INNOVATIVE METHODS FOR DETECTING AND MANAGING *CERCOSPORA BETICOLA*

By

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ABSTRACT

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In many parts of the world, including the Great Lakes region of the United States, Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola*, is a major foliar disease of sugar beet (*Beta vulgaris* subsp. *vulgaris*). CLS management has become challenging in recent years due, in part, to an increase in fungicide resistance. If CLS is to be successfully managed in the future, more information about its epidemiology and alternative management strategies will be necessary. Thus, my first study employed sentinel beets to detect *C. beticola* spores in the field during the first half of the growing season. In both years of the study, spores were detected earlier than any previous reports. The spore levels detected fluctuated during both seasons and were correlated with factors such as rainfall and relative humidity. This information could be used to improve the timing of fungicide applications. The second study examined the potential of bicarbonate salts to be used as fungicides for *C. beticola* management. Ammonium, potassium, and sodium bicarbonate were tested in vitro. Each inhibited the growth of *C. beticola*, with ammonium bicarbonate causing complete growth inhibition at 1% (w/v). Sodium bicarbonate at 2% (w/v) significantly reduced CLS in the greenhouse compared to a control, so it might be useful in the field. The third study tested the effects of heat treatment in the form of a propane torch on *C. beticola* in vivo, with the goal of using such a technique to reduce inoculum levels of the fungus. Heat treatment of infected leaves completely inhibited sporulation and decreased fungal viability by over 80%. The new insights and methods developed from this research have the potential to aid in future CLS management.

This thesis is dedicated to Mom and Dad. Every day you push me to be my best, and you believed in me even when I stopped believing in myself. Without your love and support, none of this would have been possible.

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KEY TO SYMBOLS AND ABBREVIATIONS

°	Degrees
/	Divided by
$	Dollars
=	Equals
*	Multiplied by
%	Percent
α	Alpha
β	Beta
µm	Micrometers
µmol	Micromoles
ABC	Ammonium bicarbonate
AIC	Akaike information criterion
a.m.	Ante meridiem (morning)
ARS	Agricultural Research Service
AZ	Arizona
B1	Media preparation method in which bicarbonate is added to media prior to autoclaving without ethanol, and stored for one day before fungal transfer
C	Celsius
C.	*Cercospora*
CA	California
CAN	Canada
CLS	Cercospora leaf spot

cm	Centimeters
CO	Colorado
Co.	Company
Corp.	Corporation
CT	Connecticut
CYTB	Cytochrome b
DAT	Days after transfer
DIV	Daily infection value
DMI	Demethylation inhibitor
DSV	Disease severity value
E1	Media preparation method in which bicarbonate is added to media after autoclaving with ethanol, and stored for one day before fungal transfer
E8	Media preparation method in which bicarbonate is added to media after autoclaving with ethanol, and stored for eight day before fungal transfer
EBDC's	Ethylene bis-dithiocarbamates
EC_{50}	Half maximal effective concentration
e.g.	exempli gratia (for example)
ELISA	Enzyme-linked immunosorbent assay
ERS	Economic Research Service
et al.	et alia (and others)
F	Fahrenheit
FL	Florida
FRAC	Fungicide Resistance Action Committee
GA	Georgia

GDD	Growing degree days
GPS	Global Positioning System
GRAS	Generally recognized as safe
i. e.	ide est (in other words)
IN	Indiana
IL	Illinois
Inc.	Incorporated
ISR	Induced systemic resistance
km/h	Kilometers per hour
KS	Kansas
l	Liters
LSD	Least significant difference
LLC	Limited Liability Corporation
Ly	Langley
m	Meters
M	Molar
m^2	Meters squared
MA	Massachusetts
MAWN	Michigan Automated Weather Network
ME	Maine
mg	Milligrams
MI	Michigan
mm	Millimeters

MN	Minnesota
MO	Missouri
MSU	Michigan State University
N/A	Not applicable
NASS	National Agricultural Statistic Service
NC	North Carolina
NJ	New Jersey
NM	New Mexico
NS	Not significant
NY	New York
OH	Ohio
PA	Pennsylvania
PBC	Potassium bicarbonate
PCR	Polymerase chain reaction
p. m.	post meridiem (afternoon)
ppm	Parts per million
psi	Pounds per square inch
QoI	Quinone outside inhibitor
REACh	Research and Education Advisory Council
RH	Relative humidity
RPM	Revolutions per minute
s	Seconds
SAR	Systemic acquired resistance

SBC	Sodium bicarbonate
sp.	Species (singular)
spp.	Species (plural)
SRAD	Solar radiation
Temp	Temperature
TN	Tennessee
USDA	United States Department of Agriculture
VA	Virginia
v/v	Volume to volume
WI	Wisconsin
w/v	Weight to volume
x	Multiplied by

CHAPTER 1

CERCOSPORA BETICOLA OF SUGAR BEET: SYMPTOMS, EPIDEMIOLGY, AND MANAGEMENT

History of Sugar Beet Production

For thousands of years, sugar and sugar-containing compounds have been prized commodities (Galloway, 1989; Winner, 1993; Draycott, 2006). There are two primary sources from which pure sugar in the form of sucrose can be obtained in an economically meaningful way, sugar cane (*Saccharum* spp.) and sugar beet (*Beta vulgaris* subsp. *vulgaris* L.) (Cooke and Scott, 1993; Draycott, 2006). Of these two sources, sugar cane is the oldest, being cultivated before recorded history (Galloway, 1989; James, 2004) and with the first documented sucrose extraction occurring around 300 B.C. (Galloway, 1989). Sucrose production from sugar beets has a much shorter history (Winner, 1993; Draycott, 2006). Beets were first domesticated by at least the year 2000 B.C., when they were grown as a leafy vegetable and for medicinal purposes (Winner, 1993; Draycott, 2006; Harveson et al., 2009), although they may have been domesticated as early as 8000 B.C. and collected from the wild even before that (Biancardi et al., 2012). The first attempts to extract sucrose from beet roots were made in the 1740's by the Prussian chemist Andreas Sigismund Marggraf (Winner, 1993; Francis, 2006; Harveson et al., 2009). At the time, this discovery of a second source of pure sucrose was of theoretical importance, but since the concentration of sucrose in the roots used was only 1.6%, it appeared to be an economically impractical source and the discovery was largely ignored (Winner, 1993; Francis, 2006).

Years later, one of Marggraf's students by the name of Franz Karl Achard would continue to work with beet sugar extraction, discovering a variety of beet that was better suited

for sucrose extraction (Winner, 1993; Francis, 2006). After identifying a suitable beet variety, he built the first sugar beet factory in modern day Poland in 1801, and conducted the first successful sugar beet campaign (the time period every year during which a sugar beet factory is processing beets) in 1802 (Winner, 1993; Francis, 2006). The fledgling sugar beet industry received a jumpstart in 1806 when Great Britain imposed a blockade on continental Europe as part of its war against France, preventing the entry of many goods into Europe including cane-derived sucrose (Winner, 1993; Francis, 2006; Harveson et al., 2009; Lartey et al., 2010b). It became politically and economically imperative for the French to find an alternative source of sucrose. In 1811, after being presented with a loaf of sugar made from European sugar beets, Napoleon decreed that 32,000 hectares (roughly 79,000 acres) of French farm land be converted to sugar beet production (Winner, 1993; Francis, 2006; Lartey et al., 2010b). Although the industry would be impacted by market fluctuations, sugar beets had found a permanent place in European and global agriculture (Winner, 1993; Francis, 2006; Draycott, 2006).

Eventually, sugar beet production made its way to the United States. After several failed attempts to start the industry during the 1800's, the first successful sugar beet factory in the United States was constructed in 1870 near the town of Alvarado, California (Winner, 1993; Francis, 2006; Harveson et al., 2009; Lartey et al., 2010b). In the years to follow, sugar beet production would take root in several other states throughout the country, including Michigan. Prior to the first successful factory in California, Michigan was the site of an early attempt to start the industry within the United States, when in 1839 the first commercial sugar beet crop was planted in the state and a factory was built in the town of White Pigeon. However, the factory closed only a few years later due to management difficulties (Schaetzl, 2008; Michigan Sugar Company, 2015a). Interest in sugar beet production in Michigan continued thanks to the

efforts of Dr. Robert C. Kedzie of Michigan State Agricultural College who conducted several experiments on the agronomics and processing of sugar beets (Schaetzl, 2008; Michigan Sugar Company, 2015a). In an effort to encourage the industry, the State Legislature passed a law in 1897 which promised to provide a $0.01 per pound payment to any factory producing beet sugar within the state (Michigan Sugar Company, 2015a). A flurry of factories soon opened, with the first being built by the Pioneer Michigan Sugar Company in the town of Essexville (Michigan Sugar Company, 2015a). Their first successful campaign was in 1898 and soon after, 23 more factories were built across the state (Michigan Sugar Company, 2015a). Over the course of the next century, a series of mergers, buyouts, and closures would leave only one beet sugar company, the Michigan Sugar Company, operating in Michigan (Michigan Sugar Company, 2015a). As of 2018, the Michigan Sugar Company is the third largest beet sugar company in the United States [Michigan Sugar Company, 2015a; Economic Research Service (ERS), 2018a]. It currently is a grower owned cooperative, made up of about 1,000 growers from Michigan and Ontario, Canada (Michigan Sugar Company, 2015a, 2015b). Every year the farmers of the cooperative raise approximately 150,000 acres of sugar beets, which produce between 3.5 to 5 million tons of beets (ERS, 2018b), resulting in roughly one billion pounds of sugar (Michigan Sugar Company, 2015a). The company currently operates four factories in the cities of Bay City, Caro, Sebewaing, and Croswell (Michigan Sugar Company, 2015b). The Caro factory, built in 1899, is the oldest continually operating sugar beet factory in the United States (Michigan Sugar Company, 2015a, c).

Sugar beet production remains an important part of the national and world economy. As of the year 2000, 7 million hectares (about 17.3 million acres) of sugar beets were grown throughout the world, mostly in temperate regions on the continents of North America, South

America, Europe, Africa, and Asia (Draycott, 2006). In the United States, sugar beets are responsible for the majority (55-60%) of domestic sucrose production (ERS, 2018a) and are grown on approximately 1.15 million acres [National Agricultural Statistic Service (NASS), 2018], across 11 states (Figure 1.1) which are divided into 5 distinct growing regions (ERS, 2018a). For many growers, sugar beets are considered a high value crop, with the total cash payments to farmers for sugar beets in 2016/2017 coming to about $1.64 billion (ERS, 2018a). The demand for sucrose continues to rise (Draycott, 2006), and its uses include being an ingredient in food as a source of calories, a sweetener, preservative, and fermentation agent (Galloway, 1989; Cooke and Scott, 1993); an ingredient in pharmaceuticals (Cooke and Scott, 1993); and for ethanol production (Ogbonna et al., 2001). Byproducts from sugar beets, including the leaves (McKay and Pool, 1918), pulp, and molasses (Harland et al., 2006), can be used for animal feed and various other purposes.

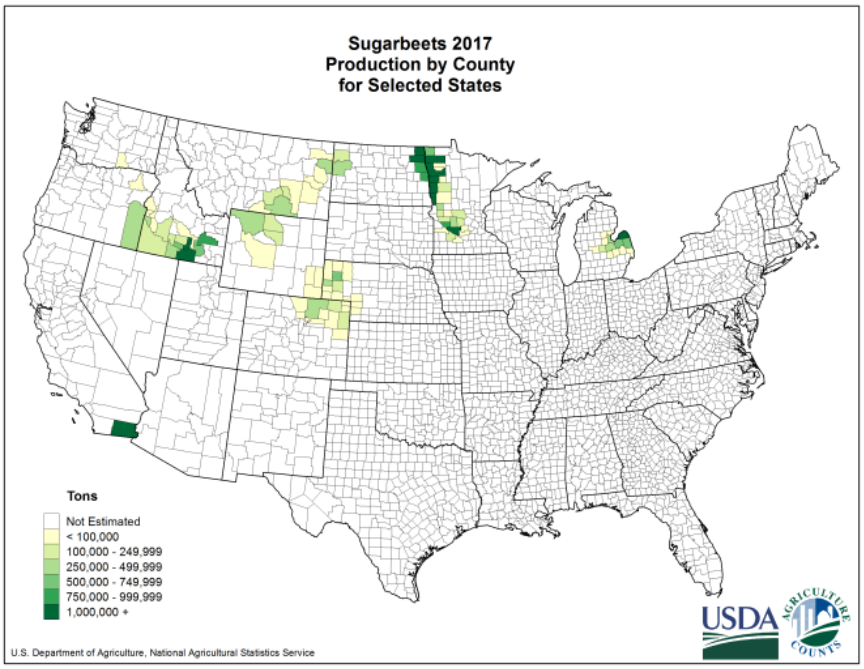


Figure 1.1: Sugar beet production by county in the United States as of 2017. Sugar beets are grown in 11 different states and five distinct growing regions. Most sugar beet production occurs in temperate regions, with the exception of southern California (used with permission, courtesy of the National Agricultural Statistics Service, USDA).

Biology and Classification of Sugar Beet

Domesticated beet is a diverse species, consisting of plants with a wide array of leaf shapes, root styles, and colors (Gil and Vear, 1958; Lewellen et al., 2009). They belong to the class Dicotyledoneae, subclass Caryophyllidae, order Caryophyllales, and family Chenopodiaceae (Lewellen et al., 2009), and can be further divided into four subgroups, which include leaf beet (such as Swiss chard), garden beet (also called table beet or beetroot), fodder beet (mangles), and sugar beet (Gil and Vear, 1958; Lewellen et al., 2009). The sugar beet group is the youngest of these; the product of selection from fodder beet which began only 200 years ago (pages 1 and 2). The storage organ of sugar beet is made up primarily of true root (90%) and has the highest sucrose concentration of all the groups, between 14-20% of the fresh weight (Elliot and Weston, 1993; Lewellen et al., 2009). There is significantly less color variation in sugar beet roots than other beet groups, with white being the only color of commercially available varieties, while the color of the other groups can range from white to yellow, orange, red, and pink (Gil and Vear, 1958; Winner 1993; Lewellen et al., 2009). Domesticated beets were likely selected from wild sea beets, the modern descendants of which are classified as *Beta vulgaris* subsp. *maritima* (Francis, 2006; Lewellen et al., 2009; Biancardi et al., 2012). They probably originated in southwest Asia, from the eastern shores of the Mediterranean to India, and from the Red Sea to the Black and Caspian Seas (Biancardi et al., 2012), but their current range extends along the coast of Europe up to Southern Sweden, west to the Canary Islands, south to North Africa, and east to India (Lewellen et al., 2009).

Sugar beet is a biennial plant, requiring two years to complete its life cycle (Gil and Vear, 1958; Lewellen et al., 2009). The first year consists of vegetative growth, during which time leaves develop in a rosette pattern and the root increases in size as well as sucrose concentration

(Gil and Vear, 1958; Lewellen et al., 2009). Sucrose is stored in the vacuoles of parenchyma cells located around the cambial rings of the beet root, with the area of the six inner most cambial rings being responsible for the majority of sucrose storage (Elliot and Weston, 1993). Beet plants possess a C3 photosynthetic pathway, utilizing the Calvin cycle to fix the carbon that is used for plant growth and sucrose accumulation (Elliot and Weston, 1993; Lewellen et al., 2009). It is partly this photosynthetic pathway which makes beets better suited for cooler, temperate climates as opposed to C4 plants such as sugar cane, which are better suited for tropical climates (Ehleringer and Sandquist, 2010). When beets are grown for sugar production, the plants are defoliated and the roots harvested at the end of the first year (Figure 1.2) (Elliot and Weston, 1993). After being stored for some time, anywhere from 1 to 150 days in Michigan (Michigan Sugar Company, 2015d), the roots are sliced and the sucrose is extracted by means of hot water diffusion (Lewellen et al., 2009). In the case of beets intended for seed production, the beets are grown a second year (Elliot and Weston, 1993). For seed production to occur, the beets must undergo vernalization (an extended cold period, optimally at 6°C) and exposure to long day lengths before their second year of growth (Elliot and Weston, 1993). This change in temperature and light induces them to create a stalk (a process known as bolting) upon which the seeds are produced (Elliot and Weston, 1993).

Figure 1.2: Harvest of sugar beets for sucrose production. When beets are being used for sugar production, they are harvested after their first year.

Background of Cercospora Leaf Spot

Sugar beet production worldwide is hindered by a variety of diseases, both of the foliage and the roots (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Hanson, 2009). Due to various factors, particularly climate, host and pathogen biology, and the agronomic practices being used, every growing region is faced with its own unique set of disease challenges (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Hanson, 2009). In many beet growing regions of the world, including Michigan and Ontario, Canada (together referred to as the Great Lakes region), Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola* Sacc., is the most serious foliar disease of sugar beets (Holtschulte, 2000; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). *C. beticola* has a worldwide distribution, being found most places where sugar beets are grown (Holtschulte, 2000; Jacobsen, 2010), but according to Bleiholder and Weltzien [1972a (in German) as cited by Holtschulte, 2000], its impacts are most keenly recognized in regions that experience warm, wet conditions during the growing season, with average monthly temperatures at or above 20°C and average monthly rainfall of at least 8 cm. It has been reported that under the most severe epidemics, this disease can result in greater than a 40% loss to growers (Jacobsen and Franc 2009). During two especially bad epidemics in 2015 and 2016, it is estimated that the Michigan Sugar Company lost $100 million in revenue to this disease alone (James Stewart, personal communication). Several tactics have been employed in an attempt to curb the effects of CLS, among which are cultural practices, host resistance, and fungicide applications (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). While these management practices have been effective in the past, new challenges, especially the development of fungicide resistance (Bugbee, 1995; Weiland and Smith, 1999; Weiland and

Halloin, 2001; Briere et al., 2003; Kirk et al., 2012; Khan, 2015; Trueman et al., 2017), have made managing CLS more difficult. Therefore, if the impact of CLS is to be successfully mitigated in the future, it will require the integration of both traditional and novel management strategies.

Classification, Biological Description

It is hypothesized that the geographic origin of *Cercospora beticola* is either the Mediterranean area or central Europe (Groenewald et al., 2005). The pathogen was officially discovered and characterized in 1876 by P. A. Saccardo (Lartey et al., 2010b). Around the time of its characterization, *C. beticola* was already an important pathogen, being found throughout Europe, North America, and South America, and causing several destructive epidemics in central Europe (Lartey et al., 2010b). *Cercospora beticola* is a fungus currently classified as a member of the phylum Ascomycota, belonging to the class Dothideomycetes, the order Capnodiales, and the family Mycosphaerellaceae (Jacobsen and Franc, 2009).

Cercospora beticola hyphae are septate, have a diameter of 2-4 µm, and can be either hyaline or light yellowish brown in color (Jacobsen and Franc, 2009). To complete asexual reproduction, it will first form dense masses of dark brown hyphae called pseudostromata which can be as wide as 60 µm (Groenewald et al., 2013). Unbranched conidiophores (Figure 1.3, C) will develop at the apex of these pseudostromata (Jacobsen and Franc, 2009). The conidiophores are brown in color, and range in size from 4-6 µm in diameter and 16-200 µm in length with 1-6 septa (Groenewald et al., 2013). From the conidiophores, conidia which are slender, straight or slightly curved, and hyaline in color are produced (Figure 1.3, A and B) (Pool and McKay, 1916b; Coons et al., 1930; Groenewald et al., 2013). Conidial length and septation vary based on environmental influences (Jacobsen and Franc, 2009), being longer when conditions are warm

(25-35°C) and shorter as the weather becomes colder (10-15°C) (Pool and McKay, 1916b; Franc, 2010). In general, conidia are 2-5 µm wide and 27-250 µm long, with 3-28 septa (Groenewald et al., 2013). Historically, there has been some confusion about the classification of species within the genus *Cercospora*, including *C. beticola* (Crous and Braun, 2003; Groenewald et al., 2006a; Lartey et al., 2010b). According to Crous and Braun (2003), the genus currently contains 659 species.

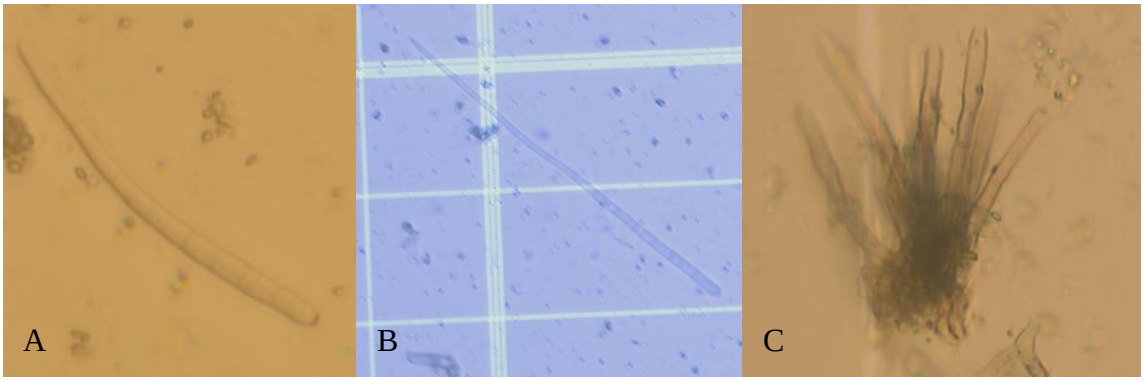


Figure 1.3: Conidia. *Cercospora beticola* conidia (A, 400x magnification and B, 200x magnification) and conidiophores, possibly attached to a pseudostromata (C, 200x magnification).

The asexual reproduction of *C. beticola* by means of conidia has been known for over 100 years (Pool and McKay, 1916b), but no teleomorph (sexual stage) has been observed (Jacobsen and Franc, 2009; Franc, 2010). In a phylogenetic analysis conducted by Goodwin et al. (2001), they found that none of the other *Cercospora* species within the monophyletic group to which *C. beticola* belongs have known teleomorphs. It has been suggested that since none of the other *Cercospora* species in this group have known teleomorphs, it is likely that *C. beticola* lacks this stage as well (Weiland and Koch, 2004). However, several recent studies found genetic evidence which supports the idea that there may be a teleomorph of *C. beticola* (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016). Eight populations were surveyed in these studies, including six from Europe, Iran, and New

Zealand (Groenewald et al., 2006b; Groenewald et al., 2008), one from the Red River Valley region of the United States (Bolton et al., 2012c), and one from New York (Vaghefi et al., 2016). Seven of the eight populations had high levels of genetic variation. Additionally, these seven populations possessed two distinct mating types (MAT1-1-1 and MAT1-2-1), which were present in approximately even proportions within the populations (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016). Bolton et al. (2012b) also provided evidence that these genes are not merely genetic artifacts, but are still functional. In their study, Vaghefi et al. (2016) found that, after clone correction, all the loci which were examined were in linkage equilibrium. This evidence strongly suggests that these populations are undergoing sexual reproduction (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016). If a teleomorph does exist, it would be a *Mycosphaerella* (Stewart et al., 1999; Crous et al., 2000; Goodwin et al., 2001), and such a life cycle stage would have major implications for our understanding and management of *C. beticola*.

Symptoms

The symptoms which *Cercospora beticola* causes on a sugar beet leaf are quite striking (Jacobsen and Franc, 2009). The primary symptom is the formation of necrotic lesions upon tissue above ground, most commonly the leaf blades and petioles (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Bennett and Leach, 1971; Jacobsen and Franc, 2009), but also cotyledons (Pool and McKay, 1916b; McKay and Pool, 1918; Coons et al., 1930; Jacobsen and Franc, 2009), as well as exposed root and crown tissue (Giannopolitis, 1978; Jacobsen and Franc, 2009). The lesions are small, usually between 3-5 mm in diameter, and are generally circular when formed on a leaf blade (Townsend, 1914; Coons et al., 1930; Bennett and Leach, 1971; Jacobsen and Franc, 2009), although their growth may be stopped by leaf veins in which

case they may have a slightly irregular shape (Townsend, 1914). Lesions formed on petioles are similar in most respects to those on the leaf blades but are usually oblong rather than circular (Jacobsen and Franc, 2009), and those on roots are sunken into the tissue (Giannopolitis, 1978). The center of a lesion consists of a combination of dead host tissue and hyphae, and is often a tannish brown or sometimes an ash gray color (Townsend, 1914; Coons et al., 1930; Bennett and Leach, 1971; Weiland and Koch, 2004; Jacobsen and Franc, 2009). Usually, a distinct border is formed around the lesion, which can be darker brown or even a red to purple color, as shown in Figure 1.4, A (Townsend, 1914; Coons et al., 1930; Bennett and Leach, 1971; Steinkamp 1979; Jacobsen and Franc, 2009). The exact color, size, and shape of the lesions can vary based on the variety of beet infected (Steinkamp, 1979; Weiland and Koch, 2004; Franc, 2010) as well as the pathogen isolate infecting it (Ruppel, 1972; Franc, 2010).

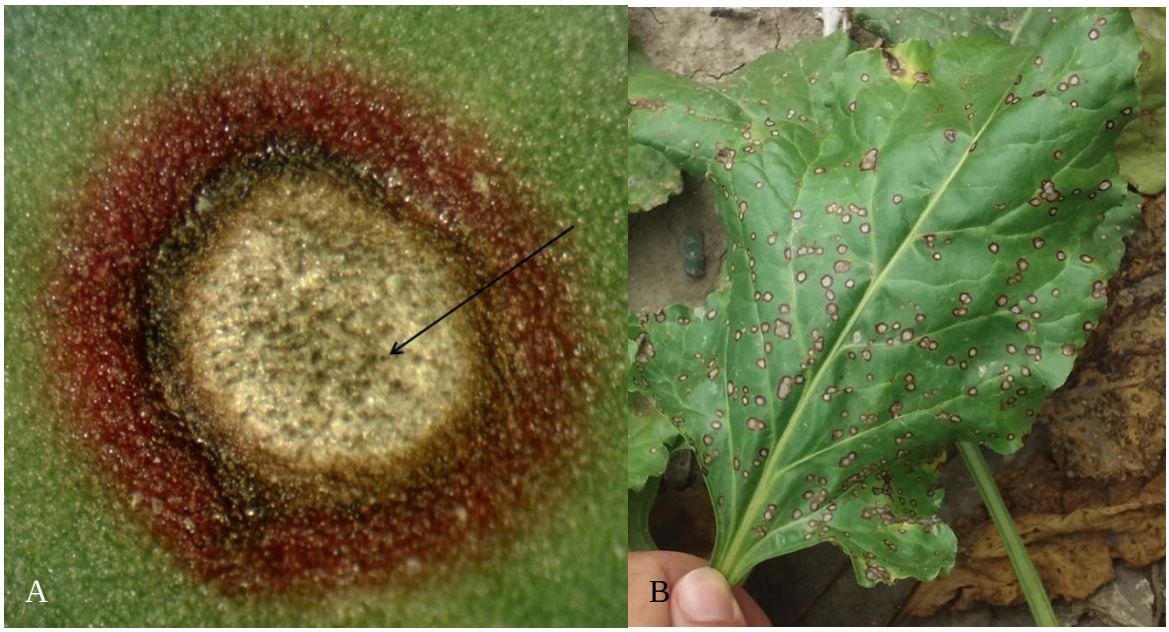


Figure 1.4: Cercospora leaf spot lesions on sugar beet. A, Cercospora leaf spot lesion caused by *Cercospora beticola* on a sugar beet leaf. The lesion is circular, 3-5 mm in diameter, has a red border, tan center, and pseudostromata (indicated by arrow) scattered throughout (25x magnification). B, multiple lesions caused by *Cercospora beticola* scattered across a sugar beet leaf.

Pseudostromata are formed and often are visible throughout the center of a CLS lesion. The apex of these structures, as well as the conidiophores, can protrude from the leaf mostly through old stomata and possibly cracks in the leaf surface (Steinkamp et al., 1979; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Jacobsen and Franc, 2009; Franc, 2010). When observed with the naked eye or under a hand lens, the pseudostromata appear as black "dots" within the lesion (Coons et al., 1930; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Jacobsen and Franc, 2009; Franc, 2010), giving the lesion center an appearance which may be similar to that of a bowl of soup with pepper flakes scattered throughout. When conidia are produced from conidiophores on the end of the pseudostromata (page 8), they appear under a hand lens as silver needles (Figure 1.8, page 23), and can give the entire lesion a sliver or gray color (Townsend, 1914; Coons et al., 1930; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009; Franc, 2010). Of the symptoms and signs produced by an individual lesion, the presence of pseudostromata and silvery, needlelike conidia are the two most important for diagnosis of the disease, as no other foliar pathogen or abiotic stress will form these on sugar beet (Duffus and Ruppel, 1993; Asher and Hanson, 2006).

Yield Impact

It is possible for several CLS lesions to be present on a single leaf at a given time (Figure 1.4, B) (Townsend, 1914; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009). Once enough lesions have formed, they can merge together or coalesce, forming one or more large necrotic areas (Duffus and Ruppel, 1993; Jacobsen and Franc, 2009). Due to the effects of phytotoxins produced by *C. beticola*, the leaf may begin to turn yellow (Bennett and Leach, 1971; Jacobsen and Franc, 2009). The combined effects of these phytotoxins, enzymes, and a large number of lesions, usually between 400-1,000, (Pool and McKay, 1916a, 1916b) will ultimately result in

leaf death (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Weiland and Koch, 2004). After death, the leaf will remain attached to the rest of the plant (Figure 1.5) (Townsend, 1914; Bennett and Leach, 1971; Jacobsen and Franc, 2009). During severe epidemics, a sugar beet plant can lose all of its leaves to this disease, and at times entire fields can suffer from severe defoliation (Figure 1.6) (Coons et al., 1930).

Figure 1.5: Defoliation. A sugar beet plant with a significant number of senesced leaves due to *Cercospora beticola* infection. Notice how the dead leaves remain attached to the beet, and how symptoms are still noticeable on the living tissue.

It is primarily through the destruction of leaf tissue that the disease impacts yield (Townsend, 1914; Coons et al., 1930; Franc, 2010). While CLS will not actually kill the sugar beet plant (Townsend, 1914; Coons et al., 1930), the loss of photosynthetic area reduces the size of the beet roots as well as the total amount of sucrose that is produced and stored (Townsend, 1914; Coons et al., 1930; Smith and Ruppel, 1973; Shane and Teng, 1992). Additionally, when the beet regrows its leaves in response to defoliation, the sucrose reserves it formed prior to infection are depleted; a loss which the beet may or may not be able to recuperate (Coons et al., 1930; Bennett and Leach, 1971; Franc, 2010). It is possible for these regrown leaves to become infected, resulting in further losses (Coons et al., 1930). Beyond the direct reduction in yield,

CLS also can impact the quality of the sugar beets (Townsend, 1914). The phytotoxins created by the pathogen (page 20) are able to cause an increase of impurities in the root, leading to sugar loss during the extraction process (Coons et al., 1930; Smith and Martin, 1978; Shane and Teng, 1992; Jacobsen and Franc, 2009). CLS may lead to damage to the crop after harvest, as it has been suggested that increased levels of CLS were responsible for greater storage losses (Smith and Ruppel, 1971). There is some debate, though, as to the nature of these losses and the true impact of infection by *C. beticola* on beet storage (Linda Hanson, personal communication). For this thesis, disease level is defined as a combination of the incidence and severity of CLS.

Figure 1.6: Infected field. An entire field of sugar beet suffering from a severe Cercospora leaf spot epidemic. Some regrowth has already taken place in this field, but many leaves killed by *Cercospora beticola* are still visible.

Life Cycle of *Cercospora beticola*

Inoculum Sources

Before infection by *Cercospora beticola* may occur, it is necessary for an initial source of inoculum to be present. It has been shown that *C. beticola* primarily overwinters in the form of pseudostromata, as these structures are more resilient to environmental degradation than conidia (Pool and McKay, 1916b). Pseudostromata survive for 1-2 years [Pool and McKay, 1916b;

McKay and Pool, 1918; Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Khan et al., 2008], while conidia were only able to survive for 1-4 months under field conditions (Pool and McKay, 1916b). Infected leaf debris from the previous year's sugar beet crop is a major source of initial inoculum (Townsend, 1914; Coons et al., 1930; McKay and Pool, 1918; Bennett and Leach, 1971), as it is common practice for the leaf tissue to remain in the field after defoliation (Smith, 2001). Long-term survival of the fungus is dependent upon having intact leaf debris [Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Bennett and Leach, 1971; Khan et al., 2008] and the speed at which this debris degrades will increase when it is buried (Khan et al., 2008). Therefore, the depth at which leaf debris is buried in the soil plays an important role in the survival of *C. beticola* [Townsend, 1914; Pool and McKay, 1916b; Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Khan et al., 2008]. In a recent study, Khan et al. (2008) found that when leaf debris was buried either 10 cm or 20 cm in the soil, survival was reduced after 10 months when compared with infected debris left at the surface (buried 0 cm). After 22 months, only the leaf debris on the surface contained any viable fungal structures (Khan et al., 2008). When pseudostromata do survive the winter, the conidiophores may or may not remain viable, so in the spring these structures may have to be regrown before conidia production can begin (Pool and McKay, 1916b).

Even though infected sugar beet leaf debris is a major source of initial inoculum, there are other sources which could potentially contribute to the overall inoculum load. One such source could be infected residue from alternative hosts (Townsend, 1914; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009). *Cercospora beticola* is not host specific (Groenewald et al., 2006a), as it can infect all species of *Beta* and several species outside this genus (Jacobsen and Franc, 2009). These include a number of important garden and field

crops, including lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), celery (*Apium graveolens*), table beet, fodder beet, Swiss chard, and safflower (*Carthamus tinctorius*) (Townsend, 1914; McKay and Pool, 1918; Groenewald et al., 2006a; Lartey et al., 2010a). Some species of flowering ornamental plants, including statice (*Limonium sinuatum*), German statice (*Goniolimon tataricum*), corn daisy (*Chrysanthemum segetum*), and crown daisy (*Chrysanthemum coronarium*) are hosts for *C. beticola* (Groenewald et al., 2006a; Groenewald et al., 2013). Several species of weeds also are hosts, including redroot pigweed (*Amaranthus retroflexus*), common lambsquarter (*Chenopodium album)*, common burdock (*Arctium minus*), broadleaf plantain (*Plantago major*), Pennsylvania smartweed (*Polygonum pensylvanicum*), species of mallow (*Malva* spp.), and bindweed (*Convolvulus arvensis*), just to name a few (Vestal, 1933; Groenewald et al., 2006a; Lartey et al., 2010a).

In addition to plant debris, infected seed also may serve as an inoculum source for *C. beticola* [Townsend, 1914; McKay and Pool, 1918; Coons et al., 1930; Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Bennett and Leach, 1971; Jacobsen and Franc, 2009]. This may have been a more significant avenue for infection in the past, but between measures taken to prevent seed stalks from becoming infected (Bornscheuer et al., 1993; Kockelmann and Meyer, 2006) and modern seed processing (Kockelmann and Meyer, 2006), it is less likely to be an issue for modern sugar beet production. In a 30 year study conducted by India's Plant Quarantine Division of the National Bureau of Plant Genetic Resources, 1,111 different seed samples being imported to India were surveyed for various pathogens, including *C. beticola* (Agarwal et al., 2006). They found 22 pathogens, including ten fungi, but *C. beticola* was never found during the entire 30 years of the study, indicating that infected seed likely is not a major source of *C. beticola* inoculum. Another possible source of inoculum could be a sexual

stage (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016), which may be occurring on one of the aforementioned hosts or a host which is currently unknown (Townsend, 1914; Vestal, 1933; Groenewald et al., 2006a; Jacobsen and Franc, 2009; Lartey et al., 2010a; Groenewald et al., 2013). However, since such a life cycle stage has not yet been confirmed (Jacobsen and Franc, 2009; Franc, 2010), the extent of its importance as a source of inoculum for *C. beticola* and its impact on CLS epidemiology is unknown.

When weather conditions are appropriate, new conidia will be produced by conidiophores on overwintered pseudostromata (Pool and McKay, 1916b). For conidia production, as well as germination and infection, both temperature and relative humidity are critically important, as it is a combination of both factors which enables these fungal processes (Pool and McKay, 1916b; Wallin and Loonan, 1971; Shane and Teng; 1984a, 1984b; Duffus and Ruppel, 1993; Asher and Hanson, 2006). Bleiholder and Weltzien [1972b (in German with English summary) as cited by both Duffus and Ruppel, 1993 and Asher and Hanson, 2006], have shown that under conditions of high relative humidity (98% or greater), sporulation can occur anywhere from 10 to 35°C. Mature conidia are dispersed by rain splash (Pool and McKay, 1916b; Carlson, 1967), the wind (McKay and Pool, 1918; Lawrence and Meredith, 1970), and other factors (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Franc, 2010), generally for 100 meters or less (McKay and Pool, 1918) (conidia dispersal is discussed in depth on pages 23 and 24).

Infection

In the event that a mature conidium lands on a leaf of an appropriate host, infection may occur (Townsend, 1914; Pool and McKay, 1916a, 1916b). This process begins when weather parameters, particularly daytime air temperatures from 27-32°C, nighttime temperatures of at

least 16°C, and relative humidity above 60% for at least 15-18 hours (Pool and McKay, 1916b; Duffus and Ruppel, 1993; Asher and Hanson, 2006) stimulate one or more germ tubes to sprout and begin growing across the leaf surface (Figure 1.7) (Townsend, 1914; Pool and McKay, 1916a; Solel and Minz, 1971; Rathaiah, 1976; Rathaiah, 1977). If the condition of high humidity, i.e. above 80% (Cioni et al., 2014) to 90% (Shane and Teng, 1984a, 1984b), is met, the required temperature and time can fluctuate as shown by Shane and Teng (1984a, 1984b). For instance, a very short time period is required for germination and infection under high humidity and high temperatures (Shane and Teng, 1984a). Pool and McKay (1916a) demonstrated that germination can take as little as 3 hours at 26°C for conidia in water. At lower temperatures, these processes require more time (Shane and Teng, 1984a). According to Jacobsen and Franc (2009), infection can occur at any temperature between 12-40°C, as long as the relative humidity is above 90% or there is moisture on the leaves.

Infection by *C. beticola* occurs primarily through the plant's stomata, either open or closed (Pool and McKay, 1916a; Solel and Minz, 1971; Rathaiah, 1976; Rathaiah, 1977). *C. beticola* germ tubes are hydrotropic, which enables them to find a stoma by growing towards areas of higher humidity (Solel and Minz, 1971; Rathaiah, 1977). As a result of this hydrotropic behavior, infection is encouraged by fluctuations in relative humidity, specifically conditions of high humidity at night, broken up by 1-6 hours of low humidity during the day (Rathaiah, 1976; Rathaiah, 1977). Once a stoma is located, if it is open the germ tube is able to penetrate it directly (Pool and McKay, 1916a; Solel and Minz, 1971; Rathaiah, 1976, Rathaiah, 1977). At one point it was believed that a stoma had to be open in order for successful invasion to occur (Pool and McKay, 1916a), but later studies show that infection is possible even through a closed stoma, with this forced entry being achieved through means of an appressorium (Solel and Minz,

1971; Rathaiah, 1976; Rathaiah, 1977). It is possible for infection to occur on either the adaxial or abaxial portions of the leaf, but as there is a higher concentration of stomata on the abaxial surface (Weiland and Koch, 2004), infection most commonly occurs there (Pool and McKay, 1916a; Weiland and Koch, 2004). It is common for the more mature leaves to become infected first, with the disease spreading to younger leaves over the course of the epidemic (Townsend, 1914; Pool and McKay, 1916a; Jacobsen and Franc, 2009). Pool and McKay (1916b) also reported that green, healthy leaves are more easily infected as opposed to less healthy leaves which have already yellowed. Vereijssen et al. (2004, 2005) suggested that the roots may be an alternative avenue for foliar infection, with the pathogen traveling systemically from the roots to the leaves where symptom development occurs. Attempts to repeat this experiment by Khan et al. (2008) and Jacobsen (2010) were unsuccessful, so if it is occurring, the exact nature and importance of this kind of infection is uncertain.

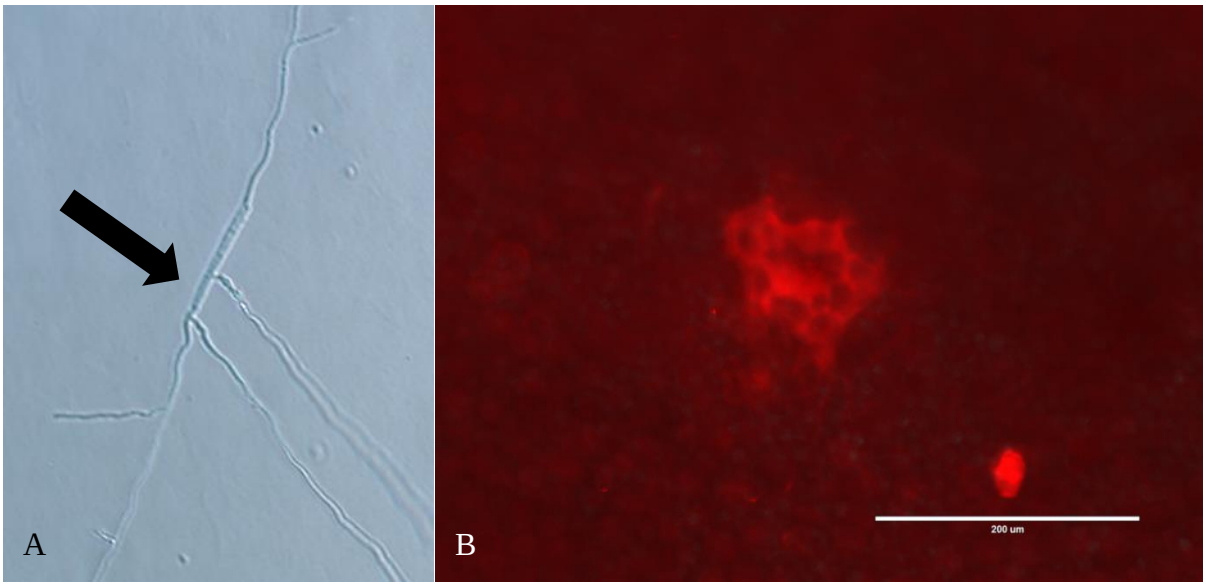


Figure 1.7: Conidium germination and early disease development. A, germinated conidium of *Cercospora beticola*, with four different germ tubes growing from it. The conidium is indicated by the arrow (200x magnification). B, *C. beticola* growth within a sugar beet leaf. At this point in disease development, the fungus is growing asymptomatically, and is only visible here due to the auto fluorescence of the cercosporin (Leisman and Daub, 1992) it has produced (photo credit to L.E. Hanson and D. M. Bublitz).

Cercospora beticola Growth and Disease Development

Upon successful invasion of the leaf by *C. beticola*, hyphae begin to grow intercellularly around the parenchyma cells of the leaf (Pool and McKay, 1916a; Steinkamp et al., 1979) (Figure 1.7, B). For at least 5 days (more commonly 9-13 days), this growth occurs asymptomatically to the naked eye (Pool and McKay, 1916b; Rathaiah, 1976; Steinkamp et al., 1979; Weiland and Koch, 2004; Franc, 2010), but during this time, necrosis of host cells is occurring, beginning with those closest to the site of infection (Pool and McKay, 1916a; Steinkamp et al., 1979). The death of the host cells is accomplished primarily through the actions of the toxins produced by *C. beticola*, particularly cercosporin or beticolins, as well as enzymes *C. beticola* creates, such as cellulase, pectinase, and esterase (Weiland and Koch, 2004). Cercosporin is an especially important virulence factor (Steinkamp et al., 1981; Weiland et al., 2010; Daub et al., 2010). When cercosporin is in the presence of light, it induces the production of active oxygen species which cause significant damage to cellular membranes, resulting in the contents leaking out of the host cell and eventual cell death (Weiland et al., 2010; Daub et al., 2010). Steinkamp et al. (1981) have shown that the injection of cercosporin into a leaf was enough to cause symptoms on sugar beets similar to CLS. Without this virulence factor, the damage caused by *C. beticola* would be greatly reduced (Weiland et al., 2010; Daub et al., 2010). Less is known about beticolins.

Cercospora beticola hyphae primarily grow intercellularly, and while they will not actively invade living host cells, they may passively enter them after necrosis has occurred (Steinkamp et al., 1979). In the early stages of infection, there is a cellular gradient of necrosis, with those cells closer to the site of infection being more damaged than those toward the outside (Steinkamp et al., 1979). Once the lesion is mature, such a gradient no longer exists. Instead,

there is a distinct boundary layer between the cells affected by the pathogen and those which have not been impacted (Steinkamp et al., 1979). Under most circumstances, it takes about 9-13 days after infection for symptoms to appear (Pool and McKay, 1916b; Weiland and Koch, 2004; Franc, 2010), but if the host is particularly susceptible and the temperature ideal, symptoms can be observed as soon as 5 days after infection (Rathaiah, 1976; Steinkamp et al., 1979; Franc, 2010). During lesion development, since fungal growth is occurring within the leaf (Steinkamp et al., 1979), relative humidity no longer is an important weather factor (Shane and Teng, 1985), but air temperature still is, with the optimal being 25-35°C (Jacobsen and Franc, 2009). For many growing areas, initial symptom development usually occurs after row closure (Windels et al., 1998; Enz et al., 1996; Wolf and Verreet, 2002). This usually is the case in the Great Lakes region, as the disease often is found during the first two weeks of July (Michigan Sugar Company, unpublished), although it has been found before row closure, as early as the beginning or middle of June (Wilbur et al., 2018a, 2018b).

Conidia Production

With the formation of a lesion, *C. beticola* will take advantage of the nutrients provided by the late cells of its host to begin producing secondary conidia (Pool and McKay, 1916a, 1916b; Weiland and Koch, 2004). *C. beticola* is a polycyclic pathogen, with several disease cycles occurring in a single year if the weather permits (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010). The production of secondary conidia requires similar warm and wet conditions as primary conidia production (page 17) (Pool and McKay, 1916b; Duffus and Ruppel, 1993; Asher and Hanson, 2006). For the development of multiple, severe epidemics, Mischke [1960 (in German) as cited by Duffus and Ruppel, 1993] reported that the ideal weather conditions would be 3-5 days during which the temperature never falls below 10°C

and there are at least 10-12 hours each day when the relative humidity is 96% or above. However, other studies have shown being too warm (above 40.5°C) can be harmful to the fungus (Pool and McKay, 1916b). Under the proper weather conditions, conidia can be produced on a lesion in 6 hours to 3 days after the lesion is visible (Carlson, 1967; Rossi, 2000; Franc, 2010). While fungal growth varies based on weather conditions (pages 17, 18, and 21) and host variety (pages 28-31), under ideal weather conditions the entire life cycle of *C. beticola* normally is completed in 10-13 days (Coons et al., 1930). Lesions usually reach maximum conidia production 10 days after being formed (Pool and McKay, 1916b). Conidia are created on both surfaces of the leaf; however, it generally is the abaxial surface which produces the largest number of conidia, possibly due to the higher humidity below the leaf (Pool and McKay, 1916b). It often is the youngest lesions which bear the greatest number of conidia, but not always (Pool and McKay, 1916b). Lesions may produce one or several crops of conidia, and some lesions can sporulate for nearly an entire month (Pool and McKay, 1916b). *C. beticola* lesions are quite prolific; when it is taken into consideration that a 5 mm diameter lesion (Jacobsen and Franc, 2009) has an area of approximately 0.1963 cm^2, and Bleiholder and Weltzien [1972b (in German with English summary)] reported that 15,600 conidia can be produced per square centimeter of lesion in 24 hours, that means a single lesion can create as many as 3,062 conidia in a 24 hour time period. To give some perspective as to the number of conidia that can be produced, it has been estimated that there can be 250,000,000 conidia on a single heavily infected sugar beet plant at one time (Pool and McKay, 1916b).

Figure 1.8: Sporulating *Cercospora beticola* lesion. Conidia being produced abundantly from pseudostromata within a *Cercospora beticola* lesion on a sugar beet leaf (63x magnification).

Conidia Dispersal

Cercospora beticola is capable of producing many conidia (Pool and McKay, 1916b), and there are several efficient methods by which it is able to disperse them (Townsend, 1914; Pool and McKay, 1916b; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Franc, 2010). Arguably the most significant method by which these spores are dispersed is by the action of rain or overhead irrigation water (Townsend, 1914; Pool and McKay, 1916b; Carlson, 1967). When the water drops from either of these sources come in contact with a leaf, they are able to separate the conidia from the conidiophore and then move the dislodged conidia to a new location (Pool and McKay, 1916b; Meredith, 1967; Carlson, 1967). A study conducted by Carlson (1967) in which both spore traps and potted sugar beets were used to measure the dispersal of conidia found a strong relationship between the number of spores detected and rain events that occurred during the study. Another avenue for spore movement is the wind (Townsend, 1914; McKay and Pool, 1918; Coons et al., 1930; Meredith, 1967; Lawrence and Meredith, 1970). Detachment of the conidia from the conidiophores can take place directly by the action of the wind or it can be triggered by the strong hygroscopic movements conidiophores undergo when they experience a sudden decrease in relative humidity

(Meredith, 1967; Meredith, 1970). This hygroscopic movement can result in the conidia being violently thrown a few micrometers into the air, just far enough for wind currents to take them away (Meredith, 1967). As compared with other fungal spores, the conidia of *C. beticola* are fairly large (Groenewald et al., 2013). As such, rain splashing and wind currents are normally able to move them over relatively short distances, with most being moved for less than 100 meters (McKay and Pool, 1918).

In addition to these major avenues for spore dispersal, there are other methods by which spore dissemination may occur (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Franc, 2010). For instance, conidia dispersal may occur by means of arthropod movement (Townsend, 1914; McKay and Pool, 1918; Meredith, 1967). Wooly bear caterpillars (*Spilosoma virginica*) (McKay and Pool, 1918) and mites (unknown species) (Meredith, 1967) seem to be particularly well suited for the task, as their setae are able to pick up the conidia and deposit them elsewhere (Meredith, 1967). Another potential means of dispersal could be movement of conidia on the clothes of farm workers as they move throughout a field or from one field to the next (Townsend, 1914; McKay and Pool, 1918). With the decrease in the requirement for manual labor to remove weeds (Francis, 2006) this method may not be as influential as it once was, but agronomists visiting a problem field should keep it in mind. Since infected leaf tissue is a major inoculum source (pages 14 and 15) (Townsend, 1914; McKay and Pool, 1918), any method of transporting such leaves, including equipment (Townsend, 1914), tare dirt, or windblown leaves (Townsend, 1914), could be a means of pathogen dispersal.

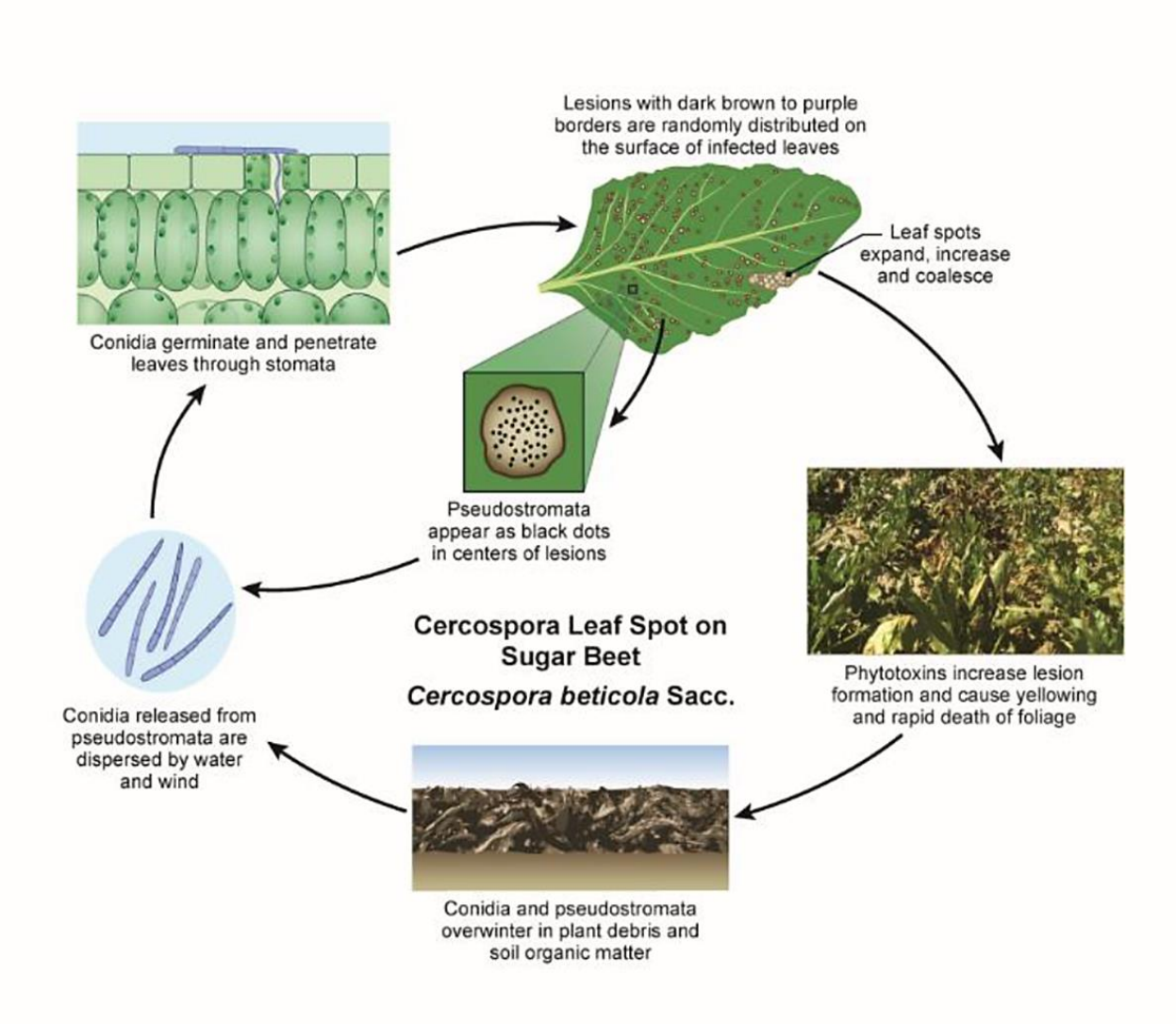


Figure 1.9: Life cycle summary of *Cercospora beticola*. The known life cycle of *C. beticola* begins in the spring with conidia production by overwintered inoculum. When these conidia land on a suitable host, infection may occur if weather conditions are appropriate. Lesions then form on the host, which produce more conidia that serve as secondary inoculum. The fungus overwinters as pseudostromata in infected leaf debris (used with permission, courtesy of L. E. Hanson; illustration by Marlene Cameron).

Management of Cercospora Leaf Spot

Cultural Practices

In order to prevent Cercospora leaf spot from causing significant yield loss to sugar beets, it is best to take an integrated approach to management, using several strategies such as cultural practices, host resistance, and fungicide applications (Duffus and Ruppel, 1993; Asher and

Hanson, 2006; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). Cultural practices encompass several different actions a grower can take to decrease disease levels, some of which involve changes in farming practices. One of the most widely accepted and arguably most successful cultural practices is the use of crop rotation (Townsend, 1914; McKay and Pool, 1918; Coons et al., 1930; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc 2009; Khan and Khan, 2010; Jacobsen, 2010; Michigan Sugar Company, 2018). Crop rotation has been successful in curbing the amount of CLS because it provides temporal separation between the host and pathogen. As stated previously, infected plant residue from the previous year is the most important source for initial inoculum (Townsend, 1914; McKay and Pool, 1918) and *C. beticola* conidia generally spread 100 meters or less (McKay and Pool, 1918). Therefore, rotating to non-host crops gives the residual plant material from an old beet field time to decompose, thus removing the majority of the initial inoculum from a field before the new beet crop is planted (Townsend, 1914; McKay and Pool, 1918; Coons et al., 1930; Khan et al., 2008; Jacobsen and Franc, 2009). In the past, a 2-3 year rotation has been suggested (Townsend, 1914; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009), but for the growers of the Michigan Sugar Company, a 3 year rotation is required, and a 4 year rotation is strongly recommended (Michigan Sugar Company, 2018). Another cultural practice which exploits the relatively low of mobility of *C. beticola* conidia is the spatial separation of a newly planted field from an old beet field (McKay and Pool, 1918). Since most of the conidia move less than 100 meters, separating new and old fields by this distance through the use of a buffer strip, another field, a road, ditch, or fence row could serve to reduce the amount of initial inoculum to which the current crop is exposed (McKay and Pool, 1918).

There are other cultural practices which focus on reducing or eliminating initial inoculum. Since many weeds can serve as alternative hosts for *C. beticola* (Townsend, 1914; Vestal, 1933; Groenewald et al., 2006a; Jacobsen and Franc, 2009; Lartey et al., 2010a; Groenewald et al., 2013), practicing proper weed management in and near the field when beets are grown, as well as the year before, could reduce the impact of this inoculum source (Jacobsen and Franc, 2009). Another method by which inoculum can be reduced is by burying infected plant residue through tillage practices such as moldboard plowing (Townsend, 1914; Pool and McKay, 1916b; McKay and Pool, 1918; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Khan et al., 2008; Jacobsen and Franc 2009; Jacobsen, 2010). In an early study on *C. beticola* survival, it was shown that when infected leaf debris was kept on or near the soil surface (buried 0.0 to 12.7 cm), the fungus was able to survive significantly longer than when it was buried deeper (15.24 to 20.32 cm) (Pool and McKay, 1916b). The recent study by Khan et al. (2008) showed similar results. After 10 months, there was a significant decrease in the survival of *C. beticola* in leaf tissue buried either 10 cm or 20 cm as compared to 0 cm, and after 22 months, only the unburied leaves contained viable fungal tissue (Khan et al., 2008). McKay and Pool (1918) as well as Khan et al. (2008) suggested the mechanism for this reduction in fungal survival is an acceleration of leaf decomposition, as leaf residue is necessary for long term survival of the fungus [Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Bennett and Leach, 1971]. While plowing old beet fields could be an effective way to decrease *C. beticola* inoculum (Townsend, 1914; McKay and Pool, 1918; Khan et al., 2008; Michigan Sugar Company, 2018), it has the disadvantage of potentially leading to an increase in soil erosion (Montgomery, 2007; Michigan Sugar Company, 2018), causing many farmers to shy away from this practice.

Even though deep plowing may not be the most sustainable approach from a soil management perspective (Montgomery, 2007; Michigan Sugar Company, 2018), removing beet leaves from the field altogether could be an effective strategy (McKay and Pool, 1918). Once removed, leaves could be fed to livestock, as passing through the digestive system of animals will effectively kill the fungus (McKay and Pool, 1918). Similarly, experiencing a heat treatment at the proper time and temperature combination, or going through the silage making process can reduce survival (McKay and Pool, 1918). However, current harvest techniques involve leaving foliage in the field after defoliating (Smith, 2001), so reintroducing this strategy would require the development of new machinery as well as new harvest techniques.

Host Resistance

Along with cultural practices, host resistance offers another potent option for management of Cercospora leaf spot (Bennett and Leach, 1971; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Skaracis and Biancardi, 2005; Asher and Hanson, 2006; Jacobsen, 2010; Franc, 2010). For CLS, there are examples of pathogen races and race-specific resistance (Whitney and Lewellen, 1976; Duffus and Ruppel, 1993; Skaracis and Biancardi, 2005), such as sugar beets with the Cb gene having strong resistance to C2 strains of *C. beticola* (Whitney and Lewellen, 1976; Lewellen and Whitney, 1976; Weiland and Koch, 2004; Skaracis and Biancardi, 2005). However, there are concerns over the possibility of race specific resistance being overcome due to its specific nature (Skaracis and Biancardi, 2005) and the high levels of genetic diversity that exist within many populations of *C. beticola* (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016). Because of these concerns, breeding efforts for CLS resistance focus primarily on quantitative resistance (Smith and Gaskill, 1970; Smith, 1985; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Skaracis and Biancardi,

2005; Asher and Hanson, 2006). It is estimated that there are approximately four to five major genes as well as several minor genes which contribute to this quantitative resistance (Smith and Gaskill, 1970; Weiland and Koch, 2004; Skaracis and Biancardi, 2005). Even though this type of host resistance for CLS is incomplete and does not result in immunity to the disease, it still is effective at reducing disease severity (Duffus and Ruppel, 1993; Weiland and Koch, 2004; Skaracis and Biancardi, 2005; Jacobsen, 2010), and because of its quantitative nature, there is a fairly low probability of *C. beticola* overcoming it (Poland et al., 2008).

The host resistance currently used in sugar beets benefits growers not only by increasing yields and decreasing impurities during high disease pressure (Miller et al., 1994; Rossi, 1999), but more importantly slowing the rate of CLS epidemics (Rossi, 1995, 1999; Weiland and Koch, 2004; Franc, 2010). As shown by Rossi et al. (1999, 2000), some of the impacts resistant varieties have on *C. beticola* include a decrease in the number of lesions formed by up to 79%, an increase in the latent period of infection by as much as 12 days, a decrease in lesion size by up to 45%, and a decrease in spore yield of the lesions by up to 35%. Host resistance also has an effect on the conidia produced, as those from lesions on resistant hosts were found to be narrower, containing fewer septa, germinating at a slower rate, and producing shorter germ tubes as opposed to those produced on susceptible hosts (Rossi et al., 2000). For growers, planting a resistant variety may allow them to conduct fewer fungicide applications and provide them with some flexibility in their spraying schedule, as well as aiding in long term inoculum management [Rossi et al., 1999, 2000; Franc, 2010; Jacobsen, 2010; Michigan Sugarbeet Research and Education Advisory Council (REACh), 2016; Michigan Sugar Company, 2018]. However, host resistance should not be viewed by growers as a standalone management strategy (Duffus and Ruppel, 1993; Weiland and Koch, 2004; Skaracis and Biancardi, 2005; Asher and Hanson, 2006;

Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018), but rather as one of the many tools in their CLS management toolbox.

Figure 1.10: *Cercospora beticola* susceptible vs. resistant sugar beet varieties. A comparison of a susceptible sugar beet variety (left) and a resistant variety (right) in the USDA Cercospora leaf spot nursery, which was inoculated to encourage disease development. Strong resistance to *C. beticola* exists in some sugar beet varieties (used with permission, courtesy of L. E. Hanson).

Planting resistant sugar beet varieties is a rather effective CLS management strategy, but the resistant varieties which are currently available are often lower yielding than susceptible varieties (Miller et al., 1994; Weiland and Koch, 2004; Skaracis and Biancardi, 2005). In the past, yield reductions as high as 18% were reported (Miller et al., 1994), but the yield drag may not be as high in modern resistant varieties (Jacobsen, 2010; Gummert et al., 2015). A recent study conducted by Gummert et al. (2015) reported a yield reduction of only 5-7% in resistant varieties when CLS was absent. In the future, the use of molecular techniques may provide novel avenues for developing CLS-resistant varieties of sugar beets, such as the development of genetically modify sugar beets with the ability to detoxify cercosporin (Weiland and Koch, 2004; Daub et al., 2010). Cercosporin is an important virulence factor for *C. beticola* (Steinkamp et al., 1981), so if the impact of this toxin was nullified, the ability of the pathogen to cause economic damage could be substantially reduced (Weiland et al., 2010; Daub et al., 2010). However, since

C. beticola produces other toxins and enzymes beside cercosporin (Weiland and Koch, 2004), the extent to which cercosporin detoxification would impact *C. beticola* virulence has yet to be determined.

Fungicide Applications

While host resistance and cultural practices play a major role in reducing the severity and economic impact of CLS, these strategies alone are not necessarily enough to keep CLS at an acceptable level (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Jacobsen, 2010). Another tactic which has been very successful in helping to manage CLS is the application of fungicides (Khan and Smith, 2005; Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). One of the earliest experiments using fungicides to reduce CLS occurred in 1914, when Bordeaux mixture was successfully used to manage the disease (Townsend, 1914). Since then, several different fungicides have been used against CLS (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). These fungicides can be divided into two major groups, protectant and systemic fungicides (Crowdy, 1971; Lamey et al., 1987; Brent and Hollomon, 2007b; Jacobsen, 2010). Protectants function by preventing infection from taking place, often by causing damage to germinating spores (Crowdy, 1971; Lamey et al., 1987; Jacobsen, 2010). Therefore, if these chemistries are to be effective, it is imperative to apply them prior to infection taking place. Since there is no meaningful movement of these chemicals into or through the leaf, it is especially important to have good leaf coverage during application (Crowdy, 1971; Lamey et al., 1987; Jacobsen 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). For sugar beet production in the Great Lakes region, three classes of protectant fungicides with different modes of action, as designated by their Fungicide Resistance Action Committee

(FRAC) code, commonly are used (FRAC, 2005). These include ethylene bis-dithiocarbamates (EBDC's, FRAC group M3), copper based fungicides (FRAC group M1), and organotins (FRAC group 30) (FRAC, 2005; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). These products are not quite as effective as the systemic fungicides, must be reapplied at shorter intervals (10-14 days), and can leave the foliage exposed to the pathogen should they be washed off or if new growth occurs quickly (Crowdy, 1971; Poindexter, 2007; Michigan Sugarbeet REACh, 2016). However, both the EBDC's and coppers target multiple sites of action within *C. beticola*, which leads to a minimal risk of resistance development to these fungicides (Crowdy, 1971; FRAC, 2005; Brent and Hollomon, 2007b).

Systemic fungicides offer several advantages over protectant fungicides, but also bring their own set of challenges (Crowdy, 1971; Lamey et al., 1987; Brent and Hollomon, 2007b; Jacobsen, 2010). One of the differences that distinguish systemic fungicides from protectants is that they are able to enter the leaf and can be transported to other parts of the leaf or even the plant (Crowdy, 1971; Lamey et al., 1987; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016). Systemic fungicide groups and even different products within those groups can vary in their range of movement within the plant (Jacobsen, 2010), but they all have some degree of internal movement (Crowdy, 1971; Lamey et al., 1987; Jacobsen, 2010). Some types of systemic fungicides can move to and protect new plant tissue (Crowdy, 1971), and most systemic fungicides provide protection from the pathogen for a longer period of time than protectant fungicides (Brent and Hollomon, 2007b), thus allowing for fewer fungicide applications (Crowdy, 1971). Additionally, because of their movement capability, achieving complete spray coverage is not as important for these fungicides as it is for protectants (Crowdy, 1971). Unlike protectants, many systemic fungicides have a so called "curative" quality, which means they are

able to kill the pathogen even after it has infected the plant (Crowdy, 1971; Jacobsen, 2010). The systemic fungicides used for CLS of sugar beet may be able to kill the fungus for 24-72 hours after infection has occurred, depending on the particular fungicide in question (Jacobsen, 2010). Another important quality of systemic fungicides is that they generally have very specific modes of action, usually targeting just one enzyme or biological process (Crowdy, 1971; Brent and Hollomon, 2007b). Having just a single site of action can lead to a greater risk of fungicide resistance (Crowdy, 1971; Brent and Hollomon, 2007b). Some groups of systemic fungicides which have been used against *Cercospora beticola* in Michigan include the benzimidazoles (FRAC group 1), sterol demethylation inhibitors (DMI's, FRAC group 3), and the quinone outside inhibitors (QoI's), also called strobilurins (FRAC group 11) (FRAC, 2005; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018).

Another method which has been used to manage CLS is the application of biological control agents (Collins and Jacobsen, 2003; Jacobsen et al., 2004; Jacobsen, 2010). This management strategy involves the application of a different microorganism to the foliage in a method similar to that of a fungicide application. The microorganism then grows on the plant to the detriment of the pathogen, with disease reduction being achieved by means of direct predation of the pathogen, the production of antagonistic compounds which inhibit pathogen growth, out competing the pathogen for resources or habitat, and/or causing a defense response in the host, either through systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Gerhardson, 2002). In the case of CLS on sugar beets, one example of a biological control is the use of *Bacillus* species to inhibit the growth of *C. beticola* (Collins and Jacobsen, 2003; Jacobsen et al., 2004; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016), but there are others which potentially could be used. In one study, the *Bacillus* sp. being used caused an ISR

which resulted in a decrease of *C. beticola* infection (Jacobsen, 2010). While biological controls generally are not as popular (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018) or as effective as chemical fungicides when used alone (Michigan Sugarbeet REACh, 2016), if they are applied in combination with other management strategies they can help reduce the severity of CLS (Collins and Jacobsen, 2003; Jacobsen et al., 2004; Jacobsen, 2010).

Fungicide Resistance

Historically, fungicides have been an effective management strategy for CLS (Khan and Smith, 2005; Poindexter, 2007). However, the development of resistance by *C. beticola* in many growing areas, including in the Great Lakes region, to several fungicides (Weiland and Halloin, 2001; Kirk et al., 2012; Trueman et al., 2017) has decreased the efficacy of this strategy and has made CLS management as a whole more difficult (Khan, 2015; Michigan Sugarbeet REACh, 2016). The Great Lakes region usually is not the first sugar beet growing region in which *Cercospora beticola* develops resistance to a particular fungicide; such resistance often first has been reported in Greece (Georgopoulos and Dovas, 1973; Giannopolitis, 1978; Karaoglanidis et al., 2000). In the Great Lakes region, the first fungicide class to which *C. beticola* resistance was detected was the benzimidazole group in 1998 (Weiland and Halloin, 2001). The resistance is conferred by a mutation in the gene for β-tubulin (Davidson, 2006; Hanson, 2010). In 2011, the QoI's were the next fungicide group for which resistant *C. beticola* isolates were found in Michigan (Kirk et al., 2012; Bolton et al., 2012a). This was particularly alarming, because these isolates, along with some isolates from Nebraska, were the first cases of *C. beticola* resistance to QoI's in the field worldwide (Kirk et al., 2012). It was shown by Kirk et al. (2012) and Bolton et al. (2012a) that this resistance was due to a mutation in the cytochrome b (CYTB) gene. By 2012, Rosenzweig et al. (2015) showed that resistance to both the benzimidazoles and the QoI's

was widespread in Michigan, being detected in 80% and 67%, respectively, of the samples tested. In 2016, *C. beticola* isolates resistant to the DMI's were found in Ontario (Trueman et al., 2017), and similar resistance has been found in Michigan (Rosenzweig et al., 2017). The level of DMI resistance which is found in the Great Lakes regions is high, with several isolates having EC_{50} values greater than 100 mg/l (Trueman et al., 2017), as compared to the level of resistance in other regions such as Greece and the Red River Valley region of the United States (North Dakota and Minnesota), with EC_{50} values that generally range from 1-10 mg/l (Karaoglanidis et al., 2000; Bolton et al., 2012b).

The majority of the fungicide resistance problems documented in the Great Lakes region have been associated with systemic fungicides. While there is less of a risk for resistance to develop to the protectants (Crowdy, 1971; Brent and Hollomon, 2007b), it is possible for this to occur (Khan, 2015). In the Red River Valley region, a significant amount of resistance had developed to the organotins in 1994 (Bugbee, 1995). This fungicide resistance has a substantial fitness cost (Giannopolitis and Chrysayi-Tokousbalides, 1980), so after the use of organotins was temporarily suspended, the populations became sensitive again. Thus the efficacy of organotins was restored (Secor et al., 2010). There have been no reports of resistance to either the EBDC's or coppers in the Great Lakes region, but there have been reports of EBDC resistance in *C. beticola* equal to or greater than 10 ppm in the Red River Valley (Weiland and Smith, 1999), and equal to or greater than 5 ppm in Colorado, Montana, Nebraska, and Wyoming (Briere et al., 2003). While these are non-field rate levels of resistance, the fact that any level of resistance is occurring is disconcerting, and if it is happening in these growing regions, it is possible it could happen in the Great Lakes region as well. The occurrence of fungicide resistance to so many fungicides highlights two key considerations for future CLS management; the need to develop

new fungicides with different modes of action, and more importantly, the exigency of increasing the utilization of alternative strategies in the CLS management program.

While the occurrence of fungicide resistance makes managing CLS more challenging, there are steps that growers can take to reduce the chances of it occurring and to cope with resistance after it has occurred (Brent and Hollomon, 2007a; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). A few steps which can be taken to reduce the risk of developing additional fungicide resistance include always applying the full label rate, tank mixing fungicides from two different FRAC groups (i.e., with different modes of action), never using a systemic fungicide of the same group back to back and avoiding its use twice in the same year, and increasing the number of fungicide groups which are used (Lamey et al., 1987; Brent and Hollomon, 2007a; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). Including non-fungicide disease management strategies such as plowing beet residue, planting resistant varieties, and using biological controls like *Bacillus* spp. are excellent ways to reduce disease severity as well as slow the development of fungicide resistance by potentially reducing the number of fungicide applications and the pathogen inoculum level (Lamey et al., 1987; Collins and Jacobsen, 2004; Brent and Hollomon, 2007a; Jacobsen, 2010; Michigan Sugar Company, 2018).

There are practices growers can employ to increase the overall effectiveness of their fungicides (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). One of these is achieving proper fungicide coverage of the leaves, which is especially important for protectants (Crowdy, 1971; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). To that end, applicators should use the correct amount of water and pressure when applying, consider using an appropriate adjuvant (particularly spreaders and stickers), and

applications should not be made if the leaves are wet or wilted (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). The timing of fungicide applications is another critical aspect of proper fungicide use (Crowdy, 1971; Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). Since CLS is a polycyclic disease (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010), it is most important to control the disease early in the season to limit the amount of secondary infection (Pool and McKay, 1916a, 1916b; Weiland and Koch, 2004; Poindexter, 2007). As it normally takes 9-13 days for CLS symptoms to develop (Pool and McKay, 1916b; Steinkamp et al., 1979; Weiland and Koch, 2004; Franc, 2010), fungicides should be applied just before infection occurs, not after symptom development (Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018).

Cercospora Leaf Spot Prediction Models

Applying fungicides at the correct time, prior to the development of disease symptoms, can be difficult. Therefore, in order to improve the timing of fungicide applications for management of CLS, several disease prediction models have been developed (Shane and Teng, 1983, 1984a, 1984b, 1985; Shane et al., 1985; Shane et al., 1986; Battilani et al., 1996; Windels et al., 1998; Wolf and Verreet, 2002; Racca and Jorg, 2007; Khan et al., 2007; Cioni et al., 2014; WIN, 2018). While there is some variation between the different models, at their core is a relationship between *Cercospora beticola* and the weather patterns that encourage it to infect, grow, and develop (pages 17, 18, and 21). The first of these models was the Shane and Teng model, created in the Red River Valley during the 1980's in response to a severe CLS epidemic in 1981 (Shane and Teng, 1983, 1984a, 1984b, 1985; Shane et al., 1985; Shane et al., 1986; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). While it has been changed somewhat

since its creation by those implementing the model as well as by researchers (Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014), the basic parts of the model are the same. The initial fungicide application is done when the first CLS lesion is observed in the field or the immediate area (Shane and Teng, 1985; Shane et al., 1985; Shane et al., 1986; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). Subsequent applications use weather data to predict when conditions are optimal for infection by *C. beticola* (Shane and Teng, 1984a, 1984b, 1985; Shane et al., 1985; Shane et al., 1986; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). Based on greenhouse studies conducted by Shane and Teng (1984a) and Wallin and Loonan (1971), the model looks at the number of hours that the relative humidity is above a certain percentage (from 80% to 90%), as well as the average air temperature for those hours to assign a daily infection value (DIV) for that day (Appendix A). In this model, a day is defined as a 24 hour time period, from midnight to midnight, and DIV's can range from 0 to 7 for each day (Shane and Teng, 1984a, 1984b; Windels et al., 1998). Shane and Teng realized that an infection period can span more than one day, so to fully account for the infection risk, DIV's from two consecutive days are added together (the sum of which is called the Cercospora Advisory) (Shane and Teng, 1984a, 1984b; Windels et al., 1998). If the two day total is below 6 there is low risk of infection, if it is equal to 6 the risk is moderate, and if it is 7 or above there is a high risk and a fungicide application is recommended (Shane and Teng, 1984a, 1984b; Windels et al., 1998). To get an accurate humidity and temperature reading, the measurement equipment should be placed within the beet canopy or an appropriate substitute such as a rhubarb patch, because the conditions within the canopy can be quite different from those outside of it (Enz et al., 1996; Windels, 1998). Originally, the humidity required to count an hour in these calculations was 90% (Shane and Teng, 1984a, 1984b), but has since been dropped to 87% (Windels et al., 1998;

Khan et al., 2007), then 85% (Jacobsen, 2010), and in Italy a version of the model called the CLS.Beta model uses 80% relative humidity (Cioni et al., 2014).

In the Great Lakes region, the CLS disease prediction model currently in use is the BEETcast model (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018; WIN, 2018). The exact details of the model have not been published, as the model is proprietary and currently owned by Weather Innovations Incorporated (WIN) (Jacobsen, 2010; WIN, 2018; Michigan Sugar Company, 2018). However, it has been reported that the BEETcast model utilizes both leaf wetness and temperature to assign daily severity values (DSV's) (WIN, 2018; Michigan Sugar Company, 2018). In a given day, DSV's range from 0 to 4, with 0 not being appropriate infection conditions, and 4 being ideal conditions (Jacobsen, 2010; WIN, 2018). Unlike the Shane and Teng model, BEETcast accumulates DSV's, starting on May 15 of every year (WIN, 2018). Prior to the beginning of the season, application thresholds are decided by the growers with the assistance of the Michigan Sugar Company (Michigan Sugarbeet REACh, 2016; WIN, 2018). These thresholds are based on a variety of factors, including the disease risk zone in which a given field is located, the level of host resistance, the fungicides to be used, and the inoculum level to which the field may be exposed (Michigan Sugarbeet REACh, 2016; WIN, 2018). Once the accumulated DSV's reach the first threshold, a fungicide is applied and the accumulated DSV's for the field are set back to zero (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; WIN, 2018). After the accumulated DSV's reach the next threshold, the second application occurs, and the process continues until the end of the season (Michigan Sugarbeet REACh, 2016; WIN, 2018). By using the model to time every fungicide application including the first, growers are able to make one or multiple fungicide applications before symptoms appear, allowing for improved CLS disease

management early and throughout the season (Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). While these are the predominant models in the United States, there also are a few different models which are used in Europe, including CERCOESY (Battilani et al., 1996), the Wolf and Verreet model (Wolf and Verreet, 2002), the CERCBET 3 model (Racca and Jorg, 2007), and the CLS.Beta model (Cioni et al., 2014).

By using prediction models to help time fungicide applications, it is possible to apply fungicides at biologically appropriate times throughout the season rather than arbitrary dates (Shane and Teng, 1985; Lamey et al., 1987; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014; WIN, 2018). Many years, this also will allow growers to perform fewer fungicide applications and still maintain CLS below the level causing economic damage to the crop (Shane and Teng, 1985; Lamey et al., 1987; Shane et al., 1986; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). Decreasing fungicide applications is beneficial to growers and the overall sustainability of farm operations (Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). It is beneficial in an agronomic sense by reducing the selection pressure for fungicide resistance (Khan et al., 2007), as well as environmentally by decreasing the overall pesticide load on the environment (Wolf and Verreet, 2002; Cioni et al., 2014), and is economically beneficial by saving the time and expense involved in fungicide applications (Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). While these prediction models are very helpful in determining the proper time for fungicide applications, none of them are perfect. Therefore, the improvement of disease prediction models should be a priority for research in the future.

Project Summary

This thesis outlines three major research projects designed to help improve our understanding of the biology of *Cercospora beticola*, as well as investigate potential alternative management options for CLS. The first project utilized sentinel beets as a means to determine the time(s) of *C. beticola* spore production and dispersal early in the season, as well as estimating the quantity of spores being released. This data was correlated to several weather variables, with the goal of eventually using this information to improve the disease prediction models. The second project examined the impact of three bicarbonate salts, including ammonium, potassium, and sodium bicarbonate, on the in vitro growth of *C. beticola*. Limited tests also were done to investigate the mechanism of fungitoxicity of these bicarbonates. Based on the results of the in vitro tests, a greenhouse test was conducted to determine if either ammonium or sodium bicarbonate could reduce CLS incidence when used as protectant fungicides on sugar beets. The third project examined the impact of a heat treatment, in the form of a propane burner, on the viability of *C. beticola* in lesions. Both the ability of lesions to sporulate and to grow vegetative hyphae after heat treatment were examined. If such a treatment can cause sufficient damage to CLS lesions, it could potentially be used at the end of the season to reduce the ability of the fungus to overwinter, thus decreasing the amount of initial inoculum. In the future, having an improved disease prediction model which account for the presence of *C. beticola* spores, an alternative fungicide with a different mode of action than those currently being used, and/or a method for decreasing the initial level of CLS inoculum could improve management of CLS and increase the sustainability of sugar beet farms in the Great Lakes region.

CHAPTER 2

POSTING SENTINELS TO DETECT *CERCOSPORA BETICOLA* SPORES

Introduction

In the Great Lakes region of the United States, as well as several other regions throughout North America and Europe, sugar beet (*Beta vulgaris* subsp. *vulgaris*) is an economically important row crop (Draycott 2006; NASS, USDA, 2018). One obstacle to the agronomic production of sugar beet is the occurrence of numerous pathogens which are capable of infecting the crop and causing economically significant loss (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Hanson, 2009). Among these is the fungal pathogen *Cercospora beticola*, which is responsible for causing the disease Cercospora leaf spot (CLS), the most damaging foliar disease of beets in the Great Lakes production area (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). The typical symptoms of CLS include small (3-5 mm) necrotic lesions which generally are circular on the leaf blades and oblong on the petioles (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Bennett and Leach, 1971; Jacobsen and Franc, 2009). These lesions are tan or gray, have a red or brown border (Townsend, 1914; Coons et al., 1930; Bennett and Leach, 1971; Steinkamp 1979; Weiland and Koch, 2004; Jacobsen and Franc, 2009), and have black pseudostromata scattered throughout their centers (Coons et al., 1930; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Jacobsen and Franc, 2009; Franc, 2010; Groenewald et al., 2013). From these pseudostromata, conidiophores can be formed and produce silver, needlelike conidia (Townsend, 1914; Coons et al., 1930; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009; Franc, 2010; Groenewald et al., 2013). CLS is a polycyclic disease, usually with several rounds of infection occurring in a single growing season when weather permits (Townsend, 1914; Pool and McKay, 1916b; Coons et al.,

1930; Franc, 2010). If enough lesions form on a single leaf (from 400 to 1,000 lesions) these, along with toxins and enzymes produced by the fungus, are able to kill the leaf (Townsend, 1914; Pool and McKay, 1916a, 1916b; Coons et al., 1930; Weiland and Koch, 2004). During an epidemic, it is possible for entire plants and even whole fields to become defoliated (Coons et al., 1930).

In years when severe epidemics occur, it is estimated that losses due to CLS can be as high as 40% (Jacobsen and Franc, 2009). These losses are effected by a decrease in the size of the beet root and the percent sugar within the root (Townsend, 1914; Coons et al., 1930; Smith and Ruppel, 1973; Shane and Teng, 1992), along with an increase in impurities (Coons et al., 1930; Smith and Martin, 1978; Shane and Teng, 1992; Jacobsen and Franc, 2009). Additional losses may be incurred during storage as a result of CLS (Smith and Ruppel, 1971). Management of CLS has been accomplished by employing an integrated approach with the use of cultural tactics, host resistance, and fungicide applications (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). Historically, the use of fungicides has been quite successful in managing CLS and has been widely adopted by growers (Khan and Smith, 2005; Poindexter, 2007; Hanson, 2010). In order to maximize the impact of fungicide applications, it is important that they be applied at the proper time (Windels et al., 1998; Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). This is especially true for protectant fungicides which must be applied prior to infection (Crowdy, 1971; Lamey et al., 1987; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018), but also is pertinent for the systemic fungicides which need to be applied within 24-72 hours of infection (Crowdy, 1971; Jacobsen, 2010).

Prior to the mid 1980's, fungicide application timings were based on the reapplication period recommended on the fungicide label, leading to a calendar-based spray schedule (Windels et al., 1998). Such a calendar-based spray schedule does not necessarily coincide with the times when applications would be most beneficial biologically and can result in unnecessary applications (Shane and Teng, 1985; Lamey et al., 1987; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014; WIN, 2018). Today, growers in many beet producing regions where CLS is an issue time fungicide applications based on disease prediction models (Shane and Teng, 1983, 1984a, 1984b, 1985; Shane et al., 1985; Shane et al., 1986; Battilani et al., 1996; Windels et al., 1998; Wolf and Verreet, 2002; Khan et al., 2007; Racca and Jorg, 2007; Cioni et al., 2014; WIN, 2018). In the United States, the two models primarily used include the Shane and Teng model (Shane and Teng, 1985; Windels et al., 1998; Jacobsen, 2010) and the BEETcast model (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018; WIN, 2018). Several additional models are used throughout Europe, including CERCOESY (Battilani et al., 1996), the Wolf and Verreet model (Wolf and Verreet, 2002), the CERCBET 3 model (Racca and Jorg, 2007), and a modified version of the Shane and Teng model (Cioni et al., 2014). These models use a combination of factors such as weather variables, information about the conditions necessary for pathogen infection, and agronomic information for the field and beet variety to allow for biologically-based fungicide applications (Shane and Teng, 1983, 1984a, 1984b, 1985; Shane et al., 1985; Shane et al., 1986; Battilani et al., 1996; Windels et al., 1998; Wolf and Verreet, 2002; Khan et al., 2007; Racca and Jorg, 2007; Jacobsen, 2010; Cioni et al., 2014; WIN, 2018). Model use often will allow growers to use fewer fungicide applications but maintain a comparable level of management to a calendar-based schedule (Shane and Teng, 1985; Lamey et al., 1987; Shane et al., 1986; Windels et al., 1998; Khan et al., 2007; Cioni et al.,

2014; WIN, 2018). When the use of a prediction model does allow for a reduction in fungicide applications, this has economic benefits to the grower as well as a positive environmental impact (Windels et al., 1998; Wolf and Verreet, 2002; Khan et al., 2007; Cioni et al., 2014).

In the Red River Valley and the Western growing regions of the United States, the Shane and Teng model is the primary CLS prediction model in use (Shane and Teng, 1985; Windels et al., 1998; Jacobsen, 2010). With this model, the initial fungicide application is conducted upon observation of the first CLS lesion (Windels et al., 1998), which often occurs around the time of row closure (Enz et al., 1996; Windels et al., 1998; Wolf and Verreet, 2002). Any additional sprays are based on relative humidity, air temperature, and the length of time both conditions occur (Shane and Teng, 1984a, 1984b, 1985; Shane et al., 1985; Shane et al., 1986; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). The model predicts a risk of infection when the relative humidity is 80-87% or above (minimum depends on the region), and air temperatures are between 16 and 35°C for one or more hours (Shane and Teng, 1984a, 1984b; Windels et al., 1998; Khan et al., 2007; Cioni et al., 2014). The model considers these three variables to calculate an infection risk each day, assigning points called daily infection values (DIV's) based on that infection risk, with 0 being low risk and 7 being high risk (Shane and Teng, 1984a, 1984b; Windels et al., 1998). At lower air temperatures, the length of time relative humidity must remain high to gain DIV's increases, but at higher temperatures (up to 34°C), the length of time required to gain DIV's decreases (Appendix A) (Wallin and Loonan, 1971; Shane and Teng, 1984a, 1984b). To determine if a fungicide application is needed, DIV's from two consecutive days are added, and if the sum is 7 or higher, an application is recommended (Shane and Teng, 1984a, 1984b; Windels et al., 1998).

BEETcast is the prediction model used in the Great Lakes region, and functions by a similar premise to the Shane and Teng model but with some modifications (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018; WIN, 2018). While the formulas for this model are proprietary, it is known that the model looks at both air temperature and time, but unlike the Shane and Teng model, uses leaf wetness rather than relative humidity (Jacobsen, 2010; WIN, 2018; Michigan Sugar Company, 2018). The points used in the BEETcast model are called daily severity values (DSV's) and range from 0 to 4, with 0 being low risk and 4 being high risk (Jacobsen, 2010; WIN, 2018). DSV's are accumulated throughout the growing season beginning on May 15, and fungicide applications, including the first, are done when predetermined thresholds are reached (WIN, 2018). Using the model to time every fungicide application has made it possible for growers to apply fungicides prior to the appearance of symptoms (Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). This is important for management of CLS because the fungicides which are being used in the Great Lakes region are most effective if applied prior to infection (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). Not only that, but as a result of the polycyclic nature of the disease (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010), if the first application is delayed until the appearance of the first lesions, these lesions can serve as an inoculum source for secondary infection (Pool and McKay, 1916a, 1916b; Weiland and Koch, 2004), making management much more difficult throughout the rest of the season (Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018).

During the creation of the Shane and Teng model, emphasis was placed on determining the weather conditions required for *C. beticola* infection of sugar beets (Wallin and Loonan,

1971; Shane and Teng, 1984a). The data upon which the model is based was collected during greenhouse tests conducted by Wallin and Loonan (1971) and Shane and Teng (1984a) which involved the inoculation of sugar beet plants with *C. beticola* conidia, followed by incubation at various temperature and time combinations under such a high relative humidity the leaves remained wet. The results of these studies were used to complete the table used to assign DIV's (Appendix A) (Shane and Teng, 1984a). During the studies, the investigators observed infection down to 10°C and up to 35°C, although CLS development at the extremes was substantially reduced (Wallin and Loonan, 1971; Shane and Teng, 1984a). Therefore, the lowest temperature incorporated into the model was 15.6°C (Shane and Teng, 1984a). Other investigators have shown that *C. beticola* growth can be reduced or even cease at temperatures above the optimal range, namely 34.7°C to 36°C (Pool and McKay, 1916b; Groenewald et al., 2005) and that the fungus can be killed at temperatures of 40.5°C and above for several hours (Pool and McKay, 1916b). As such, the current Shane and Teng model reduces the DIV's assigned at 35°C and does not assign any above this temperature, but it does not factor in the potential fungicidal activity of higher temperatures (Shane and Teng, 1984a). The BEETcast model, on the other hand, never takes the impact of above optimal temperatures into account (WIN, 2018). High relative humidity also is important for infection. Pool and McKay (1916b) originally suggested it should be at least 60%, but Shane and Teng tested both a 95% and 90% threshold, settling on 90% for the model (Shane and Teng, 1984a, 1984b). Since the creation of the original model, it has been adjusted to 87% in the Red River Valley (Windels et al., 1998; Khan et al., 2007), 85% in the Western growing regions (Jacobsen, 2010), and 80% in Italy (Cioni et al., 2014).

Spore germination and infection are the only parts of the *C. beticola* life cycle which the Shane and Teng model (Wallin and Loonan, 1971; Shane and Teng, 1984a) and presumably the

BEETcast model directly take into account. Neither conidia production nor conidia dispersal are factored into either model. In the current Shane and Teng model, the presence of the pathogen is only taken into account for the first fungicide application (Windels et al., 1998; Khan et al., 2007), which is triggered by the observance of the first lesion in a field or region. For the BEETcast model, the presence and level of the pathogen are never directly taken into account, although growers can adjust the application thresholds based on their estimate of the amount of inoculum due to factors such as the proximity of the current field to old beet fields and the previous year's level of CLS. (Michigan Sugarbeet REACh, 2016; WIN, 2018). However, such an adjustment is based on an estimate of the amount of pathogen present, and no direct measure of inoculum or pathogen presence is considered.

Less work has been done on determining the proper conditions for conidia production and dispersal than has been done for infection, and the literature is somewhat divided on the conditions needed for these processes. For conidia production, Pool and McKay (1916b) showed that both relative humidity and temperature are important. They postulated that a relative humidity above 60% for at least 12 hours is required, and that an air temperature of 15.5-26.7°C is ideal, but that conidia production may occur from 10-37.8°C. During an *in vitro* study, Coons and Larmer (1929) reported that conidia were produced from 17-34°C. Bleiholder and Weltzien [1972b (in German), with English summary] found conidia production ranged from 10-35°C, with 30°C being optimal. They also reported that a minimum of 88.5% relative humidity was required, with the optimal humidity being 98-100%. While Canova [1959a (in Italian), with English summary] showed that conidia germination could occur down to 15°C, he found that conidia production could occur at lower temperatures than other reports had indicated, ranging from 5-35°C, with 30°C being optimal as others had reported. Similarly, Lawrence and

Meredith (1970) detected conidia during a spore trapping study when the daily average temperature ranged from 5-25°C. In general, the conditions for conidia production are similar to those for infection, but conidia production may occur over a broader range of temperatures.

In addition to conidia production, the dispersal of conidia also is an important part of the CLS disease cycle. During this part of the disease cycle, temperature is not as relevant, and an outside force such as rain splashing (Carlson, 1967), wind (Lawrence and Meredith, 1970), or arthropod movement (Townsend, 1914; McKay and Pool, 1918; Meredith, 1967) is required to move the conidia, although the contribution of these and other dispersal mechanisms is debated. In the case of wind dispersal, a sudden drop in relative humidity can aid the process by causing the conidiophores to undergo hygroscopic movement (Meredith, 1967). This movement causes the conidia to become detached from the conidiophores and launched a few micrometers into the air, just far enough for them to be taken away by air currents (Meredith, 1967).

There have been several studies which have attempted to measure *C. beticola* conidia production and dispersal in the field through the use of various spore traps (Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018). The majority of these studies began once the polycyclic stage of the CLS disease cycle was already occurring (Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2009). One important finding from two of these studies is that a diurnal pattern exists for the aerial concentration of *C. beticola* conidia (Lawrence and Meredith, 1970; Tedford et al., 2018). Both studies showed that the concentration peaked early to mid-day, 10:00 a.m. according to Lawrence and Meredith (1970) and from noon to 6:00 p.m. according to Tedford et al. (2018), while the concentration was the lowest in the early morning, between midnight and 6:00 a.m. (Lawrence and Meredith, 1970; Tedford et al., 2018). Another important trend these studies revealed is that the amount of

conidia present in the environment fluctuates throughout the season based on weather conditions and the disease levels in the area being monitored (Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018). Several studies found a significant relationship between air temperature and the amount of conidia detected (Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018). Khan et al. (2009) and Tedford et al. (2018) both found a significant relationship between the number of conidia in spore traps and disease severity in the field, with R^2 values above 0.90 in both studies. In spite of the strong correlation between aerial conidia concentration and CLS severity, both studies found that the impact of spore concentration varied with location and year. For example, Tedford et al. (2018) found that a concentration of 100 conidia per cubic meter of air was correlated to a disease severity of 80% at one location-year, but in three other location-years, only resulted in a disease severity of 5% to 40%. While this lack of consistent effect is somewhat problematic, it still is possible that if the models were to take conidia production and dissemination into account, either by directly measuring conidia presence or by considering the weather variables needed for production and dispersal, their ability to accurately guide fungicide application timings could be improved.

Incorporating the presence of *Cercospora beticola* spores into the CLS prediction models could be further complicated if a teleomorph (sexual stage) of *C. beticola* exists (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016). It has been well documented that the primary means of *C. beticola* spread during an epidemic is the asexual reproduction of conidia (Pool and McKay, 1916b; Ruppel, 1986; Duffus and Ruppel, 1993; Asher and Hanson, 2006). This form of propagation has been readily observed in the field, and for many years was believed to be the only mechanism for *C. beticola* reproduction (Ruppel, 1986). However, recent studies by Groenewald et al. (2006b, 2008), Bolton et al. (2012c), and

Vaghefi et al. (2016) have found genetic evidence which supports the occurrence of sexual reproduction within this species. While no such teleomorph has been observed (Jacobsen and Franc, 2009; Franc, 2010), it would be classified within the genus *Mycosphaerella* if it does exist (Stewart et al., 1999; Crous et al., 2000; Goodwin et al., 2001). As with other members of the phylum Ascomycota, the teleomorph would be expected to produce ascospores, which would differ from the conidia in terms of their morphology, and potentially the weather conditions needed for their production, seasonal patterns of production, and dispersal mechanisms (Webster and Weber, 2007; Rieux et al., 2014). The presence of a *C. beticola* teleomorph therefore would impact our understanding of the biology and epidemiology of the fungus, and could have major implications for the way in which the pathogen is managed. As far as incorporating the presence of spores into the prediction models, the difference in ascospore appearance in comparison to conidia (Webster and Weber, 2007) could make them difficult to detect by the use of conventional spore traps. Unlike conidia which are long and needlelike (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009; Franc, 2010; Groenewald et al., 2013), ascospores are substantially smaller, have an oblong-oval shape, and have only one septa (Webster and Weber, 2007) This may result in only conidia release being considered, which could lead to the occurrence of disease risk periods that would still be unaccounted for in the models.

The purpose of the current study was to further investigate the production and dispersal of *Cercospora beticola* spores. Particular attention was paid to the first half of the season, as several previous studies have focused on conidia presence during the middle and later parts of the season (Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018). Additionally, Tedford et al. (2018) detected *C. beticola* conidia during the first week of

May, so the current study aimed to determine how early in the season *C. beticola* spores were present. Previous studies, including those conducted by Lawrence and Meredith (1970), Khan et al. (2009), and Tedford et al. (2018), employed spore traps for such investigation and were looking only at conidia, but the current study used sentinel beets, as was done by Carlson (1967). The term "sentinel beets" refers to potted beet plants which were grown in the greenhouse, placed in the field for a length of time, brought back to the greenhouse, and observed for symptom development as live spore traps. This method enabled the detection of both *C. beticola* conidia as well as possible ascospores. When microscopically examining conidia caught in a spore trap, distinguishing between conidia of various *Cercospora* spp. can be a challenge, as their conidia can appear very similar (Groenewald et al., 2005, 2013). Therefore, the sentinel beet method also allowed for improved differentiation between conidia of different *Cercospora* spp. Each lesion detected on the sentinel plants was considered equivalent to a single spore (Carlson, 1967; Vaghefi et al., 2017), either a conidium or ascospore, of *C. beticola* which had been produced, dispersed, and then deposited on the plant in the field. The study was conducted over the course of two field seasons, 2017 and 2018.

During the course of this study, sentinel beets were placed in three types of environments throughout the Michigan sugar beet production area, including old beet fields, woodlots, and marshes. Infected sugar beet leaf debris is a major source of CLS inoculum (Townsend, 1914; Coons et al., 1930; McKay and Pool, 1918; Bennett and Leach, 1971), thus old beet fields were selected to measure the contribution of this source. Another potentially important source of CLS inoculum is infected debris from other hosts, such as weeds like common burdock (*Arctium minus*), common lambsquarter (*Chenopodium album)*, redroot pigweed (*Amaranthus retroflexus*), broadleaf plantain (*Plantago major*), and species of mallow (*Malva* spp.)

(Townsend, 1914; Vestal, 1933; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Lartey et al., 2010a). These species often occur in marshes and woodlots throughout the Great Lakes region (Bryson and DeFelice, 2010), so sentinel beets were placed here to gauge their potential contribution to the early stages of the CLS disease cycle as well as estimate a potential background level for *C. beticola* spores. The marsh and woodlot locations also were included because if the teleomorph of *Cercospora beticola* does exist, it is likely to be occurring on a host present in such an environment, as no sexual stage has been observed on beets in over 100 years of research. High incidence of lesions on the sentinels from one of these other locations might indicate the presence of the teleomorph and provide some insight into a host.

The investigators had several goals throughout the course of this study. The first goal was to determine when and to what extent *C. beticola* spores were present during the first half of the growing season. As previous studies have mainly focused on the mid and late season spore levels (Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018), little currently is known about early season spore production. Tedford et al. (2018) suggested conidia could be present as early as May, so determining just how early and under what environmental conditions they are being produced could provide important information which may allow for early season management of CLS and eliminate the need for growers to wait until the first lesion appears. A second goal was to look at the presence of *C. beticola* spores in different environments in the Michigan beet growing area. This spore data was correlated to weather parameters in an attempt to further examine the conditions which are conducive for spore production and dissemination in the field. The detection of varying levels of spores at diverse environments, times, and weather parameters could provide clues as to the importance of

alternative hosts in the disease cycle as well as furnish indirect evidence for the occurrence of a teleomorph. Another, long-term goal of the project is to use this information on spore production and dispersal to improve the CLS prediction models. This could potentially be done through incorporating the weather parameters conducive for spore production and dispersal into the models, the use of spore traps to directly detect the presence of spores, or even by using sentinel beets as an early warning system. Such information may allow growers to improve the timing of their fungicide applications, resulting in better management of the disease and more sustainable sugar beet production.

Materials and Methods

Preparation of Sentinel Beets

The sugar beet used was a CLS-susceptible United States Department of Agriculture (USDA) germplasm, F1042 (Campbell, 2015). The seed was received untreated, and had only undergone the first polish step. Prior to planting, 25-50 seeds were placed in a 150 ml flask and surface disinfested for approximately 20 minutes with a solution of 0.6% sodium hypochlorite (Clorox Company, Oakland, CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO) in water. The seeds were rinsed three times with sterile, reverse osmosis water, after which approximately 50 ml of 0.3% hydrogen peroxide (diluted with sterile reverse osmosis water from 30% hydrogen peroxide, Sigma-Aldrich) was added to enhance germination (McGrath et al., 2000). The flasks were placed in an incubator shaker (New Brunswick Scientific, Edison, NJ) at room temperature (20-25°C) and 110 RPM. After being allowed to soak for roughly 24 hours, the hydrogen peroxide was drained and 2 µl of metalaxyl (Allegiance FL, Bayer Crop Science, Monheim am Rhein, Germany) was applied to each flask of seed to manage Pythium damping-off. The seed from each flask was planted into potting mix (SureMix Perlite, Michigan Grower Products, Inc.,

Galesburg, MI) contained in a single 2.45 liter plastic nursery pot (Poly-tainer NS300, Nursery Supplies, Inc., Chambersburg, PA). Once they reached the 2 to 4 leaf growth stage, beets were transplanted to 13 liter boxes (Stacking and Nesting Totes, Global Industrial, Port Washington, NY) into which drainage holes had been drilled. Each box contained four beets, with the exception of four weeks from April 29 to May 20 in 2017 which had three beets per box due to poor emergence. The boxes were segregated into groups of 16, herein called batches, based on planting date.

The beets were allowed to grow in a greenhouse which ranged in temperature from 20-30°C degrees. In addition to natural light, they received 12 hours of supplemental light, from 8:00 a.m. to 8:00 p.m., except for the final batch in 2018 from July 2 to 6 when there was a risk of overheating and natural light was sufficient. Beets were watered as needed for optimal growth, usually twice per week early in the season and every other day later in the experiment as the beets became larger. Plants were fertilized with 40 ml of 14-14-14 (Osmocote, Everris, Geldermalsen, the Netherlands) at the 6 to 8 leaf growth stage and 40 ml of 15-9-12 (Osmocote Plus, Everris) at the 12 to 16 leaf growth stage. In both 2017 and 2018, thrips were present in the greenhouse, causing some damage to the plants. They were managed by employing predatory mites (*Tyrophagus putrescentiae*, Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands; *Amblyseius cucumeris*, Beneficial Insectary, Guelph, Ontario, Canada), a mixture of BotaniGard (*Beauveria bassiana*, Laverlam International Corp., Butte, MT) and M-pede (potassium salts of fatty acids, Gowan, Yuma, AZ), and a mixture of Akari (Fenpyroximate, SePro, Carmel, IN) and Mainspring (Cyantraniliprole, Syngenta, Basel, Switzerland). Although some thrip damage was visible, there is no evidence it had a significant impact on the results of the experiment.

Sentinel Beet Locations

Beets were allowed to grow under greenhouse conditions at least until they reached the 12-16 leaf growth stage, afterwards referred to as "mature". Within a mature batch, each box of beets was randomly assigned to one of 14 field sites (pages 58-60), the untreated control, or the treated control. Once at a field location, boxes were secured by burying them so the soil line of the box approximately matched the soil line of the field (Figure 2.1). To reduce damage caused by various herbivores, a cage made out of chicken wire was fastened to the top of each box. For a few high risk locations, a secondary wire cage made out of chicken wire and the bracket of a 250 gallon spray container was placed around the beets already enclosed by the primary cage (Figure 2.1). Weather measuring equipment, including a weather station (Watchdog Model A150, Spectrum Technologies, Inc., Aurora, IL) for measuring air temperature and relative humidity, and a rain gauge (AcuRite, Chaney Instrument Company, Lake Geneva, WI) for measuring rainfall were placed within a meter of the box at each location (Figure 2.1).

A B

Figure 2.1: Example field locations. A is the typical set up for one of the sentinel beet sites. This particular site is in an old beet field. At each site, the box containing mature beets (12-16 leaf growth stage or older) was buried so the soil line in the box matched that of the field. The beets were inside a wire mesh cage to protect them from herbivores. B shows a woodlot site, at which beets were placed inside a secondary cage due to high herbivore pressure. At each site, a weather station (red arrows) and rain gauge (blue arrows) were present within a meter of the beets.

During 2017 and 2018 sentinel beets were placed in three different environments throughout the Michigan sugar beet production area, including fields that were planted to beets the year before, woodlots, and marshes. In each season, beets were placed at a total of 14 sites, each of which was a specific environment within a particular location (summary of sites provided in Tables 2.1 and 2.2). Six locations were selected (Figure 2.2), each containing multiple environments (Tables 2.1 and 2.2). During the 2017 season, there were six old beet fields, five woodlots, and three marshes. In response to the results from 2017, most of the marshes were eliminated in 2018, resulting in eight old beet field sites, five woodlots, and one marsh (Tables 2.1 and 2.2).

Location Information, 2017									
General Information						Agronomic Practices			
Location	Environment	Site Name	GPS Quardinates	MAWN Station	MAWN Station GPS Coordinates	CLS Pressure	Time of Harvest	Tillage	Current Crop
Akron	Beets	Akron Beets	43.601152, -83.535309	Fairgrove	43.5276, -83.4864	Low	Late	Deep Ripped	Soybeans
	Woods	Akron Woods	43.592991, -83.497374			N/A			
	Marsh	DNR Marsh	43.698082, -83.538920			N/A			
Bublitz	Beets	Bublitz Beets	43.557235, -83.559300	Fairgrove	43.5276, -83.4864	High	Late	Deep Ripped	Corn
	Woods	Bublitz Woods	43.545059, -83.572760			N/A			
Fairgrove	Beets	Fair Beets	43.541166, -83.563098	Fairgrove	43.5276, -83.4864	Moderate	Late	Chisel Plowed	Soybeans
	Woods	Fair Woods	43.543166, -83.554344			N/A			
Frankentrost	Beets	Frank Beets	43.407699, -83.80193	Richville	43.4995, -83.6980	Low	Late	Chisel Plowed	Soybeans
	Woods	Frank Woods	43.396349, -83.799325			N/A			
MSU	Beets	MSU Beets	42.706724, -84.472917	MSU HTRC	42.7110, -84.4760	Moderate	Late	None	Soybeans
	Woods	MSU Woods	42.689062, -84.468098			N/A			
	Marsh	MSU Marsh	42.689532, -84.473122			N/A			
Van	Beets	Van Beets North	43.559443, -83.713465	Munger	43.5617, -83.7541	High	Late	Deep Ripped	Corn*
	Marsh	Van Marsh	43.579041, -83.692947			N/A			

Table 2.1: Location information about 2017 sentinel beet sites. For each of the sentinel beet sites in 2017, general location information is shown, including the location name, environment, site name, the approximate global positioning system (GPS) coordinates of the site, the nearest MAWN (Michigan Automated Weather Network) station, and the GPS coordinates of that station. Three environments are listed: "beets" indicates a field where beets were grown the previous year, "woods" indicates a woodlot, and "marsh" indicates a wetland area with standing water and aquatic vegetation. For each of the old beet field sites, data about the agronomic practices for the sugar beet crop the year before are given, including a grower rating of Cercospora leaf spot (CLS) pressure (low, medium, or high), the time of harvest (early, which was before permanent piling, or late, which was during permanent piling; Michigan Sugarbeet REACh, 2013), the tillage done after the beets were harvested, and the crop being grown while sentinel beets were present (current crop). *Irrigated fields.

Location Information, 2018									
General Information						Agronomic Practices			
Location	Environment	Site Name	GPS Quardinates	MAWN Station	MAWN Station GPS Coordinates	CLS Pressure	Time of Harvest	Tillage	Current Crop
Akron	Beets	Akron Beets	43.594078, -83.544991	Fairgrove	43.5276, -83.4864	Medium	Early	Deep Ripped	Soybeans
	Woods	Akron Woods	43.592991, -83.497374			N/A			
Bublitz	Beets	Bublitz Beets	43.557770, -83.548087	Fairgrove	43.5276, -83.4864	Moderate	Late	Deep Ripped	Corn
	Beets	Ernie's Beets	43.542646, -83.578607			Moderate	Late	Deep Ripped	Soybeans
	Woods	Bublitz Woods	43.545059, -83.572760			N/A			
Fairgrove	Beets	Fair Beets	43.544332, -83.554024	Fairgrove	43.5276, -83.4864	Moderate	Late	Chisel Plowed	Soybeans
	Woods	Fair Woods	43.543166, -83.554344			N/A			
Frankentrost	Beets	Frank Beets	43.404139, -83.799939	Richville	43.4995, -83.6980	Low	Late	Chisel Plowed	Soybeans
	Woods	Frank Woods	43.396349, -83.799325			N/A			
MSU	Beets	MSU Beets	42.684256, -84.487365	MSU HORT	42.6734, -84.4870	Low	Late	None	No Crop**
	Woods	MSU Woods	42.689062, -84.468098			N/A			
Van	Beets	Van Beets North	43.558778, -83.714738	Munger	43.5617, -83.7541	High	Early	Deep Ripped	Soybeans*
	Beets	Van Beets South	43.557478, -83.713725			High	Early	Deep Ripped	Corn*
	Marsh	Van Marsh	43.579041, -83.692947			N/A			

Table 2.2: Location information about 2018 sentinel beet sites. For each of the sentinel beet sites 2018, general location information is shown, including the location name, environment, site name, the approximate global positioning system (GPS) coordinates of the site, the nearest MAWN (Michigan Automated Weather Network) station, and the GPS coordinates of that station. Three environments are listed: "beets" indicates a field where beets were grown the previous year, "woods" indicates a woodlot, and "marsh" indicates a wetland area with standing water and aquatic vegetation. For each of the old beet field sites, data about the agronomic practices for the sugar beet crop the year before are given, including a grower rating of Cercospora leaf spot (CLS) pressure (low, medium, or high), the time of harvest (early, which was before permanent piling, or late, which was during permanent piling; Michigan Sugarbeet REACh, 2013), the tillage done after the beets were harvested, and the crop being grown while sentinel beets were present (current crop). *Irrigated fields. **No crop is listed for this site, as it was a research plot that was going to be planted to dry beans. The current experiment ended prior to a crop being planted, although there were several beets which had overwintered present in that field.

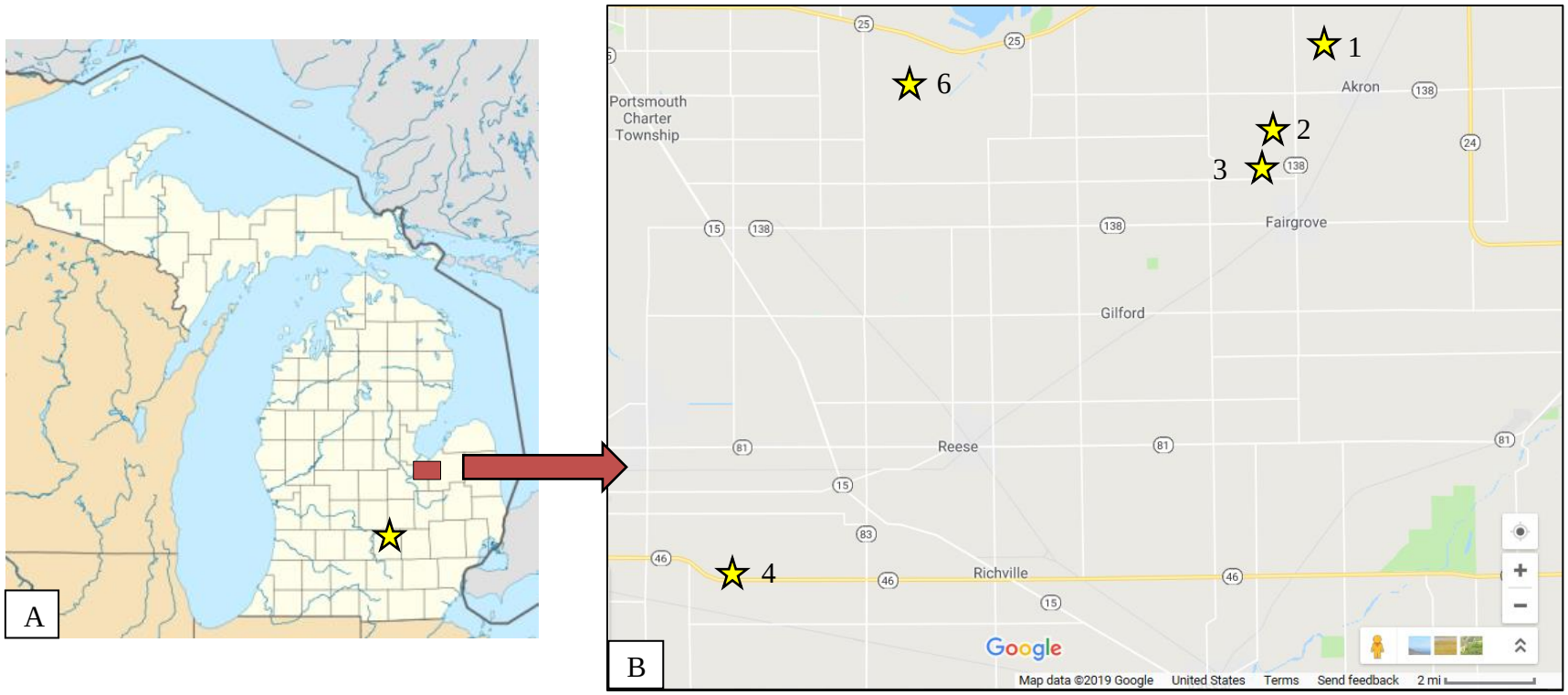


Figure 2.2: The location of sentinel beets in 2017 and 2018. In both years, beet boxes were placed in a total of six locations in the growing area, including East Lansing on farms owned by Michigan State University and on growers' land in the Saginaw Valley region. Map A shows the two general geographic regions in the state where sentinel beets were located, and map B is an expanded view of the sentinel locations in the Saginaw Valley. Each location is marked with a star, including Akron (1), Bublitz (2), Fairgrove (3), Frankentrost (4), MSU (5), and Van (6). Multiple sentinels (either in multiple or the same environments) were placed at every location. One site from 2017, the DNR marsh, is not shown, as it was further north than is shown on the map, at the Fish Point Wildlife Refuge in Akron Township. (Map A is used under the Creative Commons Attribution 3.0 Unported license, Alexrk2, 2009, https://commons.wikimedia.org/wiki/File:USA_Michigan_location_map.svg. Map B is used under the principle of "fair use", per the Google Maps & Google Earth General Guidelines; Map data: Google, 2019, https://www.google.com/maps/@43.4929766,-83.7799544,11.3z. In both images, the arrow, stars, box, and letter and number caption were added to the images.)

After being in the field for one week, each box of sentinel beets was collected and replaced with a new box from the greenhouse. In 2017, the exchange happened over the course of two days, with the MSU marsh, woodlot, and beet field sites being exchanged a day before the other 11 sites. In 2018, the exchange was conducted in a single day. Beet boxes were transported to and from the field in an enclosed vehicle. During periods of warm weather, the back of the vehicle was opened during breaks to prevent the beets from overheating. Rainfall data for the week was recorded during the exchange. Individual beet boxes were swapped on the same day of the week in both 2017 and 2018, with the exception of the exchange on June 17, 2017. This exchange was delayed by one day, so all sites for that week except for the MSU marsh, woodlot, and beet field were in the field for 8 days, and the next batch was in the field for only 6 days. In addition, the final set of beets (the week of July 8) at the Frankentrost woodlot and beet field sites in 2017 were in the field for an extra day. Both years, 13 batches were placed in the field. In 2017, the first beets were placed April 14, and the last were picked up July 16. In 2018, the first batch was placed April 11, and sustained severe damage from freezing temperatures. As a result, they were not used for data collection, so only 12 batches were used in 2018. Beets were in the field from April 11 until July 13 during 2018.

Infection and Disease Development Conditions

Once beets were retrieved from the field, they were taken back to the greenhouse and within 24 hours were placed in a humidity chamber providing appropriate conditions for *C. beticola* infection and symptom development (Wallin and Loonan, 1971; Shane and Teng, 1984a; Jacobsen and Franc, 2009). The humidity chamber was located within a walk-in cold room (Harris Environmental Systems, Andover, MA) which was 280 cm tall, 345 cm wide, and 742 cm long. The cold room provided greater control over the weather variables than was

possible in the regular greenhouse space, keeping the temperature at a constant 24.1°C. Light within the cold room was provided by six sodium halide bulbs, set on a 16:8 hour light/dark cycle. The photosynthetic photon flux density of the light within the cold room was approximately 30 µmol/(m^2*s), and within the humidity chamber it was approximately 20 µmol/(m^2*s). The humidity chamber itself was constructed out of metal carts (Super Erecta Shelving, InterMetro Industries Corp., Wilkes-Barre, PA), with 6 millimeter clear plastic sheeting (HDX, Home Depot, Atlanta, GA) for the walls, ceiling, floor, and doors of the chamber. In 2017, the chamber was 75 cm tall, 140 cm wide, and 183 cm long (Figure 2.3), and in 2018 was expanded to be 183 cm wide. The floor of the chamber was covered with newspaper which was kept wet to increase humidity, and two humidifiers were kept inside the chamber. In 2017, two 5.22 liter humidifiers (Air Innovations, model HUMID16-WHT, Miramar, FL) were used, and in 2018, two 4.54 liter humidifiers (one Hunter, model QLS-05, Memphis, TN; one Vicks, model V4600, Cincinnati, OH) were used. The humidity chamber was efficient at keeping the beets at a high relative humidity, consistently above 95%, as shown by a pair of data loggers at the end of season.

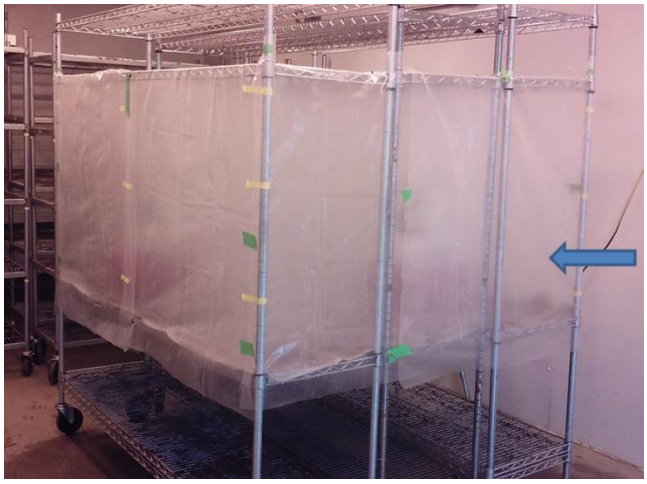


Figure 2.3: The 2017 humidity chamber used for incubation of sentinel beets. The chamber was made from metal carts and 6 mm plastic sheeting. Each humidity chamber contained two humidifiers. Areas where the plastic could be folded up to allow access (doors) were located at the front (indicated by the arrow) and the back of the chamber.

For every batch of sentinel beets, there was one box of beets that served as an untreated control, and another that served as a treated control. The treated control was inoculated with *Cercospora beticola* as described later, and served to show that conditions were appropriate for infection of the beets within the humidity chamber as well as for disease development within the greenhouse. Each year, a different system for preparing inoculum was utilized. In 2017, *C. beticola*, strain "Range A", obtained from the USDA-ARS culture collection, and originally isolated from symptomatic sugar beet in Michigan (Goodwill, unpublished) was grown on plates of half strength, clarified V-8 juice (Campbell Soup Company, Camden, NJ) agar (Miller, 1955). After starting several plates from storage, plates to be used for inoculum were started two weeks prior to inoculation by transferring hyphal plugs or 100 µm of a conidia solution from the original plates to new half strength V-8 plates. These plates were kept on a light bench at room temperature, approximately 25°C. In addition to the plates, 50 ml shake cultures consisting of a solution of 10% clarified V-8 juice in water were started one week prior to inoculation. The shake cultures were kept in an incubator shaker as above. To prepare the inoculum, the plates were flooded with sterile reverse osmosis water, scrapped with an L-shaped bacterial cell spreader (VWR International, Radnor, PA) to remove mycelium and conidia, and the suspension of fungal material was collected. The material collected from six plates and two shake cultures were combined, and mixed in a blender (Waring commercial blender, Waring Products, Inc., Torrington, CT) to ensure hyphal fragments were fine enough to be sprayed onto the leaves. The fungal suspension was then brought to 250 ml in water, and 0.25 ml of Tween 20 (Sigma-Aldrich) was added. The resulting mixture was sprayed on the leaves with hand held spray bottles (Lansing Sanitary Supply, Inc., Lansing, MI).

In 2018, inoculum was prepared by soaking infected beet leaves, as per Ruppel and Gaskill (1971). Infected leaves (approximately ten leaves) were collected from plants of another experiment, and allowed to soak in 500 ml of water for 1-3 days at room temperature (20-25°C) and ambient room lighting. After soaking, the leaves were vigorously scrubbed for at least ten minutes to remove the conidia, and the water was strained with a sieve to remove the leaf debris. The sieve which was used had a mesh size of approximately 1.1 mm by 1.8 mm. Spore counts were taken with a hemocytometer, and ranged from 8,750 to 21,250 conidia per ml, with an average of 14,750 spores per ml. Each week, the total volume of the spore suspension was about 450 ml, so no further dilution was done. Tween 20 (Sigma-Aldrich) was added to create a 0.1% final concentration, and the spore suspension was applied to the leaves of the treated control plants with a hand sprayer (Lansing Sanitary Supply, Inc.) at a rate of approximately 100 ml per plant.

The untreated control plants were included to show if the beets were exposed to *C. beticola* spores while in the greenhouse or had seed-borne inoculum. For these plants, a 0.1% solution of Tween 20 in water was prepared and applied to the leaves at an equivalent rate to what was applied for the treated control. In 2017, the controls were prepared the day before most of the beets in the field were exchanged, while in 2018 they were prepared the day after. In both cases, they were placed in the humidity chamber with the test beets the day they were prepared, and remained there for the same amount of time as the beets from the field. To determine the possible risk of cross contamination, in each year the untreated control was placed in an area of the humidity chamber where such contamination was most likely to occur, next to the treated control and at least one box from an old beet field.

The beets remained in the humidity chamber for a total of five days. For the 2017 season, they were then placed in another walk-in cold room (Harris Environmental Systems) and kept at the same temperature conditions and similar light conditions (approximately 40 µmol/(m^2*s)) as in the humidity chamber. In this cold room, however, the beets were kept at a lower relative humidity to reduce the development of bacterial leaf spot that could decrease rating efficacy. In the 2018 season, beets were moved to a standard greenhouse space. This increased the possible risk of exposing the *C. beticola* to high temperatures, particularly later in the season, which could potentially damage the fungus (Pool and McKay, 1916b). This risk was taken, however, because the higher light exposure allowed for improved symptom development and plant health (unpublished). Supplemental lighting was provided for 16 hours a day, from 6:00 a.m. to 10:00 p.m., to the plants in the greenhouse until the end of May, after which supplemental lights were not used. Plants remained in the greenhouse for at least three weeks after being removed from the humidity chamber to allow adequate time for CLS symptom development (Pool and McKay, 1916b; Carlson, 1967; Weiland and Koch, 2004; Franc, 2010). After this period, the amount of *C. beticola* which existed in the field while the sentinels were present was estimated by taking a spot count of each plant in every box. Spot counts were arbitrarily limited to 300 per plant. Except for the treated control, this number was only reached at two site-times: Bublitz Beets the week of June 18, 2017 and MSU Beets the week of May 11, 2018. Spots were confirmed as *C. beticola* for the first batch in 2017 and the first three batches in 2018 through the induction of conidia production and/or single spore isolation (Hildebrand, 1938). Random checks using the same methods also were conducted throughout the season for both years to confirm lesion species. Beets were disposed of after the spot counts were taken.

Statistical Analysis

While air temperature, relative humidity, and rainfall were collected at each site, other weather variables, including leaf wetness, solar radiation (SRAD), and wind speed, were collected from nearby Michigan Automated Weather Network (MAWN) stations (Tables 2.1 and 2.2). Spot count data and weather variables all were collected and organized in Microsoft Excel 2010 (Microsoft Corp., Albuquerque, NM). SAS 9.4 (SAS Institute Inc., Cary, NC) was used to conduct statistical analysis on the data.

There were several challenges associated with the statistical analyses for this project. One of the major challenges was that all of the beets at each site were located in the same box, so they could not be treated as replicates of the site. Such a view of the individual sentinel beets would violate the independence assumption, so they had to be used as subsamples. Since there was only one box used for the controls, they were not replicated and could not be included in the statistics. Another implication of multiple beets being in the same box is that the individual sites were not replicated, so the location cannot be considered as its own factor in the analysis. To simplify analysis, the number of lesions per box was averaged, rather than reporting four subsamples for each one. There were a few missing data points, including the Bublitz Beet site the week of June 24, 2017 which was lost due to the beets being run over by a tractor, and the first week (April 11) of the 2018 season, due to beets being frozen.

Environment Comparison

The statistical analysis of the sentinel beet data was divided into two main sections. The first of these involved comparing the data from the old beet fields with that from the marsh and woodlot locations to examine variability in the number of lesions observed on beets from the different types of environments. Before comparing the old beet field environment to the marsh

and woodlot environments, the latter two environments were combined into one group referred to as "other environments". To justify combining these environments, the three marsh and five woodlot locations from 2017 were compared to ensure they were not significantly different from each other. The experimental design for this analysis was a repeated measures, randomized incomplete block design. The proc glimmix procedure in SAS 9.4 was used for this analysis. Transformation of the data was not beneficial, so it was not done for the final analysis.

The next step in the analysis was to compare the "other environments" to the old beet field environment. The experimental design for this analysis was a repeated measures, randomized incomplete complete block design, blocked by location. The proc glimmix procedure was used for this analysis. Each year was analyzed separately, as the specific beet field used at each location was different each year. Additionally, the agronomic practices and grower reported CLS disease pressure from the prior year in the fields varied from year to year (Tables 2.1 and 2.2), so it would not be appropriate to combine the years for this analysis. For each year, a log transformation of the data was deemed necessary. To allow for log transformation, any lesion averages equal to zero were changed to 0.00001. In 2017, six beet field sites and eight "other environment" sites were compared. In 2018, eight old beet fields and six "other environment" sites were compared, blocked by location in both years.

Regression Analysis

After comparing the number of lesions on the sentinels in the old beet fields and the "other environments", the next analysis was to correlate the weather variables with the number of observed lesions. There were six variables which were compared with lesions counts, including air temperature, relative humidity, rainfall, leaf wetness, solar radiation, and wind speed (Appendix B). Prior to conducting the regression analysis, an issue which needed to be

addressed was the time increment of the variable measurements. Both the number of lesions and rainfall totals were collected on a weekly basis, while the air temperature, relative humidity, leaf wetness, solar radiation, and wind speed were collected hourly. In order to conduct the regression, it was decided to convert the hourly measurements into weekly values. For solar radiation and wind speed, the average weekly values for these variables were calculated. In the case of air temperature, relative humidity, and leaf wetness, merely taking the average or reporting the high and low would not give an accurate representation of these variables over the course of a week. Therefore, the measured air temperatures were used to calculate growing degree days (GDD) for *C. beticola*, the weekly total of which was used in the correlation. GDD's were calculated daily, similar to Holen and Dexter (1996), starting at the time of beet exchange. The base temperature used was 15.56°C (Pool and McKay, 1916b; Coons and Larmer, 1929) and no maximum temperature was used. To provide a weekly representation of relative humidity, the total number of hours with a relative humidity of 80% or greater was calculated every week, and this value was used for the correlation. Previous research shows that a relative humidity of 88.5% or above is needed for conidia production [Bleiholder and Weltzien, 1972b (in German), with English summary], so a relative humidity of 80% was selected to account for the majority of times humidity would be conducive for conidia production. Leaf wetness values were calculated as the number of hours in a week during which the leaf was wet for at least 15 minutes of the hour. As with the environment comparison, the average number of lesions per plant was used for this analysis. Individual years were analyzed separately, and the one average that came to zero, VanN on May 27 of 2017, was converted to 0.00001.

In preparation for statistical analysis, a few other modifications had to be made to the data. First, there were several onsite rainfall measurements missing from both years. In the first

year, there were not enough rain gauges for the first week. Both years, the rain gauges occasionally would be moved by the farmer or hit by a tractor, and one week flooding prohibited an accurate measurement. To compensate for this missing data, the rainfall for these site-times was estimated using the measurement from the next closest rain gauge or the nearest MAWN station (Tables 2.1 and 2.2). In 2017, estimation with the nearest rain gauge was done for the Bublitz site the week of June 10 and the Fairgrove site April 15, May 13, and June 18. Estimation with the nearest MAWN station was done for the Akron site the week of April 15, Frankentrost April 15, and Van April 15, May 6, and June 18. Similar estimates were made in 2018 for the Akron site on May 18, Ernie's on May 18, Fairgrove April 27 and May 4, MSU May 25, and Van on May 11 and May 18. For 2018, the nearest MAWN station was used only for the MSU, May 25 site-time.

Another issue which needed to be addressed was that while the weather stations used to measure air temperature and relative humidity were ordered in winter of 2017, only half of them arrived in time for the start of the field season. The rest were not in place until June 3. Therefore, since half of the data for both of these variables was not available, air temperature and relative humidity values from the MAWN stations were used in 2017. Also in 2017, the MSU beets site was dropped completely due to substantial but sporadic deer damage to the beets on multiple weeks (Figure 2.4). Finally, during analysis, it was determined that one data point in 2017 was an outlier, Bublitz beets the week of June 18 (Cook's D, 1.137). The average for this site-time was 282 lesions per plant. Rather than dropping this data point, the average number of lesions was adjusted to 100 lesions per plant. That way, it did not have such a strong influence over the data, but this important data point was still represented. No such adjustments were done

in 2018. As with the environmental comparison, there was no data from the Bublitz beets location the week of June 24 in 2017 because these beets were run over by a tractor.

Figure 2.4: Deer damage at the MSU beets site in 2017.

For all regression analyses in this experiment, proc corr was used to determine the Pearson's correlation coefficient of each individual variable with the average number of lesions observed, as well as comparing the variables with each other. The significance of these correlations also was determined. For each regression, proc reg was used to calculate the overall model with all of the factors included, as well as its significance, the significance of each variable, and the variance influence factor of each variable. Finally, proc reg was used to select the best fit model for each regression done, using both forward selection and backward elimination (α=0.15).

The first regression from each year was multiple linear regression, without any transformation. To confirm the type of relationship that existed between the average number of lesions and each of the variables examined, scatter plots comparing each variable with the average number of lesions were constructed. To account for the different types of relationships that might exist between the data, linear regression was repeated after the data was transformed. A natural log transformation was done on the data from 2017, while both natural log and square root transformations were done separately on the data from 2018. Multiple linear regression was

done on the transformed data, using the same procedure as with the original data. All of the same adjustments done to the original data in 2017 were done again for this data. After the transformation, another data point, Van on May 27 2017, was found to be an outlier, with a Cook's D value of 0.433. Therefore, this data point was removed from the analysis. No adjustments were necessary for the 2018 data.

Results

General Observations

Prior to conducting statistical analysis on the data retrieved from this project, several important observations were made which are worth noting. Perhaps the most notable of these is that lesions were observed on the sentinel beets very early in the season. The first lesions were observed on sentinel beets placed in the old beet field environments the very first week data could be retrieved in both years, the week of April 15 in 2017 and the week of April 20 in 2018. In the 2018 season, the first sentinels were placed in the field on April 11, but these beets were defoliated in a late season snow storm (Figure 2.5). However, CLS lesions were observed on the next batch of sentinel beets, placed in the field on April 20.

Throughout the experiment, the number of lesions on the sentinel beets in the old beet fields fluctuated by the week they were in the field and the location they were placed (Figure 2.6 and 2.8). In 2017, there were five peaks in the number of CLS lesions observed on the sentinels, the largest of these being the week of June 18. Interestingly, two weeks after this observation (the week of July 1), the first lesions were observed on field beets in a grower's field near the VanN site. During the 2018 season, six peaks of CLS incidence on the sentinel beets were observed, with four of these occurring during the month of May and two occurring the weeks of June 15 and 22. The first lesions in field beets during 2018 were observed on June 1 on

overwintered beets in a Michigan State University research plot near the MSU beets site (Willbur et al., 2018a). The first CLS symptoms on beets in a commercial field in the Saginaw Valley region were observed on June 15, in two different fields near the Van and Akron locations (Willbur et al., 2018b). In each year, there were sites which consistently had more lesions than others. In 2017, the Akron, Bublitz, and Frank sites had the most CLS lesions, while in 2018, it was the Akron, Frank, MSU, and VanN sites which generally developed a greater number of lesions. In every week, there were more lesions on the sentinel beets in the old beet field sites as compared to the "other environment" sites, although lesions did occur on the sentinels placed in the "other environments" (Figures 2.6, 2.7, 2.8, and 2.9).

Figure 2.5: Early Cercospora leaf spot detection, 2018. Images of the first and second batches of sentinel beets in 2018. A is of a box of sentinel beets placed in the field April 11 of 2018. No data could be retrieved from this batch because all plants were defoliated in a snow storm. CLS lesions were detected in the next batch of beets, placed in the field on April 20, leaves of which are shown in B and C (CLS lesions indicated by arrows).

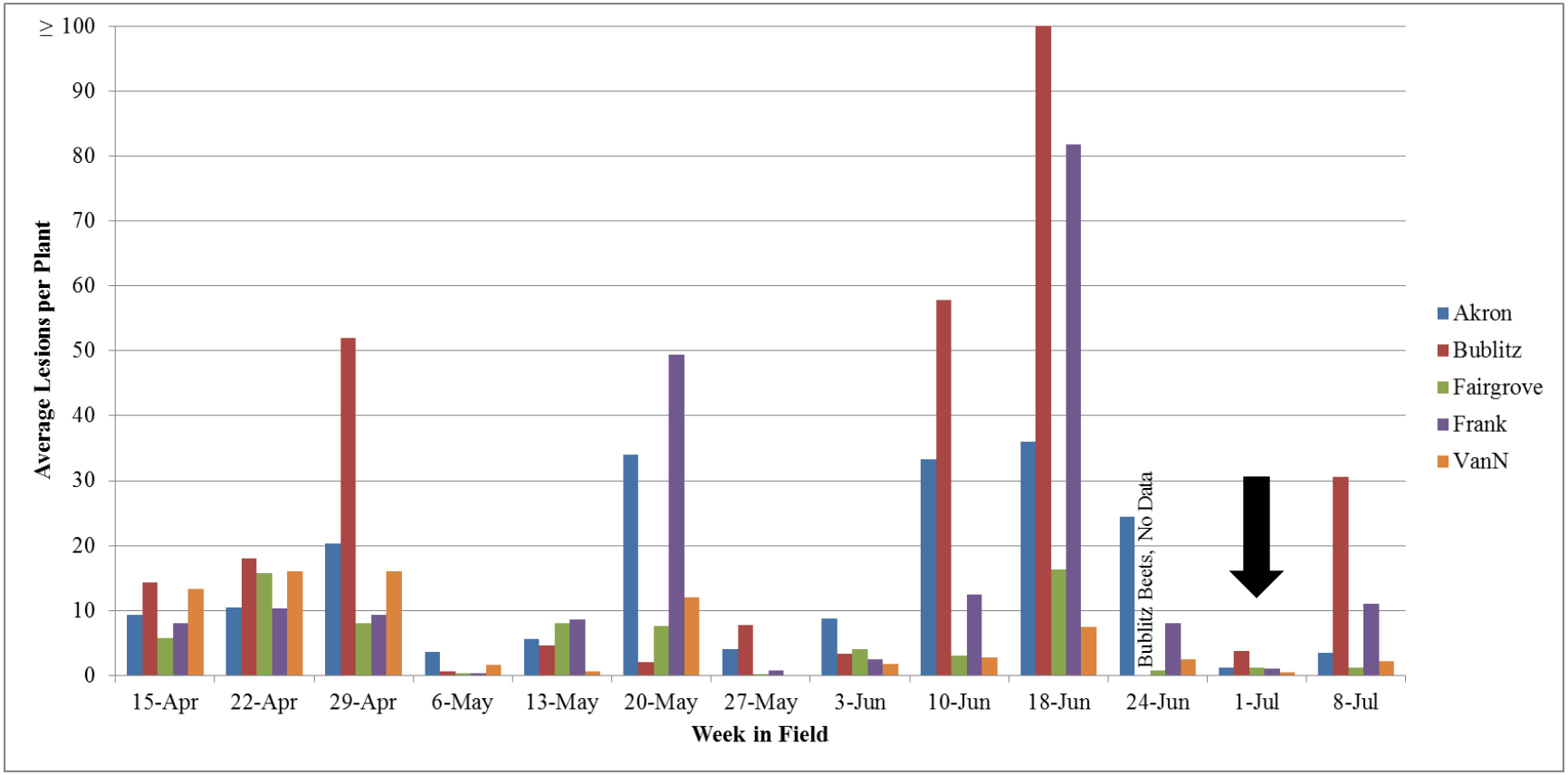


Figure 2.6: Average Cercospora leaf spot lesions observed on sentinel beets placed in old beet fields in 2017. In 2017, lesions were observed on the first sentinel beets in the field, placed on April 15. Throughout the experiment, the number of lesions observed fluctuated based on the week and the location where they were placed. The first lesions in field beets this year were observed near the Van location on the week of July 1 through 7, as indicated by the black arrow. Each bar represents the average number of lesions observed on three (April 29 through May 20) or four individual beets at each site, which served as subsamples. No data was collected the week of June 24 at the Bublitz Beet site, as those beets were run over by a tractor.

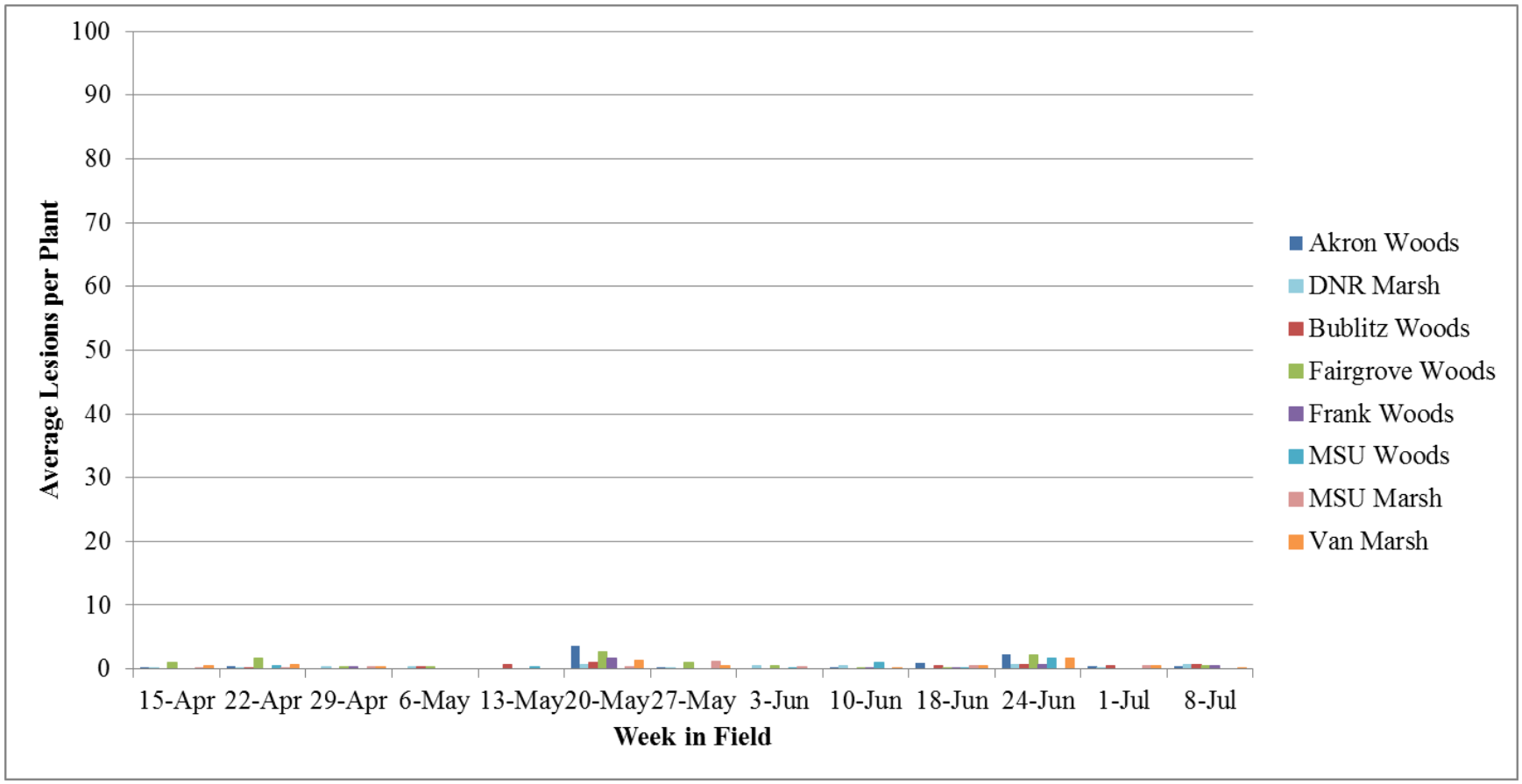


Figure 2.7: Average Cercospora leaf spot lesions observed on sentinel beets placed in woods and marsh sites in 2017. The average number of lesions observed at any non-beet field site failed to exceed four per plant. Regardless, the number of lesions observed fluctuated based on week and location. Each bar represents the average number of lesions observed on three (April 29 through May 20) or four individual beets at each site, which served as subsamples.

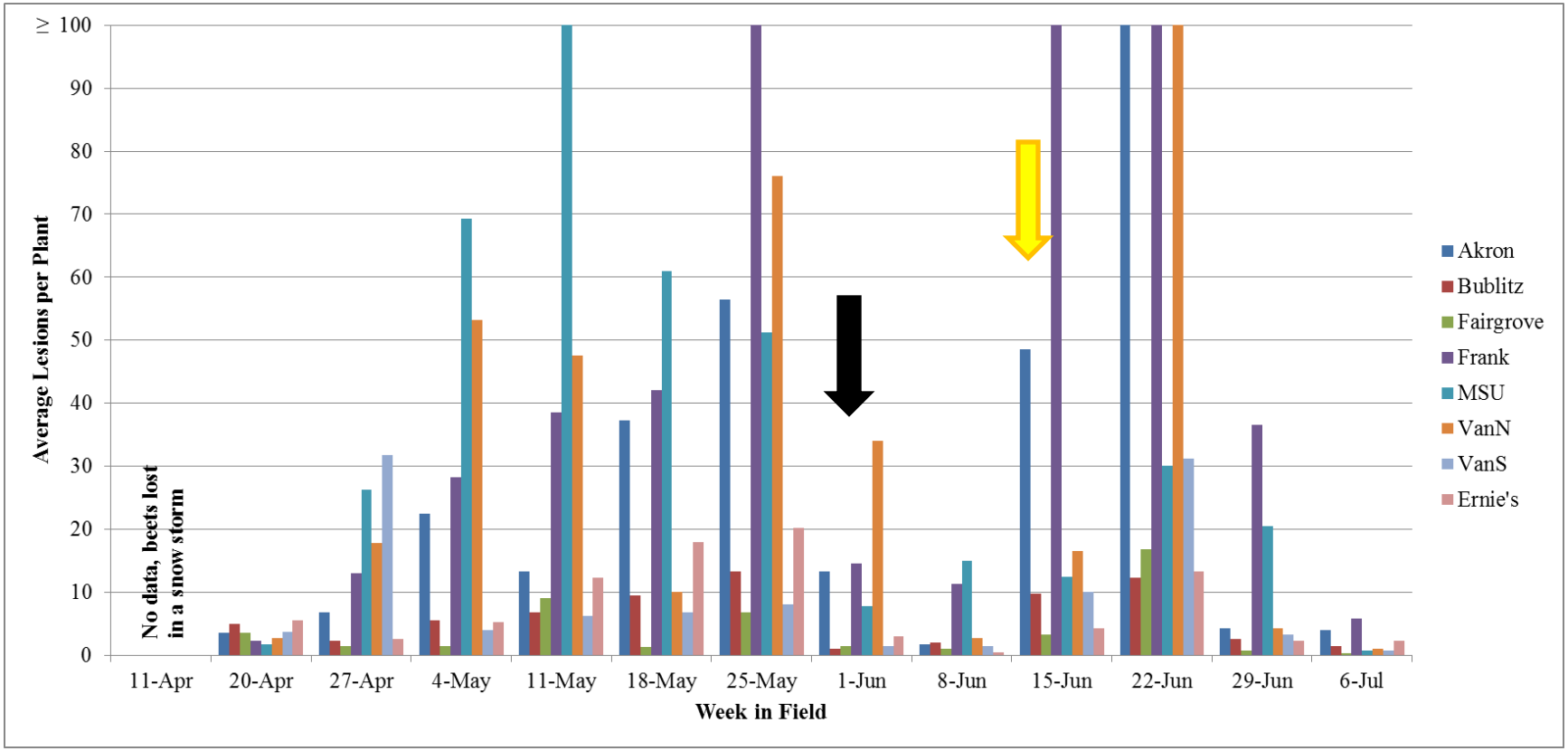


Figure 2.8: Average Cercospora leaf spot lesions observed on sentinel beets placed in old beet fields in 2018. In 2018, the first beets which were placed in the field (week of April 11) were defoliated by a late season snow storm. CLS lesions were observed on the beets that were placed in the field the next week, the week of April 20. Throughout the experiment, the number of lesions observed fluctuated by week and the location where they were placed. Some locations consistently had more lesions than others. The first lesions on field beets in 2018 were observed on overwintered research beets at MSU on June 1 (black arrow), and the first in the Saginaw Valley region were observed on June 15 near the Van and Akron sites (yellow arrow). Each bar represents the average number of lesions observed on four individual beets at each site, which served as subsamples.

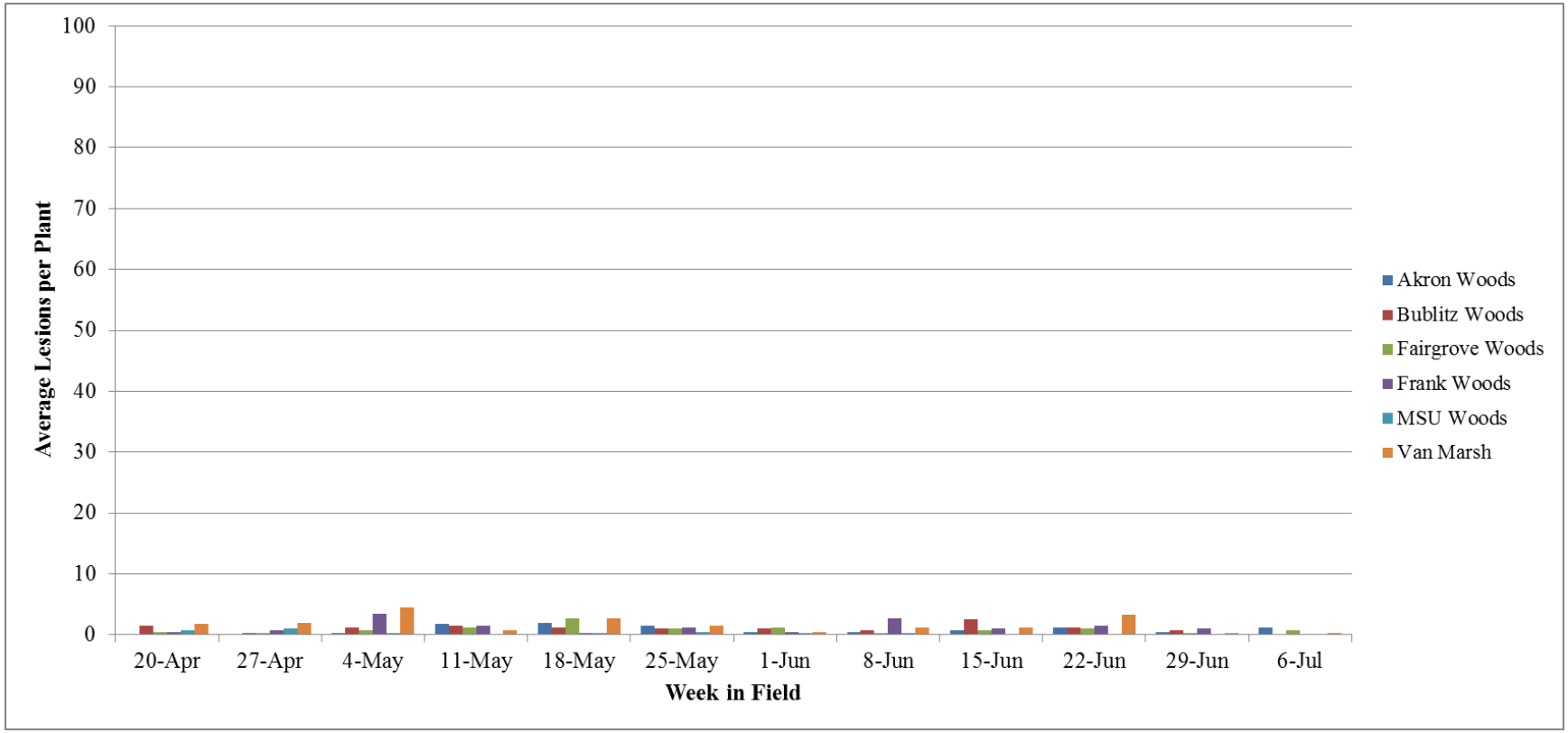


Figure 2.9 Average Cercospora leaf spot lesions observed on sentinel beets placed in woods and marsh sites in 2018. The average number of lesions on non-beet field sentinels failed to exceed five per plant, and fluctuated based on week and location. Each bar represents the average number of lesions observed on four individual beets at each site, which served as subsamples.

On the treated control beets (Figure 2.10 B), the number of lesions averaged greater than 150 lesions per plant for all but three of the weeks in 2017, and in 2018, all of the weeks averaged 300 lesions or above. Yearly averages are not given for the treated control, as the number of lesions per plant exceeded 300 on 73 out of 96 total treated control plants used over the course of the study. With the untreated controls (Figure 2.10 A), the average number of CLS lesions observed over the 2017 season was 0.91 per plant, with the greatest number observed in any week being 3.5 lesions per plant. In 2018, the average number of CLS lesions observed over the season was 3.42 per plant, while the greatest was 14.25 per plant. The controls were not replicated, and as such were not statistically compared with the other treatment groups.

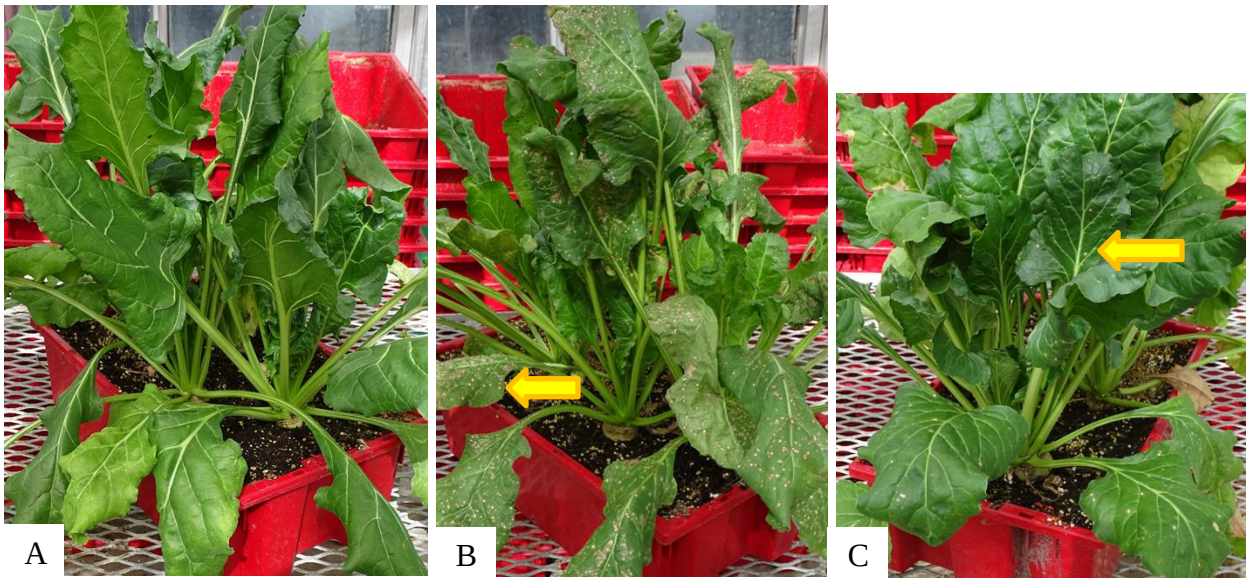


Figure 2.10: Three examples of sentinel beets from 2018. Sentinel beets from different treatment groups the week of June 22 in 2018. A is the untreated control, B is the treated control, and C is from the Akron beets site. Example CLS lesions are indicated by the arrows.

Environment Comparison

When comparing the woodlot and marsh environments from 2017, the variance structure selected for the data was first order auto-regressive with heterogeneous variance, based on the fact that this structure had the lowest Akaike information criterion (AIC) value at 118.05. Other structures were tested, including compound symmetry (AIC=150.20), first order auto-regressive

(AIC=152.20), compound symmetry with heterogeneous variance (AIC=119.21), and spatial power covariance (AIC=152.2), but first order auto-regressive with heterogeneous variance was selected. According to this model, the effect of the environment was not significant (p=0.3712). Therefore, it was concluded that the marsh and woodlot environments were not significantly different and could be combined.

With the results from the marsh and woodlot sites being combined into a single environment type referred to as "other environments", beet field and "other environment" sites were compared. The variance structure selected for 2017 was compound symmetry, with an AIC of 937.63. Other structures tested included first order auto-regressive (AIC=939.63), compound symmetry with heterogeneous variance (AIC=948.25), first order auto-regressive with heterogeneous variance (AIC=950.16), and spatial power covariance (AIC=939.63), but compound symmetry was selected based on lowest AIC. The effect of environment was significant (p=0.0003), as was the effect of week (p=0.0015), but not the interaction between the two (p=0.85302; α=0.05). The difference between the beet field environment and "other environments" was apparent upon visual examination of the 2017 data, as the average number of lesions on sentinel beets among all the site-times was 15.48 lesions per plant in the old beet field sites, and 0.50 per plant in the woods and marsh sites. The average number of lesions in any single box of sentinel beets placed in either a marsh or woodlot this year never exceeded four, while there was one from an old beet field that exceeded 100 lesions per plant (Figures 2.6 and 2.7). The data from 2018 required a different variance structure, first order auto-regressive with heterogeneous variance (AIC =610.10). Other structures tested include compound symmetry (AIC=713.18), first order auto-regressive (AIC=673.47), compound symmetry with heterogeneous variance (AIC=640.59), and spatial power covariance (AIC=673.47). As with the

2017 data, there was a significant effect of environment ($p<0.0001$) and of week ($p=0.0003$). In 2018, there also was a significant interaction between environment and week ($p=0.0030$; $\alpha=0.05$). In 2018, there was an average of 25.48 lesions per plant on the sentinel beets placed in the old beet field environment over all site-times, whereas the ones in the "other environments" had an average of 1.00 lesions per plant. For 2018, no single box of sentinel beets in a woods or marsh exceeded an average of five lesions per plant, while there were several from the old beet fields that exceeded 100 lesions per plant (Figures 2.8 and 2.9).

Regression Analysis

Once it was determined there were significantly more lesions observed in the old beet field environment than the "other environments", the next focus for analysis was correlating the observed lesions in old beet fields to weather variables. Specific weather data is not shown in this chapter, but as an example, the weather from both years at the Akron Beets site is provided in Appendix B.

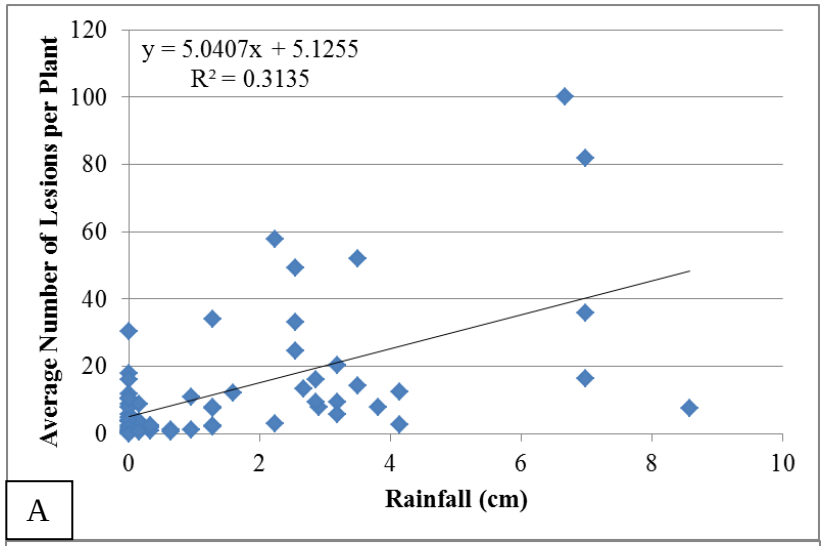
The first regression examined from each year was multiple linear regression, without any transformation. In 2017, three variables were significantly correlated to the number of lesions observed on the sentinel beets, including relative humidity ($p=0.0372$), rainfall ($p<0.0001$), and solar radiation ($p=0.0117$). In the overall model, rainfall ($p<0.0001$) was the only variable which contributed significantly to the model ($\alpha=0.05$), which was significant at $p=0.0002$ and had an adjusted R^2 of 0.2908. Rainfall was the only variable selected ($p<0.0001$) for the best fit model, which also was significant ($p<0.0001$) and had an adjusted R^2 of 0.3024 (Table 2.3). Both the forward selection and backward elimination methods agreed on this model. In 2018, three variables were significantly correlated to the average number of lesions ($\alpha=0.05$), including relative humidity ($p<0.0001$), rainfall ($p<0.0001$), and leaf wetness ($p=0.0032$). Two of these

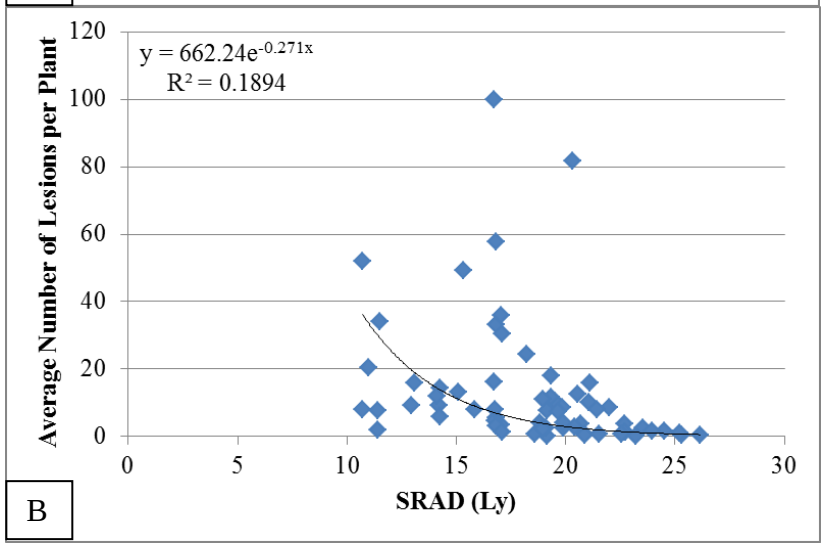
variables, relative humidity (p=0.0432) and rainfall (p=0.0041), contributed significantly to the overall model, which was significant (p<0.0001) and had an adjusted R^2 of 0.2168. This best fit model included both relative humidity (p=0.0012) and rainfall (p=0.0032), with an adjusted R^2 of 0.2392 (Table 2.3). Both the forward selection and backward elimination methods agreed on this model.

To test if linear regression was the best regression method for each of the variables examined, scatter plots comparing each variable with the average number of lesions were constructed (Figures 2.11 and 2.12). In 2017, there was a linear relationship between rainfall and the average number of lesions. However, the relationship between lesion number and solar radiation, as well as the relationship between lesion number and relative humidity, were exponential, not linear (Figure 2.11). In 2018, none of the relationships between lesion number and the weather variables were linear. Rather, lesion number's relationship with relative humidity was exponential, while the relationships with rainfall and leaf wetness were quadratic (Figure 2.12).

Sentinel Beet, 2017 Linear Regression													
Individual Varaibles Compared to Lesion Number				Total Model					Best Fit Model				
Variable	p-value	R	R^2	Variance Inflation	Individual p-value	p-value, Model	R^2	Adjused R^2	Variables Included	p-value, Variables	p-value, Model	R^2	Adjused R^2
MAWN GDD	0.8115	-0.0304	0.0009	1.23	0.7760	0.0002	0.3583	0.2908	Site Rain	<0.0001	<0.0001	0.3135	0.3024
MAWN RH	0.0372	0.2611	0.0682	2.37	0.6074								
Site Rain	<0.0001	0.5599	0.3135	1.36	<0.0001								
Leaf Wetness	0.1451	0.1842	0.0339	2.89	0.2978								
SRAD	0.0117	-0.3133	0.0982	3.39	0.0539								
Wind Speed	0.9738	-0.0042	0.0000	2.35	0.1345								
Sentinel Beet, 2018 Linear Regression													
Individual Varaibles Compared to Lesion Number				Total Model					Best Fit Model				
Variable	p-value	R	R^2	Variance Inflation	Individual p-value	p-value, Model	R^2	Adjused R^2	Variables Included	p-value, Variables	p-value, Model	R^2	Adjused R^2
GDD	0.8890	0.0144	0.0002	1.42	0.3627	<0.0001	0.2663	0.2168	RH	0.0012	<0.0001	0.2552	0.2392
RH	<0.0001	0.4262	0.1816	1.89	0.0432				Site Rain	0.0032			
Site Rain	<0.0001	0.4064	0.1652	1.34	0.0041								
Leaf Wetness	0.0032	0.2983	0.0890	2.40	0.5930								
SRAD	0.2690	-0.1139	0.0130	2.15	0.8439								
Wind Speed	0.0740	-0.1832	0.0336	1.67	0.8443								

Table 2.3: The results from multiple linear regression conducted on the untransformed 2017 and 2018 sentinel beet data. The 2017 results presented here were derived from 64 observations, which came from five sites at 13 different times. Bublitz on June 18 was an outlier, and adjusted from 282 lesions to 100 lesions. The 2018 results were derived from 96 observations, which came from eight sites at 12 different times. Upon completion of linear regression for each year of data, three sets of results were collected, including results on the individual variables compared to the average number of lesions counted on the sentinels, the model correlating all of the variables to the number of lesions (Total Model), and the best fit model, as determined by forward selection and backward elimination. Six variables were used in the regression, including air temperature in the form of growing degree days (MAWN GDD or GDD), relative humidity at or above a set point of 80% (MAWN RH or RH), site rain, leaf wetness as a count of hours during which the leaf was wet for at least 15 minutes, solar radiation (SRAD), and wind speed.





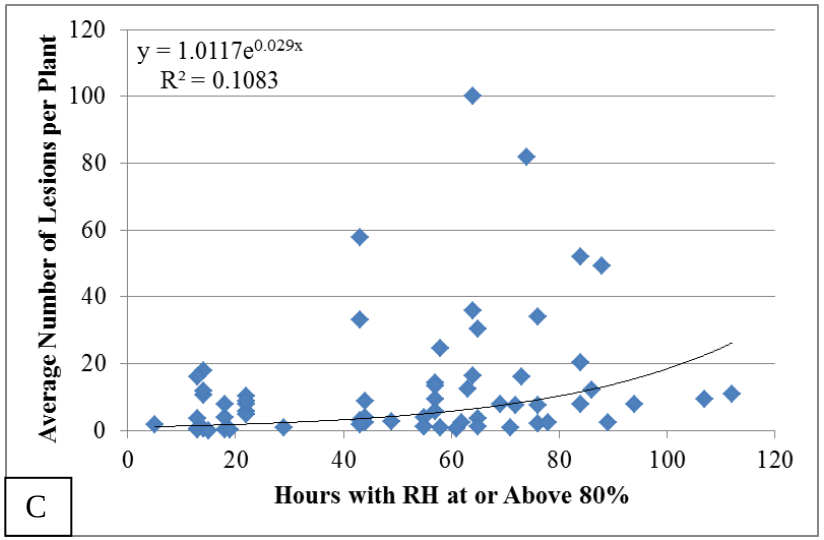
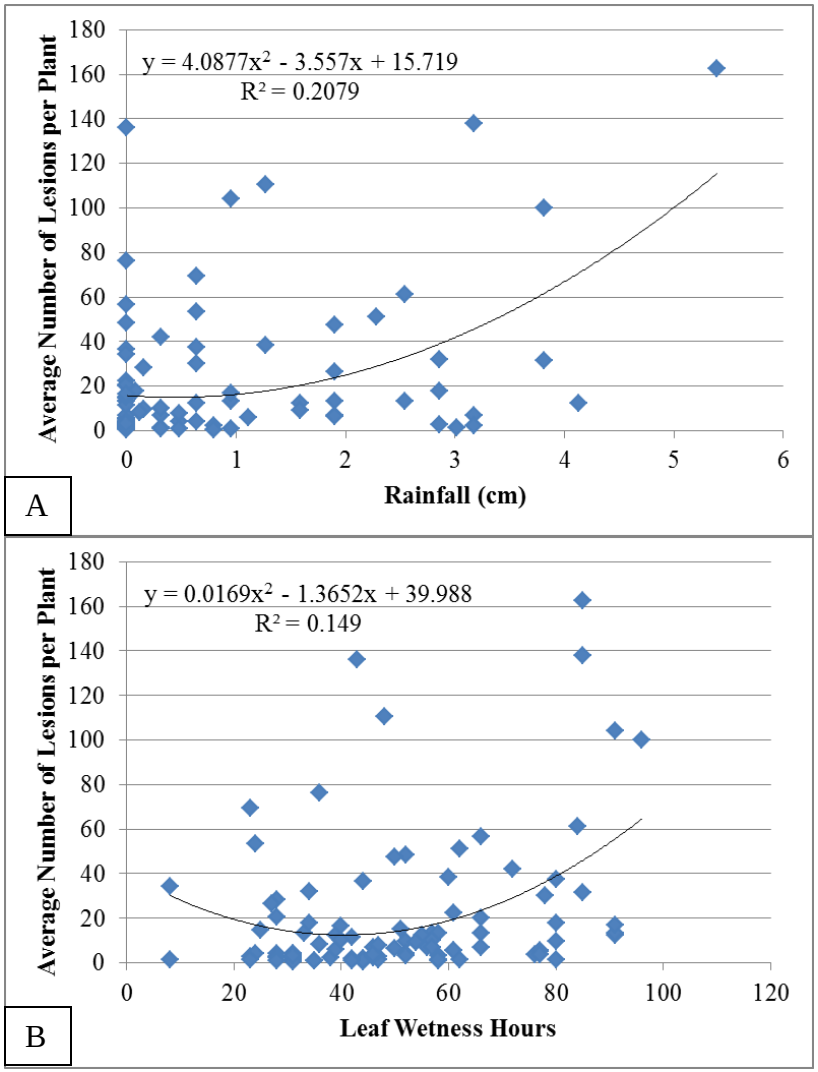


Figure 2.11: Scatter plots comparing significant weather variables with Cercospora leaf spot lesion numbers in 2017. Shown are scatter plots comparing the average number of Cercospora leaf spot (CLS) lesions observed per sentinel beet with significant weather variables from 2017 (Table 2.3), including rainfall (A), solar radiation (SRAD, B), and relative humidity at or above 80% (RH, C). In each plot, the best fit trend line for the variable is given, along with the equation and the R^2 for that line. A linear equation provided the best fit line for rainfall, while an exponential was the best for solar radiation and relative humidity at or above 80%.



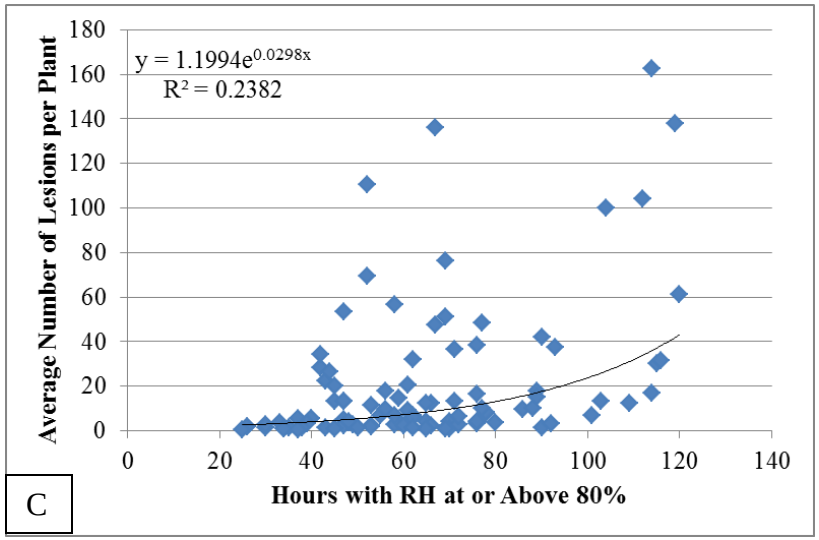


Figure 2.12: Scatter plots comparing significant weather variables with Cercospora leaf spot lesion numbers in 2018. Shown are scatter plots comparing the average number of Cercospora leaf spot (CLS) lesions observed per sentinel beet with significant weather variables from 2018 (Table 2.3), including rainfall (A), leaf wetness (B), and relative humidity (RH, C). In each plot, the best fit trend line for the variable is given, along with the equation and the R^2 for that line. An exponential equation provided the best fit line for relative humidity at or above 80%, while a quadratic equation was the best fit for rainfall and leaf wetness as a count of hours during which the leaf was wet for at least 15 minutes.

After the natural log transformation, four variables were significantly correlated to the average number of lesions observed on the sentinel beets in 2017 (α=0.05), including relative humidity (p=0.0089), rainfall (p=0.0021), solar radiation (SRAD, p<0.0001), and wind speed (p=0.0187). Of these, only solar radiation contributed significantly (α=0.05) to the overall model, which was significant at p=0.0007 and had an adjusted R^2 of 0.2592. Three variables were selected for the best fit model, which were relative humidity (p=0.0318), leaf wetness (p=0.0387), and solar radiation (p=0.0001). The best fit model was significant at p<0.0001, and had an adjusted R^2 of 0.2782 (Table 2.4). Unlike the other regression models, the 2017 natural log transformed regression model had two different best fit models. The first, above, was selected by backward elimination. The second was selected by forward selection. This model had two variables, site rain and solar radiation. However, the backward selection model was selected, because both models had the same p-value (<0.0001), and the backward selection model had a higher adjusted R^2, 0.2782 rather than 0.2497.

In the natural log transformed 2018 data, three variables were significantly (α=0.05) correlated to the number of lesions. These include relative humidity (p<0.0001), site rain (p=0.0023), and leaf wetness (p=0.0110). Only relative humidity was significant in the overall model (p<0.0001), which was significant at p<0.0001 with an adjusted R^2 of 0.2643. As in the 2017 natural log transformed data, three variables were selected for the best fitting model (α=0.15), including relative humidity (p<0.0001), leaf wetness (p=0.1168), and wind speed (p=0.0198). This model was significant at p<0.0001, and had an adjusted R^2 of 0.2743 (Table 2.4). Both the forward selection and backward elimination methods agreed on this model.

In 2018, the original data also was square root transformed, after which relative humidity (p<0.0001), rainfall (p=0.0002), and leaf wetness (p=0.0050) were found to be significantly

correlated with the number of lesions (α=0.05). Relative humidity (p=0.0028) and site rain (p=0.0274) significantly contributed to the overall model (α=0.05), which was itself significant at p<0.0001, with an adjusted R^2 of 0.2277. Relative humidity (p=0.0002) and site rain (p=0.0149) were the only variables selected for the best fit model. This model was significant at p<0.0001 and had an adjusted R^2 of 0.2449 (Table 2.5). Both the forward selection and backward elimination methods agreed on this model.

Sentinel Beet, 2017 Natural Log Transformed Regression													
Individual Varaibles Compared to Lesion Number				Total Model					Best Fit Model*				
Variable	p-value	R	R^2	Variance Inflation	Individual p-value	p-value, Model	R^2	Adjused R^2	Variables Included	p-value, Variables	p-value, Model	R^2	Adjused R^2
MAWN GDD	0.4737	-0.0919	0.0084	1.49	0.3696	0.0007	0.3309	0.2592	MAWN RH	0.0318	<0.0001	0.3131	0.2782
MAWN RH	0.0089	0.3271	0.1070	4.30	0.2961				Leaf Wetness	0.0387			
Site Rain	0.0021	0.3811	0.1452	2.51	0.5435				SRAD	0.0001			
Leaf Wetness	0.1763	0.1725	0.0298	3.03	0.1371								
SRAD	<0.0001	-0.4979	0.2479	3.40	0.0148								
Wind Speed	0.0187	0.2956	0.0874	2.63	0.7013								
Sentinel Beet, 2018 Natural Log Transformed Regression													
Individual Varaibles Compared to				Total Model					Best Fit Model				
Variable	p-value	R	R^2	Variance Inflation	Individual p-value	p-value, Model	R^2	Adjused R^2	Variables Included	p-value, Variables	p-value, Model	R^2	Adjused R^2
GDD	0.7815	0.0287	0.0008	1.19	0.6493	<0.0001	0.3108	0.2643	RH	<0.0001	<0.0001	0.2973	0.2743
RH	<0.0001	0.4949	0.2449	1.59	<0.0001				Leaf Wetness	0.1168			
Site Rain	0.0023	0.3072	0.0944	1.20	0.2214				Wind Speed	0.0198			
Leaf Wetness	0.0110	0.2584	0.0668	2.02	0.4118								
SRAD	0.0786	-0.1804	0.0325	1.98	0.5885								
Wind Speed	0.4409	-0.0796	0.0063	1.56	0.0616								

Table 2.4: The results from multiple linear regression conducted on the natural log transformed 2017 and 2018 sentinel beet data. The results for 2017 were derived from 63 observations, from five sites at 13 times. The 2017 data was adjusted in the same way as the non-transformed data. After transformation, the observation Van May 27 was found to be an outlier (Cook's D of 0.433), so it was eliminated from the analysis. The 2018 results were derived from 96 total observations, from eight sites at 12 times. Three sets of results were collected, including the individual variables compared to the average number of lesions counted on the sentinels, the model correlating all of the variables to the number of lesions (Total Model), and the best fit model by backward elimination. Six variables were used in the regression, including air temperature in the form of growing degree days (MAWN GDD or GDD), relative humidity at or above 80% (MAWN RH or RH), site rain, leaf wetness as a count of hours during which the leaf was wet for at least 15 minutes, solar radiation (SRAD), and wind speed. *The forward selection and backward elimination selected different best fit models, but the model shown was selected because it had a higher R^2 value and both had the same p-value of <0.0001.

Sentinel Beet, 2018 Square Root Transformed Regression													
Individual Varaibles Compared to				Total Model					Best Fit Model				
Variable	p-value	R	R^2	Variance Inflation	Individual p-value	p-value, Model	R^2	Adjused R^2	Variables Included	p-value, Variables	p-value, Model	R^2	Adjused R^2
GDD	0.7933	0.0271	0.0007	1.32	0.3992	<0.0001	0.2765	0.2277	RH	0.0002	<0.0001	0.2608	0.2449
RH	<0.0001	0.4602	0.2118	1.74	0.0028				Site Rain	0.0149			
Site Rain	0.0002	0.3727	0.1389	1.29	0.0274								
Leaf Wetness	0.0050	0.2846	0.0810	2.26	0.5122								
SRAD	0.1864	-0.1360	0.0185	2.18	0.7469								
Wind Speed	0.1501	-0.1480	0.0219	1.63	0.5061								

Table 2.5: The results from multiple linear regression conducted on square root transformed 2018 sentinel beet data. The results presented here were derived from 96 observations, which came from eight sites at 12 different times. Since two of the significant variables in 2018, rainfall and leaf wetness, were quadratic, the square root of the data was taken prior to conducting linear regression. Three sets of results were collected, including results on the individual variables compared to the average number of lesions counted on the sentinels, the model correlating all of the variables to the number of lesions (Total Model), and the best fit model, as determined by forward selection and backward elimination. Relative humidity at or above 80% (RH), site rain, and leaf wetness as a count of hours during which the leaf was wet for at least 15 minutes were all significantly correlated with lesion number after the transformation (α=0.05).

Discussion

The results of the sentinel beet project have several implications for our understanding of the biology and epidemiology of *Cercospora beticola*. One of the most important of these is how early in the season *C. beticola* spores were detected. In both years, CLS symptoms were detected on the earliest batch of usable beets, which were in the field during the week of April 15 and April 20 in 2017 and 2018, respectively. The occurrence of an extended period of subfreezing temperatures and a snow storm in 2018 caused severe damage to the first batch of sentinel beets, placed on April 11, but CLS lesions were observed on the very next batch of beets, in the field from April 20 to April 27 (Figure 2.5). This is the earliest in the growing season when viable *C. beticola* spores have been tested for and detected in the Great Lakes region and similar sugar beet producing areas. These results strongly support the findings of Tedford et al. (2018) who began spore trapping at their Ridgetown location in Ontario, Canada on May 1 in both years of their study, and detected *C. beticola* conidia on May 2 in 2014 and May 9 in 2015. The findings of the current study also support the early report of CLS symptoms on overwintered beets at a Michigan State University research plot in 2018 (Willbur et al., 2018a).

If viable *C. beticola* spores are being produced, dispersed, and deposited this early in the season, it is interesting that the first lesions usually do not appear in the region until much later. Most years, the first CLS lesions in the Great Lakes region are found by the Michigan Sugar Company during the first two weeks of July (Michigan Sugar Company, unpublished). In their study, Tedford et al. (2018) did not observe CLS lesions in the sugar beet plots surrounding their spore traps until 91 days after the first detection of conidia in 2014 and 75 days after in 2015. It has been suggested by Skaracis and Biancardi (2005) that for the first 80-90 days after they

emerge, seedling sugar beets appear to express immunity to CLS. This would help explain the lack of symptom development on commercial sugar beets during the early part of season in spite of viable *C. beticola* spores being present. If the molecular basis for such immunity were determined, this might offer new possibilities for host resistance. However, when sugar beets (USDA F1042 germplasm) were inoculated at different growth stages, including the cotyledon, 2-3 leaf, 4-6 leaf, 7-9 leaf, 10-12 leaf, and later stages (data not shown), no difference in susceptibility was observed, as all the beets which were tested developed CLS symptoms. An example of the symptoms which developed on a sugar beet inoculated at the 2-3 leaf growth stage is shown in Figure 2.13. This is consistent with McKay and Pool (1918) who found sugar beet seedlings could be infected.

Figure 2.13: A sugar beet exhibiting symptoms of Cercospora leaf spot after being inoculated with a spore suspension of *Cercospora beticola* conidia at the 2-3 leaf growth stage. 14 days after inoculation, several Cercospora leaf spot lesions (indicated by arrow) are present on the leaves.

It is unlikely that seedling immunity is the reason why CLS symptoms are not observed early in the growing season. Since a susceptible host (sugar beet) is present along with viable *C. beticola* spores, a more plausible explanation for the lack of early season CLS symptoms is that while weather conditions are appropriate for spore production, they may not be conducive for infection (Wallin and Loonan, 1971; Shane and Teng, 1984a). Based on the results of the

current study as well as the Tedford et al. (2018) study, it is likely that the production of *C. beticola* spores occurs over a broader temperature range than is suitable for infection. In the literature it has been well established that infection generally occurs between 15.6°C and 35°C, although it is possible down to 10°C (Wallin and Loonan, 1971; Shane and Teng, 1984a). Other studies have indicated that conidia production occurs down to 10°C [Pool and McKay, 1916b; Bleiholder and Weltzien, 1972b (in German), with English summary], or at a wider temperature range, even down to 5°C [Canova, 1959a (in Italian), with English summary]. The results of the current study support the idea that *C. beticola* conidia production can occur at this broader temperature range. It is likely that spores produced at lower temperatures may not lead to infection or CLS symptom development in the field based on conditions reported for infection (Wallin and Loonan, 1971; Shane and Teng, 1984a). Future studies examining the exact weather conditions needed for this early spore production as well as the impact of these spores on *C. beticola* epidemiology may provide a valuable contribution to our understanding of and ability to manage CLS.

Another possible explanation as to why there is no significant early season CLS symptom development in the field is because of an extended latent period due to cold temperatures. The latent period, i.e. the amount of time between infection and the appearance of symptoms, is largely determined by host resistance and temperature, with more susceptible hosts and warmer temperatures causing a decrease in the latent period (Franc, 2010). The ideal temperature range for *C. beticola* growth and CLS disease development is 27°C to 31°C (Pool and McKay, 1916b; Groenewald et al., 2005), but it can occur over a wider range, from about 10°C to 33°C (Pool and McKay, 1916b; Groenewald et al., 2005). In most field situations, symptom development typically occurs in 9-13 days (Pool and McKay, 1916b; Steinkamp et al., 1979; Weiland and

Koch, 2004; Franc, 2010), but it can occur in as few as five days with a very susceptible host and in the ideal temperature range (Rathaiah, 1976; Steinkamp et al., 1979). Rossi et al. (2000) showed that increasing the level of host resistance can extend the latent period by up to 12 days. While attention has been paid to the minimum amount of time needed for the latent period (Rathaiah, 1976; Steinkamp et al., 1979), less work has been done to determine the maximum amount of time the latent period could be extended by factors such as air temperature. It is hypothesized that if environmental conditions were appropriate for infection (Wallin and Loonan, 1971; Shane and Teng, 1984a) during a brief time in the early spring but then the air temperature cooled to below the range for disease development, infection might take place but symptom development may not occur until the temperature returns to the acceptable range. Such an extended latent period in the early part of the season was suggested by Vereijssen et al. (2005). In their study, they moved symptomless sugar beets from the field to the greenhouse and provided them optimal conditions for CLS development. Two weeks later they observed CLS symptoms, and concluded that the infection had actually taken place earlier in the spring, but the symptoms were not expressed due to weather conditions outside of the acceptable range. Based on the results of this and the current studies, it may be worth conducting future experiments testing the maximum latent period, as well as the impact of such hypothesized early infection on the epidemiology of CLS.

In the current study, the number of CLS lesions on sentinel beets from the old beet field sites were significantly greater than the number found on sentinels placed in "other environments" ($p<0.0004$ in both years). This difference is evident upon visual examination (Figures 2.6, 2.7, 2.8, and 2.9), as between both seasons, the average number of lesions observed on a single site-week from the "other environments" never exceeded five lesions per plant, while

seven site-weeks from the old beet field environment surpassed 100 lesions per plant. This agrees with previous reports that infected residue from beets is a major source of inoculum for CLS (Townsend, 1914; Coons et al., 1930; McKay and Pool, 1918; Bennett and Leach, 1971). Even though fewer lesions were observed on the sentinel beets from the marsh and woodlot environments, a few lesions were observed from these environments most weeks (Figures 2.7 and 2.9). The source of the inoculum which caused this infection is unclear. It could be from minor contamination in the greenhouse or humidity chamber, as an average of 0.91 lesions per plant and 3.42 lesions per plant were observed on the untreated controls among all the weeks in 2017 and 2018, respectively. However, the placement of the untreated controls in the humidity chamber was such that they would be more likely than any of the other sentinel beets to be affected by cross contamination. Consequently, it is unlikely that such levels of cross contamination had a major impact on the results of the other treatments. It is more likely that the lesions observed on the sentinel beets from the marshes and woodlots were the result of inoculum from the field. This could be due to long distance dispersal of *C. beticola* conidia, or conidia coming from an alternative host. These two explanations are particularly intriguing, as they have implications for the importance of initial inoculum levels or alternative hosts in the CLS disease cycle. It is possible the symptoms observed on the sentinels in non-beet field environments could be caused by the *C. beticola* teleomorph (Groenewald et al., 2006b; Groenewald et al., 2008; Bolton et al., 2012c; Vaghefi et al., 2016), but this cannot be determined from the results of the current experiment.

Over the course of each season, the number of lesions observed on the sentinel beets placed in the old beet field environment varied substantially by site and week. In each year, there were sites which frequently had more lesions than others. In 2017, these were the Akron,

Bublitz, and Frank sites, and in 2018 the Akron, Frank, MSU, and VanN sites. The reason for the difference in the number of lesions observed between the sites was not determined. It was hypothesized that the agronomic practices to the beet crop the year before or even those during current cropping could have been responsible for the difference in pathogen detection, as other studies reported agronomic practices influencing CLS disease incidence (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). However, upon examination of selected agronomic data (Tables 2.1 and 2.2), no strong pattern was evident. Alternatively, this type of variation could have been caused by differences in microclimates. Research to determine the factors responsible for the differential spore levels observed between sites could provide further information about CLS epidemiology and potentially new management techniques.

The variation in disease levels between weeks could be due in part to fluctuations in weather patterns, as five of the six weather variables measured were significantly correlated (α=0.05) to the number of lesions observed in at least one of the regressions conducted (Tables 2.3, 2.4, and 2.5). The moisture variables recorded in the current study, including relative humidity, rainfall, and leaf wetness, account for three of the five weather variables that had significant correlation to lesion number. It appears as though relative humidity and rainfall are particularly important for spore production and/or dispersal, as both variables were significantly correlated to lesion number each year of the study. Additionally, site rain had the highest R^2 value of any single variable in 2017 (non-transformed data, R^2=0.3135) and was the sole variable in the best fit model for that year, while relative humidity had the highest R^2 value in 2018 (natural log transformed, R^2=0.2449). The finding of rainfall being significantly correlated with spore presence agrees with the results of Carlson (1967). In his study, he used a combination of

sentinel beets and glass rod spore traps to measure *C. beticola* conidia dispersal over the course of two summers. While he does mention the importance of air temperature, relative humidity, and wind to the CLS life cycle, his results indicate that rainfall is primarily responsible for *C. beticola* conidia dispersal.

The other two variables which were significantly correlated with lesion number in the current study were solar radiation and wind speed. Even though solar radiation was only significant in 2017, this weather variable is intriguing, as it is the only one of the six which had a negative correlation to lesion number (natural log transformed, R^2=0.2479). The reason for its significance raises some interesting questions about *C. beticola* biology. It is possible that this variable was significant because it is acting as a pseudo-measure for the moisture variables, as intense sunshine and rain do not generally occur at the same time, and the leaves may be more likely to be dry on sunny days as opposed to cloudy days. In addition, Canova [1959a, (in Italian), with English summary] reported that *C. beticola* conidia are sensitive to solar radiation, and overexposure can lead to a decrease in germination. Thus it is possible that the negative correlation of solar radiation and lesion number could be a reflection of solar radiation decreasing conidia viability in the field. This variable should be investigated more closely in the future, as it may play an important role in CLS epidemiology and should perhaps be considered in the CLS prediction models.

While wind speed was significantly correlated to lesion number after a natural log transformation in 2017, its R^2 value was fairly low in relation to the other significant variables (R^2=0.0874). This indicates that the wind may play a role in *C. beticola* conidia dispersal, but this role may be of less importance compared to that of rainfall, as was also hypothesized by Carlson (1967). This hypothesis contradicts what was found by Lawrence and Meredith (1970).

In their study, Lawrence and Meredith (1970) used a Hirst spore trap (later commercialized by Burkard Scientific; Jenkyn, 1974; Eversmeyer et al., 1976) to track the aerial concentration of *C. beticola* conidia over the course of a field season. Based on their results, Lawrence and Meredith hypothesized that wind was the principle agent of *C. beticola* conidia dispersal, and rainfall was of secondary importance. The current study found that rainfall was correlated to lesion number both years, while such a correlation only existed one year with wind speed. Additionally, the correlations between lesion number and rainfall were considerably stronger (R^2=0.3135 and 0.1652) than the correlation between lesion number and wind speed (R^2=0.0874). As such, these results support the hypothesis of Carlson (1967), that rain plays a predominant role in *C. beticola* conidia dispersal.

Air temperature (expressed as GDD) was the only weather variable tested that was not significantly correlated with lesion number either year. This is in contrast to what was found in two recent studies conducted by Khan et al. (2009) and Tedford et al. (2018). These studies, both of which used Burkard spore traps to monitor *C. beticola* conidia levels, found air temperature to be the most influential weather variable for conidia production and dispersal. Khan et al. (2009) reported a strong correlation (R^2 of 0.813 to 0.870) between air temperature and peaks in detected conidia. The correlation results from the current study and those from Khan et al. (2009) cannot be compared fairly, however, because of the experimental design and the statistical methods employed in each. In their experiment, Khan et al. (2009) relied on artificial inoculation, which may have affected the levels of initial inoculum. They also did not begin recording weather data until June 21. As such, some of the conidia they detected could have been from secondary infection, thus potentially increasing the influence of weather variables which are favorable for infection and disease development. During their data analysis,

Khan et al. (2009) only correlated the number of conidia to air temperature when the relative humidity was above 87%. Because of this, only six to eight data points per site-year were used, out of approximately 84 total observations per site-year. Eliminating such a large amount of the data may have affected the results of the analysis. While Tedford et al. (2018) also found air temperature to be the dominant weather variable influencing conidia concentration, the correlation they found between the two was not as strong (R^2 of 0.1086). In their final model, relative humidity, rainfall, and leaf wetness were included (R^2 of 0.1639), but they determined that neither rainfall nor leaf wetness made a substantial contribution to the aerial concentration of conidia they detected. In the present study, the opposite pattern was found; moisture values, specifically rainfall and relative humidity, were consistently important to the number of lesions detected, while air temperature (in the form of growing degree days) did not have a significant influence on the number of CLS lesions observed ($p>0.47$).

The differences in significant variables observed between the above experiments and the current study could be explained in part by the time increment used for the measurements. In the Lawrence and Meredith (1970), Khan et al. (2009) and Tedford et al. (2018) studies, conidia and weather measurements were taken hourly, while in current study, sentinel beets remained in the field for an entire week. This experimental design enabled an increase in the number of sites and environments tested, but necessitated the conversion of weather data from hourly to weekly values. For the temperature and relative humidity modifications, the growing degree days had a base temperature of 15°C, and the relative humidity used was limited to 80% and above. Leaf wetness was calculated as the number of hours during which the leaf was wet for 15 minutes, rainfall measured as the total for the week, and both wind speed and solar radiation were averaged. While these conversions were based on what is known about *C. beticola* conidia

biology [Pool and McKay, 1916b; Coons and Larmer, 1929; Bleiholder and Weltzien, 1972b (in German), with English summary], it is possible the correlations may have varied if a different weather average was used. There are some weather trends which may have been missed by making an average over the week. For instance, Tedford et al. (2018) were able to examine the impact of each variable six, 12, 18, and 24 hours before the spores were detected. Such precision was impossible with the current experiment, but could be quite important for CLS epidemiology.

The above differences also could be explained by the method in which spores were trapped. In both the current and the Carlson (1967) studies, sentinel beets were used to detect *C. beticola* spores, and both found rainfall to be influential. In the other three studies, Hirst-type spore traps (also referred to as Burkard spore traps) were used to collect conidia, and none of them found rainfall to be a significant factor contributing to spore concentration. Lawrence and Meredith (1970) even asserted that the methods used by Carlson were somewhat biased in favor of dispersal by rainfall and less efficient at detecting conidia in the air. As they indicated, the Hirst spore trap is built for detecting windborne conidia, and because of its small (2x14 mm) orifice, which is designed to collect spores while facing into the wind (Hirst, 1952), it is poorly suited to detect conidia dispersed by water drops. As such, it could be argued that the Hirst-type spore trap is biased in favor of wind dispersal. Furthermore, if the way sentinel beets trap spores is biased in favor of rainfall, it is a bias likely shared by commercial beets in the field, which might provide a more accurate representation of what is happening in the field. It also should be considered that while favoring detection of wind dispersed spores, the sampling efficacy of Hirst-type spore traps can be adversely affected by wind speed (Frenz, 1999), and are more efficient at trapping smaller particles, especially those less than 10 µm wide (Frenz, 1999).

Another factor which may have caused some variation between the results of the current study and the Hirst-type spore trapping studies is that in those studies, only typical *C. beticola* conidia were counted, while in the current study, both conidia and ascospores (if present) could be detected. In a study conducted by Jenkyn (1974) comparing the efficacy of Burkard spore traps, vertical cylinders, and sentinel plants at detecting airborne spores of *Erysiphe graminis*, variation between the Burkard spore traps and sentinels was found. This variation was partly attributed to ascospores which the researchers were unable to detect when examining the tape from their Burkard spore trap. Differentiating between conidia of *Cercospora* spp. also can be challenging, particularly with closely related species such as *C. beticola* and *C. apii* (Groenewald et al., 2005, 2013). In future studies, conducting either polymerase chain reaction (PCR) (Lartey et al., 2003) or an enzyme-linked immunosorbent assay (ELISA) (Caesar-TonThat et al., 2007) on the spore trap tape could aid in differentiating *C. beticola* spores from the other potential *Cercospora* spp. collected with a spore trapping method.

The current study along with the Carlson (1967) study showed that using sentinel beets can be an effective means to estimate *C. beticola* spore presence, alleviating many of the problems associated with spore traps. Of particular interest in this case is that the sentinel beets are capable of differentiating between the conidia of *C. beticola* and other *Cercospora* species, as it is primarily *C. beticola* which is capable of infecting and causing symptoms on sugar beets (Groenewald, 2013). Additionally, if a teleomorph of *C. beticola* is occurring, the sentinel beets should be equally capable of detecting conidia and ascospores, as conidia are and ascospores would likely be capable of infecting the plants (Jenkyn, 1974). While sentinel beets do have positive attributes, they also come with a unique set of challenges. Since the use of sentinel beets involves living organisms, the beets must be cared for before, during, and after being in the

field. In order for the sentinels to provide good data, they must be kept healthy, which means keeping them watered while in the field and preventing animals from causing damage to the leaves. Some animal damage did occur in the current study, particularly at the MSU beet site in 2017, but such damage can be managed with the use of a secondary cage around the beets. Another challenge associated with sentinel beets is the labor requirement, as it takes a great deal of time and effort to start them in the greenhouse, exchange them in the field, keep them at the right infection conditions, and to count the lesions. There also is the possibility of contamination in the greenhouse, either before they go to the field or after they are in the humidity chamber. There is a risk that the conditions will not be right in the chamber or greenhouse for infection and disease development. In the current experiment, the treated and untreated controls were used to account for these situations. Over the entire 2017 season, the average number of CLS lesions per plant on the treated control was over 150 for all but three weeks, while in 2018 this number was over 300 for each week. Thus, it can be concluded that the conditions in the humidity chamber were appropriate for infection and CLS disease development. On the untreated controls, an average of 0.91 lesions per plant over all the weeks developed in 2017, while in 2018, the average was 3.42 per plant. The limited infection on the untreated controls shows that the risk of the beets being exposed to a substantial number of conidia while in the greenhouse was minimal, as was the risk of cross contamination while in the humidity chamber and of the seeds being contaminated. A further complication is that since the sentinel beets in the field are exposed to any fungal genera present there, other types of disease lesions can develop on the leaves (unpublished). This can make acquiring an accurate lesion count for CLS difficult at times, thus it is necessary to have lesion counts taken by someone who has the expertise to identify CLS lesions. It also is prudent to confirm that at least a sample of the lesions are being caused by *C.*

beticola through the use of a method such as single spore isolation (Hildebrand, 1938), the induction of conidia production, PCR (Lartey et al., 2003), or ELISA (Caesar-TonThat et al., 2007).

As indicated by the current study as well as others [Pool and McKay, 1916b; Canova, 1959a (in Italian), with English summary; Bleiholder and Weltzien, 1972b (in German), with English summary; Tedford et al., 2018], the conditions for *C. beticola* infection do not exactly match those for conidia production and dispersal. However, the current models only take infection conditions into account (Shane and Teng, 1985; Windels et al., 1998; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018; WIN, 2018). Because of this and the fluctuation in the number of spores from week to week, it is possible that the current disease prediction models could be improved by taking the presence of *Cercospora beticola* spores into account. Throughout both years of the study, there were ten weeks when over 40 CLS lesions per plant were detected at one or multiple sites, and seven weeks when there were fewer than ten CLS lesions per plant detected at any site. Similar fluctuations have been detected by each study examining conidia concentration, including Carlson (1967), Lawrence and Meredith (1970), Khan et al. (2009), and Tedford et al. (2018). In spite of their artificial inoculation, Khan et al. (2009) still observed fluctuations in the number of conidia detected, with daily averages ranging from a low of zero conidia per cubic meter of air up to approximately 160 conidia per cubic meter of air. Tedford et al. (2018) observed that the production of conidia can occur over a broader range than infection, and the level of conidia present at different site-years did not lead to the same amount of disease. Based on these findings, they suggested that conidia are not a limiting factor for CLS, and argued that considering conidia concentration in the models will not improve their accuracy (Tedford et al., 2018). However, since all of these

studies have similar fluctuations in conidia levels throughout the season, it is reasonable to hypothesize that if fungicide applications were timed to coincide with conidia peaks which were followed by appropriate conditions for infection, CLS management might be improved. In the current study, one of the major CLS peaks during the 2017 season occurred the week of June 18. Two weeks later, during the week of July 1, the first lesions were found in a commercial field. It is hypothesized that if a protectant fungicide had been applied when this peak occurred, the incidence of CLS in this field may have been reduced.

Accounting for spore presence could be an important addition to the models. However, further studies should be conducted before any such modifications are done to the models, as the data presented here and in previous studies support, but are not enough to justify such a change. Should a *C. beticola* spore detection study be repeated in the future, employing both sentinel beets and some kind of spore trap may provide improved results. Burkard spore traps could be effective for such a study, but a better choice may be something similar to a Rotorod spore trap. This spore trap has been shown to be equal to the Burkard spore trap at detecting aerial particles greater than 10 µm wide, is seldom affected by the wind (Frenz, 1999), and may be more capable of detecting spores dispersed by rain splashing (Carlson, 1967; Lawrence and Meredith, 1970). Regardless of the spore trap used, using both the sentinel beets and spore traps would bring together the strengths of the individual techniques. The methods could be used to check each other, and if there would be a time when one is detecting spores and the other is not, this could indicate another future area of study. For instance, if CLS lesions were observed on the sentinel beets but no conidia were found by the spore traps, this could indicate that the teleomorph is occurring, and responsible for those lesions.

If either the Shane and Teng (Shane and Teng, 1985; Windels et al., 1998; Jacobsen, 2010) or the BEETcast (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018; WIN, 2018) prediction models are to be modified to account for spore presence, there are several ways in which such a modification could be done. One option would be to use spore traps to directly measure the presence of *C. beticola* conidia. This could give growers a more immediate representation of the level of conidia present in the environment. Such a method would require a yearly input of labor to be implemented. Another method would be to determine the environmental conditions conducive for spore production and dissemination in the field, which would then be incorporated into the model. Based on the results of the current study as well as those of Carlson (1967), reflecting an enhanced risk from periods of rain could be particularly useful. Modifying the models in such a manner would reduce the need for yearly labor inputs, but it would not provide the more immediate measure of conidia presence. Regardless of the method by which it is done, including spore presence in the models should be further investigated. It is possible this modification could increase the efficacy of the disease prediction models, and may help growers improve the timing of their fungicide applications, thus allowing them to improve CLS management and/or increase the environmental sustainability of their farms.

CHAPTER 3

THE EFFECTS OF BICARBONATE SALTS ON IN VITRO AND IN VIVO GROWTH OF *CERCOSPORA BETICOLA*

Introduction

Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola*, is a severe foliar disease of sugar beets (*Beta vulgaris* subsp. *vulgaris*) in many beet growing areas of the world (Holtschulte, 2000) including the Great Lakes region of the United States (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). The fungus primarily infects beet foliage, causing small (3-5 mm) circular lesions on the leaf blades and oblong lesions on the petioles (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Bennett and Leach, 1971; Jacobsen and Franc, 2009). Lesion centers are generally tan or ash gray in color, and often surrounded by a red or brown border (Townsend, 1914; Coons et al., 1930; Bennett and Leach, 1971; Steinkamp 1979; Weiland and Koch, 2004; Jacobsen and Franc, 2009). Pseudostromata are scattered throughout the center of the lesions, appearing as small black dots when observed under a hand lens (Coons et al., 1930; Steinkamp et al., 1979; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Jacobsen and Franc, 2009; Franc, 2010). From these pseudostromata, the fungus can produce conidiophores upon which silver, needlelike conidia are created (Pool and McKay, 1916b; Coons et al., 1930; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009; Groenewald et al., 2013). Even though *C. beticola* has an extensive distribution (Holtschulte, 2000), the disease has the greatest impact in areas where the climate during the growing season is warm and wet [Bleiholder and Weltzien, 1972a (in German) as cited by Holtschulte, 2000], with ideal conditions for infection being daytime high temperatures between 27°C and 32°C, nighttime low temperatures greater than 16°C, and a relative humidity greater than 60% for 15-

18 hours a day (Pool and McKay, 1916b; Duffus and Ruppel, 1993; Asher and Hanson, 2006). In the ideal temperature range and at a humidity of 90% or above, conidia germination and infection can take place in a shorter time, as quickly as three hours (Pool and McKay, 1916a; Shane and Teng, 1984a). CLS is a polycyclic disease (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010), and since one generation can be completed in 10-13 days under ideal conditions (Coons et al., 1930), it is possible for several disease cycles to occur in a season (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010). During the course of an epidemic, several lesions may form on a single leaf. This, along with the effect of toxins and enzymes produced by the pathogen, can lead to leaf death, with defoliation occurring if multiple leaves are affected (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Weiland and Koch, 2004).

When a severe epidemic occurs, it is possible for entire plants and even whole fields to become defoliated as a result of CLS (Coons et al., 1930). It is primarily through this decrease in photosynthetic area that the disease inflicts yield loss (Townsend, 1914; Coons et al., 1930; Smith and Ruppel, 1973; Shane and Teng, 1992; Franc, 2010), which can reach 40% if left unmanaged (Jacobsen and Franc, 2009). This loss of yield can be the result of several factors, including a decrease in the amount of sugar produced and stored by the beets, a decrease in the overall size of the beet roots (Townsend, 1914; Coons et al., 1930; Smith and Ruppel, 1973; Shane and Teng, 1992; Franc, 2010), an increase in impurities which can hinder sucrose extraction (Coons et al., 1930; Smith and Martin, 1978; Shane and Teng, 1992; Jacobsen and Franc, 2009), and possibly an increase in storage losses (Smith and Ruppel, 1971). In order to manage CLS and reduce such yield loss, a variety of different tactics are utilized, including cultural practices, host resistance, and fungicide applications (Duffus and Ruppel, 1993; Asher

and Hanson, 2006; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). While each of these management techniques can significantly reduce the impact of CLS, none of them should be viewed as a standalone solution (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Jacobsen, 2010). Rather, an integrated approach should be taken to keep CLS from inflicting economically significant damage (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Jacobsen, 2010).

While there are several options for CLS management, the application of chemical fungicides remains a particularly important strategy for growers (Duffus and Ruppel, 1993; Weiland and Koch, 2004; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Jacobsen, 2010). The fungicides available for use against CLS can be divided into two broad groups, protectant and systemic fungicides (Crowdy, 1971; Lamey et al., 1987; Brent and Hollomon, 2007b; Jacobsen, 2010). Within each of these groups, several fungicide classes are used, with differing modes of action as indicated by their Fungicide Resistance Action Committee (FRAC) code (FRAC, 2005). For the protectants, the different classes used include organotins (FRAC group 30), copper based fungicides (FRAC group M1), and ethylene bis-dithiocarbamates (EBDC, FRAC group M3). Systemic fungicide classes which commonly are or have been used against CLS within the United States include the benzimidazoles (FRAC group 1), quinone outside inhibitors (QoI) some of which are called strobilurins, (FRAC group 11), and sterol demethylation inhibitors (DMI, FRAC group 3), especially the triazoles (FRAC, 2005; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). While the application of fungicides has been an effective management strategy for CLS in the past (Khan and Smith, 2005; Poindexter, 2007), *C. beticola* in the United States has developed field level resistance to several fungicides, including the benzimidazoles (Ruppel and Scott, 1974; Weiland and Halloin, 2001),

organotins (Bugbee, 1995), strobilurins (Kirk et al., 2012; Bolton et al., 2012a), and triazoles (Bolton et al., 2012b; Trueman et al., 2017). Such resistance has decreased the effectiveness of this management strategy and has made the overall management of CLS more challenging (Khan, 2015; Michigan Sugarbeet REACh, 2016).

If CLS is to be managed successfully in the future, the development of new fungicides from chemical groups with different modes of action will be essential. One group of chemicals which may help protect beets against CLS is the bicarbonate salts (FRAC group NC; FRAC, 2005), particularly ammonium, potassium, and sodium bicarbonate. These compounds possess broad spectrum antimicrobial properties which allow them to inhibit the growth of bacterial and yeast species (Corral et al., 1988). As a consequence of their antimicrobial activity, bicarbonates have been tested as fungicides for plant pathogens (Homma et al., 1981; Ziv and Zitter, 1992; Deliopoulos et al., 2010). A few of the earliest recorded examples of bicarbonate salts being used this way in the United States include their use to inhibit storage pathogens (*Penicillium* spp.) of citrus fruit in the 1920's (Palou et al., 2001; McGovern et al., 2003) and against powdery mildew of roses (*Sphaerotheca pannosa* var. *rosae*) in the 1930's (Deliopoulos et al., 2010). Since these early tests, more extensive studies into their efficacy and physiological activity have been conducted. Homma et al. (1981) found that sodium bicarbonate reduced infection by citrus common green mold (*Penicillium digitatum*), cucurbit powdery mildew (*Sphaerotheca fuliginea*), rice blast (*Pyricularia oryzae*), and citrus melanose (*Diaporthe citri*). In the 1990's, Ziv and Zitter (1992) at Cornell University tested ammonium, sodium, and potassium bicarbonate individually in vivo against cucurbit powdery mildew, as well as Ulocladium leaf spot (*Ulocladium cucurbitae*), gummy stem blight (*Phoma cucurbitacearum*), and Alternaria leaf blight (*Alternaria cucumerina*). For each pathogen, they found that one or multiple bicarbonates

led to a significant reduction in disease incidence, with the impact of each bicarbonate varying from pathogen to pathogen. Plate tests of the bicarbonates with the latter three pathogens and *Colletotrichum orbiculare* showed the bicarbonates caused a decrease in hyphal growth (Ziv and Zitter, 1992). Around that same time, another team from Cornell University (Horst et al., 1992) tested sodium bicarbonate against powdery mildew (*Sphaerotheca pannosa* var. *rosae*) and black spot (*Diplocarpon rosae*) of rose. Like Ziv and Zitter, they reported a significant decrease in the severity of both diseases when the bicarbonate was applied (Horst et al., 1992).

In addition to the above studies, several others have found bicarbonates capable of reducing the severity of foliar diseases, including bean rust (*Uromyces appendiculatus*; Arslan et al., 2006), wheat leaf rust (*Puccinia triticina*; Arslan et al., 2006; Karabulut et al., 2006), apple scab (*Venturia inaequalis*; Ilhan et al., 2006; Jamar et al., 2007), greasy leaf spot of citrus (*Mycosphaerella citri*; McGovern et al., 2003), powdery mildew of pepper (*Leveillula taurica*; Fallik et al., 1997b), powdery mildew of *Euonymus* spp. (*Oidium euonymi-japonici*; Ziv and Hagiladi, 1993), powdery mildew of hazelnut (*Phyllactinia guttata* and *Erysiphe corylacearum*; Turkkan et al., 2018), powdery mildew of grape (*Uncinula necator*; Yildirim et al., 2002), and gray mold of several horticultural crops (*Botrytis cinerea*; Palmer et al., 1997). Bicarbonates also have shown efficacy against storage and soil borne pathogens, such as *Alternaria alternata* of pepper (Fallik et al., 1997a), silver scurf of potatoes (*Helminthosporium solani*; Olivier et al., 1998), *Sclerotium rolfsii* isolated from bentgrass (*Agrostis palustris*) and annual bluegrass (*Poa annua*; Punja and Grogan, 1982), blue and green mold of citrus (*Penicillium italicum* and *P. digitatum*; Palou et al., 2001), and black root rot of carrots (*Thielaviopsis basicola*; Punja and Gaye, 1993). In their review, Deliopoulos et al. (2010) provide a summary of these and other examples of pathogens being controlled by bicarbonate salts.

In many cases, the bicarbonates provide moderate control, resulting in a statistically significant decrease in disease severity, but often not to the level provided by some of the systemic fungicides (Avis, 2007; Deliopoulos et al., 2010). However, the bicarbonates appear to be particularly effective against bean rust (Arslan et al., 2006), wheat leaf rust (Arslan et al., 2006; Karabulut et al., 2006), and apple scab (Ilhan et al., 2006; Jamar et al. 2007). For each of these diseases, the researchers found that concentrations of one or several of the bicarbonates tested individually were able to cause complete inhibition of spore germination and germ tube elongation (Arslan et al., 2006; Karabulut et al., 2006; Ilhan et al., 2006), as well as decrease the disease incidence in vivo, in either a greenhouse or the field (Arslan et al., 2006; Karabulut et al., 2006; Ilhan et al., 2006; Jamar et al. 2007). Several of these studies reported that the bicarbonates provided a level of disease reduction that was not significantly different from or was greater than that provided by an industry standard fungicide treatment (Karabulut et al., 2006; Ilhan et al., 2006; Jamar et al. 2007).

Bicarbonates also were reported to be effective against greasy leaf spot (*Mycosphaerella citri*), which is of particular interest because *M. citri* and *C. beticola* belong to the same family. McGovern et al. (2003) conducted a field test comparing the effects of a commercial formulation of potassium bicarbonate, CD-2346 (now called Armicarb), against three industry standard treatments. They found that the treatment with CD-2346 caused a significant reduction in defoliation, and generally performed as well as two of the three industry standard treatments (McGovern et al., 2003). The efficacy of this potassium bicarbonate mixture indicates a potential for activity of one or more of the bicarbonates against a related pathogen like *C. beticola*.

In a majority of the bicarbonate studies above, it has been shown that the addition of an adjuvant can significantly increase the efficacy of the spray solution (Homma et al., 1981a; Ziv and Zitter, 1992; Horst et al., 1992; Ziv and Hagiladi, 1993; McGovern et al., 2003; Jamar et al., 2007; Deliopoulos, 2010). Ziv and Zitter (1992) found SunSpray® ultra-fine spray oil was particularly well suited for increasing the efficacy of bicarbonates against powdery mildew and other cucurbit diseases. In the case of cucurbit powdery mildew, they found that four days after inoculation, a treatment with 0.5% (w/v) sodium bicarbonate alone resulted in 89.5% of the leaf area infected, while the addition of SunSpray oil to the sodium bicarbonate decreased the infected area to 2.3% (Ziv and Zitter, 1992). The improvement in bicarbonate efficacy with the addition of adjuvants has been attributed to increased coverage and adhesion of the bicarbonate to the foliage, as well as some antifungal properties of the adjuvant itself, or even an interaction between the bicarbonate and adjuvant (Ziv and Zitter, 1992; Horst et al., 1992; Jamar et al., 2007). Homma et al. (1981a) showed that even when the same bicarbonate is used, the most effective bicarbonate-adjuvant combination can vary depending on the host-pathogen system being examined, so identifying the correct bicarbonate-adjuvant combination may be important for achieving effective management of a particular disease.

The current study investigated the antifungal properties of ammonium, potassium, and sodium bicarbonate against *Cercospora beticola*. Tests included in vitro plate screens using several isolates of *C. beticola* and an in vivo experiment to determine the impact of ammonium and sodium bicarbonate on *C. beticola* in a greenhouse with a small number of adjuvants. Tests also were conducted to investigate varied modes of action for growth reduction, including pH and potential ammonia production. If these tests show that one or more of the bicarbonates can

inhibit the growth of *C. beticola* and reduce the severity of CLS, it is possible these compounds could contribute to management of CLS in the field.

Materials and Methods

Isolates

During the summer of 2016, four isolates of *Cercospora beticola* were collected from sugar beet fields in Michigan. Two of the isolates, BE4 and BW14, were collected from fields near the town of Fairgrove; one isolate, RFR34, from Michigan State University's Saginaw Valley Research and Extension Center; and the fourth isolate, MSU165, from a research plot on the campus of Michigan State University. Individual isolates were purified from leaves by a dry-needle, single spore isolation method (Hildebrand, 1938). Later experiments were conducted with 12 additional isolates, collected in Michigan and Ontario, Canada (Table 3.1). All isolates were maintained on half strength, clarified V-8 juice (Campbell Soup Co., Camden, NJ) agar (Miller, 1955) and kept on a light bench at room temperature (approximately 25°C).

Cercospora beticola Isolates				
Isolate	Year Collected	Location of Collection	County	Experiments
MSU165	2016	East Lansing, MI	Ingham	1, 3
RFR34	2016	Richville, MI	Tuscola	1, 3, 4
BW14	2016	Fairgrove, MI	Tuscola	1, 3, 4
BE4	2016	Fairgrove, MI	Tuscola	1, 3, 4
Blum1-3	2017	Blumfield, MI	Saginaw	2, 4
CA464-1	2016	Ontario, CAN	Kent or Lambton	2, 4
VanN	2017	Munger, MI	Bay	2
VanS5-1	2017	Munger, MI	Bay	2
P4	2015	Sebewaing, MI	Huron	2, 4
H1	2015	Bay City, MI	Bay	2, 4
CA453	2016	Ontario, CAN	Kent or Lambton	2
RangeA	2013	East Lansing, MI	Ingham	2
MSU178	2016	East Lansing, MI	Ingham	2, 4
RFS113	2016	Richville, MI	Tuscola	2
LB155	2016	Fairgrove, MI	Tuscola	2
LB141	2016	Fairgrove, MI	Tuscola	2, 4

Table 3.1: *Cercospora beticola* isolates used in the bicarbonate study. A list of the *C. beticola* isolates used, including isolate designation, year collected, location of the field from which leaf samples were obtained, and which experiments the isolates were used in. Special thanks to the Michigan Sugar agriculturalists who collected CA464-1, P4, H1, and CA453, as well as Thomas Goodwill who collected RangeA.

Media Preparation

Lima bean agar (Difco, Detroit, MI; and HiMedia Laboratories, Mumbai, India) was used unless otherwise noted. After preliminary tests for preparation methods (Appendix C), the agar was prepared according to the manufacturers' instructions, autoclaved for 45 minutes at 121°C and 18 psi, and placed in a water bath until it equilibrated to 60°C. Five grams of each bicarbonate, including ammonium bicarbonate (Fisher Scientific Co., Fair Lawn, NJ), potassium bicarbonate (Fisher Scientific Co.), and sodium bicarbonate (Spectrum Chemical Manufacturing Corp., Gardena, CA) were individually decontaminated by suspension in 70% ethanol. The suspension was shaken by hand and allowed to sit for at least five minutes. Once the media had equilibrated, bicarbonate suspensions were added in a biosafety cabinet (SterilGARD Class II

TypeA/B3, The Baker Co., Sanford, ME) for a final bicarbonate concentration of 1% (weight to volume) and a final ethanol concentration of 0.7% (volume to volume). After being swirled to ensure thorough mixing, 20 ml aliquots of media were pipetted into 100 mm by 15 mm polystyrene Petri dishes (VWR International, Radnor, PA). The process was repeated for each treatment, after which the media was allowed to solidify overnight. Experiments 1 through 3 had four treatments: ammonium, potassium, and sodium bicarbonate (each at 1% w/v) and an ethanol control (0.7% v/v). The media for Experiment 4 was prepared in a similar way, but the treatments varied as described below.

Plugs were cut from *C. beticola* isolates on half strength V-8 juice media (at least two weeks old) with a flame sterilized cork borer (approximately 7 mm in diameter). One fungal plug was placed in the center of each Petri dish using a flame sterilized dissecting needle. Upon completion of the transfers, plates were removed from the biosafety cabinet and sealed with paraffin film (Parafilm, Bemis North America, Neenah, WI). Plates were kept in an incubator (Stabil-therm, Thermal Product Solutions, New Columbia, PA), and the cultures were allowed to grow in darkness at ambient temperature, approximately 23-24°C (Experiments 1 and 3), or 26-28°C (Experiments 2 and 4). Colony diameter measurements were taken with a six inch caliper (General MG, No. JDC-6, General Tools, Secaucus, NJ) every other day starting at five days after transfer and ending 17 days after transfer for each colony. In order to better represent fungal growth, the diameter of the starting plug was subtracted from the overall colony diameter measurement. Data was recorded and organized using Microsoft Excel 2010 (Microsoft Corp., Albuquerque, NM), and statistical analysis was completed using SAS 9.4 (SAS Institute Inc., Cary, NC).

Experiment 1: Impact of Bicarbonates on *Cercospora beticola* Growth

To determine the impact bicarbonates have on *Cercospora beticola* vegetative growth, four isolates (BE4, BW14, RFR34, and MSU165) were transferred to plates of the four treatments and allowed to grow as described above. Each isolate/treatment combination was replicated three times, and the entire experiment was repeated three times (runs 1, 2, and 3) at 23-24°C. The experiment was set up in a two factor, randomized complete block design with sub samples; with isolate and treatment as the factors and the runs as blocks. Upon obtaining the diameter of fungal growth, the percent reduction as compared to the ethanol control was calculated by subtracting the diameter of fungal growth on a bicarbonate plate from the diameter of a growth on a corresponding control plate and dividing by the diameter on the control plate. An ANOVA table was constructed for both the diameter and percent reduction at each time with the proc mixed procedure in SAS 9.4. Mean separation with the least significant difference (LSD, α=0.05) method was completed using the proc glimmix procedure when appropriate.

Experiment 2: Expanded *Cercospora beticola* Isolate Testing

If one or more of the bicarbonates are to be used for management in the field, it is necessary that they be effective at reducing the growth of many different *Cercospora beticola* isolates. To determine how effective they would be against a number of diverse isolates, Experiment 1 was expanded. In this test, 12 different isolates of *C. beticola* (Table 3.1) were tested in vitro with the three bicarbonates and the ethanol control. The test was set up similarly to the first, consisting of three runs, each comprised of eight isolates per run. Each treatment by isolate combination was repeated three times per run, and every isolate was tested in two different runs. During this test, the temperature of the incubator was 26-28°C. The experimental design was a two factor incomplete block design, with isolate and bicarbonate treatment as the

factors, and the runs as blocks. Diameter measurements were taken every other day, starting at five days after transfer and going until 17 days after transfer. Percent reduction in growth was calculated, and analyzed as above.

Experiment 3: pH Testing

It has been suggested that pH plays an important role in the antifungal activity of bicarbonates (Punja and Grogan, 1982; Corral et al., 1988; Palmer et al., 1997; Deliopoulos et al., 2010). Therefore, an effort was made to determine the pH of the media from each treatment group. This was initially done by dipping pH indicator paper (Micro Essential Laboratory Inc., Brooklyn, NY) into a solution of bicarbonate and tap water (at a concentration of 1% w/v) for about 15 seconds. pH indicator paper also was placed onto media plates for about 30 seconds. To gain insight into the pH of the plates throughout the experiment, bromothymol blue indicator dye (Sigma Chemical Co., St. Louis, MO) was added to the media of each treatment at a rate of 0.05 g per 500 ml of media. Isolates BE4, BW14, RFR34, and MSU165 were transferred to the plates and sealed with Parafilm one day after pouring. Observations of media color were made before the test and every other day from five days after transfer to 23 days after transfer, during which time the temperature remained in the range of 23-24°C. pH was estimated by comparison to a color chart (Figure 3.1). At the higher pH levels (above 8.1), it was difficult to discern pH from just plate color alone, so these estimates of pH were made in conjunction with knowledge from the testing done with pH paper. Every treatment by isolate combination was replicated three times, and the test was repeated once.

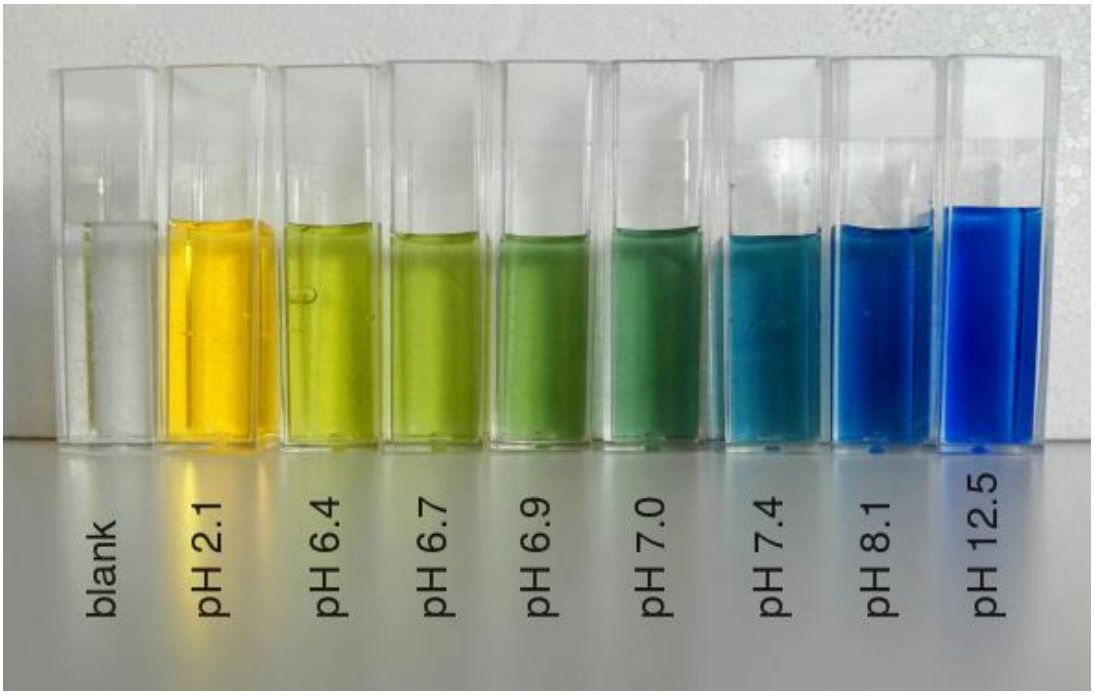


Figure 3.1: Bromothymol blue color range. The range of colors produced by bromothymol blue pH indicator dye, along with their corresponding pH levels (used under the Creative Commons Attribute-Share Alike 4.0 International license, Gregor Trefalt, 2017, https://commons.wikimedia.org/wiki/File:Bromothymol_blue_colors_at_different_pH.png).

Experiment 4: Growth on Ammonium Bicarbonate Plates

Due to the impact of ammonium bicarbonate on the growth of *Cercospora beticola*, tests were conducted to determine if the effect of the ammonium bicarbonate was fungicidal or fungistatic. After completion of the first run of Experiment 1, *C. beticola* plugs from four ammonium bicarbonate plates, one from each of four isolates, were transferred to plates of unamend lima bean agar. In addition, new fungal plugs from V-8 agar plates were transferred to the old ammonium bicarbonate plates. To test the viability of plugs transferred to ammonium bicarbonate-amended media in Experiment 2, *C. beticola* plugs from three ammonium bicarbonate plates for each of four isolates (Blum1-3, CA464, H1, P1) in the final run were transferred to new lima bean agar plates, as were fresh plugs from actively growing plates. In all, 12 plates comparing ammonium bicarbonate-treated plugs and plugs from growing colonies were prepared.

It was hypothesized some or all of the activity of the ammonium bicarbonate could be from ammonia gas being released from the plates and not the activity of the bicarbonate itself (Punja and Grogan, 1982). Therefore, strips of ammonia test paper (QA Supplies, LLC, Norfolk, VA) were wetted with tap water and hung on the plate lids with tape in order to determine the ammonia levels within day-old and week-old plates of each bicarbonate in the absence of fungi.

To determine the impact of ammonia gas on the growth of *C. beticola*, an experiment was conducted in which 100 mm by 15 mm polystyrene x-plate Petri dishes (VWR International) were filled with lima bean agar in alternating quadrants; two that were amended with 1% (w/v) ammonium bicarbonate and ethanol (0.7% v/v), and two that were amended with just ethanol (Figure 3.2). Plates of half strength V-8 juice agar were used to confirm growth in the absence of potential ammonia. Hyphal plugs from growing *C. beticola* plates were transferred to each quadrant of the x-plates and to the V-8 plates, and incubated as described above at 26-28°C. Three isolates (BE4, LB141, and Blum1-3) were tested, and each isolate by treatment combination was replicated three times. Since gas exchange could occur in the space above the x-plates, if a cause of fungicidal activity was ammonia gas, the plates in the ethanol quadrants should have been equally affected as the ones in the ammonium quadrants. Diameter measurements were taken after seven days of incubation. After 27 days of incubation, one of the plugs from the ammonium quadrants, one of the plugs from the ethanol quadrants, and one plug from the V-8 plates were transferred to fresh lima bean agar and observed for growth five days later.

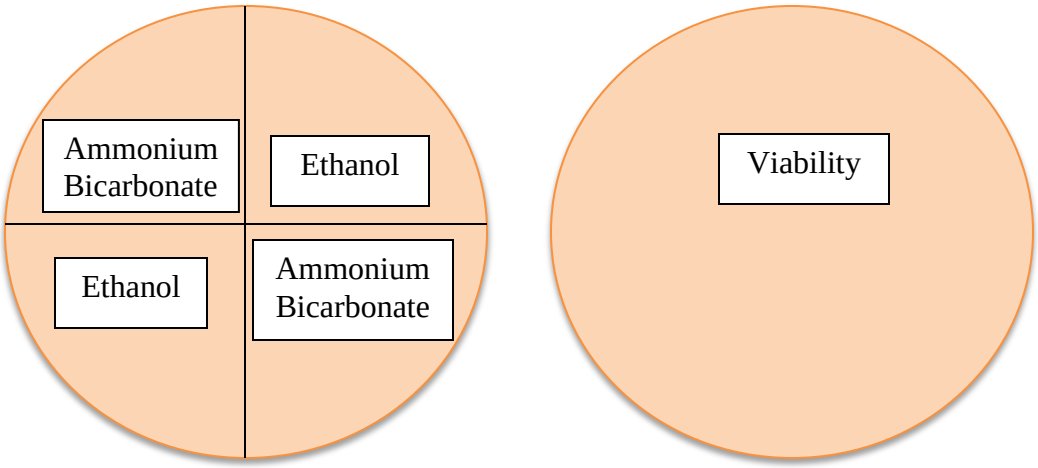


Figure 3.2: X-plate test. A diagram of how x-plates were used to test for the effect of potential ammonia gas release from the ammonium bicarbonate-amended agar on *Cercospora beticola*. Two different plates were poured. One was a half strength V-8 viability test, while the other was an x-plate with lima bean agar either amended with 0.7% (v/v) ethanol ("ethanol"), or 1% (w/v) ammonium bicarbonate in 0.7% ethanol ("ammonium bicarbonate").

Experiment 5: Greenhouse Trials

To determine the activity of bicarbonates on sugar beet, a greenhouse test was conducted. The sugar beet variety was B149N (Betaseed, Inc., Bloomington, MN) that is grown in the Great Lakes region and is highly susceptible to CLS (Michigan Sugarbeet REACh, 2017). Approximately 25 seeds were planted into each of three 2.45 liter plastic nursery pots (Poly-tainer NS300, Nursery Supplies, Inc., Chambersburg, PA) filled with potting mix (SureMix Perlite, Michigan Grower Products, Inc., Galesburg, MI). At approximately the 2 leaf growth stage, single beets were transplanted into their own 3.79 liter plastic pot (Classic 400, Nursery Supplies, Inc., Chambersburg, PA). Beets were grown under typical greenhouse conditions, with the temperature ranging from 20-30°C, and 12 hours of supplemental light from 8:00 a.m. to 8:00 p.m. in addition to natural light. Beets were usually watered Monday, Wednesday, and Friday of every week to ensure adequate moisture for growth. At the 4-6 leaf growth stage, beets were fertilized with 40 ml of 14-14-14 fertilizer (Osmocote, Everris, Geldermalsen, the Netherlands) and predatory mites (*Tyrophagus putrescentiae*, Koppert Biological Systems,

Berkel en Rodenrijs, the Netherlands) were used to manage thrips (order Thysanoptera, species unknown). Beets were used for the test once they reached at least the 10-12 leaf growth stage.

The entire experiment was repeated three times, once in February of 2018 (run one) and twice in November of 2018 (runs two and three). For the first run, beets were planted on November 8, 2017, and both the treatment and inoculum were applied as described below on the same day, February 23, 2018. The experimental design was a randomized complete block design with two factors, active ingredient and adjuvant. The active ingredient factor consisted of three treatments, including no active ingredient, sodium bicarbonate, and ammonium bicarbonate. Both bicarbonates were applied at a 2% (w/v) concentration. The adjuvant factor also had three levels, including a detergent, Tween 20 (Sigma-Aldrich, St. Louis, MO); a combination deposition aid, canopy penetrant, spreader, sticker, and drift control agent, MasterLock (WinField Solutions, LLC, St. Paul, MN); and a crop oil, SunSpray ultra-fine spray oil (HollyFrontier Refining & Marketing, LLC, Plymouth Meeting, PA), each at a 1% (v/v) concentration. Every treatment by adjuvant combination was replicated four times. The spray solutions were prepared immediately before application by adding 10 g of bicarbonate followed by 5 ml of adjuvant to 500 ml of reverse osmosis water. Hand held spray bottles (Lansing Sanitary Supply, Inc., Lansing, MI) were used to apply the solutions, with each treatment in its own bottle to prevent cross contamination. Spray solution was applied to just before runoff, which was approximately 31 ml of solution per plant, applied to both the upper and lower leaf surfaces.

Plants were allowed to dry for at least five hours before the inoculum was applied, during which time the inoculum was prepared. Six different isolates of *C. beticola* were used for inoculum, including BE4, Range A, Van N, Blum1-3, H1, and P4. Isolates were grown on half

strength V-8 juice agar on a light bench at room temperature (approximately 25°C), starting two weeks prior to inoculation with a total of 17 plates. Additionally, 15 shake cultures were grown starting one week before inoculation by adding mycelia plugs to 50 ml of 10% clarified V-8 juice (Miller, 1955) in water. Shake cultures were kept in an incubator shaker (Innova 44, New Brunswick Scientific, Edison, NJ), set at 110 rpm and 24°C. On the day of inoculation, the fungal plates were flooded with sterile water and scraped with an L-shaped bacterial cell spreader (VWR International) to release hyphae and conidia. The suspension was poured into a blender (Waring commercial blender, Waring Products, Inc., Torrington, CT) along with the contents of the shake cultures, and chopped to ensure the hyphal fragments were small enough to be applied through a hand held sprayer. A count of the conidia and hyphal fragments was taken with a hemocytometer, and the suspension was diluted to a final spore and hyphae concentration of approximately $8.0x10^4$. Tween 20 was added to the suspension at a rate of 0.1% (v/v) to ensure the conidia and hyphal fragments were able to adhere to the leaves. Consequently, all of the treatments in the first run ended up with at least 0.1% Tween 20. The final suspension was applied to the leaves with a hand held sprayer at a rate of approximately 30 ml per plant.

After inoculation, the leaves were allowed at least two hours to dry before being placed in a humidity chamber. The chamber was constructed of three metal carts (Super Erecta Shelving, InterMetro Industries Corp., Wilkes-Barre, PA) with 6 mm clear plastic sheeting (HDX, Home Depot, Atlanta, GA). The chamber was 75 cm tall, 140 cm wide, and 183 cm long (Figure 2.3, page 62). The humidity within the chamber was sustained above 95% by covering the floor of the chamber with wet newspaper and through the use of two 4.54 liter humidifiers (model QLS-05, Hunter, Memphis, TN). The humidity fluctuated throughout the day; for 16 hours starting at about 8:00 p.m., the humidifiers were turned on and the chamber was closed, then at noon it was

opened and the humidifiers were turned off. This was done to encourage infection (Rathaiah, 1976; Rathaiah, 1977) and to reduce the risk of overheating during the day. The blocks of the experiment were set up by beet placement within the humidity chamber, as there was a possibility that environmental conditions could vary based on location. Beets remained in the chamber for three humidity cycles, during which time both temperature and humidity in the chamber were monitored with weather stations (Watchdog Model A150, Spectrum Technologies, Inc., Aurora, IL). During the humidity treatment, beets were given 16 hours of artificial lighting, from 6:00 a.m. to 8:00 p.m.

Upon completion of the infective period, beets were removed from the chamber and returned to the greenhouse conditions described above, with 16 hours of artificial lighting. They were kept for four weeks, at which point spot counts were taken of each plant. An ANOVA table was constructed for the spot counts, and mean separation by the least significant difference method (α=0.05) was completed using the proc glimmix procedure.

The second and third runs of the experiment were similar to the first, except as described below. The second run was planted on August 30 and treated on November 8, while the third was planted September 13 and treated on November 12. Like the first run, the second and third were randomized complete block designs with two factors, active ingredient and adjuvant. However, the active ingredient factor had only two levels, none and sodium bicarbonate. The adjuvant factor still had three levels, but Activator-90 (Loveland Products, Inc., Greeley, CO), a non-ionic surfactant, penetrant, and antifoaming agent, was used instead of MasterLock. As a result of the reduction in factor levels, each active ingredient by adjuvant combination was repeated six times rather than four. Spray solutions were prepared in a similar way, with a 2% (w/v) concentration of bicarbonate and 1% (v/v) concentration of adjuvant. For each run, the

spray solution was applied until just before runoff. In the second run, this came to an average of 28 ml per plant. The plants in the third run were noticeably larger than in the second, so an average of 38 ml per plant was applied. Prior to treatment and inoculation, some powdery mildew was observed on a few plants of both the second and third run. For most of the experiment, this did not appear to be a significant issue, but at the very end of the experiment (right before spot counts were taken), the level of powdery mildew did become severe.

Once the applications were complete, plants were allowed at least five hours to dry before the inoculum was applied. Unlike the first run, the inoculum in runs two and three was prepared in the method described by Ruppel and Gaskill (1971). For these, beet leaves with heavy CLS symptoms were collected from the Saginaw Valley Research and Extension Center and air dried. The day before inoculation, 69.7 g of the dried leaf material was rehydrated in 1,650 ml of water. On the day of inoculation, the soaked leaves were scrubbed by hand for about ten minutes to release the spores, then the solid debris was strained with a sieve that had a mesh size of approximately 1.1 mm by 1.8 mm. Spore counts were taken with a hemocytometer, and the spore suspension was diluted in water until the final conidia concentration was approximately $1x10^4$. No Tween 20 was added to the spore suspension, because beet leaves naturally contain saponins which are released during scrubbing and allow for spore adhesion to the leaves (Ridout et al., 1994). Inoculation was done with a hand held sprayer at a rate of approximately 30 ml per plant.

Upon completion of the inoculation, beets were moved to a humidity chamber. For the second and third runs, the humidity chamber was housed within a walk-in cold room (Harris Environmental Systems, Andover, MA), measuring 280 cm tall, 345 cm wide, and 742 cm long. This allowed for improved control of the temperature, which remained at a constant 24°C.

While in the humidity chamber, all of the light was provided by six sodium halide bulbs which were programmed on a 16:8 hour light/dark cycle, with the lights being turned on at 6:00 a.m. and off at 10:00 p.m. The photosynthetic photon flux density of the light within the cold room was approximately 30 µmol/(m^2*s), and within the humidity chamber it was approximately 20 µmol/(m^2*s). The humidity chamber was constructed out of four metal carts wrapped in 6 mm thick plastic, with final dimensions of 75 cm tall, 183 cm wide, and 183 cm long. Humidity was maintained as above, except different humidifiers were used (one 4.54 liter, model QLS-05, Hunter; one 5.68, 33520 ultrasonic warm/cool mist humidifier, Hunter). As in the first run, the relative humidity was cycled from dry to humid for 8 and 16 hours, respectively, with the dry cycles beginning around noon each day. Humidity and temperature were recorded with weather stations. Beets were kept in the humidity chamber for three humid cycles and then moved back to typical greenhouse conditions as described above. This time, the beets were returned to 12 hours of artificial light. Spot counts of each plant were taken after four weeks and analyzed by the same method as the first run.

Results

Experiment 1: Impact of Bicarbonates on *Cercospora beticola* Growth

Experiment 1 examined the impact of bicarbonates on hyphal growth of four different *C. beticola* isolates in vitro. The ANOVA tables for colony diameter revealed that at all times measured, the effects of treatment and isolate were significant ($p<0.0001$ for treatment and $p<0.0006$ for isolate, at all times), and for all times except five days after transfer, the treatment by isolate interaction was significant ($p<0.05$ for all significant times, ranging from 0.0005 to 0.0494). Since the interaction between treatment and isolate was not significant five days after transfer, the data from this time point was excluded from Table 3.2. The effect of block was

significant at all times except seven days after transfer ($p<0.04$ for all significant times, ranging from 0.0001 to 0.0374). For percent growth reduction, the effects of treatment and isolate were significant at all times ($p<0.0001$ for treatment and $p<0.045$ for isolate, ranging from <0.0001 to 0.0418), while the interaction of treatment and isolate was significant at all times except five and seven days after transfer ($p<0.045$ for all significant times, ranging from 0.0145 to 0.0436). The values from five and seven days after transfer were not included in Table 3.2 because the interaction between treatment and isolate was not significant at these two time points. The effect of block was significant at five ($p=0.0015$), seven ($p=0.0116$), and nine ($p=0.0175$) days after transfer.

The bicarbonate treatments had a significant impact on *C. beticola* growth at each time of the experiment ($p<0.0001$). Ammonium bicarbonate caused the greatest decrease in growth, resulting in complete inhibition of all reps of all the isolates tested. Sodium and potassium bicarbonate also significantly decreased *C. beticola* growth ($p<0.0001$), but growth was still observed with these treatments. Between the two, sodium bicarbonate resulted in significantly less growth than potassium bicarbonate ($p<0.0004$ for diameter comparison and $p<0.0015$ for percent reduction comparison) at every time tested (Figure 3.3, Table 3.2). While there was a significant treatment by isolate interaction at all times (except for five days after transfer for the diameter comparison, and five and seven days after transfer for percent reduction, $\alpha=0.05$), for a given isolate the ammonium bicarbonate always caused the most inhibition, followed by sodium bicarbonate, and with potassium bicarbonate providing equal or less growth inhibition than sodium bicarbonate.

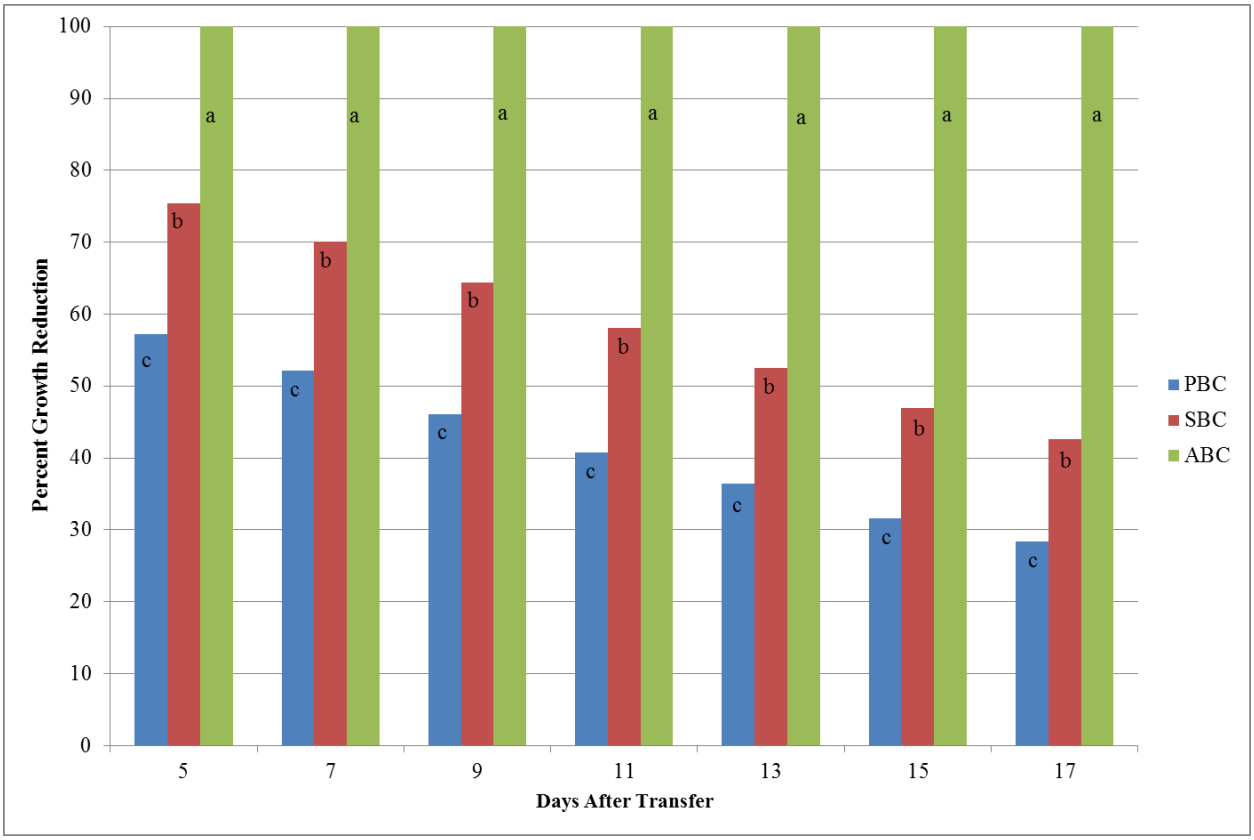


Figure 3.3: Percent reduction of *Cercospora beticola* growth in vitro. The average percent reduction in *Cercospora beticola* growth for each of three bicarbonate treatments compared to an untreated control. Each bar is an average of 36 observations; three observations from each of four isolates, replicated in three runs. Measurements were taken at seven different times, every other day from five to 17 days after transfer. Bar colors represent the different bicarbonate treatments; blue potassium bicarbonate (PBC), red sodium bicarbonate (SBC), and green ammonium bicarbonate (ABC). All are in lima bean agar at a rate of 1% (w/v). Mean separation was conducted for each time individually, so bars with the same letter at a given time point are not significantly different by least significant difference, α=0.05.

Impact of Bicarbonates on *Cercospora beticola* Growth in vitro													
Days After Transfer		7		9		11		13		15		17	
Isolate	Treatment	Colony Diameter (mm)	Percent Growth Reduction	Colony Diameter (mm)	Percent Growth Reduction	Colony Diameter (mm)	Percent Growth Reduction	Colony Diameter (mm)	Percent Growth Reduction	Colony Diameter (mm)	Percent Growth Reduction	Colony Diameter (mm)	Percent Growth Reduction
BE4	Control	19.06 A	NS	25.67 A	-	31.83 A	-	35.92 A	-	41.05 A	-	46.09 A	-
	PBC	7.87 D,E	NS	12.23 D	52.4 c	17.29 D,E	45.7 d	21.84 D	39.1 d	27.52 E	32.6 d	32.62 D	28.5 d
	SBC	4.68 F, G	NS	7.23 F,G	72.0 b	10.34 F,G	67.5 b,c	13.81 E	61.8 b,c	18.21 F	56.3 b,c	22.75 E	51.5 b,c
	ABC	0.00 H	NS	0.00 H	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a
BW14	Control	15.29 B	NS	21.02 B	-	26.52 B	-	30.64 B	-	35.70 B,C	-	39.94 B,C	-
	PBC	5.84 E,F	NS	8.62 E,F	59.1 c	11.71 F	55.7 c,d	14.29 E	53.4 c	17.81 F	50.1 c	21.25 E	46.6 c
	SBC	3.25 G	NS	4.86 G	77.1 b	6.73 G	74.4 b	8.80 F	71.5 b	11.62 G	67.9 b	14.63 F	64.4 b
	ABC	0.00 H	NS	0.00 H	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a
MSU 165	Control	19.93 A	NS	26.91 A	-	33.14 A	-	38.04 A	-	43.89 A	-	49.16 A	-
	PBC	11.45 C	NS	17.39 C	35.2 d	23.42 B,C	29.0 f	28.33 B,C	25.0 e	34.17 C,D	21.2 d	39.32 B,C	18.5 d
	SBC	7.41 E	NS	12.50 D	53.5 c	18.47 D,E	44.1 d,e	23.55 D	37.6 e,d	29.36 D,E	32.2 d	34.67 C,D	28.0 d
	ABC	0.00 H	NS	0.00 H	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a
RFR34	Control	18.32 A	NS	24.82 A	-	30.91 A	-	35.40 A	-	40.13 A,B	-	45.25 A,B	-
	PBC	10.02 C,D	NS	15.46 C	37.6 d	20.90 C,D	32.2 e,f	25.34 C,D	28.2 e,d	31.00 C,D,E	22.4 d	35.85 C,D	20.0 d
	SBC	6.64 E,F	NS	11.23 D,E	55.0 c	16.59 E	46.4 d	21.59 D	39.1 d	27.72 E	31.0 d	33.22 D	26.6 d
	ABC	0.00 H	NS	0.00 H	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a	0.00 H	100.0 a	0.00 G	100.0 a
	$LSD_{0.05}$	2.32	NS	2.92	12.26	3.7	13.13	4.27	14.05	5.18	15.01	6.01	15.76
	Standard Error	1.14		1.43	5.91	1.81	6.33	2.09	6.77	2.54	7.23	2.94	7.60

Table 3.2: Impact of bicarbonates on *Cercospora beticola* growth in vitro. A summary of the average growth for four *C. beticola* isolates, BE4, BW14, MSU165, and RFR34 (means of nine observations each). Four treatments were tested, including: ethanol control (0.7% v/v), potassium bicarbonate (PBC), sodium bicarbonate (SBC), and ammonium bicarbonate (ABC) each with 0.7% ethanol (v/v), in lima bean agar at a rate of 1% (w/v). Mean separation was done for each time individually, so within a time, values with the same letter are not significantly different by least significant difference, α=0.05. The data from five days after transfer is not shown here, as the interaction between treatment and isolate was not significant. The abbreviation "NS" means not significant.

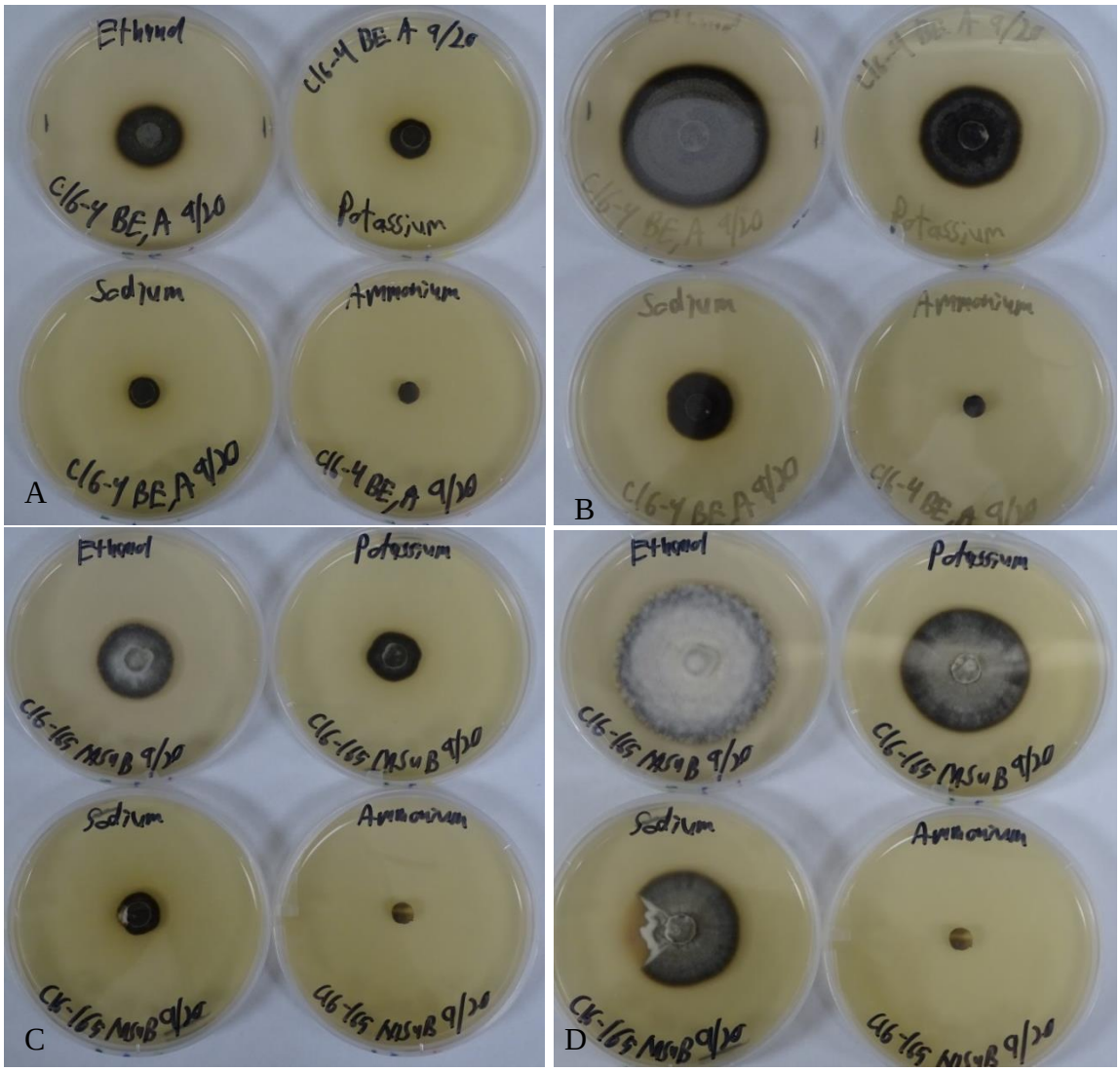


Figure 3.4: *Cercospora beticola* colonies grown on bicarbonate-amended agar. Two *C. beticola* isolates (BE4, A and B, and MSU165, C and D) from the second run of Experiment 1. A and C were taken seven days after transfer, and B and D taken 15 days after transfer. In each picture, the plate in the upper left is the ethanol control (0.7% w/v), and going clockwise the potassium bicarbonate, ammonium bicarbonate, and sodium bicarbonate plates are shown. Each bicarbonate was added to lima bean agar at a rate of 1% (w/v), with 0.7% ethanol (v/v).

Experiment 2: Expanded *Cercospora beticola* Isolate Testing

To further test the impacts of the bicarbonates on the growth of *C. beticola*, 12 more isolates were tested in an incomplete block design. The effects of treatment (Figure 3.5) and isolate were significant for the percent reduction in growth (by ANOVA; $p<0.0001$ for treatment and $p<0.0007$ for isolate) at every time. There was a significant interaction between treatment

and isolate seven (p=0.033), 13 (p=0.039), 15 (p=0.0146), and 17 (p=0.0036) days after transfer (Table 3.3), but the effect of the block was only significant five days after transfer (p=0.0082). At each time point in the study, the bicarbonate treatment had a significant effect on the growth of *C. beticola* (p<0.0001). The ammonium bicarbonate caused complete inhibition of hyphal growth, while both of the other two bicarbonates showed reduced growth. As shown in Figure 3.5, the sodium bicarbonate resulted in a significantly greater reduction in growth than potassium bicarbonate at each time point (p<0.0001 at all times except five days after transfer, when p=0.0148).

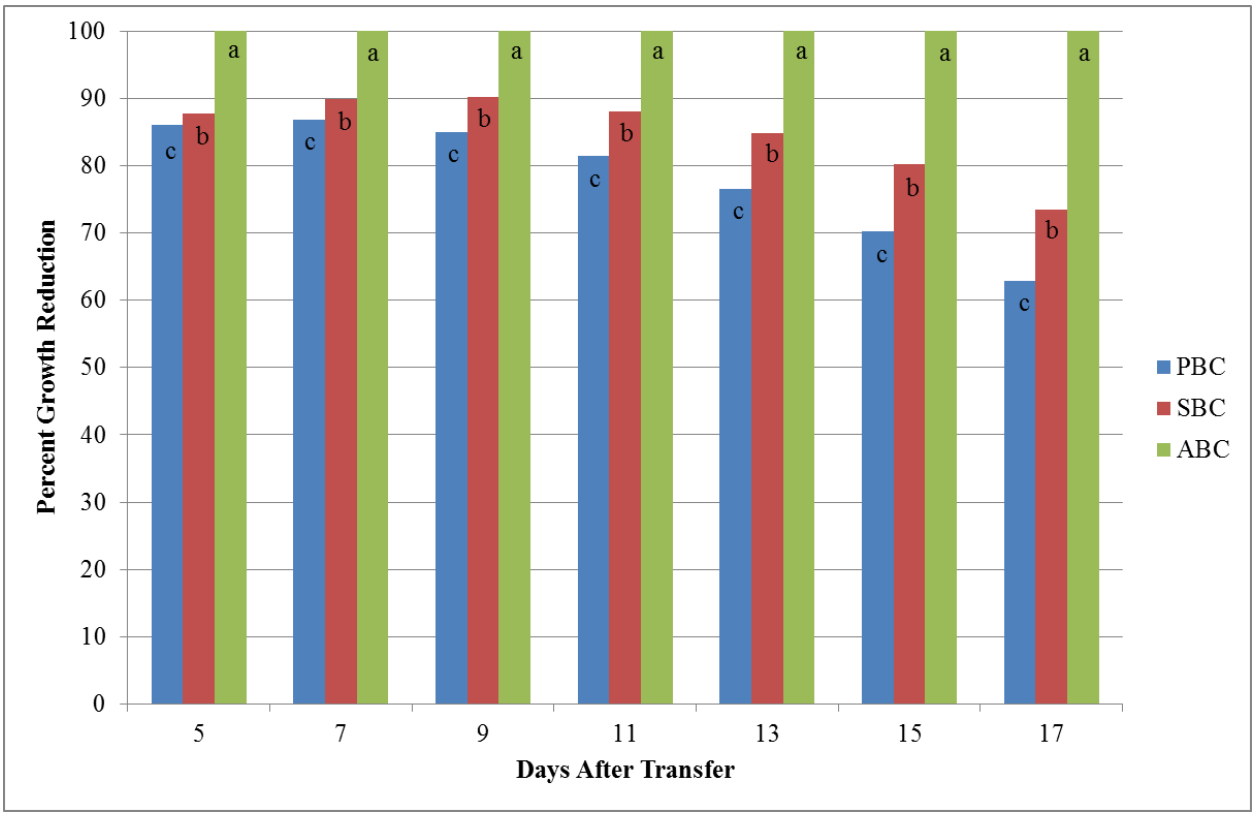


Figure 3.5: Impact of bicarbonates on *Cercospora beticola* growth, average of 12 isolates. A comparison of the percent reduction in growth of *C. beticola* caused by potassium bicarbonate (PBC, blue), sodium bicarbonate (SBC, red), and ammonium bicarbonate (ABC, green), each in lima bean agar at a rate of 1% (w/v). Each bar is the average of 72 observations; three plates each of 12 isolates, replicated twice. Mean separation was done at each time individually, so at a given time bars with the same letter are not significantly different by least significant difference, α=0.05.

Percent Growth Reduction of *Cercospora beticola* in vitro, Experiment 2									
Days After Transfer (DAT)		7		13		15		17	
Isolate	Treatment	Percent Growth Reduction	Mean Separation (α=0.05)	Percent Growth Reduction	Mean Separation (α=0.05)	Percent Growth Reduction	Mean Separation (α=0.05)	Percent Growth Reduction	Mean Separation (α=0.05)
Blum1-3	PBC	89.6	C, D, E, F	88.0	B, C, D, E, F	87.0	A, B, C, D	83.7	B, C, D, E, F
	SBC	92.2	B, C	93.2	A, B	91.9	A, B	90.0	A, B, C
	ABC	99.9	A	100.0	A	100.6	A	100.6	A
CA453	PBC	92.5	B, C	75.6	G, H, I, J	63.2	G, H, I, J	47.5	M, N, O
	SBC	93.3	B	87.8	B, C, D, E, F	79.4	B, C, D, E, F	65.2	H, I, J, K, L
	ABC	100.1	A	100.0	A	99.3	A	99.4	A, B
CA464-1	PBC	83.5	I, J	80.4	D, E, F, G	79.9	B, C, D, E, F	76.3	C, D, E, F, G, H
	SBC	86.7	F, G, H, I	88.4	B, C, D, E	87.5	A, B, C, D	86.5	A, B, C, D, E
	ABC	100.1	A	100.0	A	100.1	A	99.9	A, B
H1	PBC	85.6	G, H, I	77.7	F, G, H, I	74.4	D, E, F, G, H	70.0	F, G, H, I, J, K
	SBC	90.2	B, C, D, E, F	82.7	C, D, E, F, G	79.6	B, C, D, E, F	75.3	C, D, E, F, G, H, I
	ABC	99.9	A	100.0	A	100.6	A	100.6	A
LB141	PBC	83.9	H, I, J	78.2	E, F, G, H, I	75.8	D, E, F, G, H	72.9	D, E, F, G, H, I
	SBC	88.3	D, E, F, G	85.5	B, C, D, E, F, G	83.6	B, C, D, E	80.7	C, D, E, F, G
	ABC	99.9	A	100.0	A	100.6	A	100.6	A
LB155	PBC	86.8	E, F, G, H, I	75.5	G, H, I, J	67.9	F, G, H, I	55.9	J, K, L, M, N
	SBC	89.5	C, D, E, F	78.5	E, F, G, H, I	70.1	E, F, G, H, I	59.5	I, J, K, L, M
	ABC	100.1	A	100.0	A	100.1	A	99.9	A, B
MSU178	PBC	85.4	G, H, I	87.8	B, C, D, E, F	87.5	A, B, C, D	85.6	A, B, C, D, E, F
	SBC	86.9	E, F, G, H, I	89.5	B, C, D	89.6	A, B, C	89.0	A, B, C, D
	ABC	99.9	A	100.0	A	100.6	A	100.6	A
P4	PBC	85.8	G, H, I	69.7	H, I, J	59.1	I, J	50.0	L, M, N
	SBC	90.2	B, C, D, E, F	82.6	C, D, E, F, G	75.0	D, E, F, G, H	66.7	G, H, I, J, K
	ABC	100.1	A	100.0	A	100.1	A	99.9	A, B
RangeA	PBC	92.2	B, C	91.2	A, B, C	87.3	A, B, C, D	81.9	C, D, E, F, G
	SBC	92.9	B, C	93.5	A, B	92.7	A, B	91.4	A, B, C
	ABC	100.1	A	100.0	A	99.3	A	99.4	A, B
RFS113	PBC	81	J	69.3	I, J, K	62.2	H, I, J	54.4	K, L, M, N
	SBC	86.9	E, F, G, H, I	79.6	D, E, F, G, H	76.5	C, D, E, F, G	71.7	E, F, G, H, I, J
	ABC	100.1	A	100.0	A	100.1	A	99.9	A, B
VanN	PBC	87.8	E, F, G	59.2	K	45.3	K	32.5	O
	SBC	90.4	B, C, D, E	75.5	G, H, I, J	63.1	G, H, I, J	45.9	M, N, O
	ABC	100.1	A	100.0	A	99.3	A	99.4	A, B
VanS5-1	PBC	87.3	E, F, G, H	65.8	J, K	54.0	J, K	42.7	N, O
	SBC	91.4	B, C, D	80.9	D, E, F, G	73.0	E, F, G, H	59.7	I, J, K, L, M
	ABC	100.1	A	100.0	A	99.3	A	99.4	A, B
$LSD_{0.05}$		3.7		10.4		13.7		16.5	
Standard Error		1.8		5.1		6.7		8.1	

Table 3.3: Percent growth reduction of 12 *Cercospora beticola* isolates in vitro, Experiment 2. The average percent growth reduction compared to the untreated control for isolate by treatment combinations (means are average of six observations) on days when a significant interaction was found. Treatments include: potassium bicarbonate (PBC), sodium bicarbonate (SBC), and ammonium bicarbonate (ABC) each with 0.7% ethanol (v/v), in lima bean agar at a rate of 1% (w/v). Mean separation was done for each time individually, so within a time, values with the same letter are not significantly different by least significant difference, α=0.05.

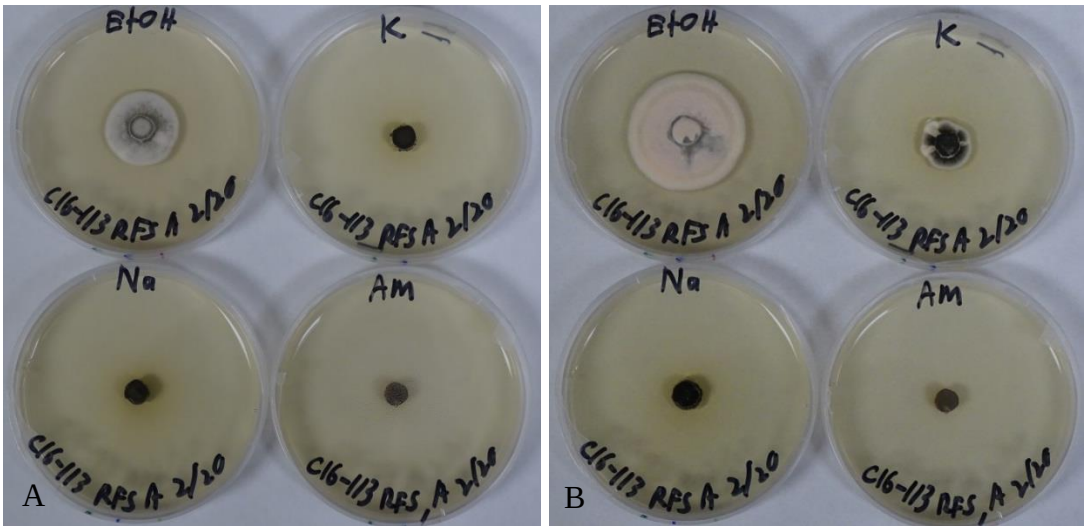


Figure 3.6: *Cercospora beticola* growth on bicarbonate-amended plates. Plates of a *C. beticola* isolate (RFS113) included in Experiment 2. A was taken seven days after transfer, and B 15 days after transfer. In each image, the plate in the upper left corner is the ethanol control (0.7% v/v), and clockwise are the potassium, ammonium, and sodium bicarbonate plates. Each bicarbonate was added to lima bean agar at a rate of 1% (w/v) with 0.7% ethanol (v/v).

Experiment 3: pH Testing

When added to tap water, both potassium and sodium bicarbonate increased pH slightly, from approximately 8.0 to 9.0 as indicated by the pH paper. The ammonium bicarbonate, on the other hand, lowered pH to about 7.5 (Figure 3.7). The bicarbonates' influence on the pH of lima bean agar was somewhat different. At one day after pouring, the pH of the control media was approximately 5.5, which is in the range of the expected pH of lima bean agar (5.6 according to the label). The pH levels of the potassium and sodium bicarbonate-amended media were approximately 10.5, and the pH of the ammonium bicarbonate-amended media was about 9.5 (Figure 3.8).

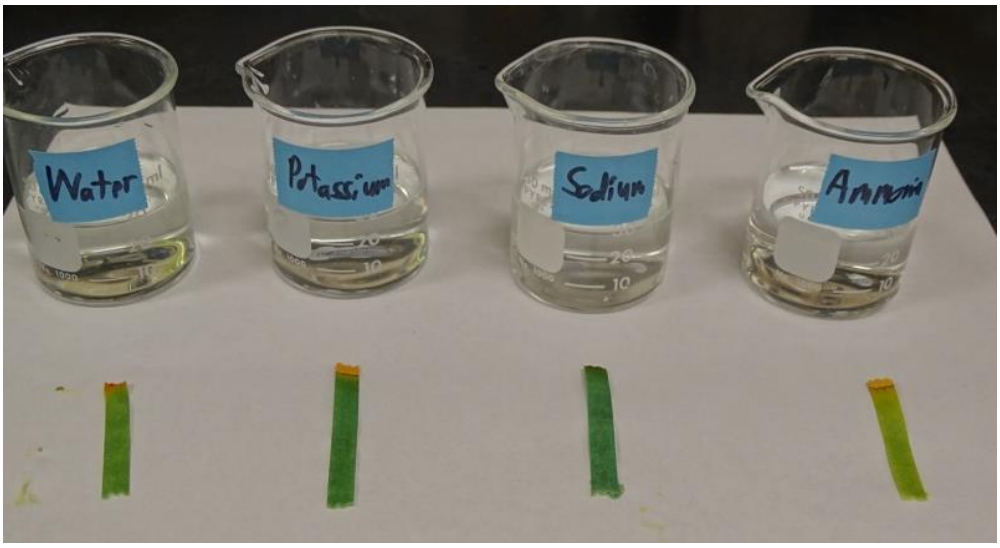


Figure 3.7: pH of 1% bicarbonate in water. The impact on pH of adding 1% of three different bicarbonates to water. On the left is tap water (pH 8.0), followed by potassium bicarbonate (pH 9.0), sodium bicarbonate (pH 9.0), and finally ammonium bicarbonate (pH 7.5).

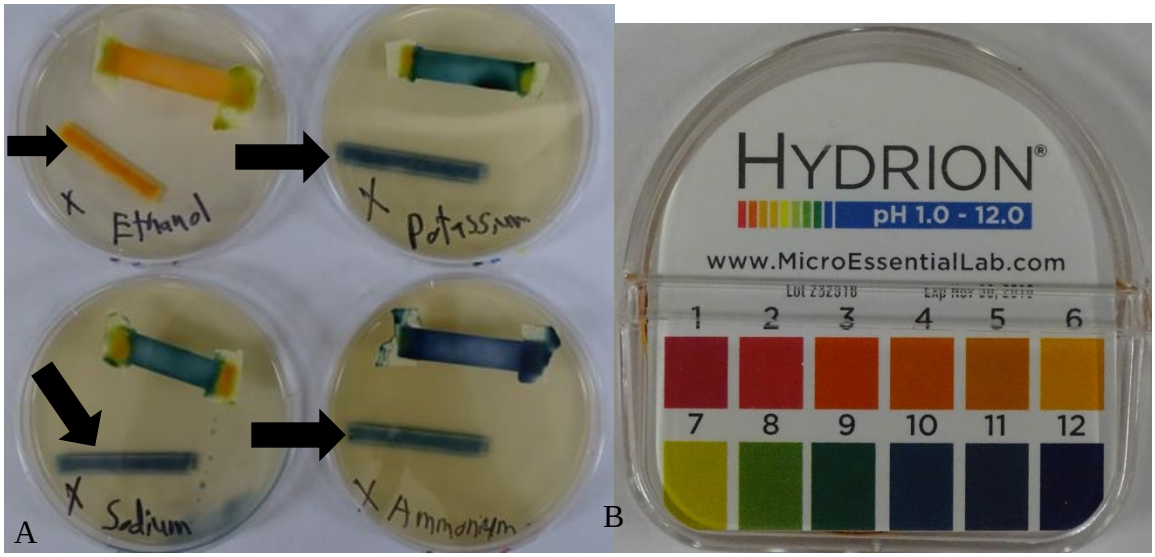


Figure 3.8: pH of bicarbonate-amended media. The pH of the lima bean agar one day (A) after pouring, as shown by pH indicator paper (designated by arrows), with color scale shown in B. The plate in the upper left is the ethanol control (0.7% v/v), and clockwise from there are potassium, ammonium, and sodium bicarbonate at 1% (w/v), with 0.7% ethanol (v/v). The pH of the control was approximately 5.5, while the plates amended with potassium and sodium bicarbonate were about pH 10.5, and the one with ammonium bicarbonate was 9.5.

The hyphae on the bromothymol blue-amended media responded to the bicarbonate treatments in a similar fashion as they did in the above tests. The hyphae from plugs on ammonium bicarbonate-amended media showed no growth, while those on sodium and

potassium bicarbonate-amended media had reduced growth in comparison to the control. The pH of the different treatment groups changed throughout the test. All the bicarbonate-amended plates started at an average pH of 10.5 on day five, while the ethanol control showed an average pH of 7.4. The pH of the ammonium bicarbonate plates remained constant at approximately 10.5 until the end of the test, 23 days after transfer. The pH of the potassium and sodium bicarbonate media varied over the course of the test, and were as low as 8.6 and 9.2, respectively, until they eventually came back up to 9.8 by day 23. The average pH of the ethanol-amended control plates remained low, staying between 7.0 and 7.4 through day 15, at which point they began to increase until the end of the test (day 23) when they reached an average of 9.7 (Figure 3.9). Throughout the test, the ability of the fungi to raise and lower the pH of the surrounding agar differed between isolates, and even within the same isolate the pH of individual colonies varied. For instance, MSU165 and RFR34 were the first to lower the pH of the sodium and potassium plates. They achieved a lower pH than either BE4 or BW14 on the potassium bicarbonate agar, while neither of the latter two isolates lowered the pH of the sodium bicarbonate agar (data not shown). On the control plates, a colony of RFR34 was the first to raise the pH of the media (Figure 3.10), but by the end of the test, the pH of nine of the 12 control plates had been raised to about 10.5 (data not shown). An example isolate (BE4) with all the treatments seven and 13 days after pouring is shown in Figure 3.11.

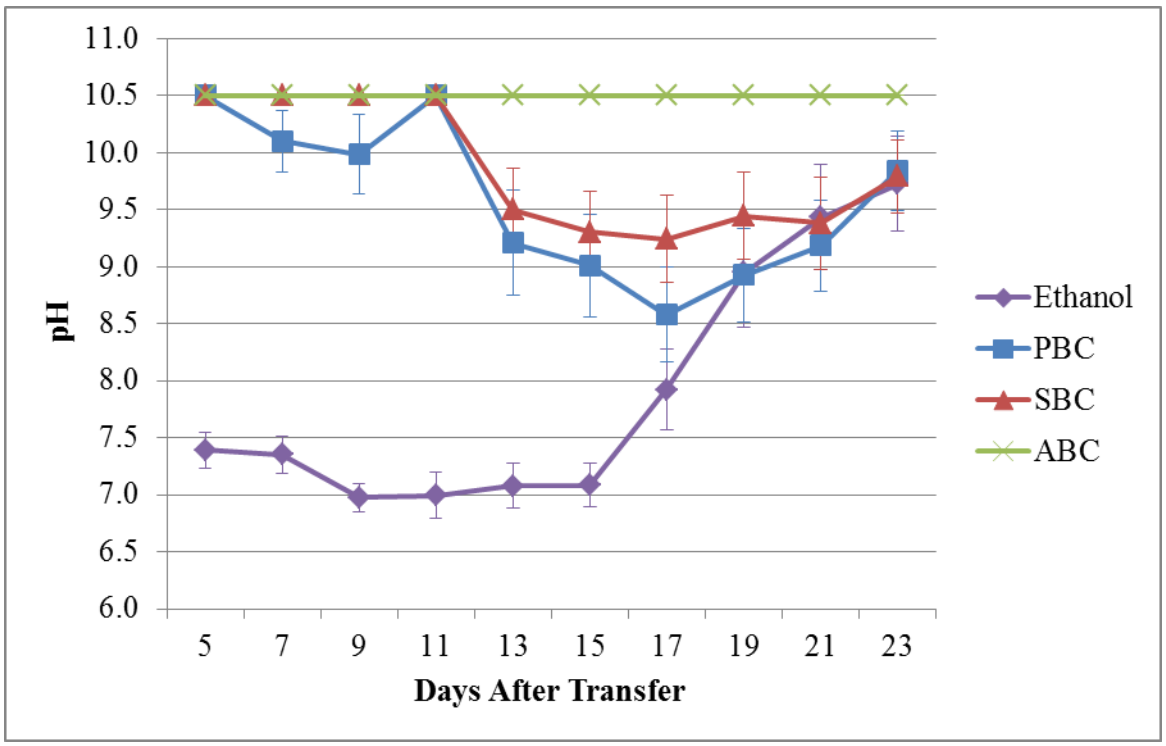


Figure 3.9: Media pH. The average of the approximate pH as indicated by bromothymol blue (0.01%) in lima bean agar amended with one of four treatments, including the ethanol control (0.7% v/v), potassium bicarbonate (PBC), sodium bicarbonate (SBC), and ammonium bicarbonate (ABC) (all three bicarbonates at 1% w/v, in 0.7% v/v ethanol), from five to 23 days after transferring *Cercospora beticola* plugs. Each point on the graph is the mean of 12 observations, three colonies from four isolates, and the error bars indicate standard error.

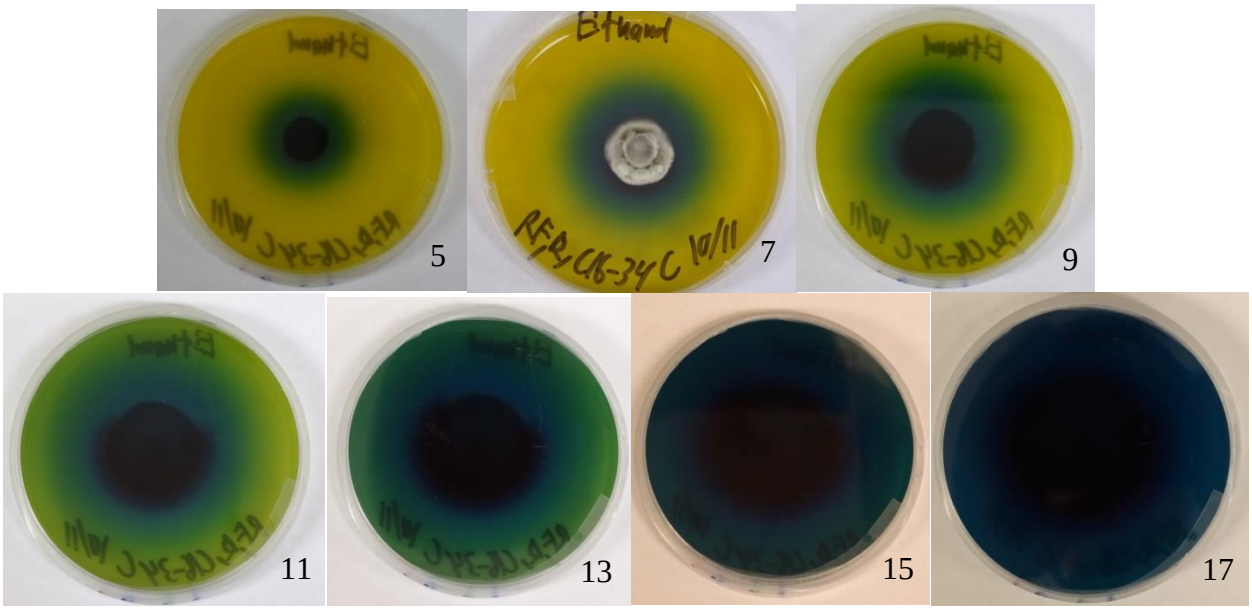


Figure 3.10: Influence of *Cercospora beticola* on media pH. These photographs are of the same colony of *C. beticola*, strain RFR34, on an ethanol control plate (0.7%, v/v) of lima bean agar amended with 0.01% bromothymol blue. They were taken every other day, starting five days after transfer ("5") until 17 days after transfer ("17"). Days after transfer are indicated by numbers.

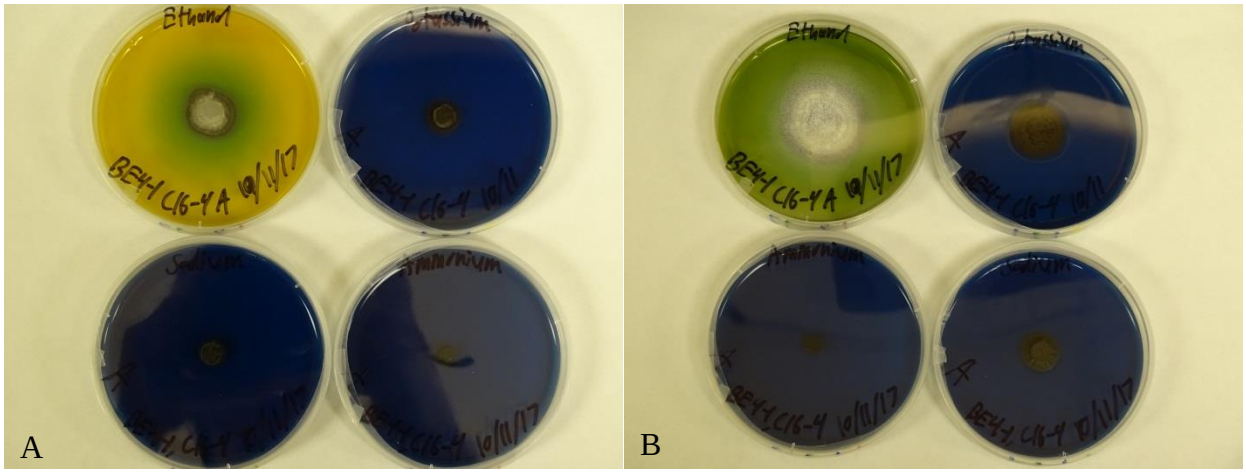


Figure 3.11: Impact of bicarbonate salts on media pH. *Cercospora beticola* strain BE4 on lima bean agar with bromothymol blue (0.01%). A, the colonies seven days after transfer, and B, 13 days after transfer. In each picture, the plate on the upper left is the ethanol control (0.7% v/v), then clockwise around each photograph are the potassium, ammonium, and sodium bicarbonate-amended plates. All bicarbonate concentrations are 1% (w/v), with 0.7% ethanol (v/v).

Experiment 4: Growth on Ammonium Bicarbonate Plates

Of the plugs transferred from or to the ammonium bicarbonate plates in Experiment 1, none showed fungal growth during the period of examination. In the test for activity of

ammonium bicarbonate plugs from Experiment 2, none of the plugs that were originally from the ammonium bicarbonate plates showed growth, while all of the plugs from healthy colonies, transferred to the same new plates, showed growth (Figure 3.12).

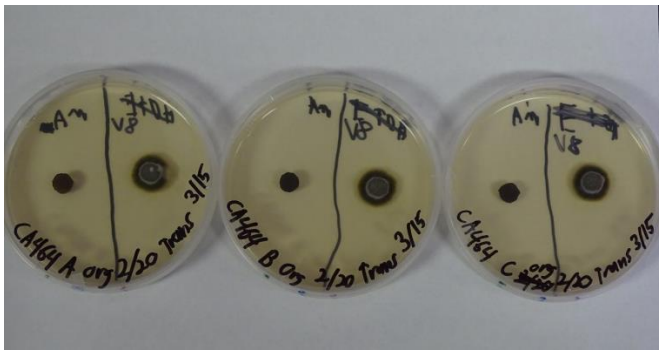


Figure 3.12: Viability test of *Cercospora beticola* from ammonium bicarbonate. Fresh lima bean agar plates (amended with 0.7% ethanol) with plugs from either an ammonium bicarbonate treatment (left) or a colony growing on V-8 agar (right). The plugs which came from the ammonium bicarbonate plates were on the original plate for 28 days, and the picture was taken five days after transfer.

The amount of ammonia gas present in the air above one plate of each treatment was estimated with ammonia test paper one day after the plates were poured, and then with a separate set of plates that were stored on the lab bench for eight days after pouring in the absence of fungi (Figure 3.13). One day after pouring, no ammonia gas was detected in the ethanol control, approximately 50 ppm was detected above both the potassium and sodium bicarbonate-amended agar, and over 100 ppm was detected over the ammonium bicarbonate-amended agar. Eight days after pouring, the amount was substantially lower, with no ammonia being detected from the ethanol control, potassium bicarbonate, or sodium bicarbonate plates. Ammonia was still detected over the ammonium bicarbonate-amended agar, at a concentration of approximately 50 ppm.

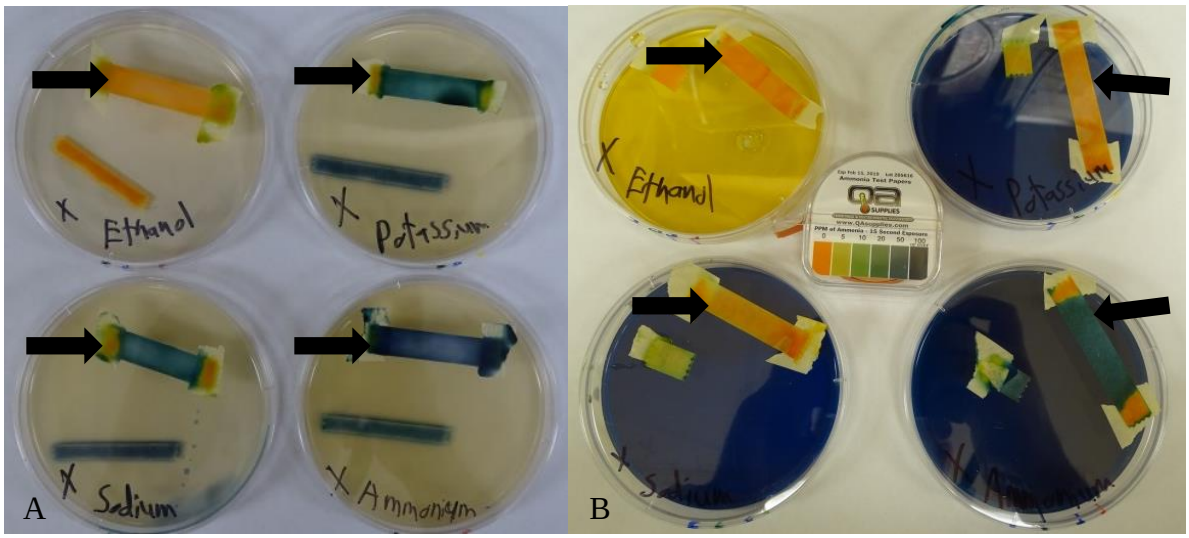


Figure 3.13: Ammonia gas concentrations above lima bean agar plates. The concentration of ammonia gas in the air above agar amended with one of four treatments one day (A) and eight days (B) after pouring. The ammonia test strips (indicated by arrows) were taped to the cover of each plate and wetted with tap water. Going clockwise around each picture, the plate in the upper left corner is the ethanol control (0.7% v/v), followed by potassium, ammonium, and sodium bicarbonate at 1% (w/v), with ethanol at 0.7% (v/v).

Due to the high levels of ammonia gas present above the ammonium bicarbonate-treated agar, the x-plate test was conducted to determine if this gas might be responsible for killing the *C. beticola* hyphae. Seven days after plugs were transferred to the x-plates and viability plates, hyphae on all plugs on the viability test plates produced growth. None of the plugs transferred to either the ammonium bicarbonate or the ethanol quadrants of the x-plates showed hyphal growth (Figure 3.14, Table 3.4). 27 days after being transferred to the x-plates, three plugs, one from each treatment on the x-plate, as well as one plug from the V-8 plate, were transferred to a fresh plate of lima bean agar. The plugs transferred from the x-plates failed to grow, while the plugs from the viability plates grew (Figure 3.15, Table 3.4).

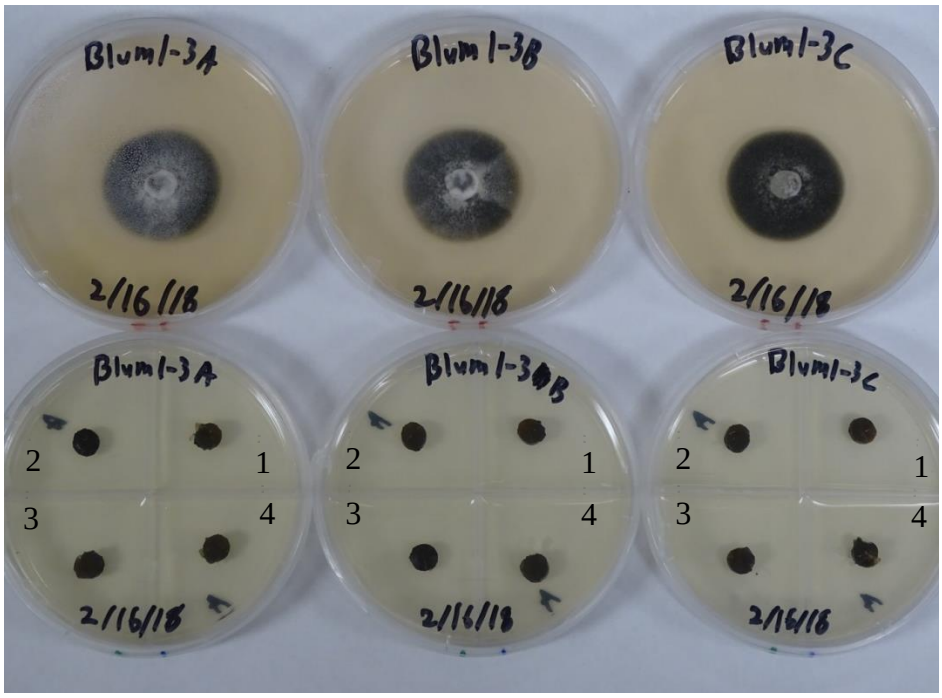


Figure 3.14: *Cercospora beticola* growth on x-plates. One of the three *Cercospora beticola* isolates (Blum1-3) included in the x-plate test. The top row shows the plates of half strength V-8 to confirm viability of fungal cultures. The bottom row shows the x-plates with lima bean agar amended with either 1% ammonium bicarbonate in 0.7% ethanol (quadrants 2 and 4) or with 0.7% ethanol (quadrants 1, 3). The hyphae grew from plugs transferred to the V-8 control plates, while the plugs transferred to quadrants of the x-plates failed to show growth after 27 days.

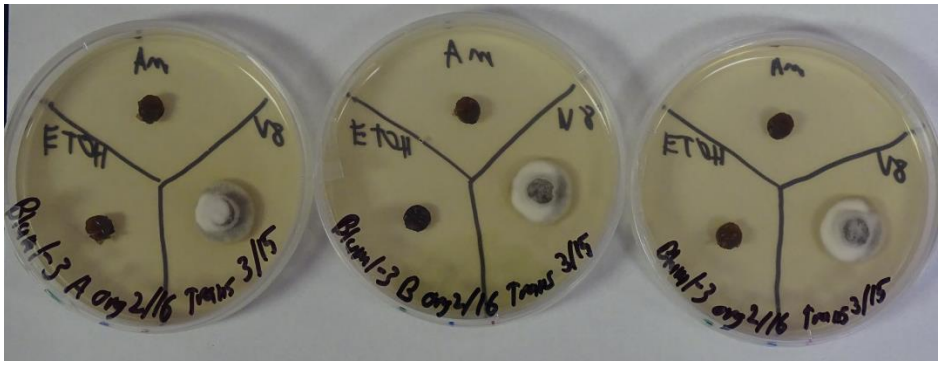


Figure 3.15: *Cercospora beticola* viability after transfer from x-plates. *C. beticola* (isolate Blum1-3) plugs from the x-plate test that were transferred to fresh lima bean agar, amended with 0.7% ethanol. In each plate, the plug at the top of the plate came from an ammonium bicarbonate quadrant of an x-plate, the plug in the left section came from an ethanol quadrant of an x-plate, and the plug on the right came from the V-8 control plate.

Cercospora beticola Growth on X-Plates			
Isolate	Treatment	Average Colony Diameter (mm), 7 Days After Initial Transfer	Growth 5 Days After Transfer to Lima Bean Agar
BE4	V-8 Control	24.7	Yes
	X-Plate Ethanol	0	No
	X-Plate Ammonium	0	No
Blum1-3	V-8 Control	21.16	Yes
	X-Plate Ethanol	0	No
	X-Plate Ammonium	0	No
LB141	V-8 Control	20.96	Yes
	X-Plate Ethanol	0	No
	X-Plate Ammonium	0	No

Table 3.4: *Cercospora beticola* growth on x-plates. Average growth of three *C. beticola* isolates in a viability test plate (V-8 juice agar), or quadrants of an x-plate with or without ammonium bicarbonate (1% w/v).

Experiment 5: Greenhouse Trials

In the first run of the greenhouse testing, the active ingredient factor had a significant effect ($p<0.0001$), while the effects of the adjuvant, block, and interaction between active ingredient and adjuvant were not significant. According to the mean separation by LSD, there was not a significant difference between having no active ingredient and ammonium bicarbonate, while the sodium bicarbonate treatment was significantly different from both ($p<0.0001$; Figure 3.16). The adjuvant type had no impact on the amount of infection, nor was there an interaction between the adjuvants and active ingredients.

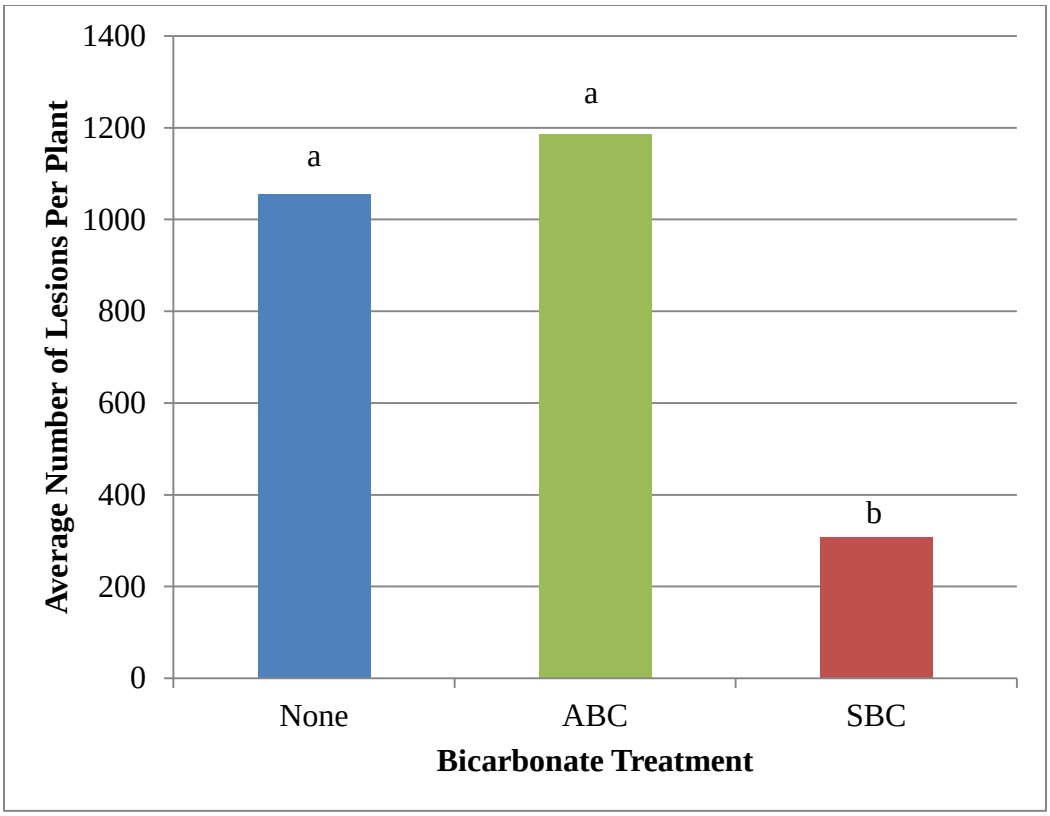


Figure 3.16: Impact of bicarbonate salts on Cercospora leaf spot, run 1. The impact of the bicarbonate treatments on Cercospora leaf spot in the first run of a greenhouse trial. Each bar is the average number of lesions per plant (12 plants per bar) between all treatments with the same active ingredient, with blue representing no active ingredient, green representing ammonium bicarbonate (ABC), and red representing sodium bicarbonate (SBC). All bicarbonates were applied at a rate of 2% (w/v). Bars with the same letters are not significantly different by least significant difference, $\alpha=0.05$.

In the second and third runs of the greenhouse experiment, the overall number of lesions observed in each treatment was less than in the first run. In both runs, the effect of active ingredient was significant ($p<0.005$). The effect of adjuvant was significant ($p=0.0325$) in the second run, but neither the interaction between the two factors nor the block effect was. The mean separation (LSD) revealed that the plants with Activator-90 had fewer lesions than either the Tween 20 ($p=0.0184$) or the SunSpray oil ($p=0.0283$). In the third run, no variable besides the active ingredient was significant. The mean separation for both runs showed that the plants treated with sodium bicarbonate had significantly fewer lesions than those with no active ingredient ($p<0.005$; Figure 3.17).

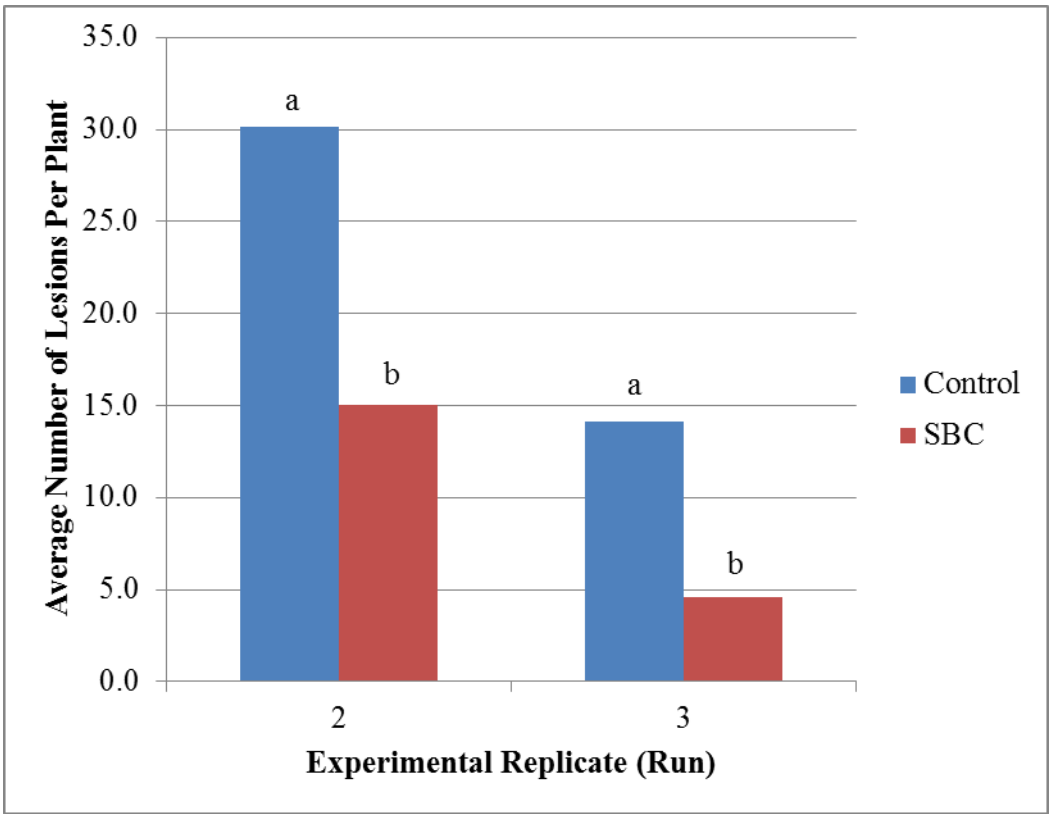


Figure 3.17: Impact of bicarbonate salts on Cercospora leaf spot, runs 2 and 3. The impact of a sodium bicarbonate treatment on the occurrence of Cercospora leaf spot for run two and run three of a greenhouse test. The bars represent the average number of lesions per plant (18 plants per bar), averaged by active ingredient, with the blue column representing the no active ingredient control, and the red column representing sodium bicarbonate (2% w/v). Mean separation was done for each run, so bars within a run with the same letters are not significantly different by least significant difference, α=0.05.

Outside of their impact on CLS development, sodium bicarbonate and some of the adjuvants tested had phytotoxic effects on the sugar beet plants in the second and third runs. The plants treated with just Tween 20 exhibited no such symptoms, and only four plants out of the 12 total which were treated with Tween 20 and sodium bicarbonate had very minor phytotoxicity symptoms. The plants treated with Activator-90, with and without sodium bicarbonate, had significant phytotoxicity symptoms, particularly darkening of leaf veins and spotty necrotic brown to black patches scattered around the leaf surface (Figure 3.18). The plants treated with just SunSpray oil did not show any symptoms of phytotoxicity, but those treated with both

SunSpray oil and sodium bicarbonate did show significant necrotic brown to black spotting on the leaves, particularly in the third run (Figure 3.19). Interestingly, no significant phytotoxicity was observed in the first run.

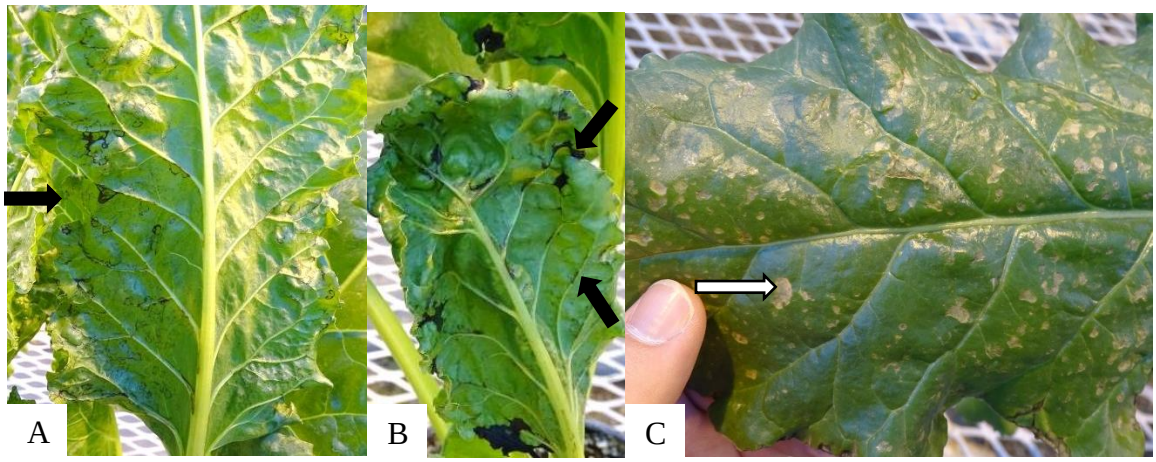


Figure 3.18: Leaf damage caused by Activator-90. Three examples of damage on sugar beet leaves which occurred in treatments with Activator-90 alone (A) or Activator-90 mixed with sodium bicarbonate (B and C). Notice the darkening of leaf veins and necrotic areas in A and B (black arrows), and the brown necrotic patches scattered around the leaf in C (white arrow).

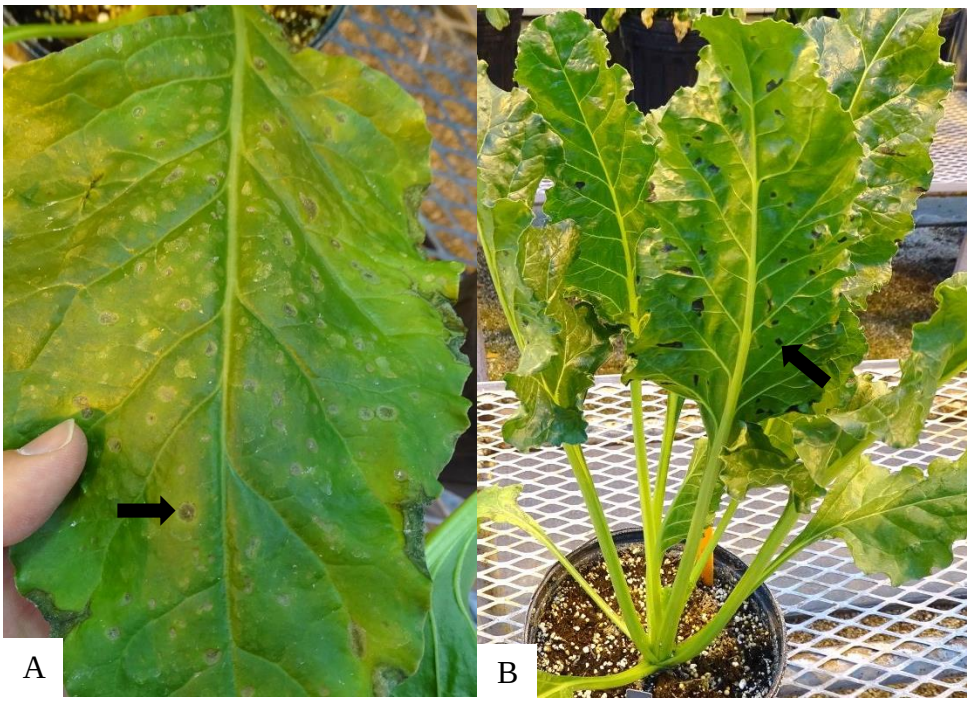


Figure 3.19: Leaf damage caused by SunSpray oil and sodium bicarbonate mixture. Two examples of leaf damage caused to sugar beets by the combination of SunSpray oil and sodium bicarbonate. Notice the tan (A) and black (B) colored necrotic areas on the leaves as indicated by arrows.

Discussion

Based on the results of the "impact of bicarbonates on *Cercospora beticola* growth" experiments (Experiments 1 and 2), each of the three bicarbonates tested can inhibit vegetative growth of *C. beticola* in vitro. Ammonium bicarbonate caused complete growth inhibition and fungal death at the concentration tested. Sodium and potassium bicarbonate inhibited but did not stop fungal growth at the same concentration. On the main effect level, sodium bicarbonate reduced fungal growth more than potassium bicarbonate ($p<0.015$ at each time point in both experiments). The results from Experiments 1 and 2 were similar to one another, except in Experiment 2 both the potassium and sodium bicarbonates provided a numerically greater level of growth inhibition than in Experiment 1. The reason for this difference is unknown. It is hypothesized the difference in temperature, 23-24°C in Experiment 1 as opposed to 26-28°C in Experiment 2, could be at least partially responsible, but this requires testing. Regardless, the results from both experiments indicate that the bicarbonates had a negative impact on the growth of *C. beticola*.

In Experiments 1 and 2, the isolate factor was significant at all times ($\alpha=0.05$), which agrees with previous studies that different *C. beticola* isolates grow at varying rates (Ruppel, 1972). Additionally, there was a significant treatment by isolate interaction at more than half of the times measured in both experiments (percent growth reduction, $\alpha=0.05$). This indicates that while all of the bicarbonates tested had a negative impact on *C. beticola* growth, they impacted individual isolates to varying degrees, with some isolates more inhibited than others. The differential response of individual isolates to a fungicide is not unexpected, as varying sensitivity of isolates to several fungicides has been reported (Bolton et al., 2012b; Rosenzweig et al., 2015; Michigan Sugarbeet REACh, 2017). In spite of this interaction, the same general pattern in

bicarbonate efficacy was found for each isolate. In Experiment 1, sodium bicarbonate provided a greater level of growth inhibition than potassium bicarbonate for the first 11 days, after which the sodium bicarbonate provided greater or equal growth inhibition compared to potassium bicarbonate. Ammonium bicarbonate provided significantly greater inhibition than either of the other two at all times. In Experiment 2, sodium bicarbonate provided greater or equal growth inhibition compared to the potassium bicarbonate. Ammonium bicarbonate showed greater inhibition than the other two for the first 11 days, after which inhibition with at least one other bicarbonate was not significantly different for four of the 12 isolates tested. A majority of the previous bicarbonate studies have not compared the impact of these compounds on individual pathogen isolates as was done in the current study, so this may be an area for further investigation. In such future tests, it will be important to test different rates of bicarbonate against a several isolates with a range of sensitivity to the bicarbonates.

Several studies have attempted to determine the precise mode or modes of action for the bicarbonates (Homma et al. 1981b; Punja and Grogan, 1982; Corral et al., 1988; Fallik et al., 1997a; Palmer et al., 1997), but the mechanisms remain elusive. Based on the aforementioned investigations, it is likely that there are multiple methods by which these compounds inhibit fungal growth (Palmer et al., 1997; Jamar et al., 2001; Deliopoulos et al., 2010). One proposed mechanism is the desiccation of fungal spores and hyphae (Homma et al., 1981b; Fallik et al., 1997a; Deliopoulos et al., 2010). The presence of a bicarbonate salt on the leaf surface or media results in a change in the osmotic potential, causing water to be removed from the fungus (Palmer et al., 1997). It also has been shown that bicarbonates may interfere with the integrity and permeability of fungal membranes (Corral et al., 1988; Avis, 2007). In either case, a loss of turgor pressure and cellular collapse occur (Homma et al., 1981b; Fallik, 1997a). Bicarbonate

salts may even interfere with extracellular enzymes, thus disrupting several physiological processes including cellular expansion (Palmer et al., 1997).

An additional mechanism which has been proposed for the antifungal properties of bicarbonates is an elevation in pH (Punja and Grogan, 1982; Corral et al., 1988; Palmer et al., 1997; Deliopoulos et al., 2010). The growth of many fungi is optimal when the surrounding environment is slightly acidic, usually from a pH of 5.0 to 7.0 (Deacon, 1997). If bicarbonate salts were to increase the pH of the media or leaf surface, this could have deleterious effects on the growth of such fungal species (Punja and Grogan, 1982; Palmer et al., 1997). Previous in vitro tests with bicarbonates have shown that they can elevate the pH of water agar to the range of 8.9-9.4, levels that could inhibit the growth of some fungi (Punja and Grogan, 1982). Punja and Grogan (1982) demonstrated that *Sclerotium rolfsii* sclerotia would not germinate above pH 7, and Palmer et al. (1997) showed *Botrytis cinerea* conidia would not germinate above pH 10. In the present study, the impact of the three tested bicarbonates on the pH of both tap water and lima bean agar was observed. In tap water, both potassium and sodium bicarbonate raised pH, from 8.0 to 9.0, but ammonium bicarbonate slightly decreased pH to 7.5. In lima bean agar, all three bicarbonates raised pH, from 5.5 in the control to 10.5 with either potassium or sodium bicarbonate, and 9.5 for ammonium bicarbonate. While the pH increase observed in lima bean agar was somewhat greater than other authors observed in water agar, the impact of this pH increase on *C. beticola* is questionable. Whereas many fungi grow best at a lower pH (Deacon, 1997) and some species will even lower the pH of their surroundings (Punja and Grogan, 1982; Deacon, 1997; Palmer et al., 1997), the bromothymol blue test showed that *C. beticola* grown on untreated lima bean agar increased the pH of the medium. Even in the early stages of growth (as few as five days after transfer), each of the 12 *C. beticola* colonies on the control plates raised

the pH of the medium immediately surrounding the colony (data not shown; similar but to a lesser extent than Figure 3.10, five days after transfer). By the end of the experiment (23 days after transfer), nine of the 12 *C. beticola* colonies on control plates had raised the pH of the entire plates to approximately 10.5, which was as high as the pH increase caused by either the potassium or sodium bicarbonate. In a study conducted by Macri and Vianello (1979), the authors found that cercosporin (then called *Cercospora beticola* toxin), was most efficient at a pH of 8. As cercosporin is a virulence factor of *C. beticola* (Steinkamp et al., 1981; Weiland and Koch, 2004; Weiland et al., 2010; Daub et al., 2010), it is reasonable to hypothesize that such an impact on the pH of its surrounding environment may enhance the activity of the toxin, thereby increasing pathogen virulence, although the author is unaware of any report in the literature confirming such pH adjustments on the part of *C. beticola*. The impact of pH on cercosporin needs to be investigated and results tested in vivo, as does the impact of pH on conidia germination and fungal growth.

Since *C. beticola* grows at higher pH conditions, an increase in the pH of its environment is likely not an important factor for the growth inhibition observed with bicarbonates. Even in cases with other fungi where an increase in pH did retard fungal growth, such an increase was not entirely responsible for the effects of the bicarbonates (Punja and Grogan, 1982; Palmer et al., 1997). Other studies have compared the growth of pH-sensitive fungi on plates in which bicarbonates were responsible for an increase in pH and plates which were increased to the same pH with another compound. Punja and Grogan (1982) found that the elevated pH hindered fungal growth, but the combination of elevated pH and bicarbonates was fungicidal. Palmer et al. (1997) also found that while elevated pH had negative effects on fungal growth, the impact of pH alone was not as harmful to *B. cinerea* as when the fungus was exposed to a bicarbonate salt.

In the future, similar tests should be done with *C. beticola* in order to better separate the impact of pH and the other bicarbonate properties on *C. beticola* growth.

When considering the modes of action for the bicarbonate salts, both the bicarbonate anion and the cation should be considered as separate units. For most bicarbonates, it is the anion which is primarily responsible for their antifungal properties (Punja and Grogan, 1982; Corral et al., 1988; Ziv and Zitter, 1992; Palmer et al., 1997; Jamar et al., 2007). In vitro tests of bicarbonates and other salts with similar cations have found that there is significantly less growth inhibition when the bicarbonate is absent (Punja and Grogan, 1982; Ziv and Zitter, 1992; Palmer et al., 1997). In the case of potassium and sodium bicarbonate in the current experiment, this appears to be the case, as both bicarbonates significantly reduced the growth of *C. beticola* (e.g. $p<0.0001$ in Experiment 1). It is even possible this is how the pH of the media influences fungal growth, not by directly disrupting fungal growth, but by influencing the amount of carbonate, bicarbonate, and carbonic acid present in the media. Punja and Grogan (1982) reported that bicarbonate solutions at a higher pH caused greater damage to fungi because more bicarbonate ion was present in solution. They stated that when a solution containing carbonic acid, bicarbonate, and carbonate is at a pH of 6.0, their ratio is 74.1: 25.9: 0, but when the pH of that same solution is raised to 9.0, the ratio changes to 0.27: 95.3: 4.22. This increase in the amount of bicarbonate ion present may increase the fungitoxic effect of the solution. Therefore, buffering the pH of the spray tank to keep the solution at a higher pH may play an important role in potential field applications of bicarbonates, even if *C. beticola* is not strongly affected by the direct increase in pH. Such an adjustment to the spray solution warrants further investigation.

While the bicarbonate anion plays a predominant role in the antifungal activity of the bicarbonate salts, the cation likely contributes to the overall toxicity as well. In each of the in

vitro experiments, sodium bicarbonate provided significantly greater fungal inhibition than potassium bicarbonate ($p<0.015$) on the main effect level, a difference that probably is in part due to the contribution of their different cations. In vivo, sodium bicarbonate caused a significant reduction in the number of CLS lesions as opposed to ammonium bicarbonate (Figure 3.16), a difference which is likely due to the contribution of the cation. Other studies also have shown that different bicarbonates cause varying degrees of inhibition on a number of species of fungi, further indicating that the cation likely plays some role, but less than that of the anion (Homma, 1981a; Ziv and Zitter, 1992; Ziv and Hagiladi, 1993; Palmer et al., 1997; Arslan et al., 2006; Turkkan et al., 2018). However, tests of potassium and sodium need to be done with *C. beticola* to determine this.

As far as which ion provides the greatest contribution to the bicarbonates' antifungal activity, it seems as though ammonium bicarbonate is different from the other two tested. In the in vitro tests with ammonium bicarbonate, it was the ammonium cation which was largely responsible for its activity in vitro. Unlike the potassium and sodium bicarbonates which did not show complete growth inhibition at a 1% (w/v) concentration, ammonium bicarbonate caused complete inhibition of growth and was fungicidal in all of the tests in which plugs were transferred to the test media the day after plates were poured. Fungicidal activity was confirmed by transferring both a *C. beticola* hyphal plug exposed to ammonium bicarbonate-amended agar and a control plug to a fresh plate. This was done a total of 12 times, and in each case, hyphae grew from the control plug, whereas no growth occurred from the plug exposed to ammonium bicarbonate. A similar study conducted by Punja and Grogan (1982) showed that at high pH (8.6-9.5), the ammonium ion from salts dissolved in media converted to ammonia gas, thus they proposed it was a combination of the anion and the ammonia gas which was responsible for the

fungicidal activity of these compounds. In the current study, levels of ammonia gas exceeding 100 ppm were detected above plates of one day-old ammonium bicarbonate-amended media. In the x-plate experiment, the hyphae from both the plugs transferred to the ammonium bicarbonate-amended quadrants and the control quadrants were killed. This indicates that a major cause of ammonium bicarbonate's fungicidal activity was the production of ammonia gas, and that *C. beticola* is sensitive enough to ammonia that it likely was responsible for the death of the fungus. The toxicity of ammonia toward several fungal species has been reported, including *Sclerotium rolfsii* (Punja and Grogan, 1982) *Penicillium digitatum, P. italicum* (Montesinos-Herrero et al., 2011), and species of *Aspergillus, Fusarium, Trichoderma,* and *Rhizopus* (Bothast et al., 1973). Thus, the sensitivity of *C. beticola* to ammonia gas is in keeping with such tests.

To determine if the bicarbonates would reduce the incidence of CLS on sugar beets in a similar manner to how they reduced fungal growth in vitro, both ammonium and sodium bicarbonate were tested in the greenhouse. Potassium bicarbonate was not tested in vivo, because it provided significantly less growth inhibition in vitro (at the main effect level) than sodium bicarbonate ($p<0.015$ at each time point in both experiments). When the interaction of treatment and isolate was considered, it provided less or equal growth inhibition compared to sodium bicarbonate (Tables 3.2 and 3.3). In addition, there is a significant price difference between the two, with pure, food grade sodium bicarbonate available in bulk for about $0.70 per pound (https://www.dudadiesel.com/choose_item.php?id=sbc50, accessed 1/9/19), and pure, food grade potassium bicarbonate costing around $2.55 per pound in bulk (https://www.piwine.com//potassium-bicarbonate-powder-bulk.html, accessed 1/9/19). Milstop (BioWorks, Inc., Victor, NY), a commercially available form of potassium bicarbonate intended for use as a fungicide, costs $12.60 per pound (https://www.arbico-organics .com/product/ milstop-foliar-

fungicide-potassium-bicarbonate/omri-certified-products, accessed 1/9/19). This price difference is enough that beet growers would be more willing to use sodium than potassium bicarbonate, assuming their efficacy is comparable.

In each of the three runs of greenhouse testing, the effect of active ingredient was significant, and the sodium bicarbonate led to a significant reduction in the number of CLS lesions as compared to the other active ingredient treatments ($p<0.005$). In the first run, active ingredient was the only factor which had a significant effect on disease ($p<0.0001$). The number of lesions on plants treated with ammonium bicarbonate was not significantly different than the number on plants without an active ingredient. The plants treated with sodium bicarbonate, on the other hand, had significantly fewer lesions than both the ammonium bicarbonate and untreated plants ($p<0.0001$).

Since ammonium bicarbonate did not provide a decrease in disease incidence in the first greenhouse run, it was dropped from the second and third runs in favor of increasing the number of replicates of the remaining treatments. Similar to the first run, the plants treated with sodium bicarbonate in both the second and third run had significantly fewer lesions than those treated with no active ingredient ($p<0.005$). Therefore, it can be concluded that sodium bicarbonate can decrease the incidence of CLS on sugar beets in the greenhouse. While sodium bicarbonate did lead to a statistically significant decrease in lesions as compared to the control, lesions still occurred on these plants at the level tested. In both of these runs, there were markedly fewer lesions on all plants, regardless of treatment, than in the first run. The reason for this decrease is uncertain. One possibility is that the conidia concentration was reduced as compared to the first (from $8.0x10^4$ to $1x10^4$), but the lower concentration should have been more than sufficient to cause adequate levels of disease (Wallin and Loonan, 1971). It also is possible that the age of

the plants may be related to the decrease in disease incidence, as Skaracis and Biancardi (2005) have suggested that sugar beet seedlings are not susceptible to CLS for 80 to 90 days after emergence. In the current experiment, the beets in the second and third runs were treated at a younger age. Specifically, the beets in the first run were 107 days old when treated, the beets in the second run were 70 days old, and the beets in the third run were 60 days old. Therefore, it is possible immunity may have been expressed in this situation. However, an age related susceptibility test was conducted (data not shown) with a different sugarbeet variety (USDA germplasm F1042), in which beets were inoculated with *C. beticola* conidia at the cotyledon, 2-3 leaf, 4-6 leaf, 7-9 leaf, 10-12 leaf, and later growth stages. No difference was observed in susceptibility, as all the beets at each growth stage in the experiment were infected. This is in agreement with McKay and Pool (1918) who showed that sugar beet seedlings can be infected by *C. beticola*. Therefore, unless there are varietal differences in seedling susceptibility, this explanation for the decrease in disease incidence during the current experiment seems unlikely. Another possibility is that a decrease in light between the first and the latter two runs could have caused a delay in symptom development. This is only a hypothesis, as no measurements were taken of light in the greenhouse. It would be possible, though, due to a decrease from 16 to 12 hours of artificial, supplemental lighting for the second and third runs. This hypothesis is predicated on the idea that cercosporin, an important virulence factor of *C. beticola* (Steinkamp et al., 1981; Weiland and Koch, 2004; Weiland et al., 2010; Daub et al., 2010), is photoactivated. It is possible that reduced light could decrease the activity of this toxin, which may reduce symptom development (Weiland et al., 2010; Daub et al., 2010). Therefore, the symptoms may have still been developing when the spot counts were taken. In spite of the overall lower CLS

pressure in these two runs, sodium bicarbonate still led to a decrease in the amount of CLS observed.

Even though ammonium bicarbonate provided excellent fungal growth inhibition in the plates, it failed to provide any disease reduction in the greenhouse test. This may support the hypothesis that it was ammonia gas which was responsible for the majority of the activity in the plates. During the in vitro tests, ammonia gas was released from the ammonium bicarbonate-amended agar and was trapped within the plates, raising the concentration of the gas to greater than 100 ppm (Figure 3.13). When the plates were stored unsealed on the lab bench for eight days after pouring, a substantial amount of this gas was able to escape, decreasing the level in the plates to around 50 ppm (Figure 3.13). When fungal plugs of *C. beticola* were transferred to eight day-old plates, complete inhibition of fungal growth was not observed (Figure 3.20; Appendix C). However, there was still inhibition caused by the ammonium bicarbonate, presumably due to the presence of the bicarbonate anion (Figure 3.20). The amount of inhibition observed on the eight day-old ammonium bicarbonate plates was not significantly different from that of the potassium bicarbonate at all times (Appendix C), and was significantly less than the sodium bicarbonate ($p<0.02$ at all times; Appendix C). For a full discussion on the test comparing the difference in fungal growth on one day-old plates with the growth on eight day-old plates, see Appendix C. If a similar ammonia gas release was occurring in the greenhouse, it is likely that elevated levels of ammonia would not be maintained around the leaf surface, thus the efficacy of the ammonium bicarbonate was reduced. It is interesting that, assuming some of the bicarbonate ion remained on the leaves of the ammonium bicarbonate treated plants, this alone was not enough to significantly reduce disease development. This suggests that the cation

may play a more important role in disease reduction on the plant as compared to in vitro fungal growth reduction for this compound.

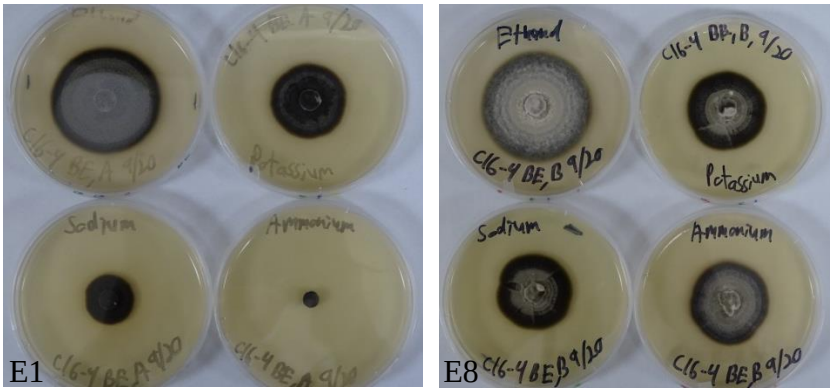


Figure 3.20: Growth of a *Cercospora beticola* isolate (BE4) transferred to plates one (E1) or eight (E8) days after pouring. Shown are lima bean agar plates with one of three bicarbonates in ethanol or an ethanol control. In each image, the plate in the upper left corner is the untreated control, and clockwise around are the potassium, ammonium, and sodium bicarbonate plates. Bicarbonate was added at a 1% (w/v) concentration, and ethanol was added at a 0.7% (v/v) concentration.

Unlike some previous reports (Homma et al., 1981a; Ziv and Zitter, 1992; Horst et al., 1992; Ziv and Hagiladi, 1993; McGovern et al., 2003; Jamar et al., 2007; Deliopoulos, 2010), the adjuvants which were examined in the current experiment did not have a strong impact on the efficacy of the bicarbonates. In both the first and third runs, the effect of adjuvant was not significant, and the interaction between adjuvant and treatment was not significant in any of the runs. The adjuvant factor was significant in the second run ($p=0.0325$), with those plants receiving the Activator-90 treatment having fewer CLS lesions than those treated with the other two adjuvants ($p<0.03$ in both cases). However, being that such an effect was only observed in one of two tests that included this adjuvant, the impact of these adjuvants needs further investigation. In the literature, there is strong evidence that adjuvants have an impact on bicarbonate efficacy for several host-pathogen systems (Homma et al., 1981a; Ziv and Zitter, 1992; Horst et al., 1992; Ziv and Hagiladi, 1993; McGovern et al., 2003; Jamar et al., 2007; Deliopoulos, 2010). It is possible that all of the adjuvants tested in the current study increased

the efficacy of sodium bicarbonate in the greenhouse by improving its coverage and adhesion to the beet leaves similar to what was reported by others (Ziv and Zitter, 1992; Horst et al., 1992; Jamar et al., 2007), and that if a treatment without any adjuvant were tested, it would have had a significantly greater level of disease than those with some type of adjuvant. Such a treatment was not done, however, because it is already standard practice in the Great Lakes region to apply an adjuvant with fungicide applications to control CLS (Michigan Sugar Company, 2018), and because of the overwhelming evidence in the literature that supports the use of some kind of adjuvant with bicarbonates (Homma et al., 1981a; Ziv and Zitter, 1992; Horst et al., 1992; Ziv and Hagiladi, 1993; McGovern et al., 2003; Jamar et al., 2007; Deliopoulos, 2010). Therefore, to conclude that each of the tested adjuvants would have led to better disease reduction than having no adjuvant is hypothesis. Another plausible explanation is that the adjuvant which was included in the inoculum (0.1% Tween 20 in the first run, beet saponins in the second and third) masked the effect of the other adjuvants. While the use of such adjuvants, particularly beet saponins which are released from the leaves during inoculum preparation, has been standard practice for the application of *C. beticola* inoculum (Ruppel and Gaskill, 1971; Ridout et al., 1994), a more significant adjuvant effect may have been observed if these compounds were removed. It also is possible that there is an adjuvant which was not included in these tests that would increase the efficacy of sodium bicarbonate against CLS, and that future studies could identify such a product. The limited evidence for an impact of an adjuvant (in one of two tests) supports such a suggestion.

In addition to the mode of action, having knowledge about the site of action for the bicarbonate salts is of great importance, as this helps determine the optimal timing of field applications. Homma et al. (1981b) examined the impact of bicarbonate salts at several points

over the life cycle of cucurbit powdery mildew (*Sphaerotheca fuliginea*), including conidial germination, hyphal growth, conidiophore formation, and conidia production. They found that the application of sodium bicarbonate did reduce hyphal growth, but this was not the stage most hindered by the chemical (Homma, 1981b). Other studies have indicated that the bicarbonates can have eradicant properties, expressing efficacy against established diseases by damaging actively growing hyphae with which they come in contact (Ziv and Zitter, 1992; Deliopoulos et al., 2010). This method is particularly important for fungi such as powdery mildew species which have a significant portion of their mycelia growing outside of the plant (Homma et al., 1981b). Following from this, researchers have tested applications of bicarbonates on structures such as conidia and conidiophores which are more exposed even for fungi which predominantly grow inside the leaf. Homma et al. (1981b) found that the bicarbonates have the greatest impact on the spores and conidiophores. In fact, sodium bicarbonate decreased the germination of *Sphaerotheca fuliginea* conidia by 95%, decreased conidiophore formation by 96%, and inhibited conidia production by 80% (Homma et al., 1981b). Fallik et al. (1997a) also observed a decrease in spore germination, germ tube elongation, hyphal growth, and spore production of both *Alternaria alternata* and *Botrytis cinerea*, but germ tube elongation was the most hindered. Other studies have found similar inhibition of spore germination and germ tube elongation in several species of fungi (Arslan et al., 2006; Ilhan et al., 2006; Karabulut et al., 2006). For fungi with more internal growth such as *Cercospora beticola* (Pool and McKay, 1916a; Steinkamp et al., 1979), most of the fungal tissue will be protected by the leaf, so the bicarbonates would most likely serve as protectants, preventing infection by damaging the fungal spores and germ tubes. This is how they were tested against apple scab (Ilhan et al., 2006), wheat rust (Karabulut et al.,

2006; Arslan et al., 2006), and bean rust (Arslan et al., 2006), as well as CLS in the current experiment.

The impact of bicarbonates on conidiophore and conidia formation was not examined during the current study. In vitro, it did appear as though the *Cercospora beticola* colonies growing on bicarbonate-amended media may have had reduced conidia production, but no measurement was taken of this. The greenhouse study examined the role of sodium and ammonium bicarbonate in reducing infection by *C. beticola*, but no bicarbonate was applied to plants with preexisting *C. beticola* infection. Future studies should be conducted to investigate the effect of bicarbonates on conidiophore and conidia formation. If the bicarbonates prove to decrease one or both of these, it is possible they could have a niche use as a rescue treatment in fields where CLS has gotten out of control.

The bicarbonates offer several advantages when compared with conventional fungicides. One advantage is the low level of human and environmental hazard of these compounds (Jamar et al., 2007; Deliopoulos et al., 2010). These salts are commonly used as ingredients in food, being used to induce aeration and foaming in baked goods, adjust pH, as leavening agents (Lindsay, 1985), and as preservatives (Corral et al., 1988). The risk these compounds pose to human safety is sufficiently low that the Environmental Protection Agency declared both sodium and potassium bicarbonate to be exempt from residue tolerance standards (Palou et al., 2001; Jamar et al., 2007). Additionally, sodium bicarbonate is classified as a GRAS (generally recognized as safe) compound by the Food and Drug Administration (Corral et al., 1988; Palou et al., 2001). Horst et al. (1997) described bicarbonates as "biocompatible fungicides", which they defined as "fungicides that exhibit low mammalian and environmental toxicities." As a consequence of their low human toxicity, their restricted entry and preharvest intervals are much

shorter than many other pesticides (Deliopoulos et al., 2010). For instance, the restricted entry interval for Milstop, a commercial preparation of potassium bicarbonate, is one hour, and there is no preharvest interval (Milstop 2019 label); as compared to Inspire XT, a DMI commonly used to manage *C. beticola*, which has a restricted entry interval of 12 hours and a preharvest interval of 21 days (Inspire XT 2018 label). Another major advantage of some bicarbonates, particularly sodium bicarbonate, is their low cost, which can make them an economically sound alternative to more expensive fungicides (Ziv and Zitter, 1992; Deliopoulos et al., 2010).

One disadvantage of the bicarbonates compared to conventional fungicides is that they are often not quite as effective, particularly when compared to the systemic fungicides (Avis, 2007; Deliopoulos et al., 2010). While bicarbonates have not been compared with any other fungicides in the current experiment, it is possible that the systemic fungicides would provide a greater level of control (Khan and Smith, 2005; Poindexter, 2007), assuming the isolates used were not resistant to them (Ruppel and Scott, 1974; Bugbee, 1995; Weiland and Halloin, 2001; Kirk et al., 2012; Bolton et al., 2012a; Bolton et al., 2012b; Khan, 2015; Michigan Sugarbeet REACh, 2016; Trueman et al., 2017). However, the bicarbonates do have an advantage when it comes to fungicide resistance (Avis, 2007; Deliopoulos et al., 2010). Since their antifungal properties are the result of multiple modes of action, the risk of resistance development is lower than for fungicides with a single mode of action (Crowdy, 1971; FRAC, 2005; Brent and Hollomon, 2007b; Avis, 2007; Deliopoulos et al., 2010). Therefore, if any of the bicarbonates show field efficacy, including them in a spray schedule by either tank mixing or alternating them with conventional fungicides could be an effective means to help manage fungicide resistance and to keep CLS levels below the economic threshold (Lamey et al., 1987; Ziv and Zitter, 1992;

Brent and Hollomon, 2007a; Deliopoulos et al., 2010; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018).

While the bicarbonates have many attributes which are desirable for a fungicide, one important concern about their use is the possibility they may be phytotoxic. Such phytotoxicity has been observed on several plant species, including roses (Horst et al., 1992), cucurbits (Ziv and Zitter, 1992), hazelnut (Turkkan et al., 2018), peppers (Fallik et al., 1997b), dry beans (Arslan et al., 2006), apples (Ilhan et al., 2006; Jamar et al., 2007), and wheat (Arslan et al., 2006). This phytotoxicity often is the result of too high of a concentration being used, emphasizing the importance of finding the correct concentration and dosage before they are used as pesticides. In the current study, very little if any phytotoxicity was observed during the first run, while some of the treatments in the second and especially the third run caused phytotoxicity symptoms. The treatments causing such symptoms include Activator-90 alone, Activator-90 and sodium bicarbonate, and SunSpray oil with sodium bicarbonate. In the treatments with Activator-90, it is possible the adjuvant was primarily responsible for the phytotoxicity rather than the sodium bicarbonate. This adjuvant has been used on sugar beets in the past at a rate of 0.25-0.5% (v/v) without causing such phytotoxicity (Christy Sprague, personal communication), so it is likely that the rate of 1% (v/v) used in the current study needs to be adjusted. In the case of the SunSpray oil and sodium bicarbonate, since neither component individually resulted in noticeable phytotoxicity, it is likely the combination of these two compounds that caused the symptoms. However, the combination did not cause these symptoms in the first run of the test. The age of the plants may be at least partially responsible for the occurrence of phytotoxicity, as the beets in the second and third runs were treated at a younger age. It is possible that beets are more easily damaged by these chemicals when they are younger. Reyes (1975) observed similar

age related phytotoxicity with benomyl applied to a cabbage cultivar. When benomyl was applied to cabbage seedlings at planting, one week-old, or two weeks-old, severe phytotoxicity was observed, but less was observed on eight week-old plants. Another possibility is that the phytotoxicity may be linked to the inoculum adjuvant used in each run. Tween 20 was used to improve conidial adhesion in the first run when no significant phytotoxicity occurred, while in the second and third, beet saponins were used. Since phytotoxicity was observed in both Activator-90 treatments, it is possible that this adjuvant could have interacted with the beet saponins resulting in the phytotoxicity. In the case of SunSpray oil, this is possible but less likely since the phytotoxicity was only observed when both SunSpray oil and sodium bicarbonate were applied together. While there was no significant impact of the adjuvant on disease reduction in two of the three runs, there was a noticeable difference in phytotoxicity between the adjuvant types, which needs to be considered in future work. Before these products can be recommended for use in the field, these phytotoxicity issues must be resolved, either by adjusting the rate applied, adjusting the bicarbonate/adjuvant combination applied, or by putting plant age restrictions on their use

The results of the current study have shown that ammonium, potassium, and sodium bicarbonate are each capable of decreasing the growth of *C. beticola* in vitro, and that sodium bicarbonate is able to significantly ($p<0.005$) reduce CLS symptoms on sugar beets. While the effects of these bicarbonates have been well documented with many other plant pathogenic species of fungi, this is the first report in the literature of their use on *Cercospora beticola* and on sugar beet. In vitro, both sodium and potassium bicarbonate decreased fungal growth, but sodium bicarbonate provided greater inhibition than potassium bicarbonate. Ammonium bicarbonate was fungicidal at the same concentration, and it is likely this increase in activity is

largely the result of ammonia gas released from the media. In the greenhouse, ammonium bicarbonate was not effective at reducing CLS lesions at the concentration tested, while sodium bicarbonate significantly reduced, but did not eliminate, lesion development at a 2% concentration. While sodium bicarbonate may never reduce CLS as much as the systemic fungicides did prior to resistance development (Khan and Smith, 2005; Poindexter, 2007), it can significantly ($p<0.005$) reduce the level of disease and might be an effective tank mix partner. Prior to being recommended to growers, field tests of sodium bicarbonate alone and as a tank mix should be conducted. Its impact on conidiophore and conidia production also should be examined, as well as the use of a pH buffer in the spray solution to increase the amount of bicarbonate available to inhibit CLS development. This study has shown that sodium bicarbonate can decrease the amount of visible CLS symptoms, and could serve as a fungicide with a different mode of action than the ones already in use, thus potentially aiding sugar beet growers in achieving successful management of CLS.

CHAPTER 4

HEAT TREATMENT AS A NOVEL MANAGEMENT STRATEGY FOR *CERCOSPORA BETICOLA* OF SUGAR BEET

Introduction

Sugar beet (*Beta vulgaris* subsp. *vulgaris*) is an important cash crop for the Great Lakes region (Michigan and Ontario, Canada) as well as a number of other regions throughout the United States and the world (Draycott 2006; NASS, USDA, 2018). Unfortunately, sugar beets are susceptible to a multitude of diseases which can result in a substantial economic loss to growers by means of direct yield loss as well as additional management costs (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Hanson, 2009). In the Great Lakes region, Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola*, is the most severe foliar disease of sugar beet (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). If left unmanaged, the economic losses from this disease are considerable, resulting in as much as a 40% reduction in yield (Jacobsen and Franc, 2009).

Generally, *C. beticola* will infect the foliage of a sugar beet, causing necrotic lesions which are nearly circular on the leaf blades (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Bennett and Leach, 1971; Jacobsen and Franc, 2009). These lesions can reach 3-5 mm in diameter (Jacobsen and Franc, 2009), and often have a red or brown border with a tan or ash gray center (Townsend, 1914; Coons et al., 1930; Bennett and Leach, 1971; Steinkamp 1979; Weiland and Koch, 2004; Jacobsen and Franc, 2009). Scattered throughout the center of these lesions are small (approximately 60 µm wide) black structures called pseudostromata (Coons et al., 1930; Steinkamp et al., 1979; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Jacobsen and Franc, 2009; Franc, 2010; Groenewald et al., 2013). These pseudostromata are extremely

important for the life cycle of *C. beticola*, as they serve not only as the primary overwintering structures for the fungus (Pool and McKay, 1916b), but also are the structures upon which conidiophores are created (Jacobsen and Franc, 2009). Under the proper weather conditions, which include temperatures of 5-35°C and 60% or higher relative humidity [Pool and McKay, 1916b; Canova, 1959a (in Italian) with English summary; Bleiholder and Weltzien, 1972b (in German), with English summary], these conidiophores will produce conidia which usually are 27-250 µm in length (Groenewald et al., 2013) and appear needlelike and silver to gray in color (Townsend, 1914; Coons et al., 1930; Duffus and Ruppel, 1993; Jacobsen and Franc, 2009; Franc, 2010). CLS is a polycyclic disease (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010), and is encouraged by warm, wet conditions, particularly daytime high temperatures from 27-32°C, low temperatures remaining above 16°C, and at least 60% relative humidity for 15 hours or more (Pool and McKay, 1916b; Duffus and Ruppel, 1993; Asher and Hanson, 2006). Under these conditions, several disease cycles can occur in a single year, causing a great number of lesions throughout a field and even on a single leaf (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Franc, 2010). It has been estimated that if 400 to 1,000 lesions occur on a leaf, the combined effect of these lesions as well as their toxins and enzymes will cause leaf death (Townsend, 1914; Pool and McKay, 1916b; Coons et al., 1930; Weiland and Koch, 2004). During severe epidemics, it is possible for entire plants and even whole fields to become defoliated (Coons et al., 1930). It is by this defoliation that the disease causes such a decrease in yield (Townsend, 1914; Coons et al., 1930; Smith and Ruppel, 1973; Shane and Teng, 1992; Franc, 2010). The loss of photosynthetic area leads to a reduction in the total mass of the beet root, a decrease in the percent sugar stored within that root (Townsend, 1914; Coons et al., 1930; Smith and Ruppel, 1973; Shane and Teng, 1992; Franc, 2010), an

increase in the impurities within the root (Coons et al., 1930; Smith and Martin, 1978; Shane and Teng, 1992; Jacobsen and Franc, 2009), and possibly an increase in storage losses (Smith and Ruppel, 1971). Additional yield loss may be incurred if the beets regrow their leaves, particularly if this new growth also becomes infected with CLS (Coons et al., 1930; Bennett and Leach, 1971; Franc, 2010; Michigan Sugarbeet REACh, 2013).

If Cercospora leaf spot is to be effectively managed, an integrated approach should be taken, one which includes the use of cultural practices, host resistance, and fungicide applications (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). In recent years, CLS management has become much more difficult in the Great Lakes region, with two exceptionally severe epidemics occurring in 2015 and 2016, during which time it is estimated that this disease cost the Michigan Sugar Company approximately $100 million (James Stewart, personal communication). One critical reason why this disease has become more difficult to manage is because *C. beticola* has developed resistance to several fungicides (Weiland and Halloin, 2001; Kirk et al., 2012; Trueman et al., 2017). In the past, fungicide applications have been one of the most essential aspects of CLS management, and were very effective at reducing the impact of this disease (Khan and Smith, 2005; Poindexter, 2007). A variety of fungicides are used against *C. beticola*, including systemic/translaminar fungicides such as benzimidazoles, strobilurins, and triazoles; as well as protectants such as organotins, EBDC's, and copper based fungicides (Fungicide Resistance Action Committee, 2005; Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). However, resistance has developed in the Great Lakes region to several of these fungicides, including the benzimidazoles (Weiland and Halloin, 2001), strobilurins (Kirk et al., 2012), and triazoles (Trueman et al., 2017). Field level resistance has

also been developed by *C. beticola* to organotins in the Red River Valley region (North Dakota and Minnesota) of the United States (Bugbee, 1995), so there is a risk of such resistance occurring in the Great Lakes region. The development of fungicide resistance has caused this management strategy to be less effective and has made overall CLS management more difficult (Khan, 2015; Michigan Sugarbeet REACh, 2016). Even if new chemicals were created or released for the management of CLS, the ability of CLS to develop resistance to a variety of fungicides raises questions about the long term sustainability of this management strategy.

Future management of CLS could be aided by the development of novel, preferably non-fungicide management techniques. Host resistance has offered one avenue for CLS management, and there are already sugar beet varieties available which offer high levels of CLS resistance (Michigan Sugarbeet REACh, 2017). However, the type of host resistance which is utilized against *C. beticola* is quantitative, and governed by four to five major genes as well as several minor genes (Smith and Gaskill, 1970; Weiland and Koch, 2004; Skaracis and Biancardi, 2005). Consequently, this resistance is difficult to breed for, and does not provide immunity to the pathogen (Duffus and Ruppel, 1993; Weiland and Koch, 2004; Skaracis and Biancardi, 2005; Jacobsen, 2010). Additionally, sugar beet varieties which are resistant to CLS often are lower yielding and produce less sugar than their susceptible counterparts (Miller et al., 1994; Weiland and Koch, 2004; Skaracis and Biancardi, 2005), so these varieties are less appealing to growers. This yield drag has been decreasing in recent years (Jacobsen, 2010; Gummert et al., 2015), but more work still is needed to bring high yielding resistant varieties to growers.

Cultural practices can offer another avenue for growers to mitigate the impact of CLS. When considering the epidemiology of *Cercospora beticola*, it is important to remember that it has physiological and physical constraints which can be utilized to manage it. One such

constraint is that it has a fairly limited survival in the soil. *C. beticola* generally overwinters as pseudostromata, as these are its most resilient structures to the effects of the environment (Pool and McKay, 1916b). Pseudostromata typically are only able to survive for 1-2 years under field conditions [Pool and McKay, 1916b; McKay and Pool, 1918; Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Khan et al., 2008], and their survival in the field is dependent upon intact leaf debris [Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Bennett and Leach, 1971]. Additionally, *C. beticola* conidia have a fairly short range of dispersal, usually up to 100 meters or less (McKay and Pool, 1918).

Given that infected leaf debris from a previous sugar beet crop is the primary source of *C. beticola* inoculum (Townsend, 1914; Coons et al., 1930; McKay and Pool, 1918; Bennett and Leach, 1971), separation of the current beet crop either temporally or spatially from old beet fields will aid in decreasing CLS incidence (McKay and Pool, 1918). One tactic which is currently used is crop rotation (Townsend, 1914; McKay and Pool, 1918; Coons et al., 1930; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc 2009; Khan and Khan, 2010; Jacobsen, 2010; Michigan Sugar Company, 2018). By allowing a two or three year rotation between the current and previous beet crop, the majority of *C. beticola* inoculum will decompose before the new crop is planted (Townsend, 1914; McKay and Pool, 1918; Coons et al., 1930; Khan et al., 2008; Jacobsen and Franc, 2009). Currently, a three year rotation is required by the Michigan Sugar Company, but a four year rotation is strongly encouraged (Michigan Sugar Company, 2018). CLS disease incidence also can be reduced if growers avoid planting next to a previous year's beet field by choosing a different location or leaving at least a 100 meter buffer strip between the fields (McKay and Pool, 1918). Since susceptible weeds such as common lambsquarter (*Chenopodium album)*, redroot pigweed (*Amaranthus retroflexus*), and

mallow species (*Malva* spp.) can serve as alternate hosts (Vestal, 1933; Lartey et al., 2010a), proper weed control in and around a field both the year before and the year when it is planted to beets also can aid in CLS management (Jacobsen and Franc, 2009).

Beyond merely separating current sugar beet fields from the inoculum source, several cultural practices have focused on eliminating the inoculum itself. In contemporary sugar beet production, it is common practice for sugar beet leaves to be left in the field after harvest (Smith, 2001), but in the early years of the American sugar beet industry, the leaves were harvested and fed to livestock (Townsend, 1914; McKay and Pool, 1918). Leaves were dried, hayed, and fed directly to livestock, or they were made into silage prior to being used as feed. Regardless, the leaves along with any overwintering *C. beticola* structures were removed from the field, and both the process of being made into silage as well as passing through the digestive tract of livestock resulted in the death of the fungus (McKay and Pool, 1918). Deep tillage such as moldboard plowing also has been an effective means to destroy infected sugar beet leaves (Townsend, 1914; McKay and Pool, 1918; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Khan et al., 2008; Jacobsen and Franc 2009; Jacobsen, 2010). Pool and McKay (1916b) and Khan et al. (2008) have shown that burying infected leaf debris decreases the longevity of *C. beticola*. This is most likely due to accelerating the decomposition rate of the leaf tissue, which hinders the survival of *C. beticola* (Khan et al., 2008). While moldboard plowing and feeding infected leaf debris to animals are both effective strategies to manage CLS, neither practice is widely accepted by contemporary sugar beet growers due to concerns about increased soil erosion caused by deep tillage (Montgomery, 2007; Michigan Sugar Company, 2018) and because feeding leaves to livestock is no longer a widespread practice (Smith, 2001).

One aspect of *C. beticola* biology which often has been overlooked in terms of field management is its sensitivity to high temperatures. In some early tests of *C. beticola*'s temperature range, Pool and McKay (1916b) concluded that the ideal temperature for in vitro fungal growth was just below 30.8°C. They also discovered that fungal growth was temporarily retarded at temperatures above 30.8°C, such as 34.7°C and 35.8°C, and temperatures at or above 40.5°C for extended periods of time (several hours) were lethal to the fungus. Likewise, Groenewald et al. (2005) found that 27°C was the ideal temperature for growth, and observed no growth at 36°C or 40°C. When sugar beet leaves were harvested for animal fodder, it was customary in some regions to dry the leaves in a pulp drier before they were stored and fed to livestock (McKay and Pool, 1918). Therefore, McKay and Pool (1918) tested the effects of short term heat upon the viability of CLS lesions, in which infected beet leaves were treated with temperatures from 60°C to 120°C for 15, 30, or 60 minutes. They found that such heat was fatal to the pathogen, as fungi in lesions were killed when treated at 100°C for 30 minutes, while 15 minutes was sufficient at temperatures of 115°C. In addition to the lethal impact of heat on *C. beticola*, their data also indicates an inverse relationship exists between the temperature and duration of exposure (McKay and Pool, 1918). As a consequence of the inverse relationship between temperature and duration, it is possible that a very short heat treatment could reduce the overwintering ability of or even kill *Cercospora beticola*, granted the heat treatment was high enough. Therefore, the current study was a preliminary test to examine the possibility of using a propane field burner to heat-treat infected sugar beet leaves prior to harvest as a means of reducing the level of *C. beticola* inoculum. If such a treatment was able to kill a significant portion of the *C. beticola* present on these leaves, it is possible that the amount of CLS observed in nearby fields the following year could be greatly reduced.

To determine if such a heat treatment might be effective at influencing CLS, the current study used a handheld propane weed torch to simulate the effects of a full-size propane burner on sugar beets grown in a greenhouse. (For a more detailed discussion about the different types of heat treating devices, specifically burners and flame contact units such as the handheld torch, see Parish, 1990.) The first goal of the study was to determine if a torch could be used to defoliate sugar beet plants. If this were the case, growers might be more willing to adopt such a practice, as it would allow them to remove CLS inoculum without adding an extra step to harvest. The second goal was to determine if a heat treatment of this kind would have any long term negative consequences on the health of sugar beet roots. The third goal was to determine the impact of this torch on CLS viability. Viability was determined by checking lesions for their ability to sporulate after being heat treated, as well as the ability of *C. beticola* to grow from lesions when transferred to agar after treatment. If heat treatment with a propane torch causes a significant decrease in *C. beticola* viability without having a major impact on sugar beet health, field tests with a full-size burner would be warranted, and such a technique may be an effective strategy for managing CLS.

Materials and Methods

The sugar beet variety B149N (Betaseed, Inc., Bloomington, MN) was selected for this experiment because it is commercially available in Michigan and is quite susceptible to *C. beticola* (Michigan Sugarbeet REACh, 2017). Seeds were started in single, 2.45 liter plastic nursery pots (Poly-tainer NS300, Nursery Supplies, Inc., Chambersburg, PA) of potting mix (SureMix Perlite, Michigan Grower Products, Inc., Galesburg, MI). When the beets reached about the 2 leaf growth stage, they were transplanted to individual 3.79 liter plastic pot (Classic 400, Nursery Supplies, Inc.). Beets were grown at standard greenhouse conditions, which

included a temperature ranging from 20-30°C and 12 hours of artificial light from 8:00 a.m. to 8:00 p.m. as a supplement to natural light. Watering generally took place every Monday, Wednesday, and Friday, and beets received 40 ml of slow release 14-14-14 fertilizer (Osmocote, Everris, Geldermalsen, the Netherlands) around the 4-6 leaf growth stage. Predatory mites (*Tyrophagus putrescentiae*, Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands) were used to manage thrips (order Thysanoptera, species unknown).

This experiment consisted of two parts. The first was a defoliation test, in which five beets were used to develop the method for heat treating beet leaves, to determine if the propane torch could be used as a defoliator, and to see if the heat treatment would cause any long term negative health effects on beet roots. Beets for the defoliation test were planted on September 13, 2018 and were not inoculated with *C. beticola*. The second part of the experiment was a CLS test, in which sugar beets expressing symptoms of CLS were heat treated. Two runs of the CLS test were conducted, the first planted on August 30 and inoculated with *C. beticola* on November 8, and the second planted on September 13 and inoculated with *C. beticola* on November 12. Inoculum was prepared in a similar way to that described by Ruppel and Gaskill, (1971). Beet leaves showing severe CLS symptoms were collected from the Saginaw Valley Research and Extension Center the summer before the experiment took place. They were air dried on a greenhouse bench, and stored in a burlap bag until the spore suspension was prepared. The day before the experiment, leaves were soaked in water to induce conidia formation. On the day of inoculation, the rehydrated leaves were vigorously rubbed together by hand for about ten minutes to aid in spore release, and then the leaf material was removed with a sieve (mesh size approximately 1.1 mm by 1.8 mm). Spore counts were taken with a hemocytometer, and the final spore concentration was brought to approximately $1x10^4$ per ml. The spore suspension was

applied to the leaves of the plants with a handheld sprayer (Lansing Sanitary Supply, Inc., Lansing, MI) until runoff (approximately 30 ml per plant).

The leaves were allowed at least two hours to dry, after which they were placed in a humidity chamber. The humidity chamber was constructed out of four metal carts (Super Erecta Shelving, InterMetro Industries Corp., Wilkes-Barre, PA), surrounded by 6 mm plastic sheeting (HDX, Home Depot, Atlanta, GA). The dimensions of the chamber were 75 cm tall by 183 cm wide by 183 cm deep (similar to Figure 2.3). To maintain a relative humidity of at least 95%, the floor of the chamber was lined with wet newspaper, and two 4.54 liter humidifiers (Hunter, model QLS-05, Memphis, TN) were run while the chamber was closed. The chamber was housed within a walk-in cold room (Harris Environmental Systems, Andover, MA), measuring 280 cm tall, 345 cm wide, and 742 cm long, which kept the plants at a constant 24°C. Lighting within the chamber was provided by six sodium halide bulbs, set to a 16:8 hour light dark cycle, turning on at 6:00 a.m. The photosynthetic photon flux density of the light within the cold room was approximately 30 µmol/(m^2*s), and within the humidity chamber it was approximately 20 µmol/(m^2*s). In order to encourage infection, the humidity was fluctuated throughout the day (Rathaiah, 1976; Rathaiah, 1977), with an 8 hour dry period starting at noon, and a 16 hour wet period starting at 8:00 p.m. Weather stations (Watchdog Model A150, Spectrum Technologies, Inc., Aurora, IL) were used to record the temperature and relative humidity within the chamber. After three humidity cycles, the beets were returned to the standard greenhouse conditions described above.

Both runs of the CLS test were made up of six plants, three heat treated and three untreated controls. Since heat treating was to occur off site, the beets that were randomly selected for heat treatment were transported off site in an enclosed vehicle a day or two before

treatment. They were kept in a well-lit, heated area at about 22°C. The untreated control beets remained in the greenhouse. For the CLS tests, up to five leaves from each plant were marked with uniquely colored twist ties around the petioles, and the number of lesions on each of these leaves was counted. Heat treating of the leaves occurred outside, with a VT 2-23C Weed Dragon, 100,000-BTU propane vapor torch (Red Dragon, Flame Engineering, Inc., LaCrosse, KS) for approximately the same intensity as they would be with a field torch. According to the manufacturer, the temperature of the handheld torch reaches approximately 1,090°C, and each plant was exposed for about 15 seconds, with the exception of one plant in the defoliation test for which an attempt was made to defoliate it completely. This beet was exposed to the heat of the torch for a substantially longer time period, approximately 120 seconds. Beets from the defoliation test were treated on November 24, 2018, beets from the first CLS test on December 9, and beets from the second CLS test on December 16. Care was taken to only move the beets outdoors for treatment when the air temperature was above 0°C, and immediately after heat treating, beets were returned indoors. Within 24 hours, beets were brought back to the greenhouse, and kept at the conditions previously described.

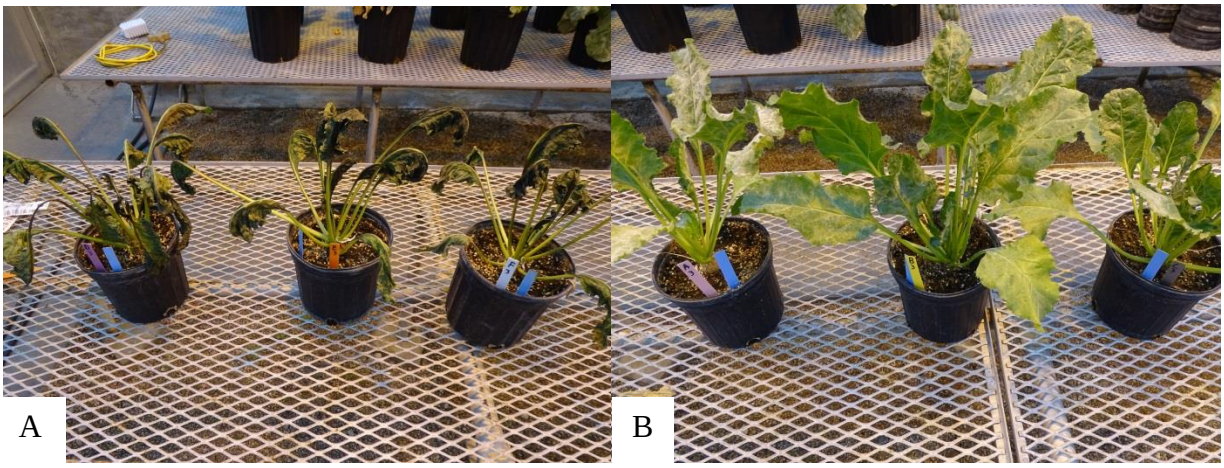
The day after heat treating for the CLS test, about five leaves were marked on the three untreated beets, and spot counts were taken from them. The marked leaves from each treated and untreated plant were then removed, each placed in a sealable plastic bag (Meijer re-closable double zipper gallon storage bags, Meijer Distribution, Inc., Grand Rapids, MI) with a moist paper towel, and kept on the lab bench to encourage conidia production. Counts were taken of the number of sporulating lesions four days after being heat treated. Data was recorded and organized using a spreadsheet (Excel 2010, Microsoft Corp., Albuquerque, NM). The experimental design of the CLS test was a one factor, completely randomized design, with five

subsamples. The two levels were heat treated and untreated, and the entire experiment was repeated twice. An ANOVA table was constructed for each run using the proc mixed procedure, and mean separation with the least significant difference (LSD, α=0.05) method was done with proc glimmix of SAS 9.4 (SAS Institute Inc., Cary, NC).

On the fourth day after heat treatment, 11 lesions (ten for the control in the first run) were selected from the treated and non-treated plants. They were cut from the leaf with dissecting scissors, surface disinfested in 0.6% sodium hypochlorite (Clorox Company, Oakland, CA) for 30 seconds, rinsed in sterile reverse osmosis water for 30 seconds, and plated on half strength V-8 juice (Campbell Soup Co., Camden, NJ) agar (Miller, 1955). The number of lesions from which *C. beticola* hyphae grew were counted three weeks after transfer. Plants were monitored visually for regrowth, root health, and any abnormal symptoms due to the potential heat stress. No statistical analysis was done on these observations.

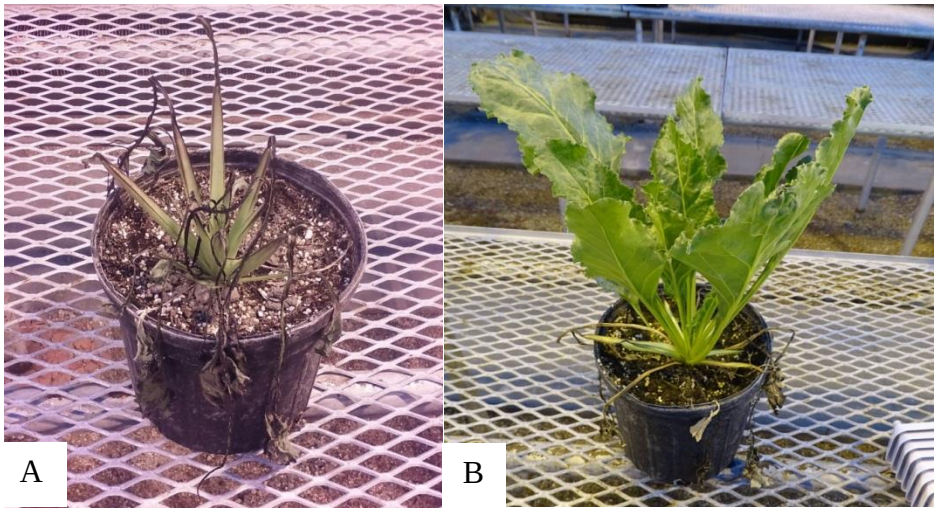
Results

Upon receiving a heat treatment for approximately 15 seconds per plant, the leaves of treated plants immediately wilted and took on a water soaked appearance. Within three to six hours, these leaves curled and turned black (Figure 4.1). In the CLS test, some of the lesions appeared to have been charred in the center. It was evident, however, that defoliation with a torch will not be possible, as it took about 120 seconds of heat to remove all the leaf blades, and even then the petioles were not removed (Figure 4.2). In spite of the intense heat, no visible damage was observed on the beet roots, and new leaf growth appeared on all of the heat treated beets, even the plant receiving 120 seconds of heat (Figure 4.2), typically within three to five days after treatment.



A B

Figure 4.1: Beet leaves heat treated for 15 seconds. Sugar beets that were heat treated with a handheld propane weed torch for 15 seconds (A) and the control beets that were not heat treated (B), one day after treatment. Upon being heat treated, beet leaves curled in a distinct shepherd's hook pattern and turned a dark, almost black color.



A B

Figure 4.2: Beet leaves heat treated for 120 seconds. A sugar beet plant that received approximately 120 seconds of heat treatment with a handheld propane weed torch. A was taken two days after heat treatment. B was taken 25 days later. Notice how new leaves have grown.

The ANOVA tables for each run of the CLS test showed that for both runs, the heat treatment significantly decreased the ability of CLS lesions to produce conidia ($p=0.0027$ and $p=0.0142$). In the first CLS test, the mean number of sporulating lesions per leaf from five leaves of the control plants was 9.53, while in the second it was 8.5 lesions per leaf (Figure 4.3). In each run, the mean number of sporulating lesions on the heat treated leaves was 0.0 (Figure 4.3). A total of 95 lesions were heat treated and 138 lesions were untreated in the first CLS test, while

in the second, 119 received the heat treatment and 133 were untreated. In each run, none of the heat treated lesions sporulated, while 89% to 100% of untreated lesions did after three days of being in the plastic bags.

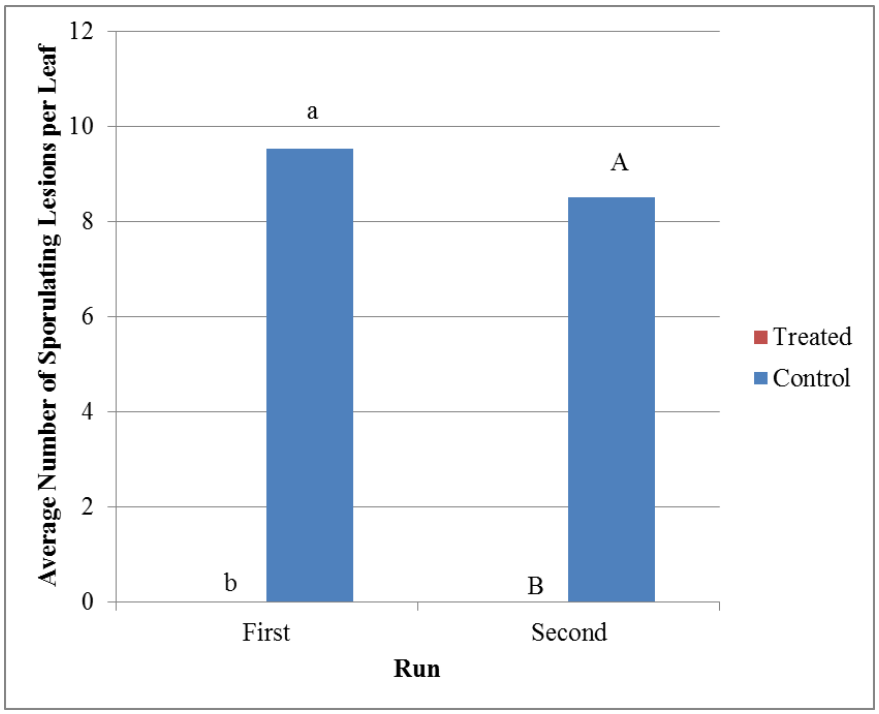


Figure 4.3: Average number of sporulating *Cercospora beticola* lesions per leaf. The average number of sporulating lesions per leaf from heat treated sugar beets (red bars) versus control plants (blue bars). Counts were taken after three days in a humidity chamber. In the first run, a total of 95 lesions were treated and 138 were untreated, while in the second, 119 were treated, and 133 were untreated. Bars from the same run with different letters are significantly different by least significant difference, α=0.05.

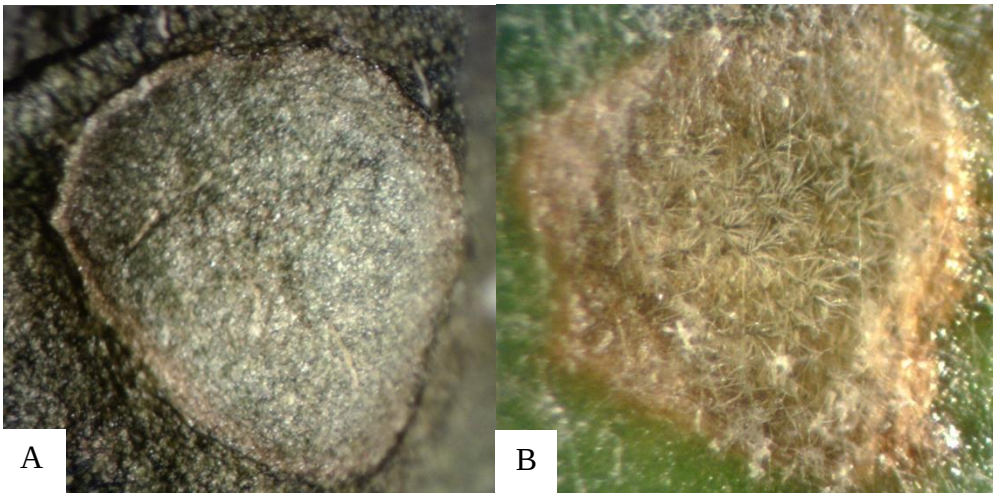


Figure 4.4: Heat treated and untreated *Cercospora beticola* lesions. Two *C. beticola* lesions, one that was heat treated for approximately 15 seconds and placed in a humid chamber for three days (A, 30x magnification) and one that was untreated (B, 35x magnification). The untreated lesion is producing conidia abundantly, while the heat treated lesion shows no visible sporulation. Also notice how the leaf tissue around the untreated lesion is still green, while the leaf tissue around the heat treated lesion is black or gray.

In addition to the heat treated lesions failing to sporulate, in most cases the hyphae were no longer viable. Between the two runs, all 21 of the untreated lesions which were transferred to the half strength V-8 agar showed fungal growth and formed colonies on the agar (Figure 4.5). For the heat treated lesions, however, the growth was not nearly as extensive. In the first CLS test, only two of the 11 transferred lesions showed hyphal growth and formed colonies on the media. Thus, the heat treated lesions showed a mortality rate of about 82% (Figure 4.5). In the second CLS test, only one of the 11 transferred lesions showed hyphal growth, a mortality rate of approximately 91% (Figure 4.5).

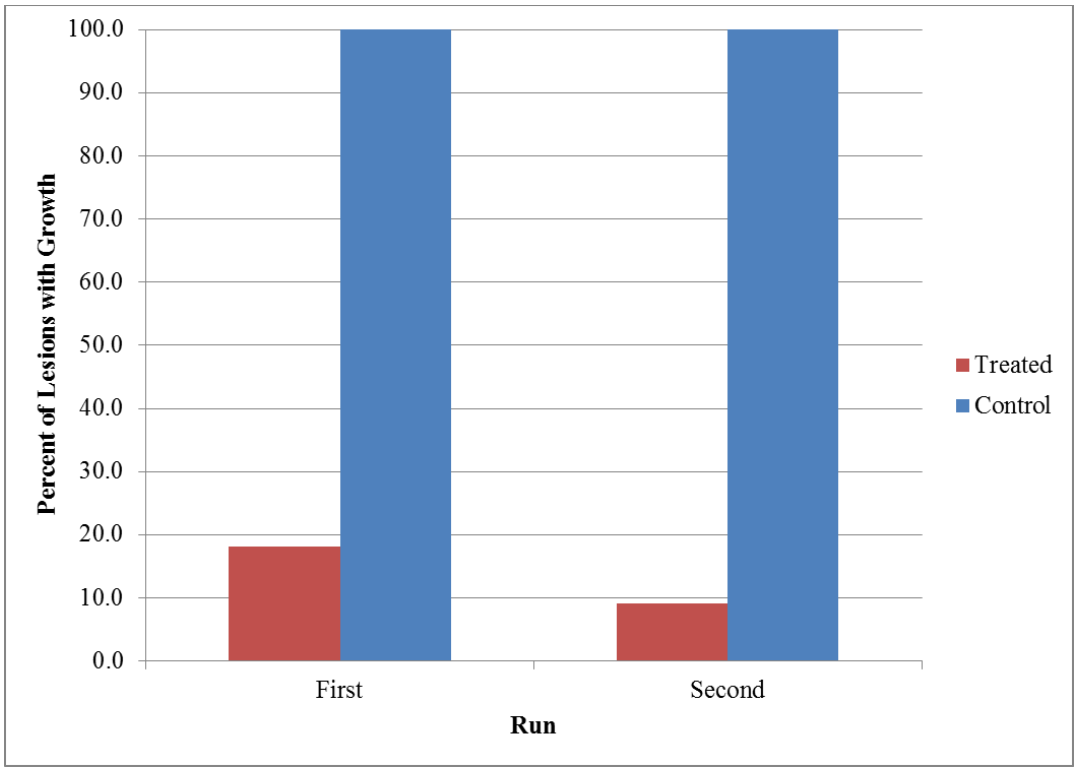


Figure 4.5: Impact of heat treatment on *Cercospora beticola* lesion viability. Shown above is the percent of *C. beticola* lesions from which hyphal growth occurred three weeks after transfer to half strength V-8 media. Red bars indicate the percent of viable lesions from leaves that were heat treated, and blue bars represent the percent from untreated leaves. Each bar represents 11 lesions, with the exception of the first blue bar which represents ten lesions.

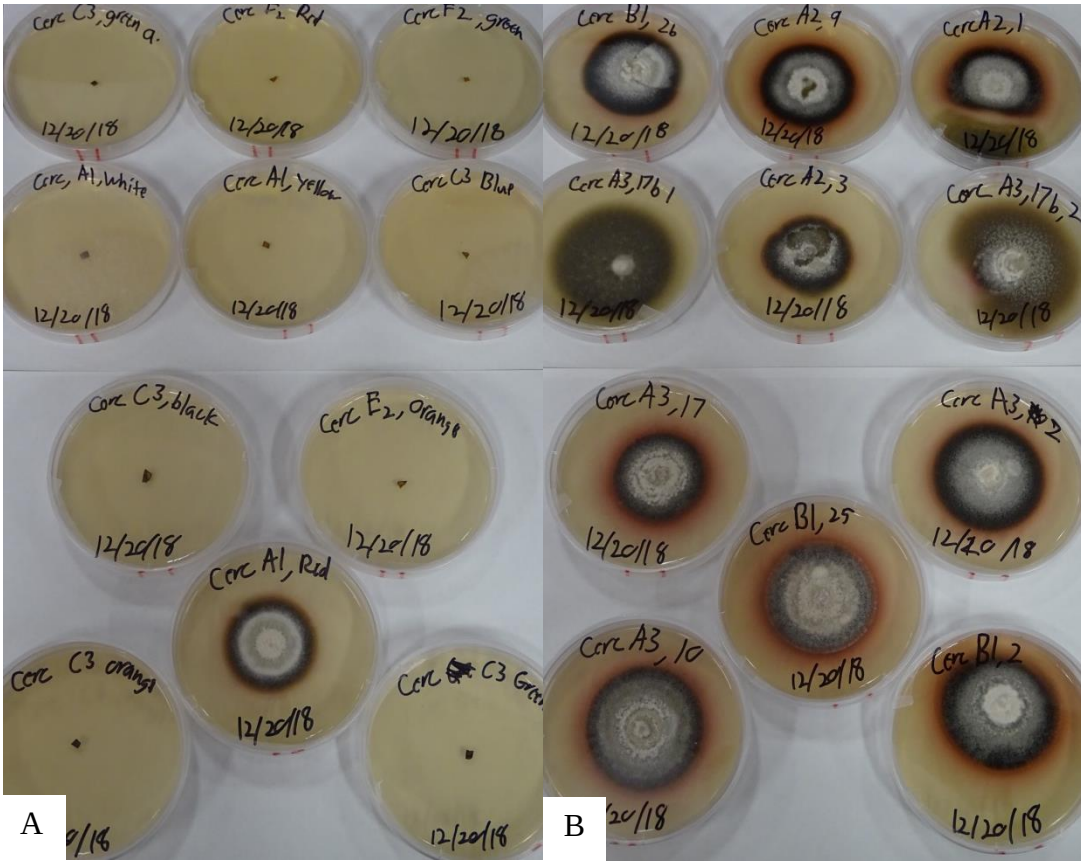


Figure 4.6: Growth of hyphae from heat treated and untreated *Cercospora beticola* lesions on half strength V-8 agar. *C. beticola* lesions were transferred from both heat treated (A) and untreated (B) sugar beet leaves to half strength V-8 agar. The pictures are from the second CLS viability test, and were taken 25 days after treatment. Three of the colonies started from lesions which were not heat treated have an unidentified fungal species growing with them on the plate, but *C. beticola* grew from every untreated lesion that was transferred.

Discussion

In recent years, CLS has become increasingly difficult for sugar beet growers in the Great Lakes region to manage. Fungicide resistance has reduced the efficacy of one of the most effective tools for CLS management (Khan, 2015; Michigan Sugarbeet REACh, 2016), and the other management tactics alone are not necessarily enough to keep CLS levels below the economic threshold. Heavily infected beet leaves from years of suboptimal CLS management can serve to increase the initial inoculum for the following year (Townsend, 1914; Coons et al., 1930; McKay and Pool, 1918; Bennett and Leach, 1971; Duffus and Ruppel, 1993), making

management more challenging. It is reasonable to hypothesize that high levels of initial inoculum could potentially overwhelm some of the management strategies currently being used (Weiland and Koch, 2004). While the current experiment is certainly not sufficient to judge the validity of heat treating CLS infected leaves on a field scale, the results indicate that a brief, intense heat treatment, such as that caused by a propane field burner, may be an effective strategy to reduce the amount of CLS inoculum, as both sporulation (Figure 4.3) and fungal viability (Figure 4.5) were reduced after a 15 second exposure to approximately 1,090°C.

Perhaps the most important result from the current study is that heat treating the leaves did not appear to cause substantial damage to the sugar beet roots, as was indicated by visual inspection of the plants and new leaf growth. If such injury had occurred, this strategy could not be used in the field. After the heat treatment, each of the beets in this study began to regrow leaves, with no visible impact to the root. Further studies will need to be conducted to examine any possible internal effects on the roots, particularly their ability to be stored. While the apparent lack of root damage is encouraging, these tests indicate that a field burner likely will not be able to completely defoliate beets in the field. Such defoliation would have been ideal, because it would have allowed this inoculum reduction strategy to be accomplished without adding an extra step to the harvest process. If an attempt were to be made to defoliate beets on a field scale with this method, it would probably take too much time and fuel (Murphy, 1968), and there would hypothetically be a greater risk of causing injury to the roots. In the current study, even when a plant was exposed to about 120 seconds of heat treatment, the petioles still remained on the beet. Such foliage remaining on the beets is unacceptable for storage, as it causes an increase in tare for the growers (Smith, 2001) and a decrease in air flow within the piles which may lead to storage problems (Bugbee, 1993; Campbell and Klotz, 2006; Michigan

Sugarbeet REACh, 2013). It is possible that the leaf tissue which remains on the roots could be removed without an extra step, perhaps by mounting scalpers (Smith, 2001) on the back of the burner or on the tractor which is pulling the harvester. This may provide better defoliation than a flail defoliator, as flails may not be as effective at removing leaves which have been damaged by heat treatment (Michigan Sugarbeet REACh, 2013). If field tests of a full-scale agricultural burner show promise, further studies will need to be conducted to determine the best method for defoliation. In addition, the proper amount of time between heat treatment and harvest would need to be determined. Previous studies have shown that sugar beets should not be defoliated more than 30 minutes before they are harvested because defoliated sugar beets increase in temperature much quicker than beets that have not been defoliated (Michigan Sugarbeet REACh, 2013). Because of this, beets may have to be harvested soon after being heat treated. However, the foliar heat treatment might increase the temperature of the beet roots, so this time may have to be extended to allow them to cool before harvest. Prior to this management strategy potentially being implemented, the impact it has on root temperature either directly or indirectly must be determined, as roots piled at too high of temperatures, namely 13°C and above, experience greater respiration rates and an increased susceptibility to storage rot pathogens compared to beets piled at cooler temperatures (Campbell and Klotz, 2006; Michigan Sugarbeet REACh, 2013).

It appears as though the heat treatment was very detrimental to the CLS lesions' ability to produce conidia as well as the overall viability of *C. beticola* in the lesions. None of the 214 lesions that were on treated leaves produced conidia over the period of the test. The impact which heat treatment had on sporulation could be due to the conidiophores and the pseudostromata being entirely and partially, respectively, exposed to the outside environment

(Steinkamp et al., 1979; Duffus and Ruppel, 1993; Weiland and Koch, 2004; Jacobsen and Franc, 2009; Franc, 2010). As such, they may be particularly sensitive to the effects of intense heat, and it is hypothesized such a treatment could destroy this exposed tissue, but this needs further investigation. Transferring heat treated lesions to half strength V-8 agar plates revealed that the treatment reduced *C. beticola* viability within the lesions by over 80%. Between both runs, only three out of the 22 transferred heat treated lesions showed viable fungal growth and produced colonies on media. While this is an excellent rate of growth inhibition, the fact that there was fungal growth from some of the lesions indicates that survival may be possible in the field. A reason for this survival could be that while a majority of *C. beticola* reproductive structures are exposed to the outside environment, the rest of the fungus is growing within and is somewhat protected by the leaf (Pool and McKay, 1916a; Steinkamp et al., 1979), perhaps allowing some of the hyphae to survive. It also is possible there could have been differences in the amount of time individual lesions were exposed to the heat, leading to some with more limited exposure surviving.

Although the hyphae of fewer than 20% of the lesions tested survived in this preliminary test, the implications of this survival on the efficacy of a field burner are unclear. In addition to causing severe damage directly to CLS lesions, the handheld torch caused significant damage to the beet leaves. Even if a percentage of the hyphae within CLS lesions are able to survive the effects of the heat treatment, the damage done to the leaf tissue by this heat (Parish, 1990) will likely cause the leaves to degrade faster than leaves that are not heat treated. Since intact leaf debris is important for *C. beticola* survival [Canova, 1959b (in Italian) as cited by Duffus and Ruppel, 1993; Bennett and Leach, 1971], increasing the speed of leaf decay could decrease the longevity of the fungus in a similar way as moldboard plowing (Townsend, 1914; McKay and

Pool, 1918; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Khan et al., 2008; Jacobsen and Franc 2009; Jacobsen, 2010; Michigan Sugar Company, 2018). This technique may even be more effective than plowing, in that if conidiophores and pseudostromata are damaged, these structures will have to be reformed in order to produce conidia (Pool and McKay, 1916b). It is hypothesized that there may not be enough energy left in the heat treated leaves for such regrowth. If that is indeed the case, such survival would be irrelevant to the epidemiology of *C. beticola*, as it is unlikely the surviving hyphae will infect new sugar beets under normal field conditions without the production of conidia (Pool and McKay, 1916b). Additionally, pseudostromata may not be reformed before winter, and since these are the primary overwintering structures for the fungus (Pool and McKay, 1916b), successful overwintering may be prevented or reduced even if it does survive the heat treatment. It should also be noted that, while effort was taken in the current study to simulate what would happen in the field with a full-scale field burner, this test was not a perfect rendition of these conditions. An actual field burner may leave more or fewer viable lesions than the handheld torch, so field tests with such an implement will need to be conducted to determine this.

These results support those of McKay and Pool (1918), that *C. beticola* is sensitive to short bursts of intense heat. In their study, McKay and Pool examined the impact of temperatures around 100°C for several minutes on *C. beticola*. Unlike that study, the current study examined the effects of very intense heat (1,000°C and above) for a much shorter duration on *C. beticola* growth and sporulation. If heat treatments are to be tested as an option for field management of CLS, a laboratory study to determine the exact time and temperature combinations required for fungal mortality at these higher temperature ranges would complement such a field test nicely.

Even though the use of agricultural burners would be a new method of CLS management, their use in agriculture as a whole is far from novel. Some of the earliest flame cultivators intended for weed control were built in the 1940's (Knake et al., 1965), and reports of using open fires in agriculture date back thousands of years (Hardison, 1976). Today, the primary use of agricultural burners and torches is for weed control, particularly in organic production (Parish, 1990). Depending on the crop, torches can be used as either pre- or postemergence weed control measures (Parish, 1990). Corn in particular is well suited for postemergence heat treating, as it is less damaged by the effects of heat than are other crops like soybeans (Knake et al., 1965). Agricultural torches also have been used to control some plant pathogens (Hardison, 1976). A few pathogens which heat treating has been tested against include various rusts of grass (*Poa pratensis*) such as leaf rust (*Puccinia poa-nemoralis*), stem rust (*P. graminis*), and stripe rust (*P. striiformis*); rust of mint (*P. menthae*); and several potato diseases, such as Verticillium wilt (*Verticillium albo-atrum*), tuber rot caused by *Alternaria solani*, and late blight (*Phytophthora infestans*) (Hardison, 1976). For Verticillium wilt from *V. dahliae*, Mpofu and Hall (2002) found that a combination of soil fumigation and vine heat treating was the only treatment out of eight which were tested that was able to decrease the population density of the pathogen, reduce the area under the disease progress curve, and significantly improve potato yield. Heat treating potato vines has been recommended for controlling late blight (Fry, 1998), and also has been used to defoliate potato vines before harvest (Murphy, 1968), particularly for organic production. Thus there is precedent for the utility of heat treatment in agriculture and specifically disease management.

Management of CLS by means of a heat treatment could have several advantages as compared to some of the other management tactics currently being utilized. One advantage is

that it would not require the addition of chemical pesticides to the environment, so in that way it may be more environmentally sustainable than using fungicides to manage CLS (Hardison, 1976; Parish, 1990). However, an in depth investigation to determine the overall environmental impact of heat treating sugar beet leaves, paying particular attention to its effect on compounds on or in the sugar beet leaves, is required before any statement can be made about its environmental sustainability. Depending on the cost and amount of propane needed, this may be a relatively inexpensive management strategy for growers to implement (Flame Engineering, Inc., personal communication; Multi-Trail Enterprises, personal communication). Additionally, in current commercial fields most of the CLS lesions present at the time of harvest are those that have probably survived one or multiple fungicide applications (Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018), and would consequently be likely to have fungicide resistance (Brent and Hollomon, 2007b; Rosenzweig et al., 2015). Therefore, it is possible heat treating CLS infected leaves could help to decrease the incidence of new fungicide resistance developing as well as reduce the rate at which resistance is spreading, although studies would have to be conducted to determine if this would be the case. Finally, heat treating sugar beet could help to manage herbicide resistance, as the resistant weeds would be passed through the burner along with the sugar beet leaves, possibly destroying some of their seed (Walsh and Newman, 2007). As with its hypothesized effect on fungicide resistance, tests would have to be done to determine to what degree this practice might impact the population of resistant weeds.

Like most agricultural practices, there would be some important disadvantages of heat treating for CLS management. From the growers' perspective, one of the most important of these is that it may require another step during harvest. There would be a time investment for growers, as they would either need to learn how to properly and safely use such a device or wait

during harvest for a trained operator to provide this service. From an epidemiological standpoint, an important consideration is that if this tactic is to be effective, all or a majority of the growers in an area likely would need to adopt the practice. For instance, if four of five growers in a particular area adopt the new technique but the fifth does not, the infected leaf debris from the fields which are not heat treated could serve as inoculum for all the fields in the area (Townsend, 1914; Coons et al., 1930; McKay and Pool, 1918; Bennett and Leach, 1971), making this strategy much less effective. Even if this practice is widely adopted, it will almost certainly not eliminate all of the CLS inoculum. Inoculum from weed hosts (Townsend, 1914; Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009) in fields planted to a different crop or field edges would be unaffected by the heat treatments. Similarly, inoculum from weeds and beets in home gardens or ornamental chard plantings would not be addressed. While this strategy will not eliminate all of the CLS inoculum, it could reduce the amount of inoculum present in the environment, aiding in overall CLS management.

The development of novel management techniques may play an important role in mitigating the future economic impact of CLS on sugar beet. Based on the findings of the current study, it appears as though heat treating infected sugar beet leaves could be one of those new techniques and potentially serve as an important part of an integrated management plan for CLS. The tests run in the current study indicate that an intense heat treatment eliminated the immediate ability of CLS lesions to produce conidia, decreased fungal viability from lesions on agar plates, and caused damage to the leaves which is hypothesized to lead to faster degradation in the field. Before such a heat treatment could be accepted as a management technique for CLS, extensive field testing will be required to verify that field burners will not cause damage to sugar beet roots. Furthermore, it must be proven that heat treating in the field will lead to a decrease in

Cercospora beticola inoculum. From the evidence gathered in this preliminary experiment, such field tests are warranted. Should these tests be fruitful, field burners may play a critical role in the long term, sustainable management of CLS, and may even serve as an important tool in implementing integrated pest management for this and other pests of sugar beet.

CHAPTER 5

CONCLUSIONS, IMPLICATIONS, AND FUTURE RESEARCH DIRECTIONS

For many beet growers in the Great Lakes region and throughout the world, Cercospora leaf spot (CLS) is a major problem (Duffus and Ruppel, 1993; Holtschulte, 2000; Asher and Hanson, 2006; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018) and likely will remain so for several years to come. An increase in the incidence and level of fungicide resistance (Ruppel and Scott, 1974; Bugbee, 1995; Weiland and Halloin, 2001; Kirk et al., 2012; Bolton et al., 2012a; Bolton et al., 2012b; Khan, 2015; Rosenzweig et al., 2015; Michigan Sugarbeet REACh, 2016; Rosenzweig et al., 2017; Trueman et al., 2017), a shift away from moldboard plowing (Townsend, 1914; McKay and Pool, 1918, Montgomery, 2007; Michigan Sugar Company, 2018) and a reluctance to plant resistant varieties in favor of higher yielding, susceptible varieties (Miller et al., 1994; Weiland and Koch, 2004; Skaracis and Biancardi, 2005) all have contributed to the problem. Adopting an integrated approach to CLS management (Duffus and Ruppel, 1993; Asher and Hanson, 2006; Jacobsen and Franc, 2009; Jacobsen, 2010) will be important for future management schemes, with new higher-yielding resistant varieties playing an especially important role (Jacobsen, 2010; Gummert et al., 2015). Along with the development of these new resistant lines (Gummert et al., 2015), current and novel management strategies will need to be employed judiciously. To that end, this thesis investigated *Cercospora beticola* spore production early in the growing season, the efficacy of bicarbonate salts as fungicides against *C. beticola*, and the impact of a brief, intense heat treatment on infected sugar beet leaves.

The primary goal of the sentinel beet project was to examine the time of and the conditions conducive for *C. beticola* spore production in the early part of the sugar beet growing

season. Both years, *C. beticola* spores were detected earlier in the season than had previously been examined or detected, the week of April 15 in 2017 and the week of April 20 in 2018. This agrees with and is even earlier than the detection of *C. beticola* conidia in Ontario, Canada, the first week of May by Tedford et al. (2018). The implications of this early spore production on CLS epidemiology need further investigation. It is possible that since conditions which are conducive for infection rarely occur this early, such spore production may be of limited consequence. However, if appropriate infection conditions do occur early in the season, the presence of spores at this time could lead to earlier infection and CLS development, similar to that reported in 2018 (Wilbur et al., 2018a, 2018b), potentially leading to a more severe epidemic later in the season (Pool and McKay, 1916a, 1916b; Weiland and Koch, 2004; Poindexter, 2007; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018). Should such early season infection occur, it is hypothesized that enacting measures to manage the disease at this time, such as taking extra precautions to avoid planting near known inoculum sources or perhaps an early season fungicide application, may help to improve CLS management.

Each year of the study, variation in the number of lesions detected on sentinel beets between sites and between weeks was observed. The cause of variation between sites was not determined, but investigation into this could provide possible avenues for inoculum reduction and overall management of the disease. Variation among weeks likely was caused in part by climatic differences. In both years, lesion numbers on the sentinel beets were significantly correlated to both rainfall and relative humidity, with R^2 values ranging from 0.10 to 0.31. This indicates these variables may contribute in a meaningful way to the production and/or dispersal of *C. beticola* spores. The correlation with rainfall is in agreement with Carlson (1967), and the correlation with relative humidity is in agreement with several studies on spore formation [Pool

and McKay, 1916b; Bleiholder and Weltzien, 1972b (in German), with English summary]. No correlation was found between lesion number and air temperature (GDD), which is in contrast with the findings of Khan et al. (2009) and Tedford et al. (2018). Thus further investigation into the relationship between spore concentration and weather variables is warranted.

Since substantial fluctuations were observed in spore concentration in this and previous studies (Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018), it is possible that considering spore concentration in either the Shane and Teng (Shane and Teng, 1985; Windels et al., 1998; Jacobsen, 2010) or the BEETcast (Jacobsen, 2010; Michigan Sugarbeet REACh, 2016; Michigan Sugar Company, 2018; WIN, 2018) prediction models could improve their accuracy and the timing of fungicide applications. Information about *C. beticola* spores could be included by means of a direct measurement of spores, or the inclusion of weather variables which favor conidia production and dispersal. Prior to this, studies should be conducted to gather more data about the relationship between weather variables and spore concentration, as well as potentially resolve the difference in significant weather variables observed between the current (early season) and previous (generally later in the season) studies (Lawrence and Meredith, 1970; Khan et al., 2009; Tedford et al., 2018). Such a study should incorporate both sentinel plants and spore traps, thus employing the strengths of both measurement techniques. Rather than the Hirst-type traps used in the above studies, it may be beneficial to use glass rod or Rotorod spore traps. These traps are effective at measuring both aerial-dispersed conidia (Frenz, 1999) and rain-dispersed conidia (Carlson, 1967; Lawrence and Meredith, 1970), while the Hirst-type spore traps are better suited to detect aerial-dispersed conidia (Hirst, 1952).

The focus of the bicarbonate study was to determine if ammonium, potassium, and/or sodium bicarbonate could be used to help manage CLS. In vitro, each of these compounds at a 1% (w/v) concentration led to a reduction in *C. beticola* growth, with sodium bicarbonate resulting in a significantly greater reduction than potassium bicarbonate ($p<0.015$). Ammonium bicarbonate led to complete inhibition of fungal growth at this concentration. The results of an x-plate test indicate that ammonia gas was a major contributor to this fungicidal activity. The impact of ammonia gas on *C. beticola* growth is intriguing; even though it may not provide a practical means for CLS management in the field, it potentially could be used to help manage other pathogens of sugar beet, such as those which cause storage rots (Bothast et al., 1973; Montesinos-Herrero et al., 2011). The bicarbonates caused an increase in the pH of the lima bean agar used in this experiment. However, pH did not appear to be the primary driver of the reduction in *C. beticola* growth which was observed on the bicarbonate-amended plates, as the fungus itself increased agar pH to a similar level as the bicarbonates, even in the absence of bicarbonate (Figures 3.9 and 3.10).

In the greenhouse, plants treated with sodium bicarbonate at a 2% (w/v) concentration had significantly fewer lesions than the control ($p<0.005$), while ammonium bicarbonate at a 2% (w/v) concentration did not cause a significant disease reduction. While one of three adjuvants tested had a significant impact on disease in one out of three runs of the experiment ($p=0.0325$), the results of the test as a whole do not indicate that the adjuvants tested had a strong impact on CLS or bicarbonate efficacy. Phytotoxicity was observed on the beets to certain adjuvant-bicarbonate combinations during the second and third runs of the experiment. Based on these results, it is possible that sodium bicarbonate could be an effective treatment for CLS, but field

testing is required before it can be recommended to growers, particularly to determine the proper adjuvants to use and to resolve the potential issues with phytotoxicity.

The heat treatment study was a preliminary study to examine the impact of a brief, intense heat treatment on CLS lesions and beet leaves. After heat treatment, there was a significant reduction in the ability of CLS lesions to produce conidia ($p<0.015$), as no conidia production was observed on any of the heat treated CLS lesions. An 81% to 91% reduction in the growth of *C. beticola* hyphae from heat treated lesions was observed 25 days after treatment. Additionally, substantial damage was caused to the leaves as a result of the treatment, which may increase the speed of leaf breakdown in the field (Parish, 1990). These results indicate that further testing of heat treatment as a means of CLS management is warranted. A field test of one or more commercial burners is planned to evaluate the effectiveness of heat treatment in the field and to ascertain the impact of such a treatment on the health and storability of the sugar beet roots. Along with this field test, an in vitro test should be conducted to expand on the work of Pool and McKay (1916b) and determine the exact temperature and time combinations which could lead to *C. beticola* death, especially in this high temperature range. It is hypothesized that an end of the season heat treatment could cause sufficient damage to the leaves (Parish, 1990) and *C. beticola* itself to reduce overwintering and thus the level of inoculum for the following season.

The information collected during these three studies has helped contribute to the overall understanding of *Cercospora beticola* biology. There are several possibilities for future research on these topics. From the sentinel beet project, these include investigating how long the latent period between *C. beticola* infection and CLS disease development can be extended due to suboptimal conditions, and investigating the relevance of early season spore production to *C.*

beticola epidemiology. The current experiment should be repeated with both sentinel beets and Rotorod spore traps, and the information provided from it should then be used to improve the prediction models. For the bicarbonate project, the bicarbonates used in this experiment should be tested in vitro at different pH's. The bicarbonates also should be tested in the field to determine if they could be used by growers. In the current experiment, ammonia gas was found to be a contributing factor for the fungicidal activity of ammonium bicarbonate. While ammonia may not be a practical means of reducing CLS in the field, it could be used against storage rot pathogens. For the heat treatment project, the next step is to test an agricultural burner in the field, evaluating its ability to decrease *C. beticola* viability and overwintering ability, as well as to ascertain its impact on sugar beet health and determine any adjustments that must be made to sugar beet harvest. It is possible that the findings of the current work, as well as future projects inspired by it, may contribute to CLS management efforts. This information may contribute to the improvement of the CLS prediction models, help to manage the disease during the season, and provide new tools with which the level of CLS inoculum may be reduced.

APPENDICES

APPENDIX A

SHANE AND TENG DIV TABLE

Hours per Day when RH≥87	Daily Infection Values (DIV's)																																					
24	1	2	4	5	5	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
23	1	2	3	4	5	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
22	1	1	3	4	5	6	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
21	0	1	2	4	4	5	5	5	5	5	5	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
20	0	1	2	3	4	5	5	5	5	5	5	5	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
19	0	0	1	3	4	5	5	5	5	5	5	5	5	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
18	0	0	1	2	3	4	4	4	4	4	4	5	5	5	5	5	5	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
17	0	0	1	2	3	4	4	4	4	4	4	4	5	5	5	5	5	5	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
16	0	0	0	2	3	4	4	4	4	4	4	4	4	4	4	4	5	5	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
15	0	0	0	1	3	4	4	4	4	4	4	4	4	4	4	4	4	5	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
14	0	0	0	1	3	3	3	3	3	3	3	4	4	4	4	4	4	4	5	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
13	0	0	0	0	3	3	3	3	3	3	3	3	4	4	4	4	4	4	5	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
12	0	0	0	0	2	3	3	3	3	3	3	3	3	4	4	4	4	4	4	5	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
11	0	0	0	0	2	3	3	3	3	3	3	3	3	3	3	3	3	3	4	5	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
10	0	0	0	0	2	2	2	2	2	2	2	3	3	3	3	3	3	3	4	4	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	3
9	0	0	0	0	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	4	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	3
8	0	0	0	0	1	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	3
7	0	0	0	0	1	2	2	2	2	2	2	2	2	2	2	2	2	2	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3
6	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	2	2	2	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3
5	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
4	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
3	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Average Temperature																																						
°F	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95		
°C	15.6	16.1	16.7	17.2	17.8	18.3	18.9	19.4	20.0	20.6	21.1	21.7	22.2	22.8	23.3	23.9	24.4	25.0	25.6	26.1	26.7	27.2	27.8	28.3	28.9	29.4	30.0	30.6	31.1	31.7	32.2	32.8	33.3	33.9	34.4	35.0		

Table A.1: Shane and Teng, DIV table. This is a copy of the table used to calculate daily infection values (DIV's) for the Shane and Teng model. The column on the left is the number of hours the relative humidity (RH) is above a predetermined level, in this case 87%. The two rows on the bottom are the average air temperature. To assign DIV's for a day, the first step is to measure the number of hours relative humidity is above the predetermined threshold. Next, the average air temperature during that time is calculated. Where these values intersect is the assigned DIV (Shane and Teng, 1984a; Windels et al., 1998).

APPENDIX B

WEATHER VARIABLES FROM AN EXAMPLE SITE USED IN THE SENTINEL BEET STUDY; AKRON BEETS

One component of the sentinel beet project (Chapter 2) was correlating six weather variables to the number of CLS lesions observed on the sentinel beets placed at the old beet field sites. The weather variables measured included air temperature, relative humidity, rainfall, leaf wetness, solar radiation, and wind speed. Weekly and hourly measurements of these variables were taken throughout both the 2017 and 2018 seasons. Due to the amount of weather data collected, it was not presented in Chapter 2. Here, the data from the Akron beets site in both 2017 and 2018 is shown as an example to provide an indication of how these variables behaved throughout the seasons. Additional information can be found at the MAWN website (https://mawn.geo.msu.edu/). The weather variables are summarized in Tables B.1 and B.2 as follows. Air temperature, in degrees Celsius, is summarized as the weekly average, high, and low, along with weekly growing degree days, calculated as described by Holen and Dexter (1996) with a base temperature of 15.56°C (Pool and McKay, 1916b; Coons and Larmer, 1929) and no maximum temperature. The percent relative humidity is summarized with the weekly average, high, and low, as well as the total number of hours per week when the relative humidity was 80% or greater [Bleiholder and Weltzien, 1972b (in German), with English summary; Cioni et al., 2014]. Rainfall is reported as total centimeters of rain per week, as measured with rain gauges at the sentinel beet site and at the Fairgrove MAWN station. Solar radiation (SRAD) is shown as average langleys per week. Leaf wetness, expressed as a percent of the hour the leaf is wet, is reported as the average, high, and low for the week, and the total number of hours during the week that the leaf was wet for at least 15 minutes. Wind speed in kilometers per hour is

shown as the weekly average, high, and low. In addition to the weather variables themselves, the total daily infection values (DIV) for each week are shown, calculated as described by Shane and Teng (1984a) and Windels et al. (1998).

Akron 2017, Weather Data									
Weather Variable	Air Temperature, MAWN (°C)				Percent Relative Humidity, MAWN				Shane and Teng DIV's
Week	GDD	Average	High	Low	Number of Hours at or Above 80% RH	Average	High	Low	Total
15-Apr	26.1	11.4	26.5	-0.3	57	68.8	92.1	34.0	0
22-Apr	33.2	13.1	25.9	1.4	14	59.6	87.5	23.0	0
29-Apr	7.2	7.6	19.7	2.2	84	75.0	97.5	29.3	0
6-May	11.1	9.9	19.7	-1.4	13	50.2	88.8	18.1	0
13-May	60.0	16.0	29.8	2.6	22	55.2	90.5	20.6	0
20-May	31.8	15.0	22.9	9.3	76	73.4	93.5	33.4	0
27-May	54.5	17.5	26.9	6.9	18	52.9	91.1	23.1	0
3-Jun	68.0	18.3	30.3	8.1	44	61.3	91.4	23.7	0
10-Jun	128.0	23.8	33.0	16.1	43	62.3	92.3	25.5	10
18-Jun	44.4	18.9	28.6	10.6	64	73.2	92.8	45.2	4
24-Jun	56.7	18.2	27.1	11.1	58	70.8	92.9	37.5	5
1-Jul	77.7	21.0	30.3	12.1	55	65.3	93.9	34.4	3
8-Jul	87.5	21.5	31.1	13.8	65	72.1	93.3	33.3	14

Akron 2017, Weather Data Continued										
Weather Variable	Rainfall (cm)		SRAD (Ly)	Percent of Hour Leaf is Wet				Wind Speed (km/h)		
Week	On Site Total	MAWN Total	Average	Number of Hours with ≥15 Minutes Leaf Wetness	Average	High	Low	Average	High	Low
15-Apr	3.2	3.2	14.2	57	30.5	100	0	15.5	36.4	1.0
22-Apr	0.0	0.0	19.5	26	14.3	100	0	15.8	42.0	0.8
29-Apr	3.2	3.7	11.0	79	41.5	100	0	16.2	36.0	1.1
6-May	0.0	0.0	20.7	64	34.4	100	0	9.4	23.7	0.8
13-May	0.0	0.0	16.8	48	27.3	100	0	16.8	47.3	0.8
20-May	1.3	0.5	11.5	89	50.4	100	0	13.8	31.5	1.6
27-May	0.0	0.0	18.8	20	10.4	100	0	12.6	38.9	0.8
3-Jun	0.0	0.4	19.8	54	26.6	100	0	10.9	33.6	0.8
10-Jun	2.5	3.4	16.8	51	22.9	100	0	12.7	31.4	1.1
18-Jun	7.0	6.6	17.1	62	39.3	100	0	10.1	33.2	1.1
24-Jun	2.5	0.0	18.2	87	45.5	100	0	12.1	24.6	1.8
1-Jul	1.0	0.0	22.7	51	27.1	100	0	7.7	23.0	0.8
8-Jul	0.2	0.0	17.1	77	41.1	100	0	7.5	19.0	1.3

Table B.1: Akron beets 2017, weather data. Weather data for each week for the 2017 Akron beets site, including: air temperature in the form of growing degree days (Holen and Dexter, 1996) as well as the weekly average, high, and low; relative humidity (RH) in the form of the number hours per week it was above 80% as well as the weekly average, high, and low; the total daily infection value (DIV) for the week (Shane and Teng, 1984a; Windels et al., 1998); total rainfall as determined by a rain gauge at the sentinel beet site (site total) and the nearest MAWN station (MAWN total); average solar radiation (SRAD); leaf wetness (percent of hour leaf is wet) reported as the total number of hours during which the leaf was wet for at least 15 minutes, as well as the weekly average, high, and low; and the wind speed, reported as the weekly average, high, and low.

Akron 2018, Weather Data									
Weather Variable	Air Temperature, On Site (°C)				Percent Relative Humidity, On Site				Shane and Teng DIV's Points
Week	GDD	Average	High	Low	Number of Hours at or Above 80% RH	Average	High	Low	Total
20-Apr	24.4	9.4	24.6	-2.5	48	62.9	99.9	17.1	0
27-Apr	54.8	13.0	31.1	-3.8	55	59.0	99.9	11.2	0
4-May	59.8	14.8	29.1	0.4	43	62.3	99.9	22.8	0
11-May	49.4	13.8	27.8	3.4	71	72.6	99.9	28.4	2
18-May	59.2	16.4	34.1	4.1	93	76.0	99.9	19.4	0
25-May	130.2	25.1	35.3	15.6	58	68.2	99.9	30.4	14
1-Jun	58.2	17.1	29.3	7.2	47	66.2	99.9	28.2	0
8-Jun	84.7	20.2	30.9	9.2	26	61.2	99.9	19.9	3
15-Jun	100.3	21.7	36.7	9.6	77	75.7	99.9	30.9	13
22-Jun	83.3	19.8	32.7	7.5	112	82.7	99.9	31.4	21
29-Jun	152.9	27.1	36.4	14.7	70	73.0	99.9	32.1	22
6-Jul	101.3	21.8	32.3	7.8	59	65.2	99.9	26.7	3

Akron 2018, Weather Data Continued										
Weather Variable	Rainfall (cm)		SRAD (Ly)	Percent of Hour Leaf is Wet				Wind Speed (km/h)		
Week	On Site Total	MAWN Total	Average	Number of Hours with ≥15 Minutes Leaf Wetness	Average	High	Low	Average	High	Low
20-Apr	0.0	0.2	16.7	76	41.3	100	0	8.3	22.4	1.1
27-Apr	3.2	2.4	17.1	46	24.9	100	0	15.9	59.2	1.8
4-May	0.0	0.2	17.5	61	31.4	100	0	14.8	41.4	1.0
11-May	1.9	2.8	19.4	57	33.2	100	0	10.5	25.3	1.0
18-May	0.6	1.4	15.9	80	44.5	100	0	9.3	21.7	1.1
25-May	0.0	0.6	19.6	66	35.1	100	0	10.8	34.3	1.1
1-Jun	0.0	0.3	19.2	58	29.1	100	0	12.4	28.8	1.0
8-Jun	0.0	0.1	18.0	44	21.1	100	0	10.1	31.9	1.4
15-Jun	0.0	1.2	15.4	52	28.5	100	0	9.3	24.1	0.8
22-Jun	1.0	1.1	16.7	91	51.4	100	0	8.0	18.0	1.0
29-Jun	0.0	0.0	21.8	28	13.5	100	0	10.9	23.0	1.1
6-Jul	0.5	0.4	22.9	31	16.7	100	0	8.2	19.3	0.8

Table B.2: Akron beets 2018, weather data. Weather data for each week for the 2018 Akron beets site including: air temperature in the form of growing degree days (Holen and Dexter, 1996) as well as the weekly average, high, and low; relative humidity (RH) in the form of the number hours per week it was above 80% as well as the weekly average, high, and low; the total daily infection value (DIV) for the week (Shane and Teng, 1984a; Windels et al., 1998); total rainfall as determined by a rain gauge at the sentinel beet site (site total) and the nearest MAWN station (MAWN total); average solar radiation (SRAD); leaf wetness(percent of hour leaf is wet) reported as the total number of hours during which the leaf was wet for at least 15 minutes, as well as the weekly average, high, and low; and the wind speed, reported as the weekly average, high, and low. This year, air temperature and relative humidity values were collected on site.

APPENDIX C

MEDIA PREPARATION TEST

An observation was made that bicarbonates were less effective at inhibiting the growth of *Cercospora beticola* if the media was stored on an open lab bench for an extended period of time before being used for fungal growth assays as compared to freshly poured plates (data not shown). This, along with conflicting reports in the literature (Punja and Grogan, 1982; Ziv and Zitter, 1992) about the difference between adding the bicarbonate before autoclaving or after autoclaving, led to a test of these parameters.

The test was similar to that conducted in Chapter 3, in that it consisted of four chemical treatments, including: ammonium, potassium, and sodium bicarbonate (each at 1% w/v), and a control with no bicarbonate added. This test included a second factor, media preparation method, which had three levels. For the first method (E1), the bicarbonates were added to autoclaved media after being disinfested in ethanol (0.7% v/v final media concentration) as in Chapter 3. In the second (B1), the bicarbonates were added prior to autoclaving and without ethanol. For the third (E8), the bicarbonates were added with ethanol (0.7% v/v final concentration) after autoclaving and stored on a lab bench for eight days before fungal transfer. Four *C. beticola* isolates were tested: BE4, BW14, RFR34, and MSU165. Fungal plugs for each of the three preparation methods were transferred at the same time and the plates were kept in an incubator together at approximately 23-24°C.

The experimental design was a two factor randomized complete block design with bicarbonate treatment and preparation method as the two factors, blocked by isolate. Each treatment by preparation method by isolate combination was replicated three times, and the entire experiment was repeated once. Colony growth was measured with a caliper and the

percent reduction in growth compared to the control was determined at seven, 11, and 15 days after transfer (DAT) by subtracting the diameter of fungal growth on a bicarbonate plate from the diameter of growth on a corresponding control plate and dividing by the diameter on the control plate. An ANOVA table was constructed for each of the three DAT's with the proc mixed procedure in SAS 9.4, and mean separation by the least significant difference method (α=0.05) was done with the proc glimmix procedure.

For each of the three DAT's, the factors treatment and media preparation, as well as their interaction and the block effect, were significant ($p<0.0001$). At seven and 11 days after transfer, there was no significant difference between E1 and B1, while E8 was significantly different ($p<0.0001$) from the other two preparation methods. At 15 days after transfer, all preparation methods were significantly different from one another ($p<0.0019$).

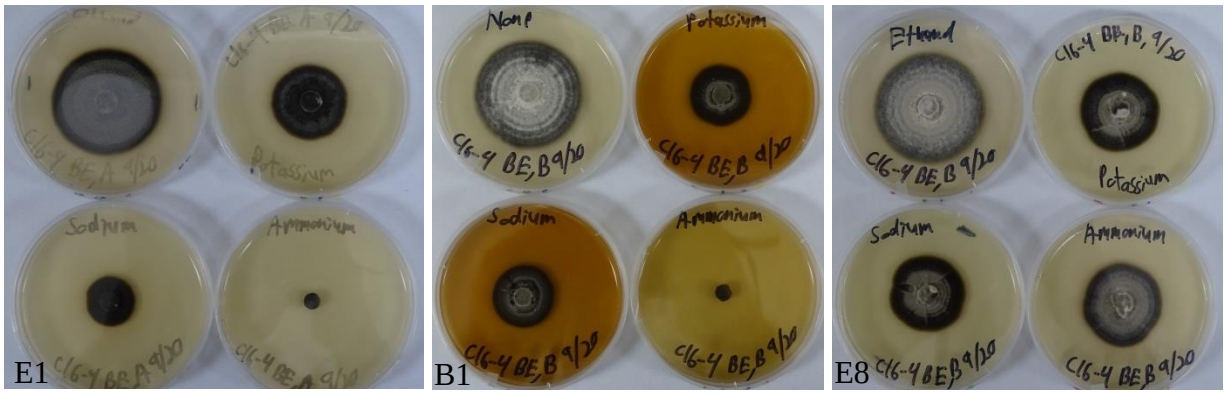


Figure C.1: *Cercospora beticola* isolate (BE4) growth on plates with three media preparation methods. Shown are lima bean agar plates with bicarbonate in ethanol added after autoclaving (E1 and E8), or bicarbonate added before autoclaving (B1). Plates were used either one day (E1 and B1) or eight days (E8) after bicarbonate was added. In each image, the plate in the upper left corner is the untreated control, and clockwise around are the potassium, ammonium, and sodium bicarbonate plates. A 1% (w/v) concentration of bicarbonate was used. Ethanol was applied at a concentration of 0.7% (v/v) to each plate in E1 and E8.

At each DAT, the ammonium bicarbonate provided complete inhibition of fungal growth with preparation methods E1 or B1. Sodium and potassium bicarbonate showed fungal growth inhibition, with sodium providing greater inhibition than potassium at each time ($p<0.0001$). For

preparation method E8, none of the treatments completely inhibited fungal growth, and sodium provided the greatest level of inhibition ($p<0.02$ for each of six comparisons). For each treatment, the colonies on plates from E8 experienced less growth inhibition than those on plates from E1 or B1.

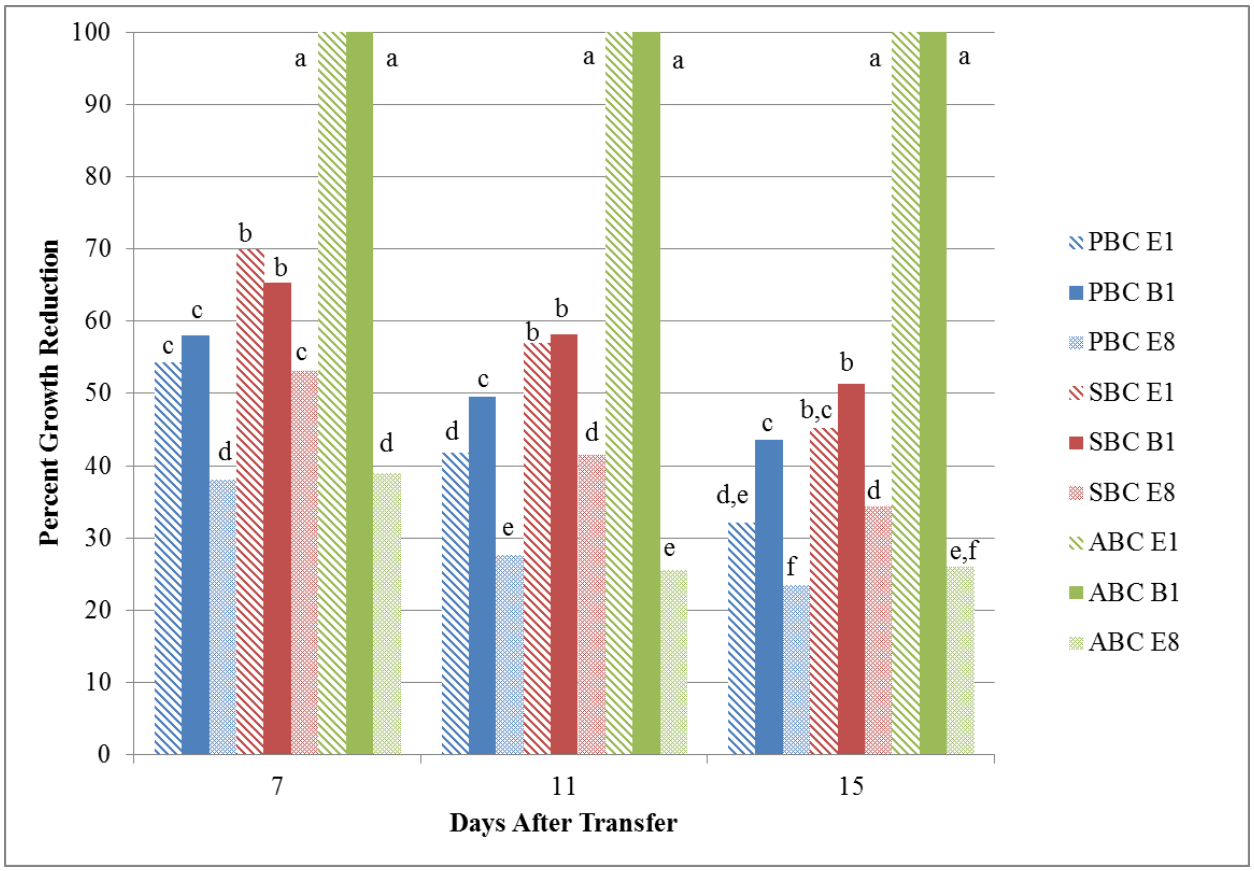


Figure C.2: Impact of media preparation on *Cercospora beticola* growth. Shown is the percent growth reduction of *C. beticola* with three bicarbonates compared to a media control at three times (seven, 11, and 15 days after transfer). The treatments were potassium bicarbonate (PBC) in blue, sodium bicarbonate (SBC) in red, and ammonium bicarbonate (ABC) in green. All bicarbonates were added at a rate of 1% (w/v). Solid bars indicate the bicarbonate added before autoclaving ("B1"), and diagonal hashing represents bicarbonate added after autoclaving with ethanol (0.7%). The one-way diagonal indicates the plates to which fungi were transferred one day after pouring ("E1"), while the double diagonal represents the plates stored for eight days before fungal transfer ("E8"). Each bar is the average of 12 measurements. Mean separation was conducted at each time; bars at the same days after transfer with the same letter are not significantly different by least significant difference, $\alpha=0.05$.

When comparing just the different preparation methods, there was no difference in fungal growth between E1 and B1. If the interaction between the bicarbonate treatment and preparation

method was considered, the ammonium bicarbonate treatments with E1 and B1 preparation methods were not significantly different from each other at any time, nor were the sodium bicarbonate treatments with the E1 and B1 preparation methods (Figure C.2). At times 11 and 15, however, the potassium bicarbonate treatments with the E1 and B1 preparation methods were significantly different from each other ($p<0.0076$). Based on these results, and the fact that B1 resulted in dark-colored media which made measuring the colonies difficult (Figure C.1), the decision was made to add the bicarbonate after autoclaving for the primary experiments.

Storing the media on the lab bench for an extra week before transferring the *C. beticola* plugs (E8) resulted in significantly less inhibition of colony growth ($p<0.0001$) as compared to E1. This was especially apparent with the ammonium bicarbonate treatment. For this treatment, plugs transferred one day after pouring, regardless of whether they were the E1 or B1 preparation method, showed complete inhibition of fungal growth. Growth from the plugs transferred to E8 plates was reduced compared to the control, but the colonies grew. This has implications for the anti-fungal activity of the bicarbonates, which are discussed in Chapter 3. At each DAT, both the potassium and sodium bicarbonate treatments with the E1 and E8 preparation methods were significantly different from each other ($p<0.007$ and $p<0.001$, respectively). Thus all of the primary experiments were done with transfers to media one day after pouring (Chapter 3).

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