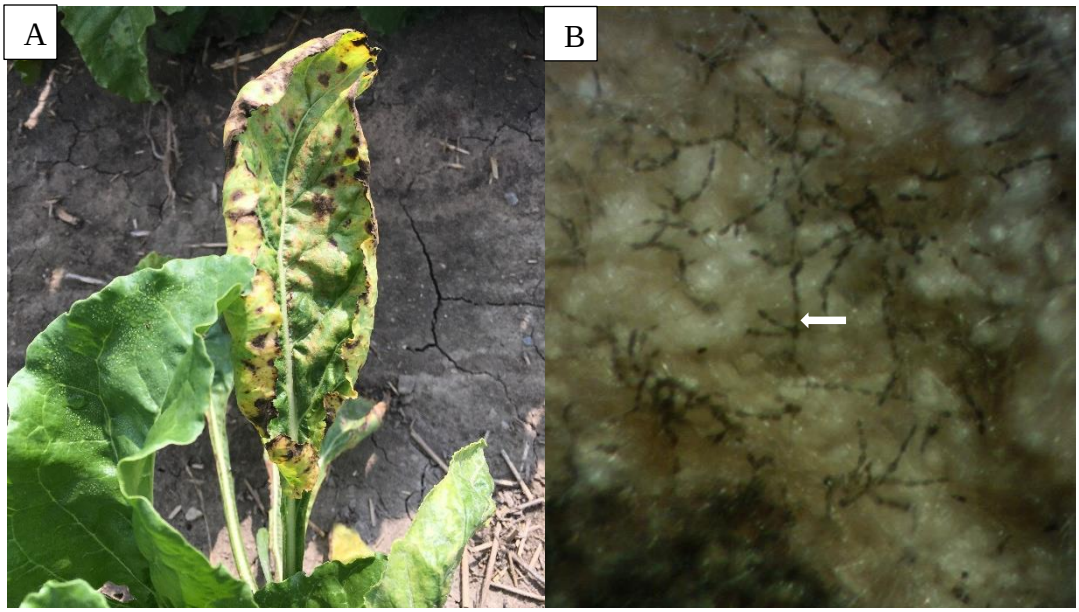

Figure 1.1: Symptoms and signs of Alternaria leaf spot on sugar beet leaves. A shows symptoms of the brown necrotic lesions and yellowing leaves. Symptomatic leaves were also curled upward at the margins. B shows a view of a lesion caused by *Alternaria alternata* (63x magnification). The white arrow indicates the pathogen conidial chains arranged on the lesion.

Disease cycle of ALS

The pathogens that can cause ALS are known as saprophytes and opportunistic pathogens and survive on the ground and in the soil on crop debris (Franc 2009, McFarlane et al. 1954). *Alternaria* species also can survive on other infected hosts in the field such as weeds (Franc 2009). The conidia of *Alternaria* spp. are melanized and the melanin can give protection against adverse environmental conditions such as UV radiation and cell wall degrading enzymes produced by other antagonistic microbes and enhance survival of the pathogen (Butler and Day 1998, Kawamura et al. 1997, Carzaniga et al. 2002). ALS also may be introduced into a field through contaminated sugar beet seeds (Srivastava 2004, McFarlane et al. 1954). The symptom production by ALS is favored by cool (7°- 30°C) and humid (>90% RH) conditions (Franc 2009, McFarlane et al. 1954, Srivastava 2004). McFarlane et al. (1954) reported that disease development by *A. brassicae* occurs at a temperature range from 7°-10° C and the disease from

this species is not prominent when the temperature is above 16° C. *Alternaria alternata* strains have a wider temperature range (15°-35°C) (Hasija 1970). Optimum disease conditions for *A. alternata* have not been studied on sugar beet, but the optimum temperature for *A. alternata* is 25°C in tomato (Malathrakis 1983) and the optimum temperature on apples ranged from 28°-31°C (Filajdić and Sutton 1991). In India, it was reported that ALS disease incidence is high with a 25°-30°C temperature range in sugar beet fields (Srivastava 2004). Once the environmental conditions are favorable for the disease, the older leaves of plants are the first parts showing the symptoms (Franc 2009). On infected tissue, *A. alternata* sporulation is enhanced by high humidity (>95% RH) and sporulation occurs on the lesions when lesions are old enough and there is high humidity (Figure 1.1B) (Bashan et al. 1991a, Franc 2009, Srivastava 2004). While spore production is stimulated by high humidity, spore release of *A. alternata* in sugar beets is enhanced by low humidity (Franc 2009). The spores from the lesions are primarily spread by wind currents (Srivastava 2004, Franc 2009). It has been reported that both rain and wind currents help to disseminate the pathogen in the field or to nearby fields (Srivastava 2004, Bashan et al. 1991a). *Alternaria* species also may contaminate seeds, and the contaminated seeds may help the pathogen to disseminate long distances (Srivastava 2004, Soteros 1979). In addition, some *Alternaria* spp. such as *A. brassicicola* are also spread by insects such as flea beetles (*Phyllotreta cruciferae*) (Dillard et al. 1998), but there is limited research on the potential role of insects in transmission of the species on sugar beet.

Host range

Alternaria spp. such as *A. alternata* and *A. brassicae* cause disease not only in sugar beet, but also in a number of crops belonging to different plant families (Lagopodi and Thanassoulopoulos 1998, Taba et al. 2009, Ghosh et al. 2002, Zhu et al. 2018, Saad and

Hagedorn 1970, Dingley 1970). Beans are one of the common hosts of *Alternaria* spp. For example, *A. alternata* can cause lesions on bean leaves and pods (Maheshwari et al. 2000, Rahman et al. 2003, Rao 1965). Although *A. solani* is the primary causative agents of leaf spot in tomato, Alternaria leaf spot in tomato also can be caused by *A. alternata* (called *A. tenuissima* in some studies) (Gleason and Edmunds 2005, Grogan et al. 1975). In addition, *Alternaria* spp. such as *A. alternata* and *A. brassicae* cause leaf spots on several hosts in the family Brassicaceae such as canola and rapeseed (Al-Lami et al. 2019a, Al-Lami et al. 2019b). Alternaria leaf spot of brassicas can cause a significant yield loss in many countries such as Australia, Canada, India, and in the European Union (Al-Lami et al. 2019a, Al-Lami et al. 2019b, Al-lami et al. 2020). A study done by Mirkova and Konstantinova (2003) found *A. alternata* can infect gerbera and the *A. alternata* inoculation on other crops such as capsicum, petunia, tobacco, potato, and carrot and confirmed previous reports that *A. alternata* can infect all these hosts. This gives evidence for a broad host range of *A. alternata*.

The diseases caused by *A. alternata* in various crops can be called by different names. For instance, symptoms caused by *A. alternata* on potato and citrus are known as Alternaria brown spot (Ding et al. 2019, Droby et al. 1984, Wang et al. 2010, Vicent et al. 2000, Marín et al. 2006, Solel 1991, Reis et al. 2006, Fourie et al. 2009). *A. alternata* also can infect fruits, causing stem end rot of mango (Amin et al. 2011), core browning and moldy core in apples (Gao et al. 2013), black rot of kiwifruit (Nemsa et al. 2012, Kwon et al. 2011), fruit rot of cucumber and capsicum (Al-Sadi et al. 2011, Anand et al. 2009), and moldy heart of peaches (Pose et al. 2010). Similarly, diseases caused by *A. brassicae* can have varied names. For example, when *A. brassicae* causes symptoms on mustards, the disease is called Alternaria blight (Prasad et al. 2003).

Since *A. alternata* was reported on a number of different crops it is possible to consider that the pathogen has a wide host range (Srivastava 2004, Meena et al. 2013, Troncoso-Rojas and Tiznado-Hernández 2014). However, some studies reported a narrow host range for certain *A. alternata* types (Nishimura 1980, Tsuge et al. 2013) with potential host specificity of the pathogen strains based at least in part on the host selective toxins they produced. In several studies, the authors proposed calling these different pathotypes producing putative hosts specific toxins as separate species such as *A. mali* on apples, *A. kikuchiana* on Japanese pear, and *A. citri* on citrus (Kohmoto et al. 1976, Kameda et al. 1973, Umer et al. 2021) while others accepted them as pathotypes of *A. alternata* such as strawberry pathotype, apple pathotype and tangerine pathotype (Maekawa et al. 1984, Zhu et al. 2017, Fu et al. 2020). Some of the host specific toxins (AM, AC, AK, AF, AT, and AL) produced by what are now accepted as *A. alternata* pathotypes and their targets are mentioned in Table 1.1. Recent studies have shown that some gene clusters encoding host selective toxins (e.g.: AK toxin, AF toxin and ACT toxin) are on conditionally dispensable small chromosomes (< 2 kb) (Johnson et al. 2001, Masunaka et al. 2005, Hatta et al. 2002, Akimitsu et al. 2013), and the strains without that chromosome lacked the ability to produce the host selective toxins but had normal growth and reproduction (Johnson et al. 2001). This is one of several factors which support that these are not separate species (Akimitsu et al. 2013, Hatta et al. 2002, Johnson et al. 2001). One issue with several of these host-specificity studies is that some have used limited hosts or only hosts all of which belong to the same host family. For instance, Kohmoto et al. (1991) tested the susceptibility of hosts with two different putative host specific toxins only on citrus species and found susceptibility on two and twenty-eight of the tested citrus types for rough lemon (ACR toxin) and tangerine pathotypes (ACTG toxin) respectively. This showed a fairly wide host range within citrus for at least one of

these “types”. Since no testing was done outside of citrus, little can be determined about the overall host range of these strains or toxins. Based on the limited testing in some of these host specificity studies and varied results from different research groups, additional clarification is needed on host specificity within *A. alternata*.

Table 1.1: Reported host selective toxins produced by proposed *Alternaria alternata* pathotypes with the disease caused and toxin target sites.

Pathotype	Host selective toxin	Disease	Target site	References
Tomato pathotype	AAL	Alternaria stem canker	Aspartate carbamyl transferase; sphinganine N-acyltransferase	Meena et al. 2017, Akamatsu et al. 1997, Abbas et al. 1994
Rough lemon pathotype	ACR	Brown spot of rough lemon	Mitochondria	Otani et al. 1991
Japanese pear pathotype	AK	Black spot of Japanese pear	Plasma membrane	Otani et al. 1991, Meena et al. 2017, Nishimura and Kohmoto 1983
Tangerine pathotype	ACT	Brown spot of tangerine	Membrane protein	Meena et al. 2017, Nishimura and Kohmoto 1983
Apple pathotype	AM	Alternaria blotch of apple	Chloroplast and plasma membrane	Otani et al. 1991, Meena et al. 2017, Li et al. 2013
Tobacco pathotype	AT	Brown spot of tobacco	Mitochondria	Meena et al. 2017, Tsuge et al. 2013
Strawberry pathotype	AF	Black spot of strawberry	Plasma membrane	Meena et al. 2017, Maekawa et al. 1984, Otani et al. 1991,
Knapweed pathotype	Maculosin	Black leaf blight of knapweed	Ribulose-1,5 bisphosphate carboxylase	Meena et al. 2017, Stierle et al. 1988

Causative agents of ALS on sugar beet

The most commonly reported causative agents of ALS on sugar beet in the United States are *A. alternata* (including some under what are now known to be synonyms, *A. tenuis* and *A. tenuissima*) and *A. brassicae* (Franc 2009, McFarlane et al. 1954, Khan et al. 2020, Rosenzweig et al. 2017, Rosenzweig et al. 2019, Gray 1995, Lastochkina et al. 2018, Robeson and Jalal 1991, Dorn 1950). In other parts of the world, some additional *Alternaria* species have been reported, such as *A. betae-kenyensis* from chard in Kenya (Simmons 2007). Misra et al. (2021) identified two potential new *Alternaria* species (*Alternaria ashwinii* and *Alternaria dilkushana*) from sugar beet in India but did not do genetic analysis and these are not yet accepted species.

Alternaria species can be separated from other pathogens of sugar beet by the production of dark, melanized, straight or slightly curved conidia with transverse and longitudinal septa arranged on dark-colored conidiophores as solitary spores or in chains (Simmons 2007, Franc 2009, McFarlane et al. 1954) with a restriction at the apex of most spores (Simmons 2007), sometimes called a beak (Woudenberg et al. 2013). Here we will concentrate on the characters of the two species that most commonly cause ALS, *A. alternata* and *A. brassicae*. As discussed later, there has been some confusion and changes in the taxonomy of *A. alternata*. The characters here are reported as per Simmons (2007) on V8 agar at 3-7 days. *A. alternata* forms relatively small conidia (13-45 x 8-18 µm) in chains of 6-20 conidia while *A. brassicae* produces larger conidia (150-205 x 20-40 µm) formed singly or in short chains of 2-3 conidia and may form secondary conidiophores from some conidia (Simmons 2007).

Taxonomy in *Alternaria alternata*

The genus *Alternaria* was initially described by Nees (1816) based on dark colored phaeodictyospores (spores having longitudinal and transverse septa) formed in chains and the

apical cell forming a tapering beak (Woudenberg et al. 2013). Based on the spore size of *Alternaria* spp. they often are differentiated into large-spore and small-spore baring species (Andersen et al. 2005, Ayad et al. 2019, Kusaba and Tsuge 1995, Simmons 2007, Woudenberg et al. 2013, Woudenberg et al. 2014). For example, Simmons (2007) divided *Alternaria* species into two sections where section I consists of *Alternaria* spp. with generally large spores, having long (>100 µm) or medium [(50-60)-100 µm] conidium length. The conidia in this group can be solitary or in chains with less than 2-3 conidia in a chain. In contrast, *Alternaria* spp. in section II have short [<50(60) µm] conidia that can be solitary or short (<50-60 µm) to medium [(50-60)-100 µm] length conidia arranged in chains with more than 2-3 conidia in a chain. Additional differences in the morphology were used to distinguished previously proposed species such as *A. alternata* and *A. tenuissima* (Simmons 2007), both of which were reported on sugar beet in different studies (Franc 2009, Khan et al. 2020). The morphology for these is distinctive enough that they were designated as different species (Simmons 2007) and some researchers still support classifying them as separate species as a convenience for those working with plant pathogens (Liu et al. 2019, Khalmuminova et al. 2020) or for other reasons such as morphological differentiation (Simmons 2007). As mentioned previously, some researchers proposed separating species within the small-spored *Alternaria* based on host range (Kohmoto et al. 1976, Kameda et al. 1973, Umer et al. 2021), a character that is not accepted for the sole characteristic to differentiate between species (Guarro et al. 1999). Other characteristics which were used included things like relative spore size, length of conidial chains, and conidial branching (Simmons 2007, Wiltshire 1993). For example, the primary difference between *A. alternata* and the former *A. tenuissima* was that *A. alternata* contains branched conidial chains whereas "*A. tenuissima*" generally produces largely unbranched chains of conidia on V8 agar or host tissue

(Simmons 2007, Wiltshire 1933). However, these characters can vary with different growth conditions which is why it is important to have consistent conditions. In addition, the conidial sizes may differ slightly, with *A. alternata* reported as 13-43 x 8-14 µm and *A. tenuissima* 32-45x 14-18 µm (Simmons 2007).

The separation of small-spored *Alternaria* from large spored *Alternaria* spp. such as *A. brassicae* is relatively easy (Kusaba and Tsuge 1995, Weir et al. 1998). However, classification of *Alternaria* spp. within the small-spored group is more complicated due to both morphological plasticity where varying growth conditions were used (Simmons 2007, Woudenberg et al. 2013, Woudenberg et al. 2015, Gannibal 2016) as well genetic similarity that does not match with selected morphological traits (Armitage et al. 2015, Woudenberg et al. 2013, Woudenberg et al. 2015). In fungal taxonomy, it is accepted that there should be a positive association between species morphology and genetic clades to identify separate species (Weir 1987, Miller and Huhndorf 2005). Several recent studies have shown that a number of the morphological characteristics that have been used to separate species within the small-spored *Alternaria* species do not relate to genetic similarities. For example, Kusaba and Tsuge (1995) performed a phylogenetic analysis based on ITS sequences of ribosomal DNA of *Alternaria* isolates and were unable to separate some small-spored *Alternaria* spp. This could be due to ITS not having sufficient resolution, as has been found with numerous fungal genera (Lloyd-MacGilp et al. 1996, Woudenberg et al. 2013, Woudenberg et al. 2015). However, the same lack of resolution has been found in several small-spored *Alternaria* using other genetic regions, and even with multi locus analysis. For instance, Andrew et al. (2009) performed a multi-locus analysis with 150 small-spored *Alternaria* isolates and reported no strict association between the phylogenetic lineage and morphology among several previously separated species, including *A. alternata* and

"*A. tenuissima*". Similar results were also reported by Woudenberg et al. (2015), Armitage et al. (2015) and Armitage et al. (2020). Based on these numerous findings, several previously recognized *Alternaria* spp. are now synonyms under *A. alternata* including a type often reported on beet, previously separated as *A. tenuissima* (Figure 1.2) (Woudenberg et al. 2015). There have been attempts made to separate some of these morpho-types in different ways. For example, Armitage et al. (2015) proposed separating *A. alternata* into subspecies, such as *A. alternata* ssp. *alternata*, *A. alternata* ssp. *arborescens*. They used 5 different genes but found inconsistency for morphology within these proposed subspecies. Similarly using whole genome analysis, clades did not show a clear separation of some of these suggested morphological groups within *A. alternata* (Armitage et al. 2020). Thus, numerous studies have not reported any reliable morphological characters for clear species division within *A. alternata*.

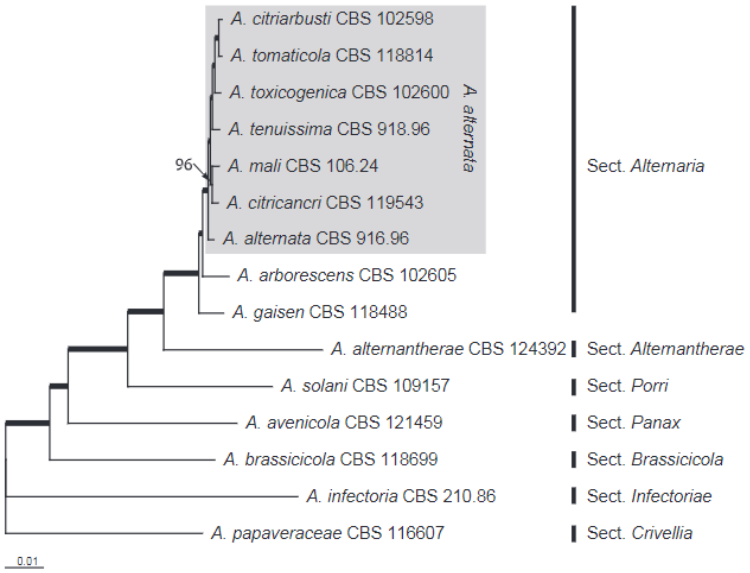

Figure 1.2: A phylogenetic tree with 9 *Alternaria* spp. including 7 *Alternaria* sections based on whole genome transcriptome reads (Woudenberg et al. 2015). Fully supported nodes are indicated by thicken lines. Previously suggested *Alternaria* spp. in the grey box are all synonyms of *A. alternata* according to Woudenberg et al. (2015).

Management of ALS

Since ALS was a minor foliar disease in the United States in the past, there were few efforts to manage the disease. Thus, there is limited information about ALS management. Generally, specific management was not needed for ALS (Franc 2009). Some research was done showing that management might reduce losses even with low disease levels (Russel 1965). However, with the increasing issues in sugar beet fields in Michigan, Minnesota, and possibly other states in the United States, there is increased interest in potential control measures (Rosenzweig et al, 2019, Khan et al. 2020). Some fungicides are labelled for foliar diseases on beets, and there is testing for efficacy of these for ALS on beet (REACh 2016, REACh 2017, REACh 2018). Previous testing also showed that some fungicides labelled for use on beet in the past might offer some control of Alternaria leaf spot, and this could impact yield in some cases (Russel 1965).

Other disease management methods include cultural control practices such as crop rotation, removing weeds that are potential hosts for either or both of the common *Alternaria* species, proper irrigation and proper fertility practices to reduce the stress on plants (Franc 2009, Srivastava 2004). Host resistance is a possible management tool as resistance has been reported (McFarlane et al. 1954, Abbas et al. 2014, REACh 2017, 2018) but there have been few efforts on resistance identification in the United States and western Europe and little is known about the genetics of resistance or susceptibility (McFarlane et al. 1954, Biancardi et al. 2005). Resistance was reported to be widespread in US commercial material in the past (McFarlane et al. 1954).

Cultural control

Some *Alternaria* species may survive on weeds (Franc 2009). Some weeds in sugar beet fields can include wild mustards, lambsquarters, pigweed, velvetleaf, ragweed, crab grass,

yellow foxtail, and wild buckwheat (Sprague 2017). Among these, some weeds such as wild mustard, pigweed, velvetleaf, ragweed, and buckwheat can be hosts of *A. alternata* and *A. brassicae* (Petersen 2004, Mazur et al. 2015, Kremer 1986, Maurya et al. 2021). In addition, McFarlane et al. (1954) reported that wild radish is a common weed in California, and a host for *A. brassicae*. Therefore, controlling weeds such as wild mustards and radishes that may be hosts of the pathogens may be helpful to reduce pathogen maintenance in the field (Franc 2009, McFarlane et al. 1954).

Another widely accepted cultural method for controlling some diseases is crop rotation (Peters et al. 2004, Bullock 1992, Kincaid 1960, Clayton et al. 1944). Crop rotation may help to reduce the level of primary inoculum in the field (Peters et al. 2004, Bockus and Claassen 1992, Jirak-Peterson and Esker 2011). However, managing ALS by crop rotation is challenging. One potential reason for difficulties in managing ALS by crop rotation is the potentially wide host range of the pathogen (Srivastava 2004), although there has been little testing for potential host specificity in the strains that impact sugar beet. This is an area that needs further investigation to determine whether there might be host specific strains for sugar beet, similar to potential specificity reported from some isolates on apple and citrus (Zhu et al. 2017, Kohmoto et al. 1991, Kohmoto et al. 1993), or more generalist strains, similar to those reported on cotton, pistachio, beans and potato (Bashan et al. 1991b, Michailides et al. 1994, Droby et al. 1984). The types of crops in the current crop rotation system vary from region to region. For instance, sugar beets are commonly rotated with wheat, soybean, and corn in Eastern North Dakota (Bayer 2019). In Michigan, sugar beets are often rotated with corn, wheat, and dry or soybeans and in some regions in Michigan potatoes or cucumbers also are included in the crop rotation, but less frequent than the first four (Sanchez et al. 2001). Since some *A. alternata* have a wide host

range, finding non-host crops may be difficult especially as several of these crops, such as dry bean, potato, soybean, corn, and wheat are hosts for some *A. alternata* (Broggi et al. 2007, Ding et al. 2019, Droby et al. 1984, Dillard and Cobb 2008, Fisher et al. 1992). Testing for host specificity in these crops is important to understand the potential for use of this disease management strategy. In addition, while the crops may all be potential hosts, there might be resistant varieties that could be selected for use (Clayton et al. 1944, Kincaid 1960). Thus, specific varieties might support rotation as a disease management strategy. However, little work has been done on host resistance in most of these crops, limiting this possible management tool.

Biological control

Several studies have tested managing *Alternaria* species using a number of varied fungal and bacterial strains as potential biological control agents (Verma et al. 2007, Tozlu et al. 2018a, Tozlu et al. 2018b). However, the effect of the biological control agents in most studies have been tested in the laboratory or greenhouse only (Tozlu et al. 2018b, Ramírez-Cariño et al. 2020). Some of the potential biological control agents that were reported to have an antagonistic effect on *A. alternata* include *Trichoderma harzianum* (on apple, tomato, and strawberry) (Tozlu et al. 2018a, Tozlu et al. 2018b), strains of *Burkholderia cenocepacia* and *Pseudomonas poae* on Aloe vera (Ghosh et al. 2016), and *Bacillus paralicheniformis* and *Trichoderma asperelloides* in tomato (Ramírez-Cariño et al. 2020). Studies on biological control of ALS in sugar beets are limited. Lastochkina et al. (2018) tested the effect of a combination of three *Bacillus subtilis* strains against *A. alternata* in sugar beet and found that the *Bacillus* strains suppressed the hydrolase enzymes such as pectinases which may help in *A. alternata* infection. There was a correlation between repressing hydrolases and less disease by *A. alternata* and the authors showed some reduction in disease in the field, but it is unclear how practicable the application

would be on large sugar beet fields. Currently no commercial biological control components are commercially available for ALS in sugar beet.

Host resistance

Screening and potentially breeding for host resistance, or possibly to exclude susceptible materials (McFarlane et al. 1954) can be an important part of disease management. Identification of resistant or susceptible varieties can be accomplished either by artificial inoculation or natural infection in the field (Auclair et al. 2004, Williams et al. 1981, Chi et al. 2019) or in the laboratory or greenhouse settings (Abbas et al. 2014, Agostini et al. 2003, Grontoft and O'Connor 1990). However, any laboratory or greenhouse testing needs to be confirmed in field conditions (Brown et al. 1999, Simon and Strandberg 1998, Odilbekov et al. 2014). A limited number of studies have been done on screening for resistance or susceptibility to ALS among sugar beet varieties. A major factor is that the disease has not been a serious issue in most production regions (Biancardi et al. 2005). McFarlane et al. (1954) performed a greenhouse inoculation with *A. brassicae* and found three highly susceptible sugar beet inbred lines and several other verities with varying levels of resistance. However, there was little, or no susceptibility found in the commercially available sugar beet varieties at the time. Therefore, they proposed that the identification of susceptibility genes and avoiding selection of susceptible varieties were more important than finding resistance. Russell (1972) found a type of ALS resistance that was associated with virus yellow tolerance. This might be resistance or might be related to the increased ALS reported in plants with virus yellows (Russel 1965, Franc 2009), which needs to be clarified. Overall, work in the United States and western Europe showed little susceptibility to ALS (Biancardi et al. 2005).

Some different reports were given in a general screening in an area with more history of issues with ALS. Abbas et al. (2014) performed a greenhouse inoculation with *A. alternata* on 12 different sugar beet varieties in Pakistan and found no complete resistance for any of the varieties tested. Variable responses were found among varieties that were classified as moderately resistant, susceptible and highly susceptible to *A. alternata*. It should be noted that Pakistan, where this work was done, has a history of ALS problems (Abbas et al. 2014), while in the US, where the McFarlane et al. (1954) testing was done, ALS has been a minor issue. The conditions and the varieties approved for these areas vary, which may be factors in these results. For the situation in Michigan, several sugar beet varieties were found with varying levels of ALS under natural infection (REACh 2017, 2018). The variety trials performed in Michigan sugar beet fields found several potentially ALS tolerant sugar beet varieties, and a small number of varieties (RR059 and SX-RR1245N) susceptible to ALS under natural infection (REACh 2017, 2018). This may be a situation that was warned of by McFarlane et al. (1954) and Biancardi et al. (2005) who warned of introducing susceptible material into an area where resistance was widespread, but this needs to be determined. The situation in Michigan indicates that screening may be important to avoid such introductions. Identification of any resistance/ susceptibility genes for ALS is important for selecting sugar beet varieties in areas where ALS might be an issue or avoiding in areas where ALS has been of little importance. There is little known about host genes important in the ALS response.

Chemical control

ALS on sugar beet has not needed control by fungicides in the United States until recently but fungicides have been used for ALS management in some other countries (Srivastava 2004) and some fungicides have been tested for ALS in the United Kingdom (Russel 1965).

There are a number of fungicides that have been used on beet primarily for management of other leaf spots (Rosenzweig et al. 2017, Rosenzweig et al. 2019, Baltaduonytė et al. 2013, Jones and Windels 1991, Solel 1970). Some fungicides show efficacy on managing ALS (REACh 2016, 2017, 2018). However, use of chemical control may be complicated as fungicide resistance has been identified in *A. alternata* for several fungicides in Michigan (Rosenzweig et al. 2017, Rosenzweig et al. 2019) and Ontario, Canada (Trueman et al. 2017). Therefore, further testing is needed on the potential of fungicides to manage ALS and ways to manage fungicide resistance.

The fungicides labelled for use on sugar beet can be grouped into two main categories such as protectant and systemic fungicides (Damicone 2014). Protectant fungicides do not move from the application site and the effectiveness depends on the presence of the fungicide on the tissue surface (Damicone 2014). Therefore, the protectant fungicides generally need to be applied prior to infection (Damicone 2014). Systemic fungicides can move into the plant once they are applied on the plant surface, but the degree of systemic movements in the host depends on the fungicide (Damicone 2014). Therefore, the most effective timing for application can vary.

In Michigan, several types of fungicides have been used for sugar beet such as quinone outside inhibitors (QoI), organo-tin compounds, demethylase inhibitors (DMI), dithiocarbamate, copper-based fungicides and methyl benzimidazole carbamate (MBC) (Table 1.2) (Kirk et al. 2008, Kirk et al. 2012, Rosenzweig et al. 2020). Among them, organo-tin, dithiocarbamate and copper-based fungicide classes are classified as protectant fungicides and the rest are systemic fungicides (Gibson et al. 1966, Georgopoulos and Skylakakis 1986). Although several fungicide applications are recommended and used for Cercospora leaf spot, which is one of the major foliar diseases in sugar beet (Holtschulte 2000, Solel and Wahl 1971), and important in the region (Rosenzweig et al. 2020, Trueman et al. 2017), fewer studies have been done on fungicide

applications for managing ALS. In addition to synthetic fungicides, Fatouh et al. (2011) reported that some citrus essential oil compounds such as Citral, methyl anthranate and Nerol reduced ALS disease severity in sugar beet by *A. tenuis* (now known as *A. alternata*) under field conditions, so other compounds might be effective for ALS management.

Table 1.2: Some of the common fungicides registered for sugar beet in the United States. The codes in each fungicide class according to the Fungicide Resistance Action Committee (FRAC). Codes starting with M are recognized as chemical multisite inhibitors while codes without M generally indicate single-site modes of action.

Group name	Frac code	Chemical group	Common name	Trade name	Mode of Action
Dithiocarbamate	M03	Dithiocarbamate	Mancozeb	Diathane, Manzate	Reaction with protein SH groups
Demethylation inhibitor	03	Triazolinthion	Prothioconazole	Proline	Inhibition of sterol biosynthesis
Quinone outside inhibitor (QoI)	11	Methoxy carbamate	Pyraclostrobin	Headline	Inhibition of respiration
		Oximino-acetate	Trifloxystrobin	Gem, Flint	
Methyl benzimidazole carbamate (MBC)	1	Thiophanate	Thiophanate methyl	Topsin	Inhibition of tubulin formation in mitosis (and the cytoskeleton)
Inorganic	M01	Inorganic	Copper	Badge	Disruption of membrane integrity and energy transport system

Fungicide resistance in A. alternata

Developing fungicide resistance in pathogens is one of the major problems in the areas where fungicides are applied to control diseases (Rosenzweig et al. 2017, Rosenzweig et al. 2019, Steffens et al. 1996, Lucas et al. 2015). A general definition of fungicide resistance is the acquired and heritable ability to grow at a concentration of fungicide at which the baseline isolates cannot grow. (by adapting several definitions e.g. Brent and Hollomon 2007, Massi et al. 2021). Scientists commonly use the term 'field resistance' when the strains are able to grow at field rates or when there is a loss of control to a fungicide or fungicides under field conditions (Staub 1991, Brent and Hollomon 2007). Therefore, resistance does not always result in loss of disease control.

Fungicide resistance can be conferred by various mechanisms. There are four main mechanisms for fungicide resistance which are a) mutation in a fungicide target gene, b) detoxification of the fungicide, c) Increased production of the fungicide target, and d) separation of the fungicide from the target (pump out, block uptake, transfer to vacuole) (Ma and Michailides 2005, Sánchez-Torres 2021). One of the ways of having resistance in fungi is an alteration of the fungicide target. This alteration is achieved by mutations in the gene. These mutations can lead to changes in the amino acid sequences that result in alteration that affects the interaction between fungal target protein and the fungicide which results in a reduction of sensitivity to the fungicide (Ma and Michailides 2005, Gisi et al. 2000, Deising et al. 2008).

For another resistance mechanism, detoxification is primarily accomplished by modifying the metabolic machinery of a fungus which can lead to metabolic degradation of one or more fungicides (Sánchez-Torres 2021). A nontoxic or less toxic form of fungicide may not harm the fungal cell (Ma and Michailides 2005, Sánchez-Torres 2021).

Increased production of the fungicide target is another mechanism of fungicide resistance. In general, there is a competition between fungicides and the primary substrate (Ma and Michailides 2005, Mair et al. 2016). The presence of higher levels of targets reduces the competition between the fungicides and the primary substrate(s) and helps to maintain the fungal cellular processes to some extent which enhances the pathogen survival (Ma and Michailides 2005, Mair et al. 2016).

A fungal body has some natural protection from foreign substances and toxic compounds by operating an efflux system (Del Sorbo et al. 2000, Ayaz et al. 2017, Urban et al. 1999). Unwanted materials can be transported outside of the fungal cell by transporters such as ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporters (Ma and Michailides 2005, McGrath, 2001, Sánchez-Torres 2021). Generally, if these efflux pumps fail to pump fungicide compounds out of the cells it can result in sensitivity to the compound. But some isolates can pump higher levels of fungicide compounds out of the cells and reduce damage through increased efflux pump production or activity (Ma and Michailides 2005, McGrath, 2001, Sánchez-Torres 2021).

With the increasing issues of Alternaria leaf spot in sugar beet fields in the United States, a few studies have been performed on fungicide sensitivity for *Alternaria* strains (primarily found to be *A. alternata* isolates) which were collected from sugar beet fields (Rosenzweig et al. 2017, Rosenzweig et al. 2019). Rosenzweig et al. (2019) isolated *A. alternata* strains from sugar beet leaves growing in east-central Michigan and southwestern Ontario in 2016- 2017 and found a number of *A. alternata* isolates resistant to DMI, QoI and organo-tin fungicides and a temporal shift towards increased prevalence of resistance for the DMI and QoI fungicide classes. Organotin, DMI, and QoI fungicides are three of the fungicide classes that have been labeled for

sugar beet and widely used in sugar beet fields to control foliar diseases such as Cercospora leaf spot (Kirk et al. 2012, Trueman et al. 2017, Weiland and Koch 2004). These resistance levels may have resulted from exposure to the fungicides on beets. In addition, some *A. alternata* have a wide host range (Srivastava 2004, Meena et al. 2013, Troncoso-Rojas and Tiznado-Hernández 2014). Therefore, it can be hypothesized that resistance to some fungicides might have developed when the *A. alternata* were exposed to fungicides on other hosts as well as sugar beets. To test for potential resistance mechanisms in *A. alternata* to QoI fungicides, several studies extracted genomic DNA for the gene that produces the target protein for this fungicide, the cytochrome *b* (*Cytb*). A comparison of DNA sequences among sensitive and resistant *A. alternata* revealed a substitution of glycine to alanine at position 143 of the amino acid sequence (G143A) in QoI resistant isolates (Vega et al. 2012, Rosenzweig et al. 2017, Nottensteiner et al. 2019, Karaoglanidis et al. 2011). This G143A mutation can confer fungicide resistance in many fungi (Ma and Michailides 2005). Although different resistant mechanisms for DMI fungicides in several fungal pathogens were found in previous studies (Albertini and Leroux 2003, Delye et al. 1997, Hamamoto et al. 2000), little or no work has been done in *Alternaria* spp. In a single study, Zhang et al. (2020) found a 6 bp insertion in the promoter region of *Alternaria* strains on potato which were resistant to DMI fungicides but have no data about whether this plays a role in resistance.

Objectives

The main objective of this dissertation is to investigate the diversity of *Alternaria* strains associated with Alternaria leaf spot in sugar beet and rotation crops, especially because of the increased incidence of ALS in the Michigan region. Studying ALS and host-pathogen interactions is important as part of an effort to understand why the disease might have increased and to manage ALS. Therefore, we have developed the objectives below to focus on the interactions between host and pathogen.

The first objective of this study is to develop a consistent and repeatable inoculation method in laboratory, greenhouse and/or in the field for *A. alternata* on sugar beet. *A. alternata* was used as this was the species most commonly found on sugar beet in the region (Rosenzweig et al. 2019, Rosenzweig et al. 2017, Hanson personnel communication). Consistent inoculation method(s) for plants is an important factor when studying host-pathogen interactions. Although studies have been done using greenhouse inoculation (Abbas et al. 2014, McFarlane et al. 1954), the authors did not include enough details to reliably repeat the methods. Developing one or more consistent inoculation methods will be helpful not only to study host pathogen interactions, but also to assess potential management tools.

The second objective of this study is to examine the host specificity/ host range of *Alternaria alternata* strains especially those associated with sugar beet. Host-pathogen interaction for *A. alternata* showed varied results in prior studies. Some studies have reported that *A. alternata* has a wide host range whereas other reports found host specificity of *A. alternata* strains. It is possible that there are generalist and more specific pathogens in the group, and it is important to learn more about how *A. alternata* interacts in the beet cropping system.

Understanding host-pathogen interactions may be useful in the future for assessing the potential for disease management such as crop rotation.

The third project is to evaluate the genetic diversity of *A. alternata*. Although most of the strains can be identified by morphological features, it is important to know how this relates to genotypes in a cropping system. In addition, the genetic diversity assessment would be helpful to better understand the genetic variability of *A. alternata*. It also could be useful to compare to material from before ALS was so severe or between areas where ALS has been an issue or not historically. This will help to understand whether a shift in strains might help to explain the increased ALS issues in Michigan. Understanding the genetic variability among *A. alternata* would be helpful in the future to study additional factors, such as fungicide resistance development in pathogen population, to determine the diversity of organisms that might be needed for host resistance screening, and to develop management strategies that can be effective against whatever range of pathogens are present.

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CHAPTER 2

DEVELOPMENT OF CONSISTENT AND EFFICIENT INOCULATION METHODS FOR ALTERNARIA LEAF SPOT IN SUGAR BEET

Introduction

Sugar cane and sugar beet are two main sources for sucrose extraction in the world (Cooke and Scott 1993, Smigocki et al. 2008). Although the production of sugar from sugar cane is limited to the tropical regions in the world, sugar beet is the primary source for local sucrose extraction in regions of North America, Europe, and North Africa (Cooke and Scott 1993, Draycott 2008,). In the United States, sugar beets are largely grown in the Great Lakes region in Michigan, the Upper Midwest (North Dakota and Minnesota), in the Great Plains (Wyoming, Colorado, Montana, and Nebraska), and the Far West (California, Idaho, Washington, and Oregon) (USDA 2021). Sugar beets are one of the important raw materials for sugar extraction in the United States where 55-60% of sugar was extracted from sugar beet and 40-55% of total sugar was extracted from sugar cane domestically (USDA-National Agricultural Services 2021). Therefore, sugar beets are one of the economically important crops in the United States. The production of sugar beet is affected by a number of diseases such as various foliar, root and seedling diseases (Duffus and Ruppel 1993, Watson et al. 1951). Among these diseases, Alternaria leaf spot (ALS) is one of the foliar diseases on sugar beets (Abbas et al. 2014, Franc 2009, McFarlane et al. 1954, Srivastava 2004). The most commonly reported causative agents of ALS on sugar beets are *Alternaria alternata* [syn. *A. tenuissima* (Woudenberg et al. 2013, Woudenberg et al. 2015)] and *Alternaria brassicae* (Franc 2009, Khan et al. 2020).The classification of *A. alternata* is under debate because of the morphological plasticity and genetic similarity of this organism (Armitage et al. 2015) where some studies have classified this species

into several *Alternaria* spp. such as *A. tenuissima* and *A. mali* (Khan et al. 2020, Sofi et al. 2013), whereas studies carried out with genetic analysis demonstrated these as synonyms of *A. alternata* (Woudenberg et al. 2015, Armitage et al. 2015). In the current work, we follow the Woudenberg et al. (2015) classification.

ALS infection and spread are favored by cool temperature (16˚- 22˚C) and high humidity (~90% relative humidity or higher) (Franc 2009). Higher temperatures can reduce the germination and infectivity of conidia of *Alternaria* species because of cellular degeneration (Abbas et al. 1995) which may be a factor in this temperature range for disease. Initially, ALS is first observed as small, circular to irregular necrotic spots on older leaves of sugar beet (Franc 2009, McFarlane et al. 1954). Subsequently, these small spots become larger (2mm-10mm) and may coalesce to form large necrotic lesions (Figure 2.1A) (Franc 2009, McFarlane et al. 1954). The necrotic spots often are zonate and dark brown with grey centers (McFarlane et al. 1954). Under favorable environmental conditions such as cool weather, the pathogen can sporulate on the necrotic lesions (Figure 2.1B). In addition, ALS is favored when plants are under stress which causes yellowing such as viral yellows by *Beet western yellow virus* (BWYV), *Beet chlorosis virus* (BCV), and *Beet mild yellowing virus* (BMYV), Fusarium yellows (Franc 2009, Russell 1965) or nutrient deficiencies (Ruppel 1986). The infection and the spread of ALS in sugar beet can lead to a reduction of photosynthetic areas on leaves, defoliation and speeding up foliar senescence (Franc 2009). This can result in reduced yield.

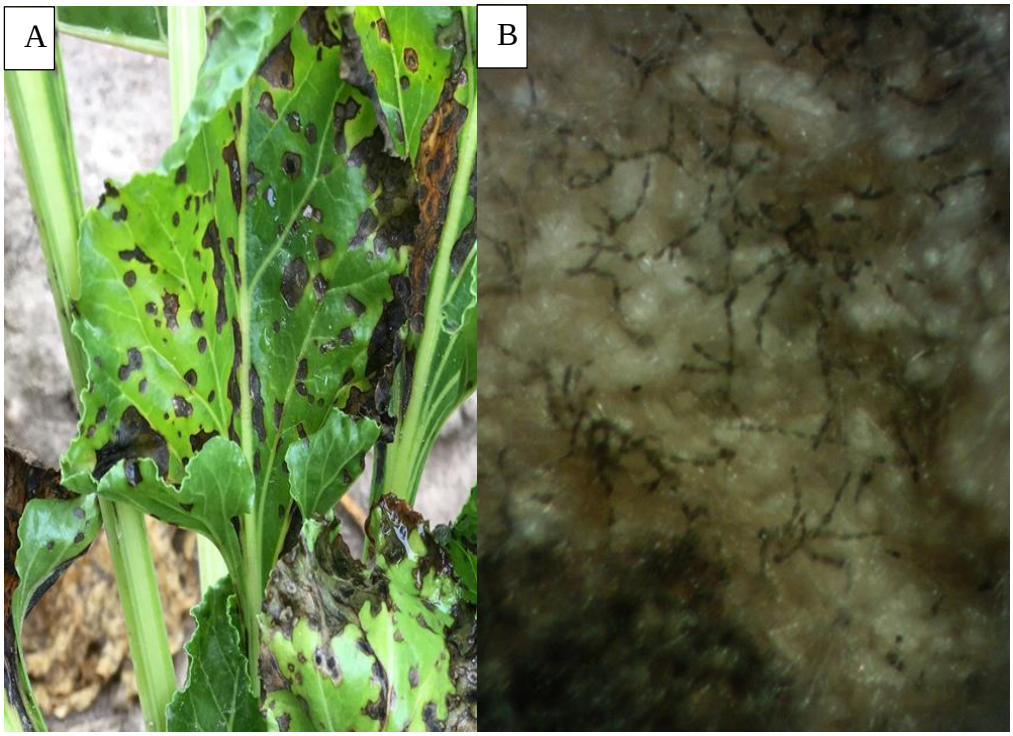

Figure 2.1: Symptoms and signs of Alternaria leaf spot in sugar beets. A-An example of necrotic lesions on sugar beet leaves. Note: the dark lesions (light brown necrotic patch in the background is not primarily Alternaria leaf spot) B-A micrograph showing sporulation with conidial chains of *Alternaria alternata* on a lesion from a sugar beet leaf with 63x magnification.

ALS has been reported everywhere sugar beet is grown (Franc 2009) including Egypt, Pakistan, Slovakia, India, and the United States (Abbas et al. 2014, El-Kholi 1994, Hudec and Rohačik 2002, McFarlane et al. 1954, Misra et al. 2020, Russell 1965). ALS is considered an economically important disease in some countries such as Pakistan and India because of the yield losses it can cause (Abbas et al. 2014, Srivastava 2004). Before 2015, ALS was considered a minor foliar disease in the United States (Franc 2009) and did not cause serious yield losses. Since then, increasing issues of ALS were reported in different states such as Michigan, and Minnesota (Khan et al. 2020, Rosenzweig et al. 2017). The ALS severity on sugar beet increased up to levels of leaf loss which could cause a potential significant yield loss in Michigan in 2015 and subsequent years (Hanson et al. 2017, Rosenzweig et al. 2019).

Since ALS was a minor foliar disease in the United States, less attention was given to this disease than other foliar diseases and limited information is available about ALS on beet (Franc 2009). With the increasing incidence more studies are needed to learn about this host-pathogen system and develop management tools. One such tool would be having a consistent and reliable inoculation method. Such an inoculation method is useful to study the host pathogen interactions (Abbas et al. 2014, McFarlane et al. 1954). In the literature, a limited number of studies have reported inoculation methods used for ALS on sugar beet. They were mostly under greenhouse and growth chamber conditions (Abbas et al. 2014, McFarlane et al. 1954). But the methods are difficult to repeat because of a lack of information. For example, Abbas et al. (2014) performed their inoculation of *Alternaria alternata* on sugar beet plants having 2-3 true leaves under greenhouse conditions. But they did not mention how much inoculum was applied to each plant or what equipment they used to apply the inoculum onto the plants. McFarlane et al. (1954) also used a greenhouse inoculation using *A. brassicae*. But the spore concentration, method of inoculum preparation and greenhouse conditions were not reported. Therefore, it is difficult to follow the methods used in past studies. Although no field inoculation for ALS in sugar beet has been developed yet, some studies have used natural infection in the field to assess sugar beet varieties for response to ALS (McFarlane et al. 1954). Although identification of resistant varieties using natural infection is convenient, it has some drawbacks such as inconsistency (Du Toit 1998, Pataky et al. 1995) and lack of data when the natural infection is low (Hansen et al. 2005, Pataky et al. 1995). Development of a consistent inoculation method is important not only for screening for resistance, but also testing or developing other management, such as fungicide screening trials (Haidukowski et al. 2005, McGregor 1982). Therefore, the current study was

carried out to develop consistent and reliable inoculation methods for ALS in laboratory, greenhouse and/or in field conditions.

Biological materials

To collect isolates, symptomatic tissue samples from four crops, sugar beet leaves, potato leaves, dry bean pods or blueberry fruits were kept in a moist chamber (a double zipper plastic bag with a moist paper towel inside) for two days to enhance fungal sporulation. When sporulation was visible, 15µl of sterile distilled water was dropped on a lesion using a sterile pipette. The sporulating lesion was gently rubbed with a sterile bacterial transfer loop and a loopful of the suspension was streaked on 2% (wt/vol) water agar plates. Plates were incubated overnight at room temperature (approximately 25˚C) with incidental light. Pure cultures of fungal strains were obtained by transferring a single germinated spore from water agar onto ½ strength clarified V8 agar (HCV8) (Miller 1955) using a sterile needle under a dissecting microscope (Olympus-LMS-225R, Leeds Precision Instruments Inc. Minneapolis, MN). Spores were considered germinated when a germ tube was longer than the width of a spore. Single spore transfers were incubated at room temperature with incidental light. Cultures were maintained on HCV8 for up to three months. For long term storage, the sterile glass fiber filter paper method (Hanson and Hill 2004) was used, and papers were stored at -20˚C in sterile magenta boxes with drierite (W. A. Hammond Drierite Company Ltd, Xenia, OH) as a desiccant. Pathogen isolates included two each from foliar lesions on sugar beet (designated B5 and B14), foliar lesions on potato (designated P2 and P23), or fruit lesions on blueberry (designated Bl7 and Bl19) or pod lesions on dry bean (designated Bn2 and Bn7). All strains were used for detached leaf assays, and isolate P23 was selected for greenhouse and field tests based on preliminary virulence

studies (unpublished data). For the inoculations, each *A. alternata* strain was grown on HCV8 agar and incubated at room temperature for 10 days in the dark.

Twenty different sugar beet germplasm of unknown Alternaria susceptibility were used for the field inoculation of ALS in the 2018 field season. In 2019, two sugar beet varieties (C059 and HM9616) were used for field inoculation to simplify the analysis. In 2020 and 2021, two different sugar beet germplasm (C869 and HM9879) were used for the inoculation because the two used in 2019 were not commercially available. Both HM9879 and HM9616 were reported as putative moderately resistant germplasm for ALS under natural infection in Michigan (REACh 2017, 2019). C869 was selected because it was well genetically characterized, a public germplasm release (McGrath et al. 2013) and was reported as susceptible for a number of other foliar diseases. In addition, C869 was from the breeding stocks at Salinas, California where a history of susceptible material for *Alternaria* spp. was found (McFarlane et al. 1954). The sugar beet commercial varieties, C059 and HM9616 were used for greenhouse inoculations and a detached leaf assay in the laboratory. Those two varieties were selected for greenhouse inoculations based on their susceptibility to ALS as predicted by response in naturally infected field plots. The sugar beet variety, C059 was reported as susceptible to ALS whereas HM9616 was a putative resistant variety to ALS (REACh 2019, 2017).

Methods

Studies on sporulation conditions for Alternaria alternata

To identify media and conditions for rapid sporulation in *A. alternata*, preliminary studies were carried out by growing *A. alternata* in three media with different lighting conditions. Initially, *A. alternata* was grown on HCV8 and incubated at room temperature for 5 days. Fungal plugs (5mm) were taken using a sterile cork-borer (No.3) and single plugs were placed on the

middle of oatmeal agar (OMA) (Borjesson et al. 1993), HCV8 or corn meal agar (CMA) (Sigma Aldrich, St. Louis, MO). Twelve replicate plates of each medium were used for this experiment. Half of the plates were placed in the dark, and half maintained in ambient light on the bench at room temperature for 10 days. Any conidia that formed on each plate were collected by flooding with 10 ml of sterile distilled water and scraping the surface using a sterile bacterial transfer loop. The concentration of conidia from each plate was estimated using a hemocytometer. The conidia concentration of each treatment was compared with an analysis of variance (ANOVA) using SAS (version 9.4) software. Pairwise comparisons were performed with the Fisher's protected least significant difference tests (LSD, α=0.05).

Detached leaf assay

The seeds of the two varieties, C059 and HM9616 were planted in 2.45 L plastic nursery pots (Poly-tainer NS300, Nursery Supplies, Inc., Chambersburg, PA) filled with commercial potting mix (SureMix Perlite; Michigan Grower Products. Inc., Galesburg, MI) in a greenhouse at the Michigan State University, East Lansing, MI. The temperature of the greenhouse ranged from 20˚-30˚C with 12 hours light supplemented with high pressure sodium bulbs from 8 am-8 pm. When the seedlings were at the two-leaf growth stage, they were transplanted to single pots with the same potting mix. Plants were fertilized with 14-14-14 fertilizer (Osmocote, Everiss, Geldermalsen, the Netherlands) when they were at the 4 to 6 leaf growth stage. Watering was done approximately every other day. Predatory mites (*Tyrophagus putrescentiae*, Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands) were used to manage thrips. When the plants were at the 8-10 leaf growth stage, leaves from the outer most whorl were cut with a scissor (including leaf blade and 2-3 cm long portion of petiole) and used for testing. The leaves were disinfected by immersing in a 0.6% sodium hypochlorite with 0.1% Tween 20 (vol/vol)

(Sigma-Aldrich) solution in water for 10 minutes and washing with sterile distilled water three times. The leaves were air dried in a biosafety cabinet (SterilGARD Class II Type A/B, the Baker Co., Sanford, ME) for thirty minutes and placed in moist chambers, which were aluminum pans (50.8 cm length, 31.7 cm width and 7.6 cm height) with lids and lined with sterile paper towels (6 towels per pan) that had been moistened (visibly wet but not dripping) with sterile distilled water. Leaves were wounded on one side of the midrib by stabbing with a sterile dissecting needle and the other side left unwounded.

The inoculum was prepared by placing 2 ml of sterile distilled water onto 10 days old *A. alternata* cultures in HCV8 plates and scraping the surface with a sterile loop. The final spore concentration of each isolate was adjusted to 1 X 10^5 spores/ml as estimated by a hemocytometer. Twenty microliters of inoculum of each *A. alternata* isolate were placed onto the wounded and nonwounded sites of each beet leaf with 5 replicates for each variety. Controls used 20 µl of sterile distilled water with and without wounding. Inoculated and control leaves were incubated in the moist chambers for lesion development. The lesion diameters, if any, were measured starting two days after inoculation and continued daily up to 5 days using a digital caliper (VWR International LLC Radnor Corporate center, Radnor, PA). The area under the disease progress curve (AUDPC) values (Meena et al. 2011) for each isolate on each variety were calculated using R studio (version 1.1.456). SAS (version 9.4) was used for statistical analysis. ANOVA tables were constructed to determine main effects (variety and isolate) and interactions (variety x isolate) with the random variable as replicates. Pairwise comparisons were performed with the Fisher's protected least significant difference test (LSD, α=0.05).

Greenhouse inoculation

The commercial sugar beet varieties, C059 and HM9616 were grown in the greenhouse with the conditions described in the detached leaf section. Plants at the 8-10 leaf growth stage were used for the greenhouse inoculation.

Wounding of half the plants was done by gently rubbing the leaves with fine sand (MAC's PACS, San Pedro, CA). Four replicate plants from each variety were used for each treatment. Inoculum of *A. alternata* strain P23 on HCV8 agar, was prepared as described in the section of detached leaf preparation. Six different treatments were used for this experiment, and it was repeated three times. The final spore concentration of the fungus was adjusted to approximately 1 x 10^4 spores/ml with a hemocytometer. Spores were suspended in sterile distilled water with 0.1% tween 20 (vol/vol) or a 0.2% malt extract solution, previously shown to enhance *Alternaria* infections in tomato (Stammler et al. 2014). The malt extract solution was prepared using Difco^{TM} malt extract broth (Becton, Dickinson and Company, Sparks, MD, USA) for a final concentration of 0.2% malt extract. The controls were sterile distilled water with 0.1% tween 20 and a sterile 0.2% malt extract solution. Plants were spray inoculated using a handheld sprayer (Lansing Sanitary Supply, Inc., Lansing MI) at the rate of 20 ml per plant onto wounded and nonwounded sugar beet plants of each variety. Once all the treatments were applied to the two varieties, the plants were placed in a humidity chamber (Bublitz 2019). The humidity chamber was in a temperature control room with a constant temperature of 20°C. Two humidifiers (Hunter, Marietta, GA) were placed inside the humidity chamber to maintain a high humidity level (>95% RH). Three days after inoculation, the plants were transferred to a regular greenhouse chamber and maintained as described for initial growth conditions. The plants were rated seven days after inoculation, using a 0-8 rating scale based on the number of leaves with

lesions (0= no spots on leaves, 1= spots on 1 leaf or one spot on up to 3 leaves, 2= multiple spots on 2 leaves, 3= multiple spots on 3 leaves, 4= multiple spots on 4 leaves, 5= multiple spots on 5 leaves, 6= multiple spots on 6 leaves, 7= multiple spots on 7 leaves and 8= multiple spots on 8 or more leaves). The statistical analysis was done using SAS (version 9.4) software. ANOVA tables were constructed to determine the homogeneity between trials, the main effects (variety and treatments), and interactions (variety x treatment) with replicates as the random effect with SAS software (version 9.4). Mean separation was obtained with Fisher's protected least significant difference tests (LSD, α=0.05) using the proc mixed procedure.

Field inoculation

Field inoculation testing for Alternaria leaf spot was started in 2018 and continued every year with modifications at the Saginaw Valley Research and Extension Center (SVREC), Frankenmuth, MI. Because of high disease pressure from Cercospora leaf spot at SVREC, in 2021 testing also was conducted at the Plant Pathology Research Farm at Michigan State University which is several miles from the major beet production region. In the field, the sugar beet seeds were planted within 20" between rows and 15ft long plots with a single row per plot. The seed planting, inoculation and rating dates and the chemical applications are given in Table 2.1. Any irrigation of the field was done as necessary for seed emergence, to maintain plant health or following inoculation for disease development.

In 2018, 20 sugar beet germplasm were grown in the field as a paired comparison for treatment (inoculated) and control (non-inoculated) and surrounded by beets with standard fungicide applications for foliar disease management. The field inoculation was carried out on July 3rd when plants were at the 8 to 10 leaf growth stage. The fungal spore suspension for inoculation was done as described in the greenhouse and detached leaf assays. The spores were

suspended in a 0.2% malt extract adjusting the final concentration of the spores to approximately 1 x 10^3 spores/ml (determined with a hemocytometer). Inoculation was done using a backpack sprayer (15L, model 63924, Chapin, wide mouth 24V rechargeable backpack sprayer, Chapin International Inc., Batavia, NY) at a rate of 20 ml/plant. The control section was sprayed with 0.2% malt extract solution. Disease rating was started 1 week after inoculation and was continued biweekly up to 6 weeks till Cercospora leaf spot (CLS) started to affect rating ALS. At the peak and later stages of CLS, several beet leaves were completely dead, and this impacts ALS rating (personal observation). A 0-10 rating scale was used where 0 – no spots, 1: 1-2 spots throughout the plot, 2: few spots on ≤ 3 plants, 3: spots on ≤ 5 plants, 4: spots on 6-10 plants, 5: spots on >10 plants, 6: spots enlarging on at least 10 plants, 7: coalescing spots, 8: 1-2 dead leaves; 9: >2 dead leaves, 10: total defoliation. The disease scores from each week were used to calculate area under the disease progress curve (AUDPC) (Meena et al. 2011). R studio (version 1.1.456) was used to calculate the AUDPC. Calculated AUDPC values on treated and control plots were used for comparisons. The same method of calculating AUDPC was used in the following years for field data. A paired comparison was carried out between treated and control using Proc t-test with SAS (version 9.4) for the statistical analysis.

Table 2.1: The sugar beet seed planting, herbicide application, inoculation, and disease rating dates in the field from 2018-2021.

Microrates: Herbicides are applied lower doses than it is on labels (Dexter and Luecke 1999). Microrates consisted of Betamix (8 oz/A), Upbeet (1/8 oz/A) and Stinger (1oz/A).

Year	Seed planting	Herbicide application	Inoculation	Rating
2018 (SVREC)	5/8/18	5/16: Microrates 5/23: Microrates 5/30: Microrates 6/5: Microrates 6/7: Dual II Magnum metolachlor- 1pt/A	7/3/18	7/10, 7/17, 7/31, 8/14
2019 (SVREC)	5/19/19	5/10: Glystar Plus- glyphosate-1qt/A 5/11: Nortron SC- 2qt/A 5/21: Microrates 5/31: Microrates 6/9: Microrates 6/17: Microrates 6/19: Dual II Magnum metolachlor-1pt/A	6/7/19	6/28, 7/12, 7/26
2020 (SVREC)	5/22/20	5/26: Quadris- 32oz/A 5/26: Nortron SC- 2qt/A 6/1: Microrates 6/8: Microrates 6/15: Microrates 6/22: Microrates 6/24: Medal II – S-metolachlor- 1pt/A	9/15/20	9/22, 9/29, 10/6, 10/13, 10/20
2021 (SVREC)	5/6/21	5/13: Nortron SC- 2qt/A 5/25: Microrates 6/1: Microrates 6/7: Microrates	9/9/21	9/23, 9/30, 10/7, 10/14

Table 2.1 (cont'd)

Year	Seed planting	Herbicide application	Inoculation	Rating
		6/15: Microrates 6/24: Warrant – 3pt/A		
2021 (East Lansing)	5/14/21	5/17: Nortron SC (Ethofumesate)-3pt/A 6/16: Upbeet (Triflusulfuron methyl)-0.5oz/A 6/16: Betamix (Phenmedipham and desmedipham)- 3pt/A	9/15/21	9/22, 9/29, 10/6, 10/13, 10/20

Based on the results obtained in 2018, an earlier inoculation was performed in 2019 (early June) to attempt to avoid high temperatures and high pressure from Cercospora leaf spot, which can make rating ALS difficult (personal observation). The two sugar beet varieties, C059 (susceptible) and HM9616 (moderately resistant), were grown in the greenhouse at Michigan State University with the same greenhouse conditions as described for the section on the detached leaf assay. When the plants were at the 2-4 leaf growth stage, they were transplanted into 13 L boxes (Bublitz 2019). Each box contained 5 plants. Once the plants had more than 8 leaves, they were transplanted to field plots (on May 13th). The two varieties were planted in the field in the same order in two sections (control and treated). Each plot had 12 sugar beet plants and each row contained sugar beet plants of the same variety. Each variety was repeated four times for each treatment and control. Inoculation was done for the transplants on June 7th. The inoculation and rating scale were the same as for 2018. Rating was started three weeks after inoculation and continued biweekly up to 7 weeks after inoculation at which time CLS started killing leaves. The statistical comparison between treated and control were the same as 2018 field inoculation.

In 2020, beet varieties C869 and HM9879 were grown in the field plots. The experiment was conducted at two different locations at SVREC where one section was surrounded by beets that were not treated with fungicides for CLS and the other surrounded with fungicide treated beets using Michigan Sugar standard spray recommendation for CLS management (designated low CLS). Each section was set up as a randomized design. Inoculation was done on September 15th to aim to avoid the peak of Cercospora leaf spot in the field and to provide favorable environmental conditions for ALS development such as cool temperature (Franc 2009). The experiment included five treatments (for each section) where the pathogen spores were

suspended in 0.2% or 0.5% malt extract solution with the controls which were water, 0.2% or 0.5% sterile malt extract solution. Four replicates were used for each variety and the treatments were applied as in 2018 and 2019 field inoculations. Disease rating was started a week after the treatments were applied and ratings were done weekly for five weeks till the temperature decreased to a level that was unfavorable for ALS development. The same disease rating scale was used as in 2018 and 2019. An ANOVA table was constructed to determine the main effect (variety and treatment) and the interaction (variety x treatment) with the random effect as replicates with the mean separation by Fisher's protected least significant difference tests (LSD; α=0.05) was completed using the proc mixed procedure.

Based on the results obtained in 2020, the experiment was repeated at two locations in 2021. The same experimental design as in 2020 was used at SVREC. The same two varieties of sugar beet were grown at the Plant Pathology Research Farm at East Lansing, MI. This is an area outside the main sugar beet production region and with lower Cercospora leaf spot pressure (unpublished data). The experiment was set up as a paired comparison with the two varieties in separate plots. This was because the adjacent field was being treated with Roundup, so the Roundup ready sugar beet variety, HM9879 was planted adjacent to that field, with the non-Roundup ready germplasm more distant to reduce the risk from herbicide drift. Each plot had 6 rows, and the middle 4 rows in each plot were used for the experiment. Each row was divided into 14 subplots. Each subplot had 10-15 sugar beet plants. Inoculation was done on September 15th to aim to avoid high temperatures. Treatments were water control, inoculum ($1x10^4$ spores/ml) in water, 0.2% malt extract control, and inoculum in 0.2% malt extract solution respectively. The same spraying method and rating scale were used as in 2020. The statistical analysis was done with a paired t test with proc ttest in SAS (version 9.4) to test between

varieties. ANOVA tables were constructed to compare the treatments on each variety using proc mixed procedure to test among treatments in each variety. The rating was started two weeks after inoculation and continued weekly for 5 weeks until cold temperatures hit the fields.

Results

Data on sporulation of Alternaria alternata

Varying sporulation was observed with three media and under dark or ambient light conditions. The highest sporulation of *A. alternata* was obtained on HCV8 agar incubated in continuous dark ($p<0.0001$) (Table 2.2). The second highest sporulation was on OMA incubated in continuous dark. *A. alternata* grown on CMA either in ambient light or the dark or on HCV8 or OMA in ambient light had significantly lower sporulation with no significant differences among these growth conditions (Table 2.2).

Table 2.2: The conidia concentration of *Alternaria alternata* in three different media with ambient light or continuous dark incubation. Values shown are the average spore concentrations for six replicates. Average conidia concentrations followed by the same letter were not significantly different by Fisher's protected LSD at 0.05.

Growing medium	Light/dark incubation	Average Conidia concentration (spores/ml)
½ strength clarified V8 agar	Light	9.5×10^3 A
½ strength clarified V8 agar	Dark	2.7×10^5 C
Oatmeal agar	Light	7.5×10^3 A
Oatmeal agar	Dark	1.3×10^5 B
Corn meal agar	Light	2.4×10^3 A
Corn meal agar	Dark	4.5×10^4 A

Detached leaf assay

All the eight isolates caused necrotic lesions on both sugar beet varieties. No lesions were observed on the water control (Figure 2.2). In the initial tests, no significant difference was observed between the AUDPC values of wounded and nonwounded leaf halves ($p>0.05$). Therefore, AUDPC values on wounded sites were used for the statistical comparison. The

homogeneity test indicated the three trials were non-homogeneous. Therefore, the three trials were compared separately. The statistical analysis indicated a significant interaction among pathogen and variety ($P<0.05$). The AUDPC values for variety C059 were significantly higher than HM9616 in 2 out of 3 trials ($P<0.05$) (Figure 2.3). More details of the detached leaf assay will be given in chapter 3.

Figure 2.2: Lesion development by *Alternaria alternata* isolates on sugar beet leaves (5 days after inoculation) in a detached leaf assay. Leaves are from variety C059 (left) and HM9616 (right). The left side of each leaf was not wounded and the right side was wounded. All the isolates caused necrotic lesions on host tissue and the controls indicated by an arrow were without any visible lesions.

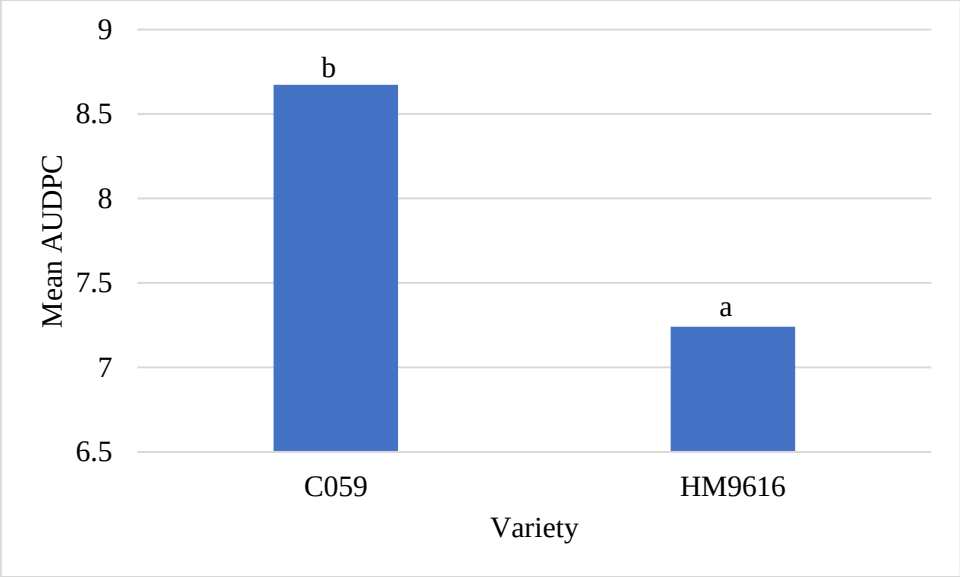

Figure 2.3: An example from one of the three tests showing average area under the disease progress curve (AUDPC) values for sugar beet varieties C059 and HM9616 in a detached leaf assay. Each bar is the average of five replicates of a total of eight *Alternaria alternata* isolates. Bars with the same letter were not significantly different by Fisher's protected LSD at 0.05.

Greenhouse inoculation

In the greenhouse, dark colored lesions were observed 5 or 6 days after inoculation on both varieties, C059 and HM9616, with any treatment containing *A. alternata* spores. The lesions were observed on both yellowing leaves and on green leaves (Figure 2.4 A, B). No lesions were observed on the two controls (sterile distilled water or 0.2% malt extract) (Figure 2.4D). The statistical analysis of the three trials indicated that all were homogeneous ($p = 0.9681$). Therefore, the three trials were analyzed together. The statistical comparison among the main factors (treatment and variety) indicated a significant interaction between treatment and variety ($P<0.0001$).

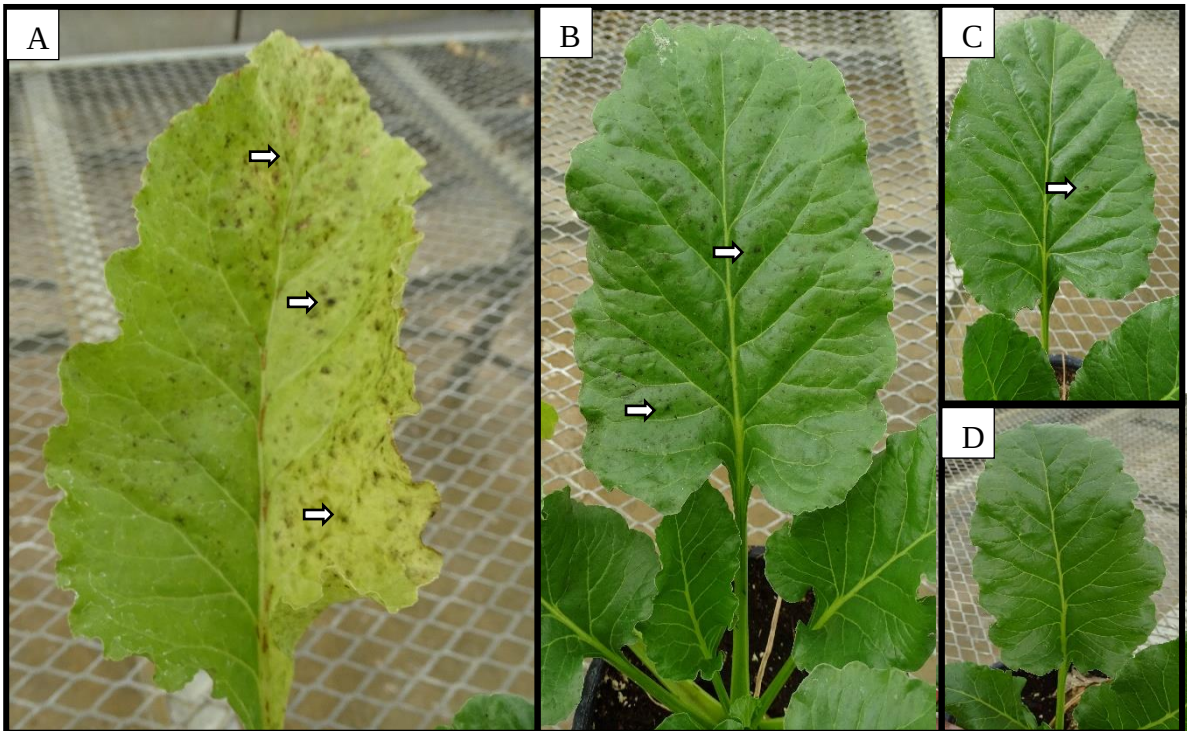

Figure 2.4: Examples of Alternaria leaf spot lesion development in sugar beet leaves 7 days after inoculation in the greenhouse. The sugar beet variety was C059. All the leaves are nonwounded. A, B: lesion development in both yellowing (A) and green (B) leaves with inoculum in 0.2% malt extract, C: inoculum in sterile distilled water, and D: 0.2% malt extract control with no visible lesions. Arrows indicate examples of lesions on leaves.

There were no significant differences between the non-inoculated controls (water and malt extract) or inoculum in water (with or without wounding) on either variety (Figure 2.5). But both treatments of spores in malt extract had significantly higher disease scores for both varieties, with or without wounding (Figure 2.5). The comparison of C059 in treatment 1 and 2 indicated that the disease score was higher in nonwounded than wounded plants (Figure 2.5). No significant difference was found between wounding or not for the inoculum in malt extract for HM9616 (P=0.085) (Figure 2.5). When a correlation analysis was performed between disease score obtained in greenhouse inoculations and AUDPC detached leaf assays, a positive correlation (p=0.0147 and r=0.8103) was observed for detached leaf assay in trial1, no correlation was observed for the second detached leaf assay, and a negative correlation (p=0.0088 and r= -0.841) was observed for the third trial.

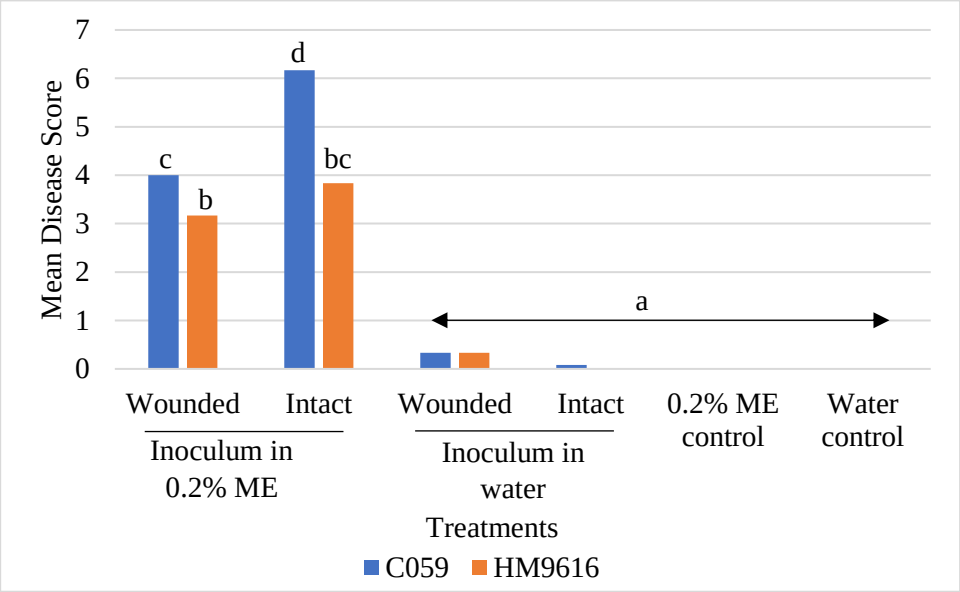

Figure 2.5: A histogram showing the mean score (0-8; where 0 is no spots on leaves) between susceptible (C059) and putative resistant (HM9616) sugar beet plants treated with *Alternaria alternata* spores. ME indicates 0.2% malt extract solution. "Intact"indicates non-wounded. Values are the average of four plants in each treatment. Bars with the same letter were not significantly different by Fisher's protected LSD at alpha=0.05.

Field inoculation

In 2018, the mean AUDPC on treated plots with *A. alternata* spores in 0.2% malt extract were significantly higher than control (malt extract alone) plots (p=0.0063) over the 6 weeks rating period (Figure 2.6). The mean AUDPC values were low in the field throughout the rating period.

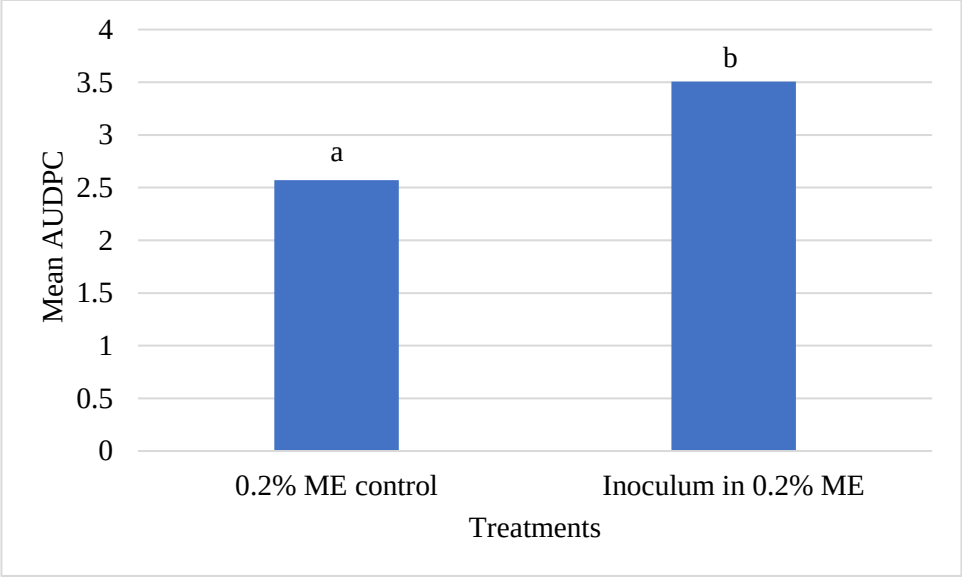

Figure 2.6: A histogram showing the mean area under the disease progress curve (AUDPC) values for field grown sugar beets in 2018. Inoculum was prepared by suspending *Alternaria alternata* spores in 0.2% malt extract solution (ME). The weekly disease scores over 6 weeks were used to calculate the AUDPC values. Beets were inoculated on July 3rd. Bars with different letters were significantly different by Fisher's protected LSD at alpha=0.05.

In 2019, the transplants, which were inoculated on June 7th, showed a significant difference for AUDPC between treatments (Figure 2.7). The mean AUDPC from the plots treated with 0.2% malt extract showed a significantly higher disease score than the plots treated with *A. alternata* spores in 0.2% malt extract solution (Figure 2.7). The mean AUDPC in both treated and controls were higher in 2019 than 2018 (Figures 2.6 and 2.7).

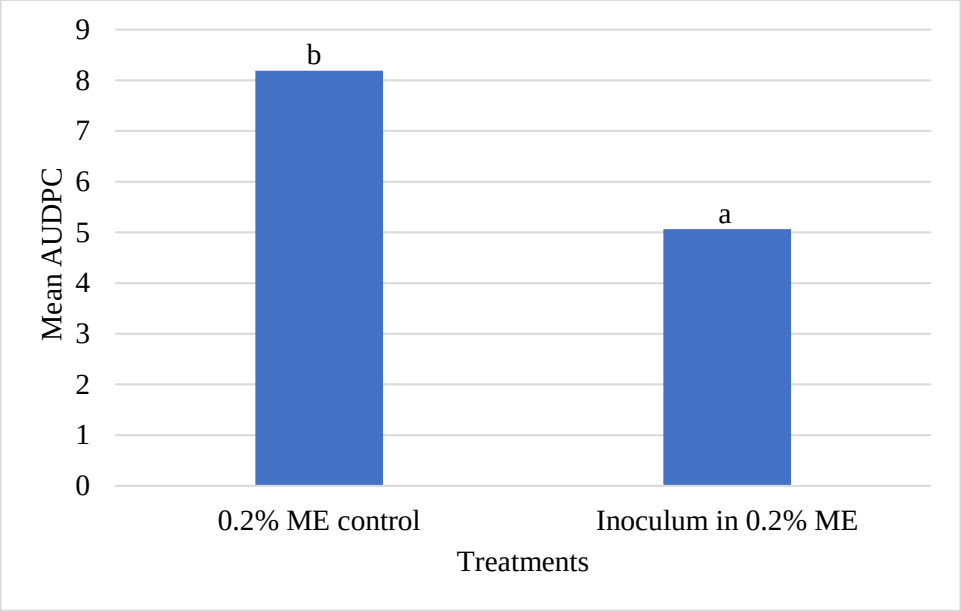

Figure 2.7: A histogram showing the mean area under the disease progress curve (AUDPC) values for transplanted sugar beets in the field in 2019. Inoculum was prepared by suspending *Alternaria alternata* spores in 0.2% malt extract solution (ME). The weekly disease scores over 5 weeks were used to calculate the AUDPC values. Beets were inoculated on June 7th. Bars with different letters were significantly different by Fisher's protected LSD at alpha=0.05.

The statistical comparison among the treatments in the field in 2020, indicated a significant interaction between variety and treatment ($p<0.0001$) in area which was surrounded by beets where Cercospora leaf spot was controlled with fungicides. Comparison among the interactions indicated that the AUDPC values for HM9879 were significantly higher than C869 (Figure 2.8A). Although no significant difference was observed between the treatments and controls in C869, plots with HM9879 inoculated with pathogen spores in 0.5% malt extract had a higher AUDPC than the other treatments including the water and 0.5% malt extract controls (Figure 2.8A). The area surrounded by beets not treated with fungicides indicated a significant difference between the varieties only ($p<0.0001$) where the AUDPC on HM9879 was significantly higher than on C869 (Figure 2.8B).

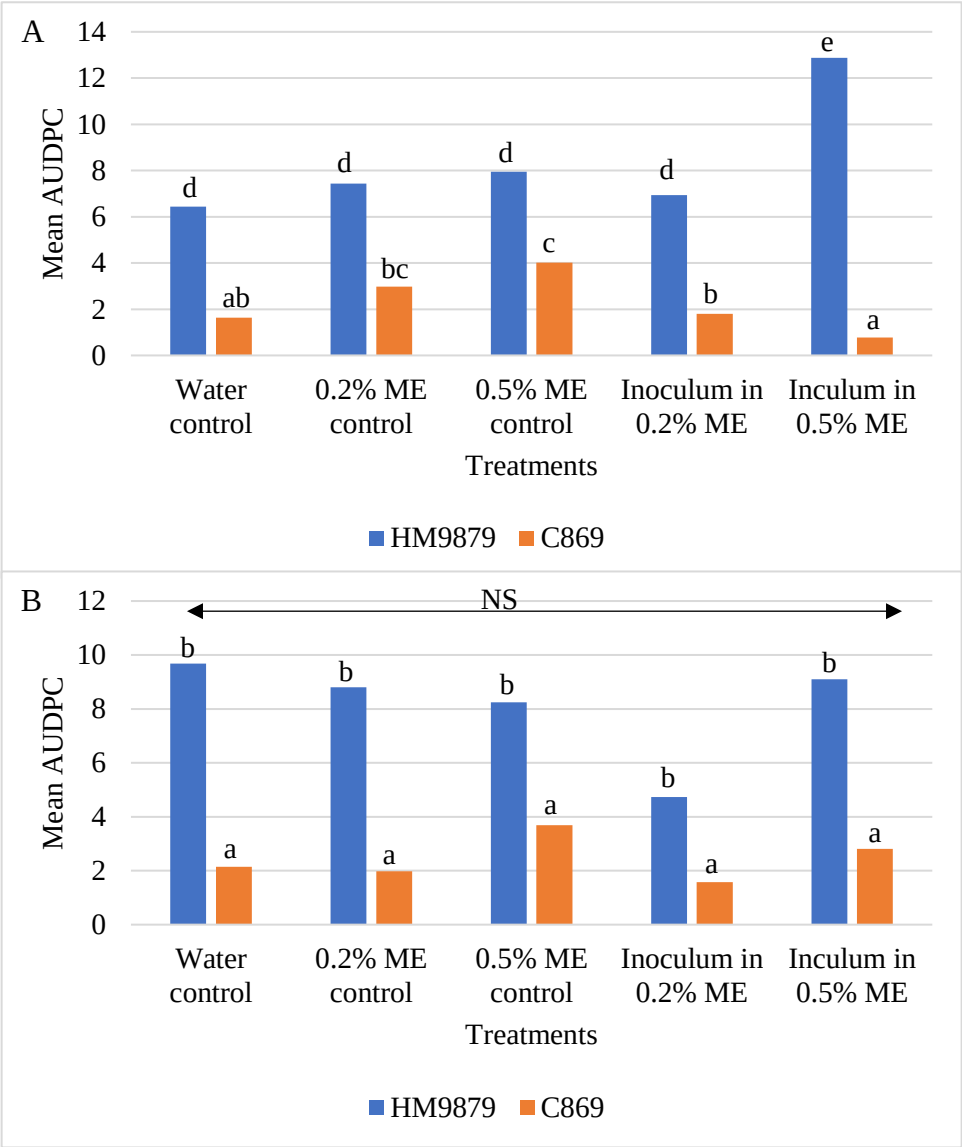

Figure 2.8: Histograms showing the mean AUDPC values of Alternaria leaf spot (ALS) in two sugar beet varieties (C869 and HM9879) in A: Cercospora leaf spot managed with fungicides and B: high Cercospora leaf spot pressure in 2020. Inoculum was prepared by suspending *Alternaria alternata* spores in malt extract solution (ME). Shown are the average AUDPC of four plots at 5 weeks after treatments were applied in both sections. The lowercase letters above each bar indicate significant difference among treatments in each variety in A and between varieties in B. Bars with the same letters were not significantly different by Fisher's protected LSD at alpha = 0.05. NS: no significant difference among treatments at alpha=0.05.

The statistical comparison among the varieties in the field at SVREC in 2021 indicated a significant interaction between variety and treatments in the area surrounded by beets where Cercospora leaf spot had been managed by fungicide applications (Figure 2.9A). Comparison indicated that HM9879 treated with pathogen spores in 0.5% malt extract had significantly higher mean AUDPC value than all the other treatments ($p<0.05$). HM9879 with water control had the next higher AUDPC and all the other treatments in both varieties were not significantly different from each other ($p>0.05$). The area surrounded by beets with no fungicide applications to control Cercospora leaf spot had a significant difference between varieties where the AUDPC values on HM9879 was significantly higher than C869 (Figure 2.9B), but no significant difference was observed among the treatments (Figure 2.9B).

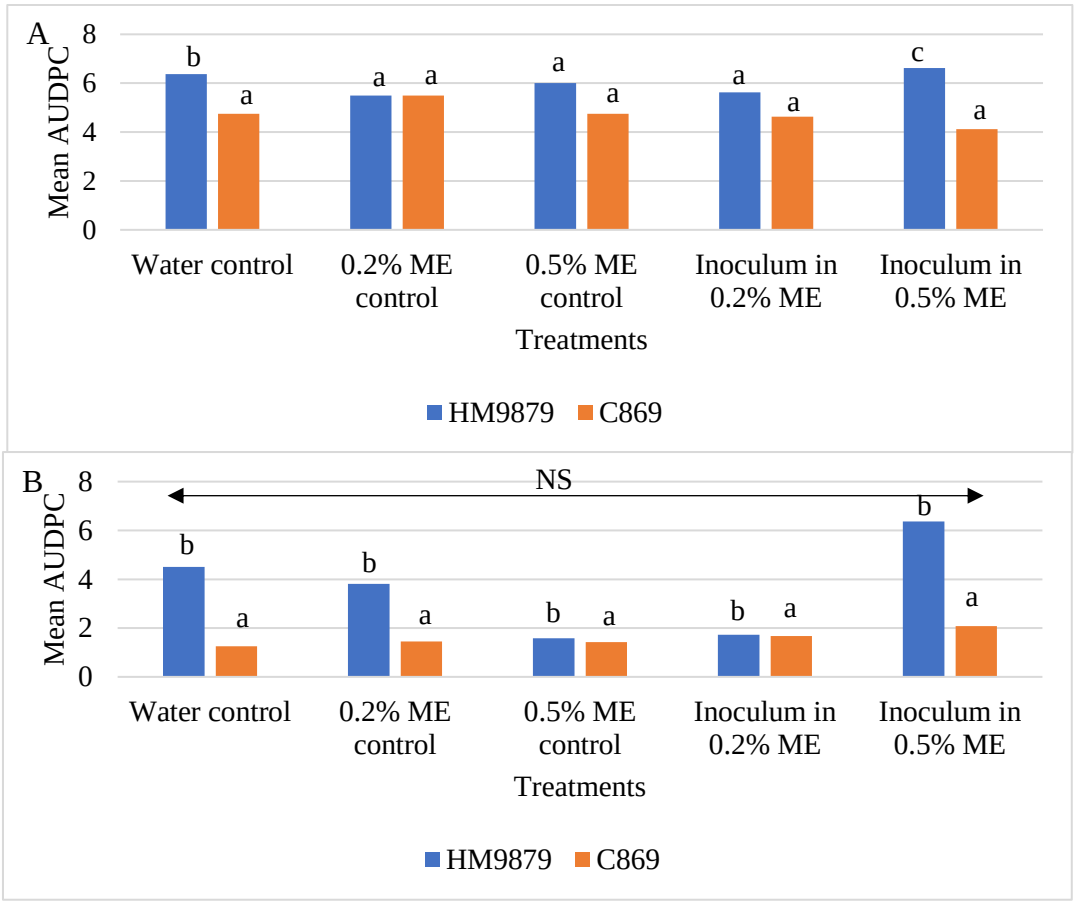

Figure 2.9: Histograms showing the mean AUDPC values of Alternaria leaf spot (ALS) in two sugar beet varieties (C869 and HM9879) in A: Cercospora leaf spot managed with fungicides and B: no fungicide applications to manage Cercospora leaf spot in 2021 at Saginaw Valley Research and Extension Center. Inoculum was prepared by suspending the spores of *Alternaria alternata* in malt extract solution (ME). Shown are the average AUDPC of four plots at 5 weeks after treatments were applied. The lowercase letters above each bar indicate significant difference among treatments for each variety in A and differences between the varieties in B. Bars with same letters were not significantly different by Fisher's protected LSD at alpha = 0.05. NS: no significant difference among treatments at alpha=0.05.

The statistical comparison between the two plots with HM9879 and C869 at the Plant Pathology Research Farm indicated a significant difference between the varieties where the AUDPC for C869 was significantly higher ($p<0.0012$) than HM9879 (Figure 2.10A). The comparison among the treatments showed a significant difference in HM9879 whereas no significant difference was observed in C869 (Figures 2.10B and 2.10C). In HM9879, a

significantly higher ($p<0.05$) diseases score was obtained when the plants were inoculated with pathogen in 0.2% malt extract than the two controls (Figure 2.10B).

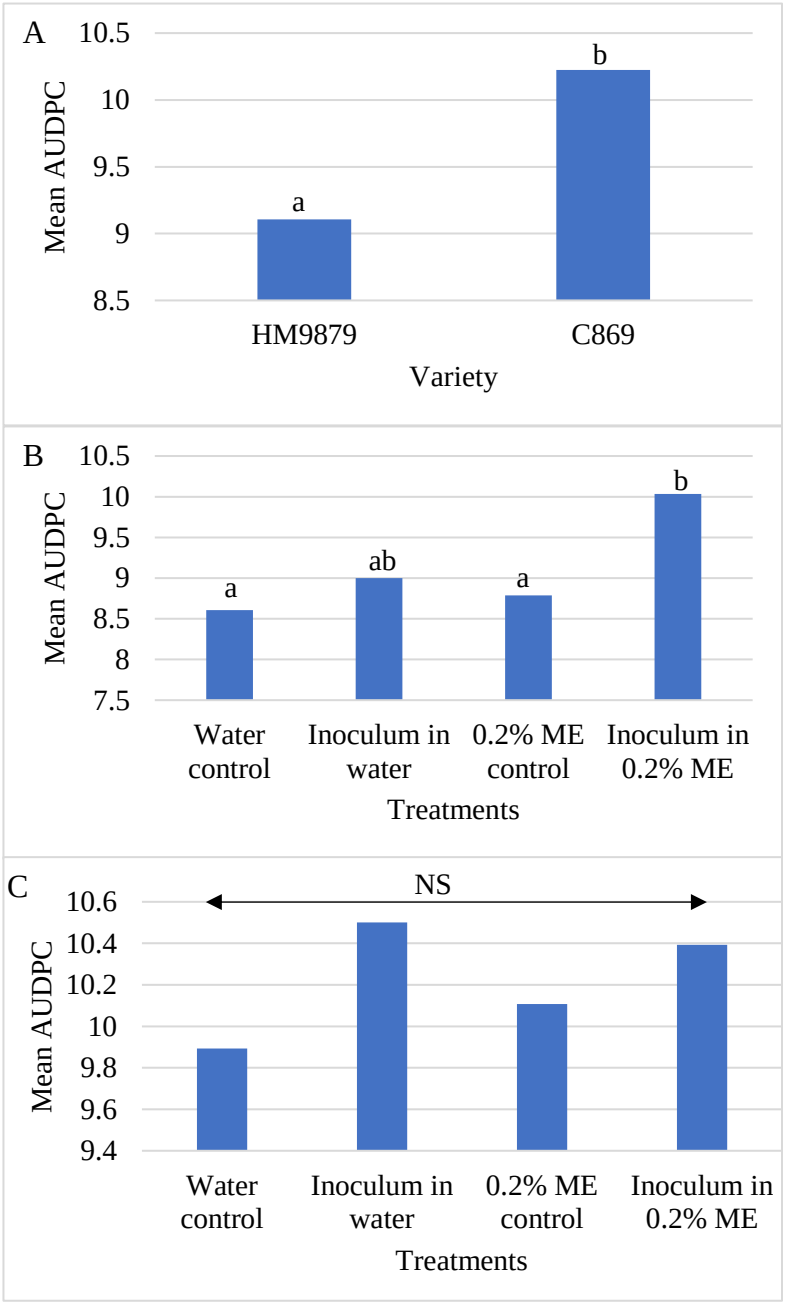

Figure 2.10: Histograms showing the mean AUDPC values for Alternaria leaf spot (ALS) in two sugar beet varieties (C869 and HM 9879) at the Plant Pathology Research Farm in 2021. A: The mean AUDPC of two varieties, B: Mean AUDPC on HM9879 with four treatments, C: Mean AUDPC on C869 with four treatments. Inoculum was prepared by suspending *Alternaria alternata* spores in water or 0.2% malt extract solution (ME).The lower case letters above each bar indicate significant difference among varieties in A and treatments in B. Bars with the same letters were not significantly different by Fisher's protected LSD at alpha= 0.05 in A and 0.1 in B and C. NS: no significant differences among treatments.

Discussion

In all inoculation methods, disease severity was higher with inoculation than in the non-inoculated controls in 1 or more tests. This indicates that there is a potential to use such inoculation methods to examine host-pathogen interactions for *Alternaria alternata* on sugar beet.

In the detached leaf assay, a significant difference between the two varieties (putative susceptible C059 and putative resistant HM9616) was observed in two out of three trials. The AUDPC in C059 was significantly higher than HM9616 (Figure 2.3). This is consistent with reports that C059 and HM9616 were susceptible and moderately resistant to ALS under natural infection (REACh 2019, 2017). These results indicate that there is a potential to obtain similar results with a detached leaf assay and natural infection in the field. When we compared the results for the detached leaf inoculation and field inoculation, no statistically significant correlation was observed ($p>0.05$). This likely is due, at least in part, to the differences in the germplasm in the tests. Further testing is needed to compare methods using the same germplasm. Previous studies have shown positive correlations between detached leaf tissue studies and whole plant responses in the field for other diseases. For example, Goth and Kaene (1997) found a positive correlation between a detached leaf assay and intact plant inoculation for *Phytophthora infestans* causing late blight in tomato and potato. Several other studies have used detached leaf assays as an effective method to screen varieties of different crops for resistance (Bouhassan and Tivoli 2004, Doullah et al. 2006), including for diseases caused by *Alternaria* species (Lohith et al. 2011). Thus, a detached leaf assay could be a relatively easy and fast laboratory method that can be used to screen sugar beet varieties for response to ALS. Results, however, need to be confirmed with additional varieties.

Eight different *A. alternata* isolates originally collected from four hosts were used in the detached leaf assay experiment (chapter 3). Using different pathogen isolates instead of a single pathogen strain may cause confounding effects to the overall experiment, but it is important to examine whether a response is consistent or isolate specific. The same isolates which were used in this experiment were also used to investigate the host range of *A. alternata* which is described in chapter three. As both the host range and genetic diversity analysis of these *A. alternata* isolates (in chapters 3 and 4) do not indicate any clear separation among these *A. alternata* isolates for their effect on sugar beet results for these can provide evidence that this method can be robust for diverse isolates. The use of several isolates with some genetic variability helps to demonstrate consistency of the inoculation method.

Considering AUDPC values instead of lesion diameters gives advantages such as taking all the factors for disease development into consideration (Royle 1994). AUDPC was used for screening for resistance among different hosts such as wheat, and tomato in the past (Pandey et al. 2003, Jeger and Viljanen-Rollinson 2001). Therefore, the AUDPC was chosen to use for analysis in all ratings where multiple observations were taken (detached leaf assay and field tests). Since plants in the greenhouse were not maintained under high humidity conditions for ALS epidemic development, only a single disease severity rating was taken, and no AUDPC could be calculated for these. Testing could be done to determine whether a consistent epiphytotic could be developed in the greenhouse for more direct comparison.

In the greenhouse, a significant difference was observed between the two treatments where *A. alternata* was applied in 0.2% malt extract compared to a non-inoculated control or *A. alternata* applied in water (Figure 2.5). This indicates that *A. alternata* with malt extract solution facilitated disease on plants compared to *A. alternata* in water. This is consistent with the finding

of Stammler et al. (2014) who reported that disease on potato from *A. solani* was significantly higher when the inoculation was done with conidia suspended in 2% malt solution than the conidia in water. It is hypothesized that malt extract may enhance disease as an external nutrient source has been reported to facilitate some pathogen infection and penetration (Wan and Li 2011), especially for weak pathogens like *Alternaria* species (Robeson et al. 1985). The exact mechanisms and optimum levels of malt extract need for further testing.

In the greenhouse trials, like detached leaf assay, there was a significant difference between the two varieties for the *A. alternata* in malt extract treatments (Figure 2.5) where the putative susceptible, C059, had a significantly higher disease score than the putative moderately resistant variety, HM9616. This occurred whether or not plants were wounded. This supports the relative susceptibility of these two varieties from naturally infected material in the field (REACh 2019, 2017) and agrees with the results of the detached leaf assay. In the present study, a positive correlation was observed between the detached leaf assay and greenhouse inoculations with C059 and HM9616 ($p=0.0147$, $r=0.8103$). Since these studies used the same germplasm, this supports the hypothesis that at least some of the lack of correlation with the field result maybe due to differences in germplasm. The positive correlation between a detached leaf assay and greenhouse screening is similar to the findings of Doullah et al. (2006) where they found a positive correlation between a detached leaf assay and seedling inoculation performed in a greenhouse on *Brassica rapa* with *Alternaria brassicicola*. Even using a single rating period, the greenhouse test also shows potential for testing Alternaria leaf spot response in sugar beet.

In the greenhouse, we found a significantly lower disease score in wounded C059 than nonwounded (Figure 2.5). This is in contrast to reports that wounding enhances disease caused by *Alternaria* species (Thomma 2003, Prusky et al. 1981, Pleysier et al. 2006, Li et al. 2017).

However, there are some precedents for similar results. For example, Hamilton-Kemp et al. (1992) tested the effect of volatiles from wounded tomato leaves and found that some of the volatile aldehydes and terpenes inhibited the growth of *Alternaria alternata*. Sugar beet leaves also contain a number of volatile compounds and aldehydes and terpenes were some of them (Macleod et al. 1981, Rabetafika et al. 2008). Thus, it may be hypothesized that the volatile compounds released from wounded sugar beet leaves may inhibit the pathogen growth which might lead to a lower mean disease score in wounded plants than nonwounded. In addition, the results indicate no significant difference in HM9616 whether the plants were wounded or not (Figure 2.5). This may indicate something about the type of resistance in HM9616 suggesting it may not have epidermal or surface related resistance (Altpeter et al. 2005, Douchkov et al. 2005). If volatiles are involved, this would need some investigation. Overall, the results indicate that wounding leaves is not necessary for screening sugar beet for response to *A. alternata*.

The field inoculations in 2018 showed a relatively low AUDPC compared to the other years. It was difficult to rate ALS in the field plots at SVREC due to interference from other foliar diseases such as Cercospora leaf spot. When the Cercospora leaf spot infection was high, the spots coalesce and form large necrotic lesions and ultimately, the leaves may die (Pool and McKay 1916, Skaracis et al. 2010, Jacobsen and Franc, 2009). Since *A. alternata* favors stressed and senesced parts, infection often was seen on those leaves which had heavy symptoms of Cercospora leaf spot. This causes trouble in rating because of the difficulty to separate leaf spots. In addition, Cercospora leaf spot initially causes symptoms on older leaves (Jacobsen and Franc 2009) which also is more susceptible to *Alternaria* (Franc 2009, Srivastava 2004). If these leaves are killed by Cercospora leaf spot, there can be less of the more susceptible tissue for ALS. The field at SVREC we used for ALS inoculation had high Cercospora pressure. Therefore, this

might be one reason for low disease scores in 2018, however the same field was used in other years as well, so this likely is not the only factor. In addition, in 2018, the temperature ranged from 26˚-32˚C during the first two weeks after inoculation. From week 2 to 4 the temperature dropped down a little to average 21˚C (Time and Date AS 1995-2021). The average temperature in 2018 during the rating period was high and that might be one reason for low disease scores in 2018. Based on this, the inoculation timing was changed for subsequent years to either earlier (2019) or later (2020 and 2021) in an attempt to have conditions more conducive to ALS and less conducive to Cercospora leaf spot (Jacobsen and Franc 2009, Franc 2009).

In 2019, the AUDPC on control plots were significantly higher than the inoculated plots (Figure 2.7). In 2019, the inoculation was done earlier than 2018 in an attempt to avoid the unfavorable environmental conditions such as high temperature and potentially reduce interactions with Cercospora leaf spot. Therefore, the time when inoculation was done in 2019 might be a good period for natural infection of *A. alternata* and that might be one reason that control plots showed significantly higher disease than the treated plots, but this would need further testing.

Interestingly, a significant difference between controls and treatments was observed at both SVREC only in the section of the field where the fungicide treatments to manage CLS were used as well as in East Lansing, selected for lower Cercospora leaf spot pressure. This supports the hypothesis that Cercospora leaf spot may have interfered in some way with ALS. Further testing is needed to determine potential effect or interactions between Cercospora leaf spot and ALS as well as to improve the field inoculation method. Based on these findings, performing the experiments with less or without Cercospora leaf spot disease pressure may be useful to improve ALS screening in the field.

In 2020 and 2021 field trials at SVREC, the variety HM9879 had a significantly higher mean AUDPC than C869 (Figures 2.8 and 2.9). In contrast, C869 had a significantly higher AUDPC than HM9879 in 2021 at the Plant Pathology Research Farm at East Lansing (Figure 2.10A). It was reported before that HM9879 was moderately resistant to ALS (REACh 2017), but no screens had been done on C869 for *Alternaria alternata*. According to the results at both SVREC in 2020 and 2021 and the Plant Pathology Farm in 2021, C869 appears to have a similar resistance to HM9879. It would be helpful to include a more susceptible variety. The susceptible variety used in the greenhouse, C059 (REACh 2016, REACh 2017, this study) was no longer commercially available starting in 2020. Identifying other susceptible materials could be useful for better comparison of screening methods and might aid in developing a consistent field test.

Conclusions

In conclusion, a detached leaf assay, a greenhouse inoculation and some varied field inoculation methods were assessed to develop consistent and reliable inoculation methods for ALS in sugar beet. The detached leaf assay and the greenhouse inoculation with the inoculum in 0.2% malt extract were both consistently significantly higher disease severity than the uninoculated control, indicating that the inoculation enhances disease production. In addition, using both methods, a significant difference between susceptible and resistant varieties was observed that was consistent with relative ratings in field screens done by Michigan Sugar Company. The disease severity was not dependent on wounding in contrast to some previous reports with other hosts. The detached leaf assay and the inoculation with spores in 0.2% malt extract in the greenhouse showed potential as repeatable inoculation methods for ALS in sugar beet. The AUDPC in the field inoculation was increased between 2018 to 2021 and interestingly, field plots with less Cercospora leaf spot pressure had a significant difference among the

treatments. For example, a consistent difference between inoculation and control in two locations was observed when the field plots had low Cercospora leaf spot pressure. However, more studies should be done for field inoculation with multiple varieties to assess the potential to differentiate among varieties for responses. Testing additional malt extract concentrations also may be helpful, since a significant difference was observed in one location between a 0.2% and 0.5% solution, with more severe disease with the higher concentration of malt extract.

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CHAPTER 3

INVESTIGATION OF HOST PATHOGEN INTERACTIONS IN *ALTERNARIA ALTERNATA* STRAINS

Introduction

Alternaria leaf spot (ALS) is a foliar disease, causing necrotic lesions on leaves on a number of crops including sugar beet (Franc 2009, Akhtar et al. 2004, Droby et al. 1984). Symptoms of *A. alternata* infection on crops also may be called by different names such as leaf blight in tomato, brown spot in potato and Alternaria leaf spot in sugar beet and sunflower (Franc 2009, Akhtar et al. 2004, Droby et al. 1984, Lagopodi and Thanassoulopoulos 1998). The lesions initially are small, circular to irregular (2-10 mm diameter) with a gray to dark brown color. Sometimes the lesions are surrounded by a chlorotic area (Franc 2009) or have a light center and dark border (Srivastava 2004). Under favorable environmental conditions such as high humidity (>90% relative humidity) and cool temperatures (16˚-22˚C) (Franc 2009, McFarlane et al. 1954,) they can coalesce to form large necrotic areas and eventually the whole leaf may die. ALS commonly is found initially on older leaves, and stressed plants or those with foliar yellowing (Franc 2009, Russell 1965). Foliar yellowing that can increase ALS includes diseases like beet yellows (Franc 2009, Russell 1965), or Fusarium yellows (Franc 2009) as well as nutrient deficiencies (Ruppel 1986).

ALS has been reported as occurring everywhere beets are grown (Franc 2009) including Egypt, India, Pakistan, Slovakia, and the United States (Russell 1965, Abbas et al. 2014, Baltaduonytė et al. 2013, El-Kholi et al. 1994, Hudec and Rohačik 2002, McFarlane et al. 1954, Misra et al. 2020). Before 2015, ALS was considered a minor foliar disease in sugar beet fields in the United States (Franc 2009). Since then, ALS has been an increasing issue in sugar beet

fields in Michigan and also has been reported as an increasing issue in other US states such as North Dakota and Minnesota (Rosenzweig et al. 2017, Khan et al. 2020). With increasing incidence of the disease, possible strategies may need to be deployed to manage ALS. Host resistance is one of the ways to manage many diseases (Biancardi et al. 2005). Because it has not been an issue in the United States and Europe, there have been limited breeding efforts for ALS resistance (Biancardi et al. 2005). Variability has been observed in beet populations for ALS response (El Kholi et al. 1994, McFarlane et al. 1954, Russel 1972) with reports of general resistance in most commercial breeding lines in the US (McFarlane et al. 1954), but only moderate resistance was reported in Pakistan (Abbas et al. 2014). Other possible management strategies such as chemical controls and cultural controls (e.g., crop rotation, proper irrigation, elimination of weeds) are also recommended to manage ALS (Franc 2009).

As the name of the disease implies, *Alternaria* spp. cause ALS (Franc 2009). Some species reported to cause ALS in sugar beet are small-spored species such as *A. alternata* (syn. *A. tenuis, A. tenuissima*) and large-spored species such as *A. brassicae* (Franc 2009, Abbas et al. 2014, Khan et al. 2020, McFarlane et al. 1954). There has been confusion about species designation among small-spored *Alternaria* (Armitage et al. 2015, Srivastava 2004), but recent genetic evidence confirms several types with diverse morphology all belong in *A. alternata* (Woudenberg et al. 2015). With the diverse strains, some studies have indicated there may be host specificity within *A. alternata* (Masunaka 2007, Abbo et al. 2018, Babu et al. 2002, EI-Morsy et al. 2006), some authors tried to give separate names to strains that varied in potential hosts (Kameda et al. 1973, Kono et al. 1986, Li et al. 2013). Recent tests confirm these are likely *formae speciales* (Woudenberg et al. 2015). In several other fungal species, there are *formae speciales* that are pathogenic on certain hosts (Shurtleff and Averre 1997, Simmons 2007). If

there are *formae speciales* in *A. alternata*, strains within the species might have a narrow host range. This could impact numerous aspects of ALS, including disease management such as potential crop rotation, finding appropriate isolates for resistance screening, or potential role of weed management.

The host-pathogen interactions for *A. alternata* have been contradictory in some past studies. Several studies reported that the members in *A. alternata* have a wide host range (Cheng et al. 2020, Meena et al. 2013, Meena and Samal 2019, Min et al. 2019) whereas other reports found host specificity for certain *A. alternata* strains (Masunaka 2007, Abbo et al. 2018, Babu et al. 2002, EI-Morsy et al. 2006). In several studies, authors proposed different pathotypes (sometimes proposed as species as mentioned earlier) some of these were found to produce putative hosts specific toxins which could lead to a narrow host range (Nishimura 1980, Kohmoto et al. 1976, Maekawa et al. 1984, Kohomoto et al. 1993, Stierle et al. 1988). However, there are concerns about certain of these studies on host specific toxins as testing has been limited. For instance, Kohmoto et al. (1991) tested the susceptibility of hosts with two proposed host specific toxins by testing the pathogenicity and virulence only on citrus species. They reported susceptibility on two and twenty-eight of the citrus species for strains with the rough lemon (ACR toxin) and tangerine pathotypes (ACTG toxin) respectively. This showed variability for host specificity even within citrus and, since other potential hosts were not tested for sensitivity to either the toxin or the isolates, pathotypes may not be host specific. To address these contradictions, studies should be conducted about host interactions in *A. alternata* that include diverse hosts.

The objective of the current study was to investigate host interactions that could be important for sugar beet production within a set of *A. alternata* strains by isolating from hosts

representing diverse plant families that are either grown in rotation with sugar beet or in the same production region and cross inoculating into four crops belonging to three different plant families that are grown in rotation with or near sugar beet.

Materials and Methods

Sampling pathogen isolates

Strains of *A. alternata* were obtained from symptomatic dry bean pods, blueberry fruits, and potato or sugar beet leaves. All crops are grown in Michigan. Isolates from blueberries (generously provided by Dr. T. Miles, Michigan State University) and potatoes (generously provided by C. Long, Michigan State University) were collected in 2019 and 2018, respectively. Isolates from sugar beet were from the Hanson (USDA, ARS) collection and had been collected in 2002 and 2014. The isolates from 2002 had been confirmed as *A. alternata* by DNA sequence analysis of the *ITS* region and GAPD (unpublished data). For isolations, tissue samples were kept for two days in a moist chamber (a double sealed gallon plastic bag with a moist paper towel inside) for sporulation at room temperature with ambient lighting. Lesions were observed through a dissecting microscope (Olympus-LMS-225R, Leeds Precision Instruments Inc. Minneapolis, MN) and 15 µl of sterile water was placed onto each lesion where spores were visible using a sterile micro-pipette. A sterile bacterial transfer loop was used to streak spores onto 2% water agar (wt/vol) and agar plates were incubated overnight in the dark at room temperature. Pure cultures from each isolate were prepared by transferring a single germinated (when the germ tube was longer than the width of the spore) conidium onto half strength clarified V8 juice (HCV8) agar plates (Miller 1955) and incubating at room temperature (approximately 25˚C) with incidental light. Cultures were maintained on HCV8 for short term.

For long-term storage, a sterile glass filter paper method was used (Hanson and Hill 2004) with isolates stored at -20°C.

Plant materials

Four different crops which were reported hosts for *Alternaria alternata* (Ding et al. 2019, Franc 2009, Tu 1985, Morris et al. 2000), were used for inoculation. The plants included sugar beet, potato, tomato, and dry beans. Potato and dry bean are rotational crops with sugar beet in some areas including Michigan (Wilson 2001, Christenson et al. 1995, Gebremedhin and Schwab 1998, Sanchez et al. 2001). Potatoes and tomatoes also are common in back gardens in many areas including Michigan. Hence, tomato, potato and sugar beet have potential for interactions in a cropping system. In contrast, blueberries are common in Michigan, but they are not generally grown in the same area as sugar beet (Longstroth and Hanson 2012, Stilgenbauer 1927). Thus, blueberry represents a potential *Alternaria* host crop with less potential direct interaction with sugar beet. Screening included two varieties of sugar beet [C059 and HM9616 reported as susceptible and tolerant to ALS respectively (REACh 2019, 2017)], one variety and a breeding line of potato [Atlantic (Webb et al. 1978) and MSW; a hybrid between Atlantic and Saginaw chipper (personal communication C. Zhang)], three varieties of dry bean representing three different classes [Cayenne; a small red (Kelly et al. 2018b), Zorro; a black (Kelly et al. 2009), and Redcedar; a kidney (Kelly et al. 2018a)] and a commercially available cherry tomato (purchased from a commercial market- Meijer, Okemos MI). The sugar beets were grown in the greenhouse (temperature range from 20˚-30˚C with 12 hours light supplemented with high pressure sodium bulbs) at Michigan State University (East Lansing, MI). The seeds were planted in 2.45-liter plastic pots filled with commercial potting mix (SureMix Pertile, Michigan Grower Products. Inc., Galesburg, MI). Once the seedlings reached the two-leaf growth stage, they were

transplanted into individual pots using the same potting medium. Plants were fertilized with 14-14-14 (Osmocote, Everiss, Geldermalsen, the Netherlands) when they were at the 4 to 6 leaf growth stage. When the plants were at the 8 to 10 leaf growth stage, the leaves from the outermost whorl were cut using scissors. Potato leaves and bean pods were obtained from field grown plants (generously provided by Dr. D. Douches and E. Wright, Michigan State University respectively).

Detached tissue assay

Several studies, such as Goth and Kaene (1997), reported that there was no significant difference between the results obtained from a detached leaf assay and intact plant inoculation on tomato and potato. Based on this information, we used a detached tissue assay for our experiment. Two isolates identified as *A. alternata* by morphology each from dry beans (Bn-2 and Bn-7), potato (P-2 and P-23), sugar beet (B-5 and B-14; both were identified genetically as well) and blueberries (Bl-7 and Bl-19) were used for this study. The isolates were grown on HCV8 and incubated ten days in the dark to enhance sporulation (chapter 2). Inoculum was prepared from each isolate by flooding the plate with 2 ml of sterile distilled water and scraping the surface using a sterile loop. The spores were collected to a sterile centrifuge tube (5 ml) with a sterile pipette. The final spore concentration was adjusted to approximately 1 x 10^5 spores/ml in sterile distilled water after counting the spores using a hemocytometer. All plant tissues were surface disinfected by immersing in a 0.6% sodium hypochlorite with 0.1% tween 20 (vol/vol) solution for 10 minutes and rinsing with sterile distilled water three times. The plant materials were air dried in a biosafety cabinet (SterilGARD Class II Type A/B, the Baker Co., Sanford, ME) for thirty minutes. Tissue was wounded by stabbing with a sterile dissecting needle and 20 µl of the spore suspension of one isolate, or a sterile water control, was placed on each wound

site using a sterile pipette. Inoculum and the controls were also applied onto unwounded sites of sugar beet and potato leaves. Since no significant difference on AUDPC between wounded and unwounded was observed, AUDPC values by wounding were considered in the analysis. Inoculated and control plant tissue was placed in moist chambers. The moist chambers were aluminum pans (50.8 cm length, 31.7 cm width and 7.6 cm height) with lids and were lined with sterile paper towels (6 per pan) that had been moistened with sterile distilled water. Moist chambers were incubated at room temperature for lesion development. The lesion diameters were taken using a digital caliper (VWR international LLC. Radnor, PA) starting two days after inoculation and continuing daily up to five days after inoculation. The pathogen was re-isolated from representative samples and were grown on HCV8 plates using the same method as for initial isolation from beet tissue. Five replicates of each isolate on each host were used in each experiment and the experiment was repeated three times. Average lesion diameters for each isolate on each host over the duration on the test were used to obtain area under the diseases progress curve (AUDPC) (Meena et al. 2011). R studio (version 1.1.456) was used to calculate the AUDPC. Calculated AUDPC values from each isolate on all hosts were used for comparisons. SAS software (version 9.4) was used for statistical analysis. Only one variety from each host, sugar beet, potato and dry bean was used to examine the final host-pathogen interaction. The significance of the main effects (host and isolate) and interactions (host x interaction) with the random effect as replications were tested by constructing ANOVA tables using the proc mixed procedure and pairwise comparisons were performed with Fisher's least significant difference tests (LSD, α=0.05). When different varieties were used, the variety with the numerically highest average AUDPC was chosen to compare among host genera.

Results

On HCV8 agar incubated in the dark, the colonies appeared as a dull grey color with poorly defined concentric rings. All isolates produced dark, multicellular, acropetal, broad base, club shaped spores (conidia) with cross walls (in most cases in two directions). Conidia were arranged as largely unbranched chains (Figure 3.1). Based on this and comparison to known isolates, all were classified as *A. alternata* [formerly *A. tenuissima* (Simmons (2007)] (Woudenberg et al. 2015). Two isolates from each host were randomly selected for testing. All isolates tested caused lesions on all four hosts tested regardless of the host from which they were originally isolated.

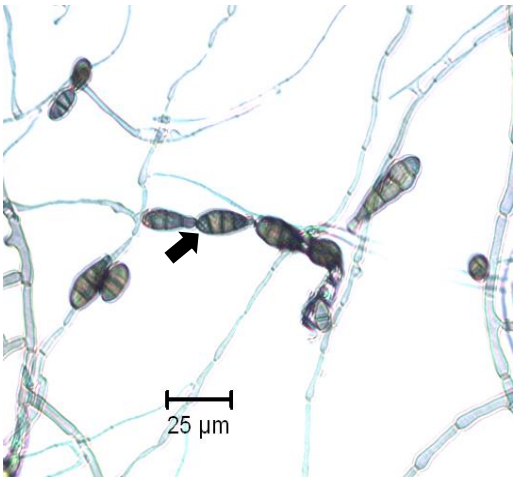

Figure 3.1: Examples of dark, multicellular, broad base, club-shaped conidia surrounded by hyphae of *Alternaria alternata* (200x magnification) from ½ strength clarified V8 agar. An unbranched conidial chain of *A. alternata* is indicated by an arrow. The chain is a partial chain, with some conidia that have become detached.

A homogeneity test was performed among the three trials and the results indicated that the trials were nonhomogeneous ($p>0.05$). Therefore, each trial was analyzed separately. Although all the hosts showed symptoms with all the *A. alternata* isolates (Figure 3.2), there was a significant difference in response among the varieties ($p<0.05$). Among the three varieties of dry bean, the variety Cayenne had a significantly higher ($p<0.01$) AUDPC than the varieties,

Zorro and Redcedar in two out of three trials. The third trial showed no significant difference between Cayenne and Zorro, but Cayenne still had significantly higher disease severity than Redcedar. Based on the higher AUDPC, Cayenne was selected for the final comparison. Interestingly, Redcedar had a significantly lower ($p<0.0001$) AUDPC than Cayenne and Zorro in all the three trials. For potato, two out of three trials showed a significant difference, but the relative disease severity varied in each trial with Atlantic and MSW each having a higher disease severity in one trial (trial 2 and 3 respectively). Therefore, we arbitrarily chose the variety Atlantic for our final comparison. For the two sugar beet varieties, C059 gave significantly higher AUDPC ratings than HM9616 in two out of three trials and was not significantly different in the third. Therefore, C059 was chosen for the final comparison.

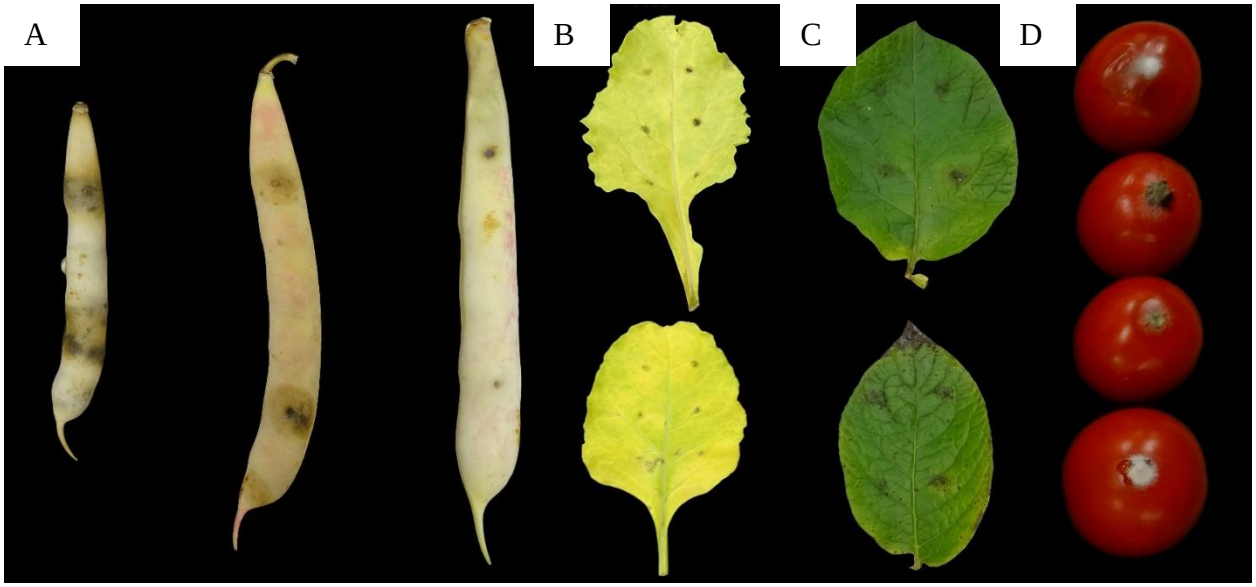

Figure 3.2: Examples of lesion development following inoculation with *Alternaria alternata* on different varieties of A. dry bean pods B. sugar beet leaves C. potato leaflets or D. tomato fruits. A) The dry bean varieties, from left to right were Zorro, Cayenne and Red cedar. B) The sugar beet varieties were C059 (top) and HM9616 (bottom). C) The potato germplasm was Atlantic (top), and MSW (bottom) and D) tomato fruits. On the leaves, the left side of the leaf was wounded prior to inoculation while the right side was inoculated without wounding.

An ANOVA test performed among the selected varieties indicated a significant host-pathogen interaction ($P<0.0001$). To investigate the interactions, each isolate and each host was compared across all four hosts and all eight isolates. While both strains from potato caused lesions on all hosts, the severity varied among the four hosts tested. The AUDPC values from lesions caused by potato isolate P-2 were not significantly different between potato or sugar beet leaves in all the three trials tested. Similarly, potato isolate P-23 had no significant differences on these crops in two out of three trials. However, the AUDPC on dry bean pods caused by the two potato isolates were significantly higher than on potato leaves in all three trials. On tomato, the AUDPC values were significantly higher from isolate P-2 than on potato in all three trials (Table 3.1). Isolates, B5 and B14, originally from sugar beet leaves, produced lower AUDPC values on sugar beet leaves than on tomato fruit and dry bean pods in all the trials while AUDPC values were not significantly different on potato leaves in two out of three trials (Table 3.1).

To further examine host pathogen interactions, all eight isolates were compared on individual hosts (Table 3.1). On sugar beet no significant difference in AUDPC was observed between isolates in the three trials (Table 3.1). In potato, no significant difference was found in trial 1 among isolates but trial 2 and 3 indicated significant differences among isolates. In trial 2, the AUDPC values from one blueberry isolate (Bl-7) were significantly higher than all isolates from other crops in potato while the other blueberry isolate (Bl-19) was not significantly different than the potato isolates. In trial 3, the AUDPC values from B-5 and again Bn-7 were significantly higher than the potato isolates (P-2 and P-23) in potato (Table 3.1). A similar scenario was observed in dry bean, and tomato, where a significant difference in AUDPC was observed among isolates but isolates originally isolated from the given host were not always significantly different from other isolates (Table 3.1).

Table 3.1: Comparison among area under the disease progress curve (AUDPC) values for eight *Alternaria alternata* isolates on four hosts, potato, tomato, dry bean, and sugar beet. Three trials were analyzed separately. All the isolate x host interaction in each trial were analyzed together. Isolates designated with a P, B, Bn, or Bl indicate the isolates were originally collected from potato or sugar beet leaves, dry bean pods or blueberry fruits respectively. Five replicates were used to determine the average AUDPC values. Lower-case letters next to each AUDPC values were used to compare each *Alternaria alternata* isolate across four hosts and upper-case letters were used to compare each host across eight *Alternaria alternata* isolates. Averages followed by the same letter were not significantly different by Fisher's protected LSD at α=0.05.

Trial	Isolate	Average AUDPC on host			
		Potato	Tomato	Dry bean	Sugar beet
1	P-2	15.95 a A	49.75 b B	76.42 c DE	11.90 a A
1	P-23	30.22 a A	30.93 a A	57.71 b CD	8.74 a A
1	Bn-2	15.11 ab A	49.90 c BC	31.30 bc AB	5.06 a A
1	Bn-7	22.52 a A	58.74 b C	12.10 a A	6.31 a A
1	B-5	23.28 ab A	38.29 b AB	84.32 c E	14.03 a A
1	B-14	14.07 a A	55.11 b C	41.58 b B	5.74 a A
1	Bl-19	14.96 a A	21.05 a A	45.16 b BC	6.43 a A
1	Bl-7	23.80 a A	58.75 b C	57.55 b C	11.16 a A
2	P-2	22.11 a AB	77.67 c B	51.46 b B	7.11 a A
2	P-23	31.75 b B	45.40 bc A	61.73 c BC	5.46 a A
2	Bn-2	4.19 a A	38.47 b A	83.81 c D	8.84 a A
2	Bn-7	21.14 a A	51.11 b A	47.56 b B	6.14 a A
2	B-5	16.65 a A	44.17 b A	144.66 c F	7.25 a A
2	B-14	6.72 a A	40.02 b A	70.17 c CD	7.03 a A
2	Bl-19	39.56 c BC	35.52 c A	9.75 b A	8.74 ab A
2	Bl-7	50.81 b C	54.75 b A	109.31 c E	8.30 a A
3	P-2	12.36 a A	53.74 b BC	59.41 b BC	6.05 a A
3	P-23	14.93 a A	73.69 b D	67.92 b CD	4.96 a A
3	Bn-2	13.42 a A	60.74 b CD	65.95 b C	5.01 a A
3	Bn-7	32.77 b B	42.52 bc AB	58.66 c B	4.09 a A
3	B-5	35.98 b B	29.52 b A	45.82 b AB	5.75 a A
3	B-14	23.73 b A	46.47 c B	33.46 bc A	6.09 a A
3	Bl-19	23.61 a A	49.77 b B	81.71 c D	9.38 a A
3	Bl-7	26.72 a AB	46.47 b B	125.55 c E	12.32 a A

Discussion

All the *A. alternata* isolates tested were virulent on all four hosts inoculated, irrespective of the host from which they were originally isolated. This agrees with the reports that the species is common on numerous different hosts (Franc 2009, Meena et al. 2013, Meena and Samal 2019, Min et al. 2019) and has a broad host range. However, our results differ from reports of potential host specificity (Masunaka 2007, Abbo et al. 2018, Babu et al. 2002, EI-Morsy et al. 2006) indicating that there may not be beet specific toxin. The results of the current study support the suggestion of a wide host range for *Alternaria alternata* as all eight isolates tested caused lesions on crops from three different families, and severity was not related to original source of the isolates. For example, isolate B-5, originally collected from sugar beet, gave significantly higher disease severity ratings on dry bean pods than two isolates from dry beans in two out of three trials.

While no association was found between host and disease with artificial inoculation, a genetic study of *A. alternata* should be carried out to determine whether there might be specificity in isolates collected from a given crop in the field (Armitage et al. 2015, Morris et al. 2000, Gherbawy 2005). For example, Abbo et al. (2018) reported a separate genetic cluster for *A. alternata* isolated from tomato compared to potato, pepper and eggplant which could indicate some host specificity in the field. Cross-infectivity tests performed in the same study showed that each isolate originally collected from the four crops caused disease on all crop types with the exception of one *A. alternata* isolate, which was collected from eggplant, and did not cause symptoms on pepper, but did cause disease on potato and tomato. Although we did not use *A. alternata* isolated from tomato in our study, the cross infectivity of *A. alternata* isolates

originally from potato agrees with Abbo et al. (2018) and we have expanded this to show similar cross-pathogenicity outside the Solanaceae.

The *A. alternata* isolates used in the current study were originally isolated from four crops, including leaves of sugar beet and potato, dry bean pods and blueberry fruits. Although they were collected from different hosts representing four plant families, the morphology of all the isolates was visually similar on the inoculated host tissues (beet, dry bean, and potato) and on HCV8.

All the isolates produced dark elongated conidia arranged as largely unbranched conidial chains (Figure 3.1). This pattern agrees with the description of *A tenuissima* [now *A. alternata* (Woudenberg et al. 2015)] by Simmons (2007). Based on the morphological similarity among isolates, and genetic evidence from two representative members of the population (unpublished data), this study supports that there is a potential to have the same *A. alternata* on diverse hosts.

For three of the potential hosts more than one variety were included in screening. Although all the *A. alternata* isolates caused lesions on all the varieties tested (Figure 3.2), there were differences in disease severity among varieties in two of the three hosts. Distributing commercial varieties that are resistant to common pathogens in the region is one of the key factors for maintaining an adequate food supply (Stuthman et al. 2007). Thus, it can be important to identify sources with potential for resistance in the field (Abbas et al. 2014). A reliable screening method for resistance is important to identify resistant varieties. Previous studies showed that a detached tissue assay can indicate resistant varieties (Akhtar et al. 2012, Browne and Cooke 2004). Results from the current testing indicate that there is a potential for host resistance in several of the crops of interest, but field confirmation generally is needed.

All *A. alternata* strains originally collected from all the four crops caused lesions on all the crops (sugar beet, potato, dry bean, and tomato) tested. All of the host types used in this study are commonly grown as commercial crops in Michigan (Bingen and Siyengo 2002, Grafius 1997, Stilgenbauer 1927, USDA-National Agricultural Statistical Services 2021). Sugar beets are often grown in rotation with dry bean and potato (Wilson 2001, Christenson et al. 1995, Gebremedhin and Schwab 1998, Sanchez et al. 2001). Since the results of our research indicate that the same *A. alternata* isolates can cause lesions on all three hosts, care may need to be taken when using these crops in rotation if Alternaria leaf spot or pod rot is an issue in the area. Crop rotation with a non-host has a potential to reduce the level of primary inoculum in the field (Bockus and Claassen 1992, Jirak-Peterson and Esker 2011). Further examination of the effect of disease in the field on crop rotation with *Alternaria* resistant varieties is needed.

Among the three dry bean varieties tested, the AUDPC on Red cedar was consistently significantly lower than Zorro and Cayenne. None of the varieties were previously tested for *Alternaria* response (personal communication Dr. J. Kelly). The results of the current study indicate that there could be potential to identify some level of resistance in bean varieties for use either to manage seed discoloration associated with pod infection (Tu 2005) or potentially for rotation with crops where Alternaria leaf spot is more problematic (Buhre et al. 2009). Therefore, the potential for resistance on varieties is worth investigating for disease management. Including resistant varieties in a crop rotation system will be better than rotating susceptible varieties in a crop rotation which might help to decrease the level of primary inoculum buildup in the field.

The sugar beet variety C059 had significantly higher AUDPC values than variety HM9616 in two out of three trials. The two varieties, C059 and HM9616 were previously

reported as susceptible and partially resistant to Alternaria leaf spot in field conditions (REACh 2017, REACh 2019), and the current results agree with these results under controlled conditions.

According to McFarlane et al. (1954), resistance to *A. brassicae* was widespread in sugar beet breeding material in the US. The historically low levels of issues with *A. alternata* in the US (Franc 2009, Ruppel 1986) likely indicate a similar widespread resistance to this pathogen. With the increasing issues with Alternaria leaf spot in the region (Rosenzweig et al. 2019), more susceptible material may have been introduced into the region, as Biancardi et al. (2005) warned against. It is important to be able to reliably identify any such more susceptible materials and remove them from use in areas where ALS is a risk.

A significant interaction was observed between host and pathogen ($p<0.0001$). While all the strains caused lesions on all the hosts, there was some variability in specific host/isolate interactions. There was no significant difference among AUDPC in sugar beet between any isolates (Table 3.1), and varied evidence for differences on potato (non-significant in one of the three trials), but there were significant differences in disease severity for dry bean (Table 3.1). Interestingly, an *A. alternata* strain isolated from blueberries (Bl-7) caused larger lesions than the two dry bean isolates tested on dry bean. With the small number of isolates tested, no conclusions can be drawn on relative virulence, but this supports a broad host range.

Conclusions

In conclusion, *Alternaria alternata* strains caused lesions on all four hosts, representing three different plant families, and the virulence was not strongly associated with the host from which they were originally isolated. This supports prior reports (Meena and Samal 2019, Meena et al. 2013, Rotem 1994) that *A. alternata* has a wide host range. This study helps to answer the

questions raised by Franc (2009) about whether isolates from such diverse crops actually may be the same species or strains.

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CHAPTER 4

A STUDY ON A GENETIC DIVERSITY OF *ALTERNARIA ALTERNATA*

Introduction

Alternaria spp. are a diverse group of fungi and are ubiquitous in the environment (Gherbawy 2005, Thomma 2003, Slifkin 1971, Anaissie et al. 1989). *Alternaria* spp. are found as saprophytes in soil and crop debris (Gherbawy 2005, Thomma 2003, Spurr 1977), endophytes (Spurr 1977, Hellwig et al. 2002) and as pathogens to humans, animals, and plants (Thomma 2003, Dang et al. 2015, Ramjegathesh and Ebenezar 2012, Droby et al. 1984, Dubey and Patel 2000, Zhu et al. 2017, Kohmoto et al. 1991, Hausbeck et al. 2000). As plant pathogens, they cause diseases in a wide variety of crops belonging to various families such as Amaranthaceae, Solanaceae, Malvaceae, Fabaceae, Rutaceae and Amaryllidaceae (Franc 2009, Ramjegathesh and Ebenezar 2012, Droby et al. 1984, Dubey and Patel 2000, Zhu et al. 2017, Kohmoto et al. 1991). Common hosts of *Alternaria* spp. include sugar beet, potato, dry bean, tomato, apple, blueberry, and pear (Franc 2009, Droby et al. 1984, Jia et al. 2013, Kawamura et al. 1999, Zhu and Xiao 2015, Zhu et al. 2017).

Alternaria leaf spot (ALS) is a foliar disease of sugar beet caused by *Alternaria* spp. (Franc 2009, Khan et al. 2020, Srivastava 2004, Russel 1965). Two of the most frequently reported *Alternaria* spp. on beets are *A. brassicae* and *A. alternata* (syn. *A. tenuissima*, *A. tenuis*) (McFarlane et al. 1954, Franc 2009, Srivastava 2004, Russel 1965). ALS has been reported wherever beets are grown (Franc 2009) including countries such as India, Russia, Pakistan, the Netherlands, Romania, Belarus, Poland, Germany, and Slovakia (Abbas et al. 2014, Srivastava 2004, Hanse et al. 2015, Hudec and Rohačik 2002, Bălău 2009, Dunning and Byford 1982, Kolomiets et al. 2010, Pusz 2007, Dorn 1950). ALS is reported as a major disease which impacts

the economics of beet production in several regions such as Russia, India and Pakistan (Srivastava 2004, Misra et al. 2021, Abbas et al. 2014, Lastochkina et al. 2018, Gannibal 2018). In the United States, however, ALS was generally a minor foliar disease on sugar beets (Franc 2009). Recently, however, ALS has caused increasing issues with potential significant losses in Michigan sugar beet fields (Rosenzweig et al. 2017, Rosenzweig et al. 2019).

Since *Alternaria* spp. have diverse lifestyles as saprophytes, endophytes or pathogens, studying the diversity of *Alternaria* spp. is important to understand the interactions between *Alternaria* spp. and host genotypes (Morris et al. 2000, Kakvan et al. 2012, Bagherabadi et al. 2015, Jiang et al. 2021, Mahmoudi et al. 2018). Because the majority of ALS in Michigan in recent years has been found to be *Alternaria alternata* (Rosenzweig et al. 2017, Rosenzweig et al. 2019), this species will be the focus of the current study. The genetic diversity of an organism gives information about whether it is a diverse population or monoclonal population (Bayman and Cotty 1991, Levy et al. 1993, Atallah et al. 2004). This information may help to decide to use multiple isolates or a single strain for different testing including inoculation (Knight et al. 2019, Burlakoti et al. 2012, Zhang and Xue 2010, Secor et al. 2010). Some studies have been carried out on genetic diversity analysis of *A. alternata* isolates collected from several regions (counties/ states) and found no clear geographic clustering among them suggesting that *A. alternata* genotypes have widespread distribution (Morris et al. 2000, Adhikari et al. 2021, Andrew et al. 2009, Esfahani 2018) whereas some studies found a positive correlation between the molecular diversity of *A. alternata* and a specific geographic location (Kakvan et al. 2012, Bagherabadi et al. 2015) indicating potential variability with geographic regions. These genetic diversity studies on *A. alternata* isolates reveal some variability within the species genotypes and

it may help to come up with management decisions such as quarantine measures (Meng et al. 2018). It is not sure what situation may be occurring in beets.

Since ALS was a minor disease before 2015 in the United States, little is known about ALS and host-pathogen interactions. Because of increasing disease incidence, there are questions on why this might have occurred, such as a population shift. With the limited information of the population, this is difficult to determine. Thus, it is important to study the *Alternaria*-sugar beet pathosystem to better understand the organism and to potentially develop management tools for this disease. Although several studies have reported on genetic diversity of *A. alternata* in other crops (Morris et al. 2000, Gherbawy et al. 2018, Bagherabadi et al. 2015), the genetic diversity in *A. alternata* in sugar beets is not known. Since increasing issues of ALS were reported in Michigan after 2015 (Rosenzweig et al. 2017, Rosenzweig et al. 2019), it could be helpful to compare the *A. alternata* genotypes before and after 2015 to determine whether ALS issues might be related to a shift in the pathogen population. The present study was carried out with 22 isolates of *A. alternata* from sugar beet from 2005 to 2019 and compared to isolates originally collected from three other host families. These hosts included two crops grown in rotation with sugar beet, dry bean and potato, (Christenson et al. 1995, Gebremetdhin and Schwab 1998, Sanchez et al. 2001), and from a crop not normally rotated with sugar beet, blueberry (Longstroth and Hanson 2012, Stilgenbauer 1927), and primarily produced in a different geographic region of the state than sugar beet. All isolates in the current study were collected in Michigan. These other hosts are included to compare diversity on the primary host of interest, the cropping system, and the region.

Methods

Alternaria alternata strain collection and pure culture preparation

Symptomatic sugar beet and potato leaves, dry bean pods, and blueberry fruits were collected in Michigan and used for isolation of fungal strains. The sugar beet and potato leaves represent vegetative tissue, and the dry bean pods and blueberry fruit represent reproductive tissue. Tissue samples were kept two days in a moist chamber (a double sealed plastic bag with a moist paper towel inside) at room temperature (approximately 25°C) with ambient light to enhance sporulation. Lesions with evidence of spores were observed through a dissecting microscope (Olympus-LMS-225R, Leeds Precision Instruments Inc. Minneapolis, MN) and 15 µl of sterile distilled water was placed on a single lesion using a pipette. Spores were collected by scraping the surface of the lesion using a sterile bacterial transfer loop. The loop was used to streak spores on to 2% (wt/vol) water agar plates. Plates were incubated overnight at room temperature (approximately 25°C) with incidental light. Pure cultures for each isolate were obtained by transferring a single germinated conidium onto a ½ strength clarified V8 agar plate (HCV8) (Miller 1955) and incubated at room temperature with incidental light. Isolates were tentatively identified to species by morphology on HCV8 (Simmons 2007). Isolates with characteristics of small-spored *Alternaria* [small (<50(60) µm to medium (50-60)-100 µm) conidia arranged in chains with more than 2-3 conidia in a chain] were selected for further analysis. The isolates collected from the four crops are given in Table 4.1. Short term storage of the isolates was done by cutting fungal plugs (5 mm diameter) from the edge of a fungal colony grown on HCV8 with a sterile cork-borer and transferring onto fresh HCV8. Plates were incubated in the dark at room temperature. Long term storage of all isolates was done using the sterile glass fiber filter paper method described by Hanson and Hill (2004).

Table 4.1: Details of 64 *Alternaria alternata* strains used for genetic analysis. The table includes the host from which the isolate was originally collected with isolate designations, the year of collection and the source of the sequence data for three genes, endoploygalacturanase (*EndoPG*), translation machinery associated protein (*Tma22*) and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*Pgs1*). All strains indicated "this study" were collected from Michigan. Isolates indicated "FERA", "EGS" and "ARIPP" were used from in-silico data and were from the UK Food and Environmental Research agency, private collection of Prof. E. G. Simmons and Russia Institute of Plant Protection respectively.

Isolate	Host	Tissue	Year	Geographic location	Source
A05-40	*Beta vulgaris*	Leaf	2005	Michigan	This study
A09-4	*Beta vulgaris*	Leaf	2009	Bay county, Michigan	This study
A09-7	*Beta vulgaris*	Leaf	2009	Frankenmuth, Michigan	This study
A09-8	*Beta vulgaris*	Leaf	2009	Frankenmuth, Michigan	This study
A09-9	*Beta vulgaris*	Leaf	2009	Bay City, Michigan	This study
A11-1	*Beta vulgaris*	Leaf	2011	Richville, Michigan	This study
A11-2	*Beta vulgaris*	Leaf	2011	Richville, Michigan	This study
A17-2	*Beta vulgaris*	Leaf	2017	Frankenmuth, Michigan	This study
A18-1	*Beta vulgaris*	Leaf	2018	Frankenmuth, Michigan	This study
A18-2	*Beta vulgaris*	Leaf	2018	Frankenmuth, Michigan	This study
A18-3	*Beta vulgaris*	Leaf	2018	Frankenmuth, Michigan	This study
A18-4	*Beta vulgaris*	Leaf	2018	Frankenmuth, Michigan	This study
A18-5	*Beta vulgaris*	Leaf	2018	Frankenmuth, Michigan	This study
A19-1	*Beta vulgaris*	Leaf	2019	Brookfield, Michigan	This study
A19-8	*Beta vulgaris*	Leaf	2019	Sebewaing, Michigan	This study
A19-39	*Beta vulgaris*	Leaf	2019	Brookfield, Michigan	This study
A19-47	*Beta vulgaris*	Leaf	2019	Sebewaing, Michigan	This study
A19-84	*Beta vulgaris*	Leaf	2019	Michigan	This study
A19-89	*Beta vulgaris*	Leaf	2019	Michigan	This study

Table 4.1 (cont'd)

Isolate	Host	Tissue	Year	Geographic location	Source
A19-113	*Beta vulgaris*	Leaf	2019	Quanicassee, Michigan	This study
A19-116	*Beta vulgaris*	Leaf	2019	Brookfield, Michigan	This study
CB2	*Beta vulgaris*	Leaf	-	Michigan	This study
A18P-1	*Solanum tuberosum*	Leaf	2018	Montcalm county, Michigan	This study
A18P-2	*Solanum tuberosum*	Leaf	2018	Montcalm county, Michigan	This study
A18P-11	*Solanum tuberosum*	Leaf	2018	Montcalm county, Michigan	This study
A18P-15	*Solanum tuberosum*	Leaf	2018	Montcalm county, Michigan	This study
A18P-22	*Solanum tuberosum*	Leaf	2018	Montcalm, county, Michigan	This study
A18P-23	*Solanum tuberosum*	Leaf	2018	Montcalm, county, Michigan	This study
A18P-25	*Solanum tuberosum*	Leaf	2018	Montcalm, county, Michigan	This study
A18P-30	*Solanum tuberosum*	Leaf	2018	Montcalm, county, Michigan	This study
ABn-1	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABn-2	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABn-3	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABn-4	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study

Table 4.1 (cont'd)

Isolate	Host	Tissue	Year	Geographic location	Source
ABn-5	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABn-6	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABn-7	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABn-9	*Phaseolus vulgaris*	Pod	2019	Ingham county, Michigan	This study
ABl-1	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-3	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-5	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-7	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-9	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-11	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-13	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-15	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-17	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
ABl-19	*Vaccinium corymbosum*	Fruit	2019	Michigan	This study
FERA348	*Malus domestica*	-	-	FERA	Armitage et al. (2015)
FERA538	*Pyrus pyrifolia*	-	-	FERA	Armitage et al. (2015)
FERA631	*Pyrus communis*	-	-	FERA	Armitage et al. (2015)
FERA632	*Pyrus* sp.	-	-	FERA	Armitage et al. (2015)
FERA650	*Pyrus* sp.	-	-	FERA	Armitage et al. (2015)

Table 4.1 (cont'd)

Isolate	Host	Tissue	Year	Geographic location	Source
FERA704	*Pyrus pyrifolia*	-	-	FERA	Armitage et al. (2015)
FERA1164	*Malus domestica*	-	-	FERA	Armitage et al. (2015)
EGS 34.015	*Dianthus* sp.	-	-	EGS	Armitage et al. (2015)
EGS 34.016	*Archis* sp.	-	-	EGS	Armitage et al. (2015)
EGS 38.029	*Malus domestica*	-	-	EGS	Armitage et al. (2015)
EGS 39.128	*Solanum lycopersicum*	-	-	EGS	Armitage et al. (2015)
EGS 90.0512	*Pyrus pyrifolia*	-	-	EGS	Armitage et al. (2015)
RGR 97.0010	*Malus domestica*	-	-	USDA	Armitage et al. (2015)
RGR 97.0024	*Malus domestica*	-	-	USDA	Armitage et al. (2015)
O 159	*Malus domestica*	-	-	ARIPP	Armitage et al. (2015)
A.brassicicola		-	-	-	Genbank; GCA_002796735.1

DNA extraction

Forty-eight *Alternaria* isolates identified as *A. alternata* by morphology were used for DNA extraction. Isolates were grown on HCV8 agar in the dark at room temperature for 8-10 days. Five plugs from each *A. alternata* isolate were taken from the edges of the fungal colony using a sterile cork-borer (5mm) and transferred to 250 ml Erlenmeyer flasks with 50 ml of HCV8 broth. Cultures were placed in an incubator shaker (New Brunswick Scientific, Edison, NJ) at room temperature (20˚-25˚C) and 110 rpm for 5 days. The mycelia were harvested using sterile forceps, washed three times with sterile distilled water and transferred to 50 ml polypropylene conical screw-cap tubes (USA Scientific, Ocala, FL). The tissues were lyophilized

in a freeze dryer (VirTis Genesis; SP Scientific; Warminster, PA). The freeze-dried samples were ground using a paint shaker (Miracle Paint Rejuvenator, St. Paul, MN) with 6 mm ceramic grinding beads (Zircoa, Inc.; Solon, OH). DNA extraction from each isolate was done using an OmniPrep for fungus kit (G-Biosciences, St. Luis, MO) following the manufacturer's instructions with the following modifications. Instead of fresh fungal tissue samples, we used ground, lyophilized tissue (20-25 mg). The mixtures were incubated at 65°C for 1 ½ hours and extracted using 500 µl chloroform: isoamyl alcohol (24:1). After adding RNase A and incubating 30 minutes at room temperature, samples were extracted a second time with 500 µl of chloroform: isoamyl alcohol (24:1). To the supernatant, 50 µl of DNA stripping solution was added and incubated at 60˚C for 10 minutes. Following extraction, the DNA concentration from each isolate was measured by a fluorometer (Qubit 4; Thermo Fisher; Waltham, MA) and adjusted to 25 µg/ml using Tris- EDTA buffer (pH=7.5) (Maniatis et al. 1982).

PCR amplification, purification, and gel electrophoresis

Three primer pairs for the endoploygalacturanase (*EndoPG*), translation machinery associated protein (*Tma22*) and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*Pgs1*) genes, as described in Armitage et al. (2015), were used for this study. We also developed primers for the *Cyp51* gene in *A. alternata.* The Geneious software was used to design the primers. The whole *Cyp51* gene of *A. alternata* was imported from NCBI and five forward and reverse primers were designed in Geneious.

PCR reaction mixtures for *Pgs1* and *Tma22* consisted of a total volume of 40 µl with the final concentrations of 1X Phusion II HF buffer, 0.004 mM of dNTPs, 0.5 µM each of the forward and reverse primer, 1 mM $MgCl_2$, 0.02 U/ µl high-fidelity DNA polymerase enzyme, and 1.25 µM of genomic DNA. A similar PCR reaction mixture for the *EndoPG* primers was

prepared without $MgCl_2$. PCR reactions were performed in a thermocycler (C1000 Touch, BIO-RAD, Hercules, CA), using the conditions for each primer from Armitage et al. (2015) with recommended annealing temperatures (Table 4.2). The final extension was at 72˚C for 7 minutes. The PCR conditions for *Tma22* and E*ndoPG* were the same except the extension and final extension periods were decreased to 30 seconds and 5 minutes respectively. The PCR products were purified using sephadex spin columns. A 10% (wt/vol) sephadex suspension was prepared by mixing sephadex (Sephadex G-50 superfine; GE Healthcare Life Sciences; Pittsburg, PA) in sterile distilled water. A volume of 300 µl of sephadex suspension was added to a spin column (polypropylene extraction tubes; Qiagen, Hilden, Germany) and centrifuged at 150 g for 1 minute to make a firm bed. Excess water was removed from the spin column by a second centrifugation at 150 g for 1 min. The spin column was moved to a sterile 1.5 ml polypropylene centrifuge tube (Eppendorf North America, Inc., Enfield, CT). Individual PCR reactions were added to sephadex columns and centrifuged at 268 g for 1 minute and the filtrate (purified PCR product) was used for further experiments. Five µl of the product from each PCR reaction was examined by running on a 2% agarose gel stained with Redsafe (iNtRON Biotechnology, Kirkland WA) for DNA visualization in 1X Tris acetate buffer (Thermo Fisher; Waltham, MA) and visualized under UV light in a gel documentation system (Eagle Eye II Cabinet, Stratagene, La Jolla, CA). For PCR products where amplification was confirmed on the agarose gels, subsamples were submitted to the Michigan State University Genomics core (East Lansing, MI USA) for Sanger sequencing.

Table 4.2: Primers used for sequencing three loci in *Alternaria alternata*. The genetic loci used in this study were endoploygalacturanase (*EndoPG*), translation machinery associated protein (*Tma22*) and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*Pgs1*). The sequence on the top and bottom rows of each locus were the forward and reverse primer sequences respectively.

Locus	Sequence (5`-3`)	Source	Annealing Temperature (˚C)	Approximate length of amplicon (bp)
EndoPG	TACCATGGTTCTTTCCGA GAGAATTCRCARTCRTCYTGRTT	Armitage et al. (2015)	55	449
Pgs1	CAGACGCCTGCCGAGTTTTAT CCTTCGTTGATGCGTTTAGG	Armitage et al. (2015)	62	480
Tma22	CAAGCTCTCTCTTTCGCGTC GAGGGAGGCCATGTTCTGCTG	Armitage et al. (2015)	65	402

Construction of the phylogenetic tree for A. alternata isolates

All the sequence data analysis and phylogenetic tree construction were done in Geneious (Biomatters, Inc.; Newark, NJ). The sequence comparison among isolates for *Cyp51* gave limited variation and thus was not used further for genetic diversity comparison. In addition to the *Alternaria* originally collected from sugar beet, potato, blueberry, and dry bean, the DNA sequences of E*ndoPG*, *Tma22* and *Pgs1* loci from 15 *Alternaria alternata* strains which were submitted by Armitage et al. (2015) were obtained from NCBI and included in this analysis. The data from the genome of *A. brassicicola* (GCA_002796735.1, WGS project: PHFN01) obtained from NCBI was used for the outgroup. The DNA sequences obtained from the MSU genomics core were imported into Geneious and the forward and reverse sequence of each gene were assembled using *denovo* assembly, edited and saved as a consensus sequence. Each gene fragment of all the isolates was aligned separately using multi-locus alignment (MUSCLE) and trimmed to get a similar length for all the isolates. The aligned concatenated sequences for all the isolates were used for the construction of a multi-locus phylogenetic tree with Geneious Tree

Builder. Neighbor-joining was used as the tree building method. The bootstrap permutation test was used with 100 replicates to estimate the significance of the branches in the phylogenetic tree.

Results

All isolates collected in the current study produced small spores (<25 µm) in largely unbranched chains on HCV8 (Figure 3.1). The morphological characteristics fit with the description of *A. tenuissima* of Simmons (2007). Based on the revision of the genus of Woudenberg et al. (2013) and Woudenberg et al. (2015) and the genetic sequences obtained, all isolates were identified as *Alternaria alternata*.

The sequence analysis of the three genes showed a high degree of genetic diversity. Isolates analyzed in the current study using three genes showed five genetic clades (Figure 4.1) with the *A. brassicicola* outgroup separate from all *A. alternata* isolates. A sixth clade was observed that consisted solely of isolates included in the *in-silico* analysis. The clades 1 to 6 consisted of seven (two from current study), thirteen (all from current study), six (five from current study), twenty-four (eighteen from current study), ten (all from current study) or three (none from current study) isolates of *A. alternata* respectively (Figure 4.1).

The current study included *A. alternata* isolates collected before 2015 when the ALS issues started to increase and after 2015. The *A. alternata* isolates collected before 2015 were found in all the clades except clades 1 and 6 (neither of which had any sugar beet isolates) and the *A. alternata* isolates collected before and after 2015 did not show a clear separation among the isolates (Figure 4.1).

All the clades consisted of a combination of *A. alternata* collected from different hosts except clade 6 which consisted only of *in-silico* data from strains originally collected from pears. *In-silico* data for *A. alternata* isolates collected from pear also were found in clades 1 and 4

(Figure 4.1). Twenty-two *A. alternata* isolates collected from sugar beet leaves in the current study were separated in to four of the six clades. The majority of *A. alternata* from sugar beets were grouped in clade 4 (9 isolates) followed by clade 5 (5 isolates) and clades 2 and 3 with 4 isolates each (Figure 4.1). Although only eight *A. alternata* isolates from potato were used in the current study they were found in all the clades except clade 6. Three isolates of *A. alternata* from potato were grouped in clade 5, and two in clade 1 with *in-silico* sequences from Armitage et al. (2015) which included *A. alternata* isolates from apple, pear, and tomato (Figure 4.1). Out of eight *A. alternata* isolates from dry bean, the majority (6 isolates) were grouped in clade 4 and the other two isolates were in clade 5. A total of ten *A. alternata* from blueberry were used to construct the phylogenetic tree and eight of them grouped in clade 2 with two isolates in clade 4 (Figure 4.1). Clade 4 had a combination of *A. alternata* isolated from eight different crops.

All the *A. alternata* isolates which were grouped into the 3 clades described in Armitage et al. (2015) had the same pattern of grouping except isolate, FERA 1164. Among the fifteen *Alternaria* isolates which were used for *in-silico* analysis, *Alternaria* isolates collected from apples were grouped in clades 1, 3 and 4 and *Alternaria* isolates from pear were grouped in clades 1, 4 and 6.

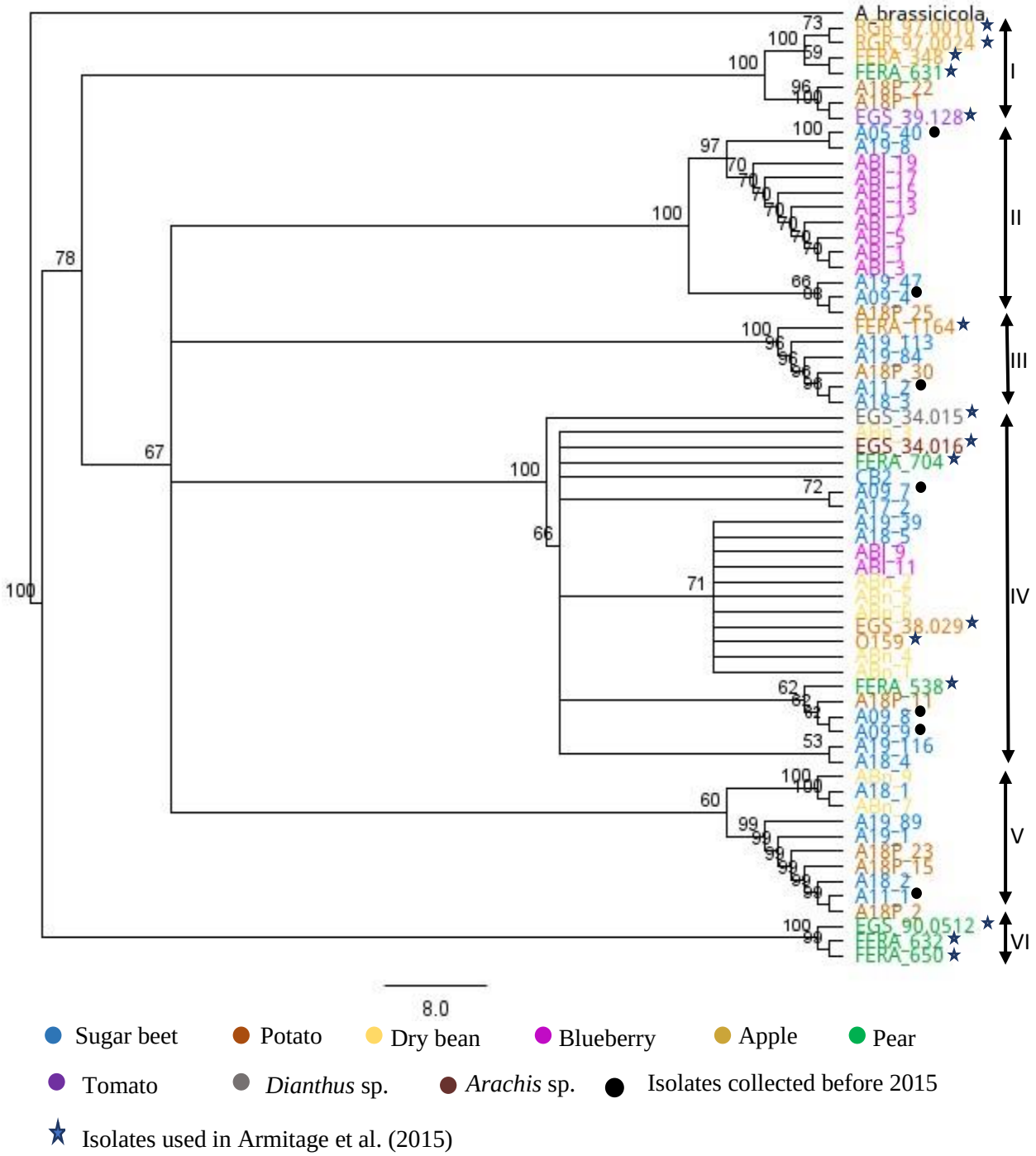

Figure 4.1: A phylogenetic tree for *Alternaria alternata* isolates from Michigan and in-silico sequences based on three genes, endoploygalacturanase (*EndoPG*), translation machinery associated protein (*Tma22*) and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*Pgs1*). The isolates were separated into six potential clades based on bootstrap support of a minimum of 60%. The outgroup, *A. brassicicola* was obtained from NCBI. The colors indicate the host from which isolates were originally collected.

Discussion

All isolates collected in the current study were morphologically "*A. tenuissima*" by the criteria of Simmons (2007) and were in the *A. alternata* clade by genetic analysis (Armitage et al. 2015, Woudenberg et al. 2015). This is consistent with reports of *A. tenuissima* from sugar beet (Khan et al. 2020) and the findings that the primary morphological characters for separating *A. tenuissima* and *A. alternata* (branching or unbranching chain) is not a good morphological character to separate strains into separate monophyletic groups (Armitage et al. 2015, Woudenberg et al. 2015). The isolates collected from Michigan in the current study fell into two of the same *A. alternata* clades as those of Armitage et al. (2015). While all were morphologically *A. tenuissima*, none of our isolates fell into the clade that Armitage et al. (2015) proposed as a potential morphological subspecies *A. alternata* ssp. *tenuissima*. The majority of the isolates fell into the proposed *A. alternata* ssp. *alternata* of Armitage et al. (2015), but two isolates fell into the clade proposed as *A. alternata* ssp. *arborescens*. These results agree with the varied morphology reported within these clades by Armitage et al. (2015) and provide further support that the branching of the spore chains is likely not to be a useful characteristic to separate species or subspecies within this group.

The study agrees with previous work showing high genetic diversity in *A. alternata* (Kakvan et al. 2012, Morris et al. 2000). The phylogenetic tree with *A. alternata* isolates in the current study collected from four different hosts and *in-silico* analysis with additional *A. alternata* isolates used in Armitage et al. (2015) showed up to six potential clades. The *A. alternata* strains used from the clades 1 and 3 in Armitage et al. (2015) showed the same clustering pattern. *A. alternata* taken from clade 2 in Armitage et al. (2015) were also clustered together in a clade in this study except for isolate, FERA 1164 which fell into clade 3 in the

current study, a clade not separated in the Armitage et al. (2015) (Figure 4.1). A possible factor for this difference is that additional isolates may supported separation of a clade.

The phylogenetic tree did not show a clear separation between pre and post 2015 *A. alternata* strains from beet (Figure 4.1). The increasing issues of ALS in Michigan was observed after 2015 (Rosenzweig et al. 2017, Rosenzweig et al. 2019). There are multiple factors that could account for having a change in disease severity such as pathogen factors, environmental factors and host factors (Schoeneweiss 1975, Colhoun 1973). Since no clear separation was observed between pre and post 2015 *A. alternata* isolates in Michigan, the results do not support a change in a pathogen population. However, this might be further confirmed by including more pre 2015 *A. alternata* isolates in a future study, but there is limited availability of such species from Michigan sugar beet.

This phylogenetic analysis indicates that *A. alternata* strains isolated from the four hosts, blueberry, dry bean, sugar beet and potato did not show a clear genetic separation correlating with host (Figure 4.1). This supports a lack of host specificity of strains from the field. This agrees with Andrew et al. (2009) where they used 150 *A. alternata* isolates collected from citrus, pistachio, walnuts and apples and found no association between their phylogenetic clades and the hosts from which they were collected. Analyzing the host specificity and genetic data indicates that there is a potential of having a common *A. alternata* on all the hosts examined, similar to what has been proposed by Nishimura (1980) and Kusaba and Tsuge (1995). Therefore, these results agree with studies supporting a wide host range of *A. alternata* (Meena and Samal 2019, Meena et al. 2013). These results also agree with those of Matic et al. (2020) where twenty-three *A. alternata* isolates were collected from different ornamental plants and both cross inoculation and genetic diversity analysis together showed a lack of host specialization. The results also

agree with our own testing with six of the *A. alternata* isolates used in this analysis that were tested for host specificity by inoculating them on four different hosts (chapter 3). That test similarly found no evidence for host specificity among the eight *A. alternata* on four hosts. Although *A. alternata* from sugar beet, potato, dry bean, and blueberry had no clear genetic differentiation, 8 out of 10 blueberry isolates were grouped together in clade 2 along with five *A. alternata* isolates originally collected from sugar beet and potato (Figure 4.1). This could indicate that certain genotypes of *A. alternata* may predominate on some hosts, but a larger number of isolates would be needed to assess this.

Some of the *Alternaria alternata* strains. that were used for *in-silico* comparison from Armitage et al. (2015) were isolated from apples, pears, and tomatoes. Just as with the isolates from Michigan, there was no clear separation in genotype based on the host from which they were originally collected (Figure 4.1). For example, our clade 1 consists of *A. alternata* from four different hosts, apple, pear, tomato, and potato (Figure 4.1). These crops belong to two different families, the Rosaceae and the Solanaceae, with varying growth habits (perennial versus annual). In addition, other isolates collected from the same hosts (apple and pear) were also clustered with some of our isolates from sugar beet and dry bean in different clades (Figure 4.1). Although several studies have reported on pathotypes such as apple and tomato pathotypes based on the toxins they produced (Li et al. 2013, Okuno et al. 1974, Nakashima et al. 1985, Akamatsu et al. 1997), our results do not show evidence for beets, potatoes, beans and blueberry specific toxin production being probable, although specific testing would be needed to confirm this.

In the current study, all the isolates were collected from Michigan. In Michigan, sugar beet, potato and dry bean are grown in the eastern and central regions, with some production of potato in the northern regions, whereas blueberries are grown in the western region of the lower

Peninsula. No clear separation was found between *A. alternata* originally collected from the more western regions and those collected in the eastern and central regions. The results do not indicate any potential geographic impact on genotypes of *A. alternata* (Figure 4.1). This agrees with previous studies where *A. alternata* strains did not cluster separately based on geographic origins (Adhikari et al. 2021, Andrew et al. 2009, Esfahani 2018, Morris et al. 2000).

Conclusions

In conclusion, 22 *A. alternata* isolates from sugar beet in Michigan showed high genetic variability using three phenotypically informative genetic loci. No major differences were observed among the *A. alternata* isolates from before ALS was observed as a major issue in the state compared to isolates collected when disease issues increased. No clear separation was observed between *A. alternata* strains related to the host from which they were originally collected nor related to collection in the western, central, or eastern part of the state. This supports a lack of host specialization of *A. alternata* among the diverse hosts examined. The isolates from the current study were all morphologically similar but fell into two phylogenetic groups previously suggested as potential morphological subspecies, as well as some other phylogenetic groups not reported in that previous study. Therefore, the results in the current study do not support such separation related to previous descriptions of *A. alternata, A. tenuissima* and *A. arborescens*, instead supporting a single species with diverse morphology, *A. alternata*.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Although Alternaria leaf spot (ALS) in sugar beet is a major foliar disease in some countries in the world, it was a minor issue in the United States before 2015 (Rosenzweig et al. 2017, Rosenzweig et al. 2019, Srivastava 2004, Franc 2009). Since 2015 ALS has caused increasing issues and potential significant yield losses in Michigan sugar beet fields (Rosenzweig et al. 2017, Rosenzweig et al. 2019) and increased incidence has been reported in other states (Khan et al. 2020). Due to its relative lack of importance, little research has been done on ALS in sugar beet (Franc 2009). With the increasing incidence, it is important to learn about host pathogen interactions which may help to manage this disease. To address that, this dissertation investigated the diversity of one of the two pathogens most commonly associated with ALS in sugar beet, *Alternaria alternata*. This was the primary pathogen found in Michigan fields screened for ALS (Rosenzweig et al. 2019) and the pathogen reported in the Red River Valley under the synonym, *A. tenuissima* (Khan et al. 2020).

The first goal of this project was to develop consistent and reliable inoculation methods in the lab, greenhouse or in the field. Having a consistent and reliable inoculation method is useful when studying host-pathogen interactions. A detached leaf assay and an inoculation with the pathogen in a 0.2% malt extract in the greenhouse show potential as inoculation methods for ALS in sugar beet. Only two varieties were tested in these detached leaf and greenhouse inoculation assays. Additional varieties should be included, and results compared for different screening methods to ensure the results give repeatable and reliable disease levels for more genetically diverse materials. In the greenhouse inoculation, only one pathogen strain (P23) was used. But the results indicate that the *A. alternata* isolates collected from sugar beet are a diverse

group (high genetic variability). Therefore, more isolates or an inoculum with combined *A. alternata* isolates should be included for the greenhouse inoculation to check the consistency of the results in greenhouse inoculation.

More optimizations are needed for field inoculation methods such as inoculation timing and testing with different carriers. It also could be helpful to use some varieties more susceptible to ALS. One of the main difficulties that we had in the field inoculation was the interference by the other foliar diseases specially Cercospora leaf spot during ALS rating. The results indicated a significant difference among treatments under lower Cercospora leaf spot pressure compared to where Cercospora leaf spot was severe. Therefore, it would be preferable if future ALS inoculation can be carried out at a location with low Cercospora leaf spot pressure or where Cercospora leaf spot is managed. Further testing may be needed for potential interactions between ALS and Cercospora leaf spot.

The second goal of this project was to examine the host range of *A. alternata* isolates, especially from sugar beet. Two *A. alternata* isolates which were originally collected from each of four different hosts (sugar beet leaves, potato leaves, dry bean pods and blueberry fruits) were cross inoculated into four hosts (sugar beet leaves, potato leaves, dry bean pods, and tomato fruits) and the disease severity as indicated by the area under the disease progress curve (AUDPC) for daily ratings over 5 days from each inoculation was compared. All the *A. alternata* isolates caused lesions on all four hosts tested and the virulence was not strongly associated with the host from which they were originally isolated. These results agree with other reports supporting a wide host range of *A. alternata* (Meena and Samal 2019, Meena et al. 2013, Rotem 1994). For this experiment, only two *A. alternata* isolates were used from each host. Since *A. alternata* is a diverse group, it would be recommended to do additional tests in which more

isolates from each host, as well as additional hosts such as soybean, tomato and hops be included. In addition, further testing should be done with intact plants in greenhouse conditions and in the field to confirm the findings in the detached leaf assay.

Studying the genetic diversity of *A. alternata* isolated from sugar beet was the third objective of this dissertation. The DNA sequences of three genetic regions [endoploygalacturanase (*EndoPG*), translation machinery associated protein (*Tma22*) and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*Pgs1*)] of 22 *A. alternata* isolates originally collected from sugar beet leaves showed a high genetic diversity. An additional 26 isolates from other crops, potato leaves (8 isolates), blueberry fruits (10 isolates) and dry been pods (8 isolates) were also included in the current study. In addition, *in-silico* data for 15 *A. alternata* isolates used by Armitage et al. (2015) were used to construct a phylogenetic tree with *A. brassicicola* as the outgroup. As well as the beet isolates showing high genetic diversity, the combined analysis indicated a high level of genetic diversity among *A. alternata* isolates, similar to what has been found for other crops like tomato, potato and apple (Morris et al. 2000, Esfahani 2018, Sofi et al. 2013). There was no clear separation among *A. alternata* isolates based on the crop from which they were originally isolated. These results support the previous reports of lack of host specialization of *A. alternata* and was further supported by the results obtained when examining the host range in the second objective. There were some possible enhanced levels of genotypes on two of the crops, dry bean and blueberry. Since we used only eight and ten *A. alternata* isolates from beans and blueberry respectively, it is not clear whether this reflects some sampling bias or a genetic predisposition for these hosts. Testing with additional isolates from these crops could clarify this. The high diversity has implications for breeding for resistance, such as the potential need to test for response to diverse genotypes. It also is a

consideration when looking at other management practices such as crop rotation. The current study only included *A. alternata* isolates collected in Michigan fields. It is important to determine whether there is similar diversity in other areas with varied cropping systems, different varieties, and production processes that might vary (e.g: irrigated versus rainfed agriculture). Including more *A. alternata* isolates representing all the counties in Michigan where sugar beets are grown for the analysis could be useful to determine the extent of *A. alternata* genotypes in Michigan. This should be compared with cropping systems, such as rotation crops (potato, wheat, corn, soybean, dry bean, cucumber, etc.). It also could be useful to construct a phylogenetic tree with *A. alternata* isolates collected from different parts or growth stages of sugar beet such as seedlings, leaves and roots. This can indicate whether the *A. alternata* genotypes in different plant parts are similar. This might give useful information to help understand the disease cycle of *A. alternata* and might provide additional options to manage ALS such as fungicide application times and seed treatments. Long term, it would be helpful to compare to diversity and genotypes present in other states and growing regions, especially those with different production practices and those where ALS has historically been a more severe issue. ALS has been a serious disease in areas such as Pakistan, India and Russia (Abbas et al. 2014, Srivastava 2004, Gannibal 2018) while it has been a minor issue in the US and western Europe (Franc 2009). Examination of strains from the different growing areas could provide information about factors that might contribute to this variability in prevalence of the disease. In addition, it is important to understand the genetic variability in diverse areas to determine appropriate materials for screening and other disease management. For example, if strains in eastern Europe and southeast Asia are different than those in western Europe and the US, quarantine might be indicated.

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