INTEGRATED STRATEGIES FOR IMPROVED MANAGEMENT OF CERCOSPORA LEAF SPOT ON SUGARBEET

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology – Doctor of Philosophy

2025

ABSTRACT

Sugarbeets account for 55 to 60% of U.S. sugar production. Cercospora leaf spot (CLS), primarily caused by the fungal pathogen *Cercospora beticola*, is a major foliar disease of sugarbeet. Since leaf tissue is a primary site of pathogen survival between growing seasons, management strategies were evaluated to reduce this source of inoculum. Fall- and spring-applied treatments were evaluated over three years at two locations. Treatments included a non-treated control, a propane-fueled heat treatment, and a desiccant application seven days pre-harvest compared to tilling immediately post-harvest. The fall-applied heat treatment significantly reduced lesion sporulation and *C. beticola* isolation in at-harvest samples ($P < 0.05$) as well as detectable sporulation for up to 70- or 90-days post-harvest ($P < 0.05$). Both fall- and spring-applied heat treatments reduced CLS area under the disease progress curve in the growing season after treatment ($P < 0.05$).

Fungicide resistance is a major concern due to the frequent applications necessary to control multiple infection cycles of CLS each season and the importance of single-site fungicides. Reduced sensitivity has been observed for *C. beticola* to multiple fungicide groups, including quinone outside inhibitors. Programs were tested to minimize *C. beticola* pyraclostrobin resistance development and maximize management of CLS. In 2019 and 2020, rotation and tank-mixture programs integrating pyraclostrobin with mancozeb treatments were evaluated in field studies. For all programs incorporating pyraclostrobin, distributions of *C. beticola* pyraclostrobin sensitivities were not significantly different from each other but differed from the non-treated control ($P < 0.05$). No additional CLS control, yield, or sugar benefits were observed for programs using pyraclostrobin.

Monitoring the development of fungicide resistance in the region is important for making

management decisions. In 2021 and 2022, the distribution of *in vitro* fungicide sensitivity was determined in Michigan *C. beticola* isolates and mutations associated with resistance to various fungicide groups were assessed. High levels of resistance to prothioconazole and pyraclostrobin were observed for *C. beticola* isolates collected from commercial sugarbeet fields. Tested isolates were most sensitive to difenoconazole, fenbuconazole, and triphenyltin hydroxide. The number of fungicide group applications affected isolate sensitivity to thiophanate-methyl and prothioconazole ($P < 0.05$). Many triazoles in this study possessed cross resistance with other DMI fungicides. This study found the E198A mutation was accurate in predicting resistance to thiophanate-methyl, while the G143A and Glu169 mutations were not sufficient to forecast resistance to pyraclostrobin and all triazole active ingredients, respectively.

Current CLS prediction models used for fungicide application timing do not consider the presence of *C. beticola* spores. CLS forecasting could benefit from the addition of aerial spore factors. In 2019-2022, eight site-years of aerial spore and environmental data were collected from Michigan and Ontario. Initial correlation and logistic regression analyses found duration of leaf wetness, air temperature, and wind speed were able to predict the risk of elevated *Cercospora* spore concentrations with 67.9% accuracy. In 2022 and 2023, a limited set of thresholds for a selected model were tested for fungicide application timing. Two models resulted in CLS, yield, and sugar metrics comparable to the grower standard despite one less fungicide application. In additional training analysis, an ensemble model included leaf wetness, air temperature, relative humidity, and wind speed variables with a testing accuracy of 73.2%. These studies aimed to develop integrated CLS management options to improve sugarbeet production sustainability and profitability.

This dissertation is dedicated to my Parents, Sister, and Husband. Mom and Dad, you have always taught me to take one thing at a time and to be mentally tough. Victoria, you have always been there for me, my best friend. Patrick, your unwavering support is truly empowering. My passion could not be realized without all of your love, encouragement and advice. You all have helped me to reach my potential. Thank you.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Jaime Willbur, for her guidance throughout my PhD. I will always look fondly on my experience in your lab. You were truly invested in the projects and work accomplished by your lab members. As an advisor you always supported me with an incredible ability to create a space of trust and creativity. You taught me determination and perseverance in difficult times. The practical skills you have taught me and the translation of those skills into coordination and mentorship of others will forever be part of my professional and personal journey. You gave me the tools to succeed. You continue to be an incredible mentor with the flexibility to adapt to the specific needs of a mentee. I am truly grateful to have you as my mentor and could not have completed my doctoral degree without your constant support.

I would also like to thank my committee members, Dr. Linda Hanson, Dr. Timothy Miles, and Dr. Andrew Jarosz for their encouragement and guidance. I would like to give a special thank you to Dr. Linda Hanson for always lending her expertise to my research projects from the next lab over. Thank you to my friends and lab mates for your advice, comradery, and creation of a positive atmosphere which fostered support and collaboration. I would like to acknowledge and thank Chris Bloomingdale for his guidance and help with all field related experiments. I would like to thank the staff at the Saginaw Valley Research and Extension Center for their help with my field studies, specifically Paul Horny, Holly Corder, and Tom Wenzel. I would like to thank Tom Wenzel and Daniel Bublitz for their assistance with the heat treatment applications. Lastly, I would like to acknowledge Sugarbeet Advancement and Michigan Sugar Company for their help with leaf sample collection. The research in the following manuscript was achieved though the support of everyone listed. I have a great deal of appreciation to all who have helped me throughout my academic experience.

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CHAPTER 1: LITERATURE REVIEW

1.1 Sugarbeets

Beets were first grown at least 2000 years ago and originate from *Beta* species from the Mediterranean shores (Draycott 2006). By the seventeenth century, fodder beet was grown on a large scale for feeding cattle (Draycott 2006; Winner 1993). Different varieties of beet were grown with different traits including beets with desirable leaf properties for consumption and others that maximized root size (Lewellen et al. 2009). High sugar acquisition became one of the defining features of the beet crop (Draycott 2006). The Silesian beet with a white root type and a high concentration of sugar was bred in the early eighteenth century and is the origin of the sugarbeet varieties grown today (Draycott 2006; Winner 1993). Currently, sugarbeets are grown in temperate climates where the seeds are planted in the spring and harvested in the fall. Sugarbeets are biennial plants, therefore the reproductive stage of growth occurs in the second year after planting under favorable conditions (Winner 1993). Typical field-scale cultivation practices for sugar acquisition do not allow sugarbeets to reach the reproductive growth phase (Draycott 2006).

During harvest, sugarbeet leaves are removed and the roots are harvested. Once harvested, the sugarbeet may be stored in piles or taken directly to a sugar processing facility. Sugarbeet are first rinsed and washed then sliced creating “cossettes”. Diffusion is used to remove the sugar from the beet slices creating “raw juice”. The “raw juice” is purified using a combination of filtration screens, heating, milk of lime, and carbonation to removing the non-sucrose material, termed impurities. The purified juice then goes through an evaporation process that removes water and increases the concentration of sucrose. To promote crystallization of the sugar, low temperature boiling is used until super saturation with the addition of powdered sugar or finely milled sugar and isopropyl alcohol followed by centrifugation. Biproducts of sugarbeet

processing includes beet pulp, molasses, and factory lime (Sugarbeet Processing 1997). The sucrose derived from sugarbeet are the same as sugarcane (Draycott 2006). Modern sugarbeet hybrids contain >18% sugar content (Panella et al. 2014).

Sugarbeet is a major crop for white sucrose production (Draycott 2006). Approximately 20% of global sugar production originates from sugarbeet (Rangel et al. 2020). Sugarbeets are responsible for over half of the sugar produced in the United States (USDA-NASS 2022). The U.S. produces approximately 30 million tons of sugarbeets annually, which equates to about six million tons of refined sugar (USDA-NASS 2022). Michigan is the fourth largest producer of sugarbeet in the United States (USDA-NASS 2022). Sugarbeet production is negatively affected by many biotic and abiotic factors, including diseases. Cercospora leaf spot (CLS), Fusarium root rot, downy mildew, powdery mildew, Ramularia leaf spot, rust, rhizomania, and Rhizoctonia root and crown rot are diseases that affect sugarbeet (Asher and Hanson 2006).

1.2 Cercospora leaf spot

Cercospora leaf spot (CLS) is one of the most important foliar diseases of sugarbeet worldwide (Weiland and Koch 2004; Jacobsen and Franc 2009; Khan et al. 2009). The causal agent of CLS is the pathogen *Cercospora beticola* Sacc., which is a fungus in the Ascomycota family and was first reported by Saccardo (1876). Cercospora leaf spot affects plants in the family *Chenopodiaceae* including sugarbeet, table beet, and chard, as well as spinach, safflower, and closely related weed hosts such as lambsquarter, goosefoot, and pigweed (Lartey et al. 2005; Weiland and Koch 2004). This foliar disease is one of the major yield limiting factors for sugarbeet production in the United States and worldwide (Holtschulte 2000).

Symptoms of CLS include foliar lesions that have a tan to gray center with a brown to purple or red border (Windels et al. 1998). CLS is responsible for significant reductions in root

yields and sucrose content (Lamey et al. 1987; Shane and Teng 1992; Weiland and Koch 2004; Jacobsen and Franc 2009; Khan et al. 2009). Severe CLS pressure in susceptible sugarbeet varieties can cause lesions or spots to coalesce resulting in premature death of many plant leaves and significant sugar losses (Rossi et al. 2000b). Sugarbeet yield losses from CLS can be as high as 40% (Jacobsen and Franc 2009). Additionally, infection by *C. beticola* can result in higher production costs due to increased concentration of impurities within the beet root which interferes with the sugar extraction process (Khan et al. 2008). Growing seasons with high humidity (> 60%) and temperatures above 15°C promote high CLS pressure (Franc 2010). Management of CLS relies on crop rotation, timely fungicide applications, disease prediction models, and the use of tolerant sugarbeet varieties (Windels et al. 1998; Khan et al. 2007; Jacobsen 2010). Integrated pest management for controlling CLS on sugarbeet is often critical to preserve current control measures. Diseases have been increasingly difficult to manage due to reduction in tillage, including *C. beticola*. Investigation and validation of novel management tools is important to maintain high sugarbeet yield for generations to come, especially due to potential increases in pathogen presence as result of reduced in tillage (Bockus and Shroyer 1998; Sumner et al. 1981).

1.3 *C. beticola* life cycle

The fungal pathogen *C. beticola* overwinters on infected leaf debris as pseudostromata and is capable of surviving in plant residue left on the surface of the soil anywhere from 20-22 months (Nagel 1938; Khan et al. 2008) to two to three years (Pool and McKay 1916; Solel 1970). Conidia are dispersed through wind and water movement and germinate after landing on susceptible tissue (Carlson 1967; Jacobsen and Franc 2009; Lawrence and Meredith 1970; McKay and Pool 1918; Meredith 1967). Following conidial germination, *C. beticola* uses an

appressorium to penetrate the leaf through the stomata (Rathaiah 1976; Rathaiah 1977). Once infection occurs, *C. beticola* grows intercellularly (Steinkamp et al. 1979) and, in later stages of infection, causes death to localized tissue leading to lesions on the leaf. The pseudostromata form in the center of lesions and produce asexual conidia that can be released for further infection of susceptible plants within a field throughout a growing season (Jacobsen and Franc 2009). Under favorable conditions as described in the following section, symptoms can develop in as little as 5 days on older leaves with spore generation from pseudostromata occurring as soon as 7 days after infection (Pool and McKay 1916; Jacobsen and Franc 2009). One cycle of *C. beticola* sporulation, from spore germination to sporulating lesion, typically takes 12 days depending on field conditions (Rossi et al. 2000a; Weiland and Koch 2004). The iterative nature of the pathogen reproduction makes this disease especially difficult to manage once established in a field, therefore management of primary inoculum is critical (Filho et al. 2016).

CLS is polycyclic due to multiple cycles of infectious inoculum produced through conidial release during a season (McKay and Pool 1918; Nagel 1945; Vereijssen et al. 2007). Pseudostromata produce white to silver spores that can often be observed using a magnifying glass or hand lens after leaves have been exposed to humid conditions in the field or in a moist chamber (Harveson 2013). The abundant needle-like conidia are typically clear and iridescent with a straight to slightly curved shape, multiseptated, and between 2.5-4.0 µm wide and 50-200 µm long (Groenewald et al. 2013; Weiland and Koch 2004). Survival of *C. beticola* overwintering structures is important for the life cycle of the pathogen (Khan et al. 2008; Pool and McKay 1916). Conidia were not viable after one month on infected material under field conditions (Pool and McKay 1916). Conidiophores and pseudostromata are melanized which is known to protect fungi from degradation (Gómez and Nosanchuk 2003) and likely contribute to

the survival of *C. beticola* overwintering structures. No sexual cycle has been observed for *C. beticola*. Observations of nearly proportional presence of both mating types in some areas and linkage disequilibrium in *C. beticola* suggest there is a possibility sexual reproduction occurs (Groenewald et al. 2006, 2008; Bolton et al. 2012b).

1.4 Environment conditions favorable for *C. beticola* and CLS development

Previous research has found that *C. beticola* conidia were produced most readily at temperatures between 15 to 23°C and relative humidity greater than 60% (Jacobsen and Franc 2009; Pool and McKay 1916). Conidia production is inhibited by temperatures less than 10°C and greater than 38°C (Jacobsen and Franc 2009; Khan et al. 2009; Pool and McKay 1916). For example, airborne conidia concentration peaked during increases in average daily temperature when the relative humidity was greater than 87% (Khan et al. 2009; Shane and Teng 1984; Wolf and Verreet 2005). Disease development for CLS is favored by day temperatures of 25 to 35°C and night temperatures at or over 16°C (Forsyth et al. 1963; Jacobsen and Franc 2009; Shane and Teng 1983). Though *C. beticola* can grow and germinate at temperatures as low as 9°C, ideal temperatures for growth were determined to be around 30°C (Pool and McKay 1916). Conditions when relative humidity is elevated, around 90 to 95%, with available leaf moisture creates an optimal environment for CLS development (Forsyth et al. 1963; Jacobsen and Franc 2009; Ruppel 1986; Shane and Teng 1983, 1984). Monitoring environmental conditions that are conducive for conidia production and release in understudied. Using this information, in addition to factors that contribute to disease development, is critical for managing CLS on sugarbeet.

Conidia of *C. beticola* can be dispersed by wind (Lawrence and Meredith 1970; McKay and Pool 1918), water splash (Carlson 1967), running water, and insects (McKay and Pool 1918; Meredith 1967). Wind has been considered a major component of *C. beticola* dispersal (Khan et

al. 2008; Lawrence and Meredith 1970; Meredith 1967). Wind- (Khan et al. 2008) and rain-dispersed (Carlson 1967) *C. beticola* conidia were proposed as the major source of inoculum spread within a field (Asher and Hanson 2006; Duffus and Ruppel 1993; Pool and McKay 1916; Ruppel 1986). It is well known that conidia readily disperse over short distances of less than 100 meters (Lawrence and Meredith 1970; McKay and Pool 1918). Conidia dispersal has been reported as being highly influenced by wind in some studies (Lawrence and Meredith 1970) and by rain in others (Bublitz et al. 2021; Carlson 1967). Understanding spore movement dynamics and epidemiology is important for disease prediction models. Anticipating the spread of *C. beticola* could assist growers in managing impending epidemics.

1.5 Integrated CLS management

Currently, CLS is managed by the use of tolerant varieties, crop rotation, and in-season fungicide applications (Asher and Hanson 2006; Jacobsen 2010; Jacobsen and Franc 2009; Ruppel 1986). Tillage of infected plant residue was a commonly used tool in the past to reduce the survival of *C. beticola* inoculum (Pool and McKay 1916; Khan et al. 2008). The use of tillage practices in current sugarbeet production is limited due to an industry shift to reduced tillage and no till systems based on current recommendations for reduced soil erosion and increased soil health (Hao et al., 2001; Tzilivakis et al., 2005). With the shift to reduced tillage practices, survival of soilborne inoculum has been shown to increase (Bockus and Shroyer 1998). At least a 3-year rotation of nonhost plants with tolerant sugarbeet cultivars is recommended to manage CLS on sugarbeet (Pool and McKay 1916; Khan et al. 2007). Field isolation of at least 91.4 meters from an active sugarbeet field and at least 33 meters from a previous years sugarbeet field is effective in reducing CLS severity (McKay & Pool 1918; Khan et al. 2008). Crop rotation is a standard practice in sugarbeet management (Jacobsen 2010; Jacobsen and Franc 2009).

However, additional control strategies are necessary to manage CLS in some areas. The current recommendations to plant at least 100 meters from previous fields may not be entirely effective, especially when beet crops are densely populated in particular areas of a state or region.

Breeding efforts for CLS tolerant varieties are vital to manage the impact CLS has on sugarbeet yield (Rossi et al. 1999; Smith and Campbell 1996). However, it is difficult to integrate the four to five genes that are reported to be responsible for *Cercospora* resistance in sugarbeet (Smith and Gaskill 1970) while maintaining high yielding cultivars (Smith and Campbell 1996). CLS-resistant sugarbeet varieties have been developed with economic performance that surpasses CLS-susceptible varieties and without yield penalties in the absence of CLS (Vogel et al. 2018). Sugarbeet varieties are currently available with high levels of tolerance to CLS by way of single gene resistance and a multi-gene CLS-resistant background, while also maintaining yield performance (Törjék et al. 2020, 2022). Despite the recent strides in achieving CLS-resistant varieties, sugarbeet producers still observe CLS symptoms in these cultivars and require a combination of integrated pest management strategies to maintain high yield. Single gene disease resistance has low durability and can be overcome by pathogens as they are highly adaptable with shorter life cycles (Manzoor et al. 2024; National Research Council 1993; Sprague et al. 2006; Van Esse et al. 2020). In some instance, disease resistance conferred by single genes can be defeated within a few years of widespread deployment in the field (Sprague et al. 2006).

The gene responsible for CR+ sugarbeet variety tolerance to CLS (BvCR4) was identified by Törjék et al. (2020, 2022). Highly CLS resistant and high yielding sugarbeet hybrids were able to be generated with this resistance. It is important to consider the possibility that CLS resistant cultivars may have variable sensitivity to other sugarbeet pathogens as well as yield

impacts (REACh (Michigan Sugarbeet Research and Education Advisory Council) 2023a; SMBSC (Southern Minnesota Beet Sugar Cooperative), 2023). Concern for the evolution of *C. beticola* stains virulent on the resistant material is present (Pilet-Nayel et al., 2017), especially if effective integrated pest management is not used (Chen et al. 2024). Highly resistant sugarbeet varieties will not be successful if used in the absence of additional management strategies (Chen et al. 2024). CLS has been observed consistently on CR+ varieties with symptoms identified 2 to 4 weeks earlier (mid- to late-June) than previous years and first detections of CLS in Michigan occurred in a CR+ variety (personal observation, 2024).

Timely fungicide applications during the growing season are critical for managing CLS (Khan et al. 2007, 2008; Secor et al. 2010a; Windels et al. 1998; Weiland and Koch 2004). *C. beticola* is a pathogen classified as medium risk for fungicide resistance development (FRAC pathogen risk list, 2019); classification is based on multiple yearly proliferation cycles, significant genetic diversity (Vaghefi et al. 2016, 2017a, 2017b), and a high number of fungicide applications each season, as many as 6-8 in some regions (Hernandez et al. 2023). Disease prediction models improve application timing for more accurate control measures depending on when conditions are conducive to high or low disease risk (Tedford et al. 2019). Investigation into environmental variables that influence infection and disease development has resulted in the creation of forecasting models for management of CLS in sugarbeet (Khan et al. 2007; Pitblado and Nichols 2005; Shane and Teng 1992; Smith and Ruppel 1973; Wallin and Loonan 1971; Windels 2010; Windels et al. 1998). Disease forecasting models are used to predict the proper timing of fungicide applications to manage CLS on sugarbeets (Windels 2010), however, they have shown variable efficacy.

1.6 Tillage-alternative and residue management strategies for disease management

Soil-disturbing disease management strategies, such as plowing and tillage, can have undesirable impacts on soil structure, nutrient levels, and some microbial populations (Hungria et al. 2009; Miura et al. 2015; Xue et al. 2018). Tillage practices are known to reduce soil fertility, soil structure and porosity, and soil organic matter (Rieke et al. 2022). Environmental impacts of tillage practices may include soil erosion, pesticide and nutrient runoff, and emission of greenhouse gases (Withers and Lord 2002; Cerdan et al. 2010; Smith et al. 2016). Minimum tillage or no-till management is recommended globally (Hao et al. 2001; Tzilivakis et al. 2005). Because major management strategies for pathogens like *C. beticola* include burying residue, alternative sanitation strategies are critical to enhance decomposition of leaves and reduce inoculum.

Studies of tillage-alternative residue management practices in apple, pear, and citrus have tested treatments with some efficacy such as application of compounds and mixes like urea, sugarbeet pulp, sugar cane pulp, and dolomitic lime, as well as shredding of leaf litter (Spotts et al. 1997; Vincent et al. 2004; van Bruggen et al. 2017). Similarly, herbicides used for preharvest defoliation or desiccation (Stahler 1953) can accelerate leaf degradation with potential to indirectly impact disease (Altman and Campbell 1977). Heating foliage to high temperatures could be considered for potential sanitation, as *C. beticola* is sensitive at or above 45.5°C depending on length of exposure (Jacobsen 2010; Pool and McKay 1916). A propane-fueled foliar heat treatment impacted CLS lesion sporulation and viability of *C. beticola* for sugarbeets that were grown in the greenhouse and inoculated (Bublitz 2019), supporting the potential for such a strategy. Strategic sanitation and inoculum management could reduce next-year disease

pressure and have long-term economic, ecological, and environmental benefits (Hernandez et al. 2023).

1.7 Risk assessment tools for CLS management

The BEETcast disease forecasting model is currently used to advise fungicide applications to manage CLS on sugarbeet in Michigan and Ontario (Pitblado and Nichols 2005). Weather INnovations host's this CLS forecasting tool, which generates daily severity values (DSVs) from 0 to 4 based on local temperature and leaf wetness data. Cumulative DSVs are used to trigger action thresholds chosen using field specific metrics such as varietal resistance and CLS pressure (www.michiganbeets.com). In other growing regions different models are used. For example, the North Dakota Agricultural Weather Network (NDAWN) model is used in the Red River Valley (Jones and Windels 1991; Shane and Teng 1984; 1985; Windels et al. 1998). The NDAWN model is used to advise on fungicide application timing based on onset of disease in addition to infection event predictions using environmental data. Canopy closure initiates weekly CLS monitoring. Once CLS symptoms have been observed, 48-hour temperature and rainfall data are used to calculate daily infection values (DIVs), ranging from 0 to 7 to indicate whether conditions are favorable for *C. beticola* infection (Windels et al. 1998). Both models are based on the risk of CLS development (Jones and Windels 1991; Pitblado and Nichols 2005; Shane and Teng 1984; 1985; Windels et al. 1998) and do not incorporate spore presence. Disease prediction models could be further refined by incorporating the risk of spore production and dispersal (Carisse et al. 2005; Gent et al. 2009; Thiessen et al. 2016).

Fungicide efficacy is impacted by application timing. For example, a demethylation inhibitor (DMI) or triazole fungicide (FRAC Group 3) was not as effective once CLS lesions were present compared to application before infection, however, a pre-mix of succinate

dehydrogenase inhibitor (SDHI; FRAC Group 7) and triazole active ingredients was effective at a threshold of one CLS lesion per leaf (Pethybridge et al. 2020). While the efficacy of foliar programs may be dependent on fungicide active ingredient, generally those initiated either prior to infection or at early leaf spot detection (<1 spot per leaf) are most effective (Liebe et al. 2023; Pundhir and Mukhopadhyay 1987). Due to several factors, including small lesion diameters, dense sugarbeet canopies, and considerable size of commercial fields, these first spots may be difficult to detect in time to accurately inform initiation of fungicide programs. The criteria for timing fungicide applications needs to be reliable and precise for effective management.

In 2018, the model-based thresholds using BEETcast were met two weeks after the first lesions were reported in Michigan, which is not ideal for the most effective management (Willbur et al. 2018). Similarly, in Canada, *Cercospora* conidia were first observed with a mechanical spore trap before the BEETcast threshold of 50 DSVs was reached in 2014 and 2015 (Tedford et al. 2018), and CLS symptoms were seen before a 50 DSV threshold was reached in 2019 (Tedford et al. 2018). Recurrent false-negative predictions are problematic and lead to costly CLS spread, defoliation, and crop or sucrose losses.

Disease forecasting models focus on predicting infection or disease development conditions (Windels 2010) without considering the presence of pathogen spores, yet studies have shown that CLS severity is associated with *C. beticola* conidia concentrations (Khan et al. 2009; Pundhir and Mukhopadhyay 1987; Tedford et al. 2018). In Michigan, evidence of infectious *C. beticola* spores has been found as early as April, with consistent early season (April to May) detections in 2017-2018 (Bublitz et al. 2021). Tedford et al. (2018) reported similar findings of airborne *Cercospora* conidia present in early May in Ontario, Canada. Early detections of *Cercospora* spores in fields previously planted to beet supports the likelihood that conidia are a

primary inoculum source in North Central and Northeastern regions. Airborne conidia of *C. beticola* play an important role in primary infection, secondary infection, and in disease pressure (Asher and Hanson 2006; Duffus and Ruppel 1993; Khan et al. 2009; Pool and McKay 1916; Ruppel 1986). The impact of environmental variables on *C. beticola* spore abundance was assessed using live beet as spore traps (Bublitz et al. 2021; Carlson 1967). Patterns of airborne *Cercospora* conidia concentrations have been assessed from April to July in Ontario and late-June to September in Minnesota and North Dakota (Khan et al. 2009; Tedford et al. 2018). Predictors of *C. beticola* inoculum dispersal prior to symptom development may provide useful information to improve disease management.

1.8 Fungicide use for CLS management

Reduced fungicide efficacy develops as a consequence of increased fungicide resistance in pathogen populations (Deising et al. 2008). The fungicide resistance action committee (FRAC; www.frac.info/) provides information on how to limit resistance development. FRAC offers recommendations for resistance management, information on annual sensitivity monitoring, and reports a list of pathogens with varying risks of fungicide resistance development. Most importantly, this group maintains a FRAC code list that categorizes fungicide active ingredients based on mode of action, target site, and risk of resistance development (e.g. FRAC code list 2022). Over the years, reduced sensitivity has been observed in *C. beticola* for various fungicide groups that have been registered and commonly used for CLS management on sugarbeet, including methyl benzimidazole carbamate (MBC, FRAC group 1), quinone outside inhibitor (QoI, FRAC group 11), demethylation inhibitor (DMI, FRAC group 3), and organotin (FRAC group 30) fungicide classes (Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974;

Giannopolitis, 1978; Cerato and Grassi, 1983; Bugbee, 1995; Karaoglanidis et al., 2000; Weiland and Halloin, 2001; Secor et al., 2010a; Kirk et al., 2012; Rosenzweig et al., 2020).

It is recommended to rotate different active ingredients with varying modes of action, limit the number of applications in one growing season, use broad spectrum tank-mix partners that are effective against a wide range of pathogenic fungi, utilize labeled rates, and employ integrated pest management strategies to reduce the risk of fungicide resistance development (Corkley et al., 2022; van den Bosch et al., 2014a, 2014b). FRAC classifies fungicides as high-, medium-, or low-risk for resistance development (FRAC pathogen risk list 2019; Georgopoulos 1987). High-risk fungicides are characterized by a history of reduced pathogen sensitivity to the products. These active ingredients typically target one site against a single gene. With proper use, these products can be effective, but fungicide resistance development occurs rapidly and more often for these products than for other types (Bosch et al. 2014). Many medium-risk fungicides are single site and typically have some evidence of reduced sensitivity in pathogen populations. Fungicides listed as low-risk have little to no proven development of fungicide resistance to date with some history of use. Low risk fungicides often have multiple target sites effecting multiple metabolic processes, which reduces the risk of decreased sensitivity in pathogen populations (Karaoglanidis and Ioannidis 2010).

A major concern in sugarbeet production is the development of *C. beticola* populations that are insensitive to many different classes of fungicides (Georgopoulos and Dovas 1973; Ruppel and Scott 1974; Giannopolitis 1978; Cerato and Grassi 1983; Bugbee 1995; Karaoglanidis et al. 2000; Weiland and Halloin 2001; Secor et al. 2010a; Kirk et al. 2012; Rosenzweig et al. 2020). Single-target site fungicides are most prevalent and have been very effective in managing pathogens (Steinberg and Gurr 2020). The tendency to apply products

with one active ingredient with a single target mode of action has contributed to the development of insensitivity to some fungicides previously recommended for CLS control (Bosch et al. 2014). The continuous use of the same fungicide active ingredient with a single mode of action to control disease imposes selection pressure on the target pathogen (Eckert and Ogawa, 1988). Isolates that are sensitive to the active ingredient are eliminated from the population leaving any insensitive isolates to survive and reproduce (Delp and Dekker 1987). Site-specific fungicides may be rendered ineffective by a single-site mutation in the pathogen, therefore, these fungicides have a high risk for pathogen resistance development (Karaoglanidis and Ioannidis 2010). Multi-site fungicides interfere with multiple metabolic processes, which is more difficult for a pathogen to overcome (Brent and Holloman 1998). The use of products with multi-site activity and rotation of single-site fungicides at the labeled rate can reduce the risk of resistance development (Secor et al. 2010b).

1.9 History and overview of *C. beticola* fungicide resistance

Ethylene bisdithiocarbamate (EBDC; FRAC Group M03) fungicides, such as the active ingredient mancozeb, and copper-based fungicides (FRAC group M01) provide broad-spectrum activity and are common tank mix partners for CLS management in sugarbeet (Karaoglanidis and Ioannidis 2010). These fungicide products have a low risk for fungicide resistance development (Brent and Holloman 1998; Karaoglanidis and Ioannidis 2010) and have demonstrated effectiveness in CLS control on sugarbeets without indication of reductions in isolate sensitivity (FRAC code list, 2022; REACh 2023b).

Methyl benzimidazole carbamate (MBC; FRAC group 1) has been used historically for CLS control (Delp 1980; Weiland and Halloin 2001). Benzimidazoles act primarily by binding to fungal β-tubulins, thus interfering with mitosis and disrupting integrity of the fungal cytoskeleton

(Davidse 1986). This is a single-site target fungicide group, therefore the risk for resistance development is high (Karaoglanidis and Ioannidis 2010). Resistance to MBC was observed and resulting economic losses were reported in Europe and the United States in 1973 and 1974, respectively (Georgopoulos 1982; Georgopoulos and Dovas 1973; Ruppel and Scott 1974). Resistance to methyl benzimidazole carbamate is associated with a single base pair mutation in the *beta tubulin* gene (E198A) in *C. beticola* (Davidson et al. 2006; Ma and Michailides 2005).

Organotin (FRAC group 30) products, such as active ingredients triphenyltin hydroxide and triphenyltin acetate, have been and are still commonly used for management of CLS in the U.S., but are not currently registered for use in Europe. Organotin insensitivity was first detected in 1978 in Greece (Giannopolitis 1978) and in 1994 in the U.S. (Bugbee 1995) followed by an epidemic that was reported in 1998 (Secor et al. 2010a). Though respiration is the target mode of action, the mechanism associated with *C. beticola* insensitivity to organotin fungicides is not currently known.

Strobilurin or quinone outside inhibitor (QoI; FRAC group 11) fungicides, with active ingredients such as pyraclostrobin or trifloxystrobin, are important for CLS management on sugarbeet (Anesiadis et al. 2003; Karadimos et al. 2005; Khan and Smith 2005; Karadimos and Karaoglanidis 2006). QoI fungicides bind to the cytochrome bc1 complex to disrupt mitochondrial respiration (Anke 1995; Bartlett et al. 2002). Strobilurin fungicides are site-specific and considered high-risk for resistance development in certain pathogen populations (e.g. FRAC code list 2022). Though highly effective at controlling *C. beticola* on sugarbeet (Karadimos et al. 2005; Khan and Smith 2005; Secor et al. 2010a), reduced sensitivity was detected for QoI fungicides in 2004 (Secor et al. 2010a) shortly after registration. Rapid resistance development followed (Kirk et al. 2012) which appears to be relatively stable for *C.*

beticola populations in the United States (Rangel et al. 2020). Potential loss of control using field rates of QoI products is attributed to reductions in pathogen sensitivity (Kirk et al. 2012; Malandrakis et al. 2006; Bolton et al. 2013). Three mutations are reported to cause reduced sensitivity to QoI fungicides, among these, the G143A mutation in the *cytochrome b* (*cytb*) gene has been associated with high levels of QoI resistance in other fungal pathogens, including *Alternaria* spp., *Mycosphaerella* spp., and *Venturia* spp. (Ma and Michailides 2005). This mutation has been found in *C. beticola* (Kirk et al. 2012; Bolton et al. 2013) but isolates with the mutation exhibit a wide range of EC_{50} values (Bolton et al. 2013; Hernandez et al. 2024).

Many different triazole or demethylation inhibitor (DMI; FRAC group 3) active ingredients are registered for use to manage CLS on sugarbeet (Byford 1996; Meriggi et al.2000). Triazoles inhibit the production of ergosterol, though different active ingredients achieve this though different mechanisms (Price et al. 2015; Siegel 1981; Sisler et al. 1983). This FRAC group is considered medium-risk for resistance development (Brown et al. 1986; Georgopoulos and Skylakakis 1986; Köller 1991). In 1995, *C. beticola* sensitivity to tetraconazole decreased in Greece (Karaoglanidis et al. 2000) and similarly decreased in the U.S. during the 2000s (Secor et al. 2010a; Bolton et al. 2012c; Rosenzweig et al. 2020). A silent mutation (Glu169) located in the *14-C alpha-demethylase* gene (*CbCyp51*) has been associated with reduced sensitivity in *C. beticola* to the DMI active ingredient, epoxiconazole (Nikou et al. 2009). Other potential mechanisms, including alternative mutations in the target site, increased copy number or overexpression of Cyp51, and increased efflux activity, in *C. beticola* could be responsible for reductions DMI sensitivity for other active ingredients (Bolton et al. 2012a; Del Sorbo et al. 2000; Ma and Michailides 2005; Spanner et al. 2021; Ziogas and Malandrakis 2015).

Field resistance of *C. beticola* to DMI fungicides poses a challenge for sugarbeet production in Michigan due to the presence of pathogen insensitivity for other registered fungicide groups.

1.10 Opportunities to improve CLS management

Studies are required to evaluate the best fungicide application practices with goals to limit resistance development in *C. beticola* while managing CLS in the field. Fungicide sensitivity monitoring in *C. beticola* populations must be continued locally and shared across growing regions to provide relevant application advice to sugarbeet growers. Investigation of mutations associated with *C. beticola* fungicide resistance may be useful to implement detection of potential resistance and continue sensitivity monitoring in the future. Preserving fungicide active ingredients and implementing impactful integrated pest management are key to sustainable management. Novel alternative management strategies, such as those that target the pathogen overwintering stage, would further provide integrated options for growers as systems shift away from tillage practices. Lastly, the risk of elevated *C. beticola* spores could be predicted by further exploring CLS epidemiology, specifically the conditions associated with conidia production and dispersal. Integrating *C. beticola* spore presence and abundance into the current disease prediction models could improve model application timing for effective CLS management.

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CHAPTER 2: AN IN-FIELD HEAT TREATMENT TO REDUCE *CERCOSPORA BETICOLA* SURVIVAL IN PLANT RESIDUE AND IMPROVE CERCOSPORA LEAF SPOT MANAGEMENT IN SUGARBEET

A version of this chapter has been published in *Frontiers in Plant Science*: Hernandez, A. P., Bublitz, D. M., Wenzel, T. J., Ruth, S. K., Bloomingdale, C., Mettler, D. C., Bloomquist, M. W., Hanson, L. E., and Willbur J. F. 2023. An in-field heat treatment to reduce *Cercospora beticola* survival in plant residue and improve Cercospora leaf spot management in sugarbeet. Front Plant Sci. 14:1-18 doi: 10.3389/fpls.2023.1100595

2.1 Abstract

INTRODUCTION: Sugarbeets account for 55 to 60% of U.S. sugar production. Cercospora leaf spot (CLS), primarily caused by the fungal pathogen *Cercospora beticola*, is a major foliar disease of sugarbeet. Since leaf tissue is a primary site of pathogen survival between growing seasons, this study evaluated management strategies to reduce this source of inoculum.

METHODS: Fall- and spring-applied treatments were evaluated over three years at two study sites. Treatments included standard plowing or tilling immediately post-harvest, as well as the following alternatives to tillage: a propane-fueled heat treatment either in the fall immediately pre-harvest or in the spring prior to planting, and a desiccant (saflufenacil) application seven days pre-harvest. After fall treatments, leaf samples were evaluated to determine *C. beticola* viability. The following season, inoculum pressure was measured by monitoring CLS severity in a susceptible beet variety planted into the same plots and by counting lesions on highly susceptible sentinel beets placed into the field at weekly intervals (fall treatments only).

RESULTS: No significant reductions in *C. beticola* survival or CLS were observed following fall-applied desiccant. The fall heat treatment, however, significantly reduced lesion sporulation (2019-20 and 2020-21, $P < 0.0001$; 2021-22, $P < 0.05$) and *C. beticola* isolation (2019-20, $P < 0.05$) in at-harvest samples. Fall heat treatments also significantly reduced detectable sporulation for up to 70- (2021- 22, $P < 0.01$) or 90-days post-harvest (2020-21, $P < 0.05$). Reduced numbers of CLS lesions were observed on sentinel beets in heat-treated plots from May 26- June 2 ($P < 0.05$) and June 2-9 ($P < 0.01$) in 2019, as well as June 15-22 ($P < 0.01$) in 2020. Both fall- and spring-applied heat treatments also reduced the area under the disease progress curve for CLS assessed the season after treatments were applied (Michigan 2020 and 2021, $P < 0.05$; Minnesota 2019, $P < 0.05$; 2021, $P < 0.0001$).

DISCUSSION: Overall, heat treatments resulted in CLS reductions at levels comparable to standard tillage, with more consistent reductions across year and location. Based on these results, heat treatment of fresh or overwintered leaf tissue could be used as an integrated tillage-alternative practice to aid in CLS management.

2.2 Introduction

Sugarbeets are the source of approximately 20% of global sugar production (Rangel et al., 2020). The United States produces 36.75 million tons of sugarbeets annually from which 6.62 million tons of sugar is refined (USDA-NASS (United States Department of Agriculture, National Agricultural Statistics Services), 2019). Beet sugar comprises approximately 55-60% of total US sugar production (USDA (United States Department of Agriculture), 2021). In the U.S., Minnesota is the number one sugarbeet producer with 12 million tons grown annually and Michigan is the number four producer with 4-5 million tons grown annually (USDA-NASS (United States Department of Agriculture, National Agricultural Statistics Services), 2019). Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola* Sacc., is the most important foliar disease of sugarbeet in much of the world (Weiland and Koch, 2004; Jacobsen and Franc, 2009; Khan et al., 2009), including Minnesota and Michigan. The formation of CLS lesions decreases photosynthetic area which leads to sugar losses. Severe infection can lead to defoliation and further sugar losses when regrowth of leaves occurs (Franc, 2010). This disease can cause reduced root weight and sugar content, and yield losses of up to 50% may occur (Lamey et al., 1987; Shane and Teng, 1992). Fungicide management for CLS costs sugarbeet growers between $300-375 per hectare in Minnesota. In Michigan, severe CLS can cause an estimated $100 million in management costs and yield losses (*personal communication*, C. Guza, Michigan Sugar Company, 2022).

Cercospora beticola overwinters largely as pseudostromata present on infected leaf residue (Pool and McKay, 1916; McKay and Pool, 1918; Khan et al., 2008). Survival was shown to be reduced by burying leaf debris, which becomes more effective with increased depth and time. Inoculum on the soil surface survives anywhere from 20-22 months (Nagel, 1938; Khan et al., 2008) to two to three years (Pool and McKay, 1916; Solel, 1970), while inoculum buried at 10 to 20 cm decreased *C. beticola* survival to 10 months (Khan et al., 2008) or less (Solel, 1970). Pseudostromata in infected leaf debris are thought to be the primary inoculum source (Pool and McKay, 1916; Jacobsen and Franc, 2009) and dispersal of *C. beticola* can occur through the movement of infested plant material (Knight et al., 2018; Knight et al., 2019). Infected debris from weed hosts can also be an important inoculum source as it perpetuates CLS infection and inoculum in years when sugarbeet are not planted (Khan et al., 2008; Franc, 2010; Skaracis et al., 2010; Tedford et al., 2018; Knight et al., 2020). Further studies have identified potential alternative inoculum sources, such as infected seed (Knight et al., 2020; Spanner et al., 2022). Once *C. beticola* conidia are produced by the primary inoculum, they are dispersed by wind, water movement, and insects (McKay and Pool, 1918; Carlson, 1967; Lawrence and Meredith, 1970; Khan et al., 2007). Conidial dispersal distance can reach up to 100 m (McKay and Pool, 1918), which means it is important to consider neighboring sugarbeet fields during management.

Management of CLS relies on at least a three-year crop rotation with non-host crops, timely fungicide applications using disease prediction models, and the use of tolerant varieties (Windels et al., 1998; Khan et al., 2007; Jacobsen, 2010). None of these management strategies are effective on their own in areas with severe disease, and an integrated approach is necessary to keep CLS from causing economic damage. Host resistance is one of the primary management tools used against CLS. Unfortunately, there are no commercial varieties that have immunity to

CLS (Smith and Gaskill, 1970; Smith and Ruppel, 1974; Rossi, 1999). While several newer varieties are highly tolerant (REACh (Michigan Sugarbeet Research and Education Advisory Council), 2020; REACh (Michigan Sugarbeet Research and Education Advisory Council), 2021), studies are ongoing to assess these varieties for root weight, sugar concentration, and various agronomic traits. As it is often difficult to maintain high recoverable sucrose yield in sugarbeet cultivars with high levels of diseases tolerance to CLS, development of tailored management programs to preserve desirable agronomic qualities are also required (Smith and Gaskill, 1970; Smith and Campbell, 1996).

While fungicides are extensively used for CLS management (Ruppel, 1986; Jacobsen and Franc, 2009), *C. beticola* is at high-risk for fungicide resistance development because of the numbers of fungicide applications each season (averages of 6-8 in severe epidemics in Michigan), high-level of genetic diversity in *C. beticola* populations (Vaghefi et al., 2016; Vaghefi et al., 2017a; Vaghefi et al., 2017b), and the numerous rounds of infection each season (McKay and Pool, 1918; Vereijssen et al., 2007). Reduced sensitivity to multiple fungicide groups including organotins, quinone outside inhibitors, demethylation inhibitors and benzimidazoles has been detected for *C. beticola* populations (Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974; Giannopolitis, 1978; Cerato and Grassi, 1983; Bugbee, 1995; Karaoglanidis et al., 2000; Weiland and Halloin, 2001; Secor et al., 2010; Kirk et al., 2012; Rosenzweig et al., 2020). Thus, there is a critical need for integrated management strategies to control CLS in sugarbeet.

One understudied and underutilized strategy for managing CLS is the reduction of primary inoculum. In Michigan, evidence of infectious *C. beticola* spores has been found as early as April, with consistent early-season detections in 2017-2019 (Bublitz et al., 2021). Tedford et al. (2018) reported similar findings of airborne conidia present in early May in Ontario, Canada. Early

detections of *C. beticola* spores from April through June in fields previously planted to beet support these as a primary inoculum source in North Central and Northeastern regions. This further provides evidence for successful overwintering of *C. beticola*, which may be present on leaf debris in the soil or on alternative hosts (Pool and McKay, 1916; Khan et al., 2008). In years between sugarbeet crops, infections of alternate hosts arising from overwintered sources would create a fresh source of inoculum in the field (Ruppel, 1986). Additionally, neighboring fields that were planted to beets the year prior will serve as an abundant source of inoculum.

Deep tillage has been shown to reduce *C. beticola* inoculum (Ruppel, 1986; Khan et al., 2008), however, this has become a less common practice in Michigan and Minnesota due to its disruption of soil structure. With the move to minimum tillage practices, alternative sanitation strategies are critical to enhance decomposition of leaves and potentially reduce inoculum. Studies of tillage-alternative residue management practices in apple, pear, and citrus have tested treatments such as urea, sugarbeet pulp, sugar cane pulp, dolomitic lime, fungal antagonists, and shredding of leaf litter (Spotts et al., 1997; Vincent et al., 2004; Heijne et al., 2006; van Bruggen et al., 2017). Similarly, herbicides used for preharvest defoliation or desiccation (Stahler, 1953) could accelerate leaf degradation with further potential to directly or indirectly impact disease (Altman and Campbell, 1977). Heating foliage to high temperatures could be further considered for potential sanitation due to the reports of 45.5°C being lethal to *C. beticola* (Pool and McKay, 1916). A propane-fueled foliar heat treatment impacted CLS lesion sporulation and viability of *C. beticola* for sugarbeets that were inoculated and grown in the greenhouse (Bublitz, 2019), supporting the potential for such a strategy. Strategic sanitation and inoculum management would reduce next-year disease pressure and have long-term economic, ecological, and environmental benefits.

This study aimed to i) assess potential end-of-season and early-season management strategies to reduce *C. beticola* inoculum levels and CLS severity in subsequent or current seasons and ii) investigate chemical and non-chemical sanitation practices as integrated tools to improve CLS management and reduce losses in root weight and sugar content. Treatments with the potential to reduce *C. beticola* overwintering and survival were tested in field experiments in Michigan and Minnesota. Three treatments were included in this study. One was a standard plow or tillage application to promote leaf degradation and reduce overwintering success in host material. The second treatment was a propane-fueled foliar heat treatment to directly reduce pathogen survival after exposure to high temperatures. The foliar heat treatment was applied in the spring to directly assess pathogen reduction (in Minnesota), or in the fall to incorporate potential increases in the rate of leaf degradation (in Michigan). The third treatment tested was a chemical desiccant to increase the rate of leaf degradation and reduce overwintering success in host material. To our knowledge, the current study is the first to test the use of in-field heat treatment and chemical desiccation for foliar disease management in sugarbeet.

2.3 Methods

2.3.1 Inoculum reduction trials of fall-applied treatments

2.3.1.1 Trial information

The experiment was conducted at the Saginaw Valley Research and Extension Center (SVREC) in Frankenmuth, MI from 2019 to 2022 (Table 2.1). This location had Tappan-Londo loam soil with 0 to 3 percent slopes (USDA-NRCS (United States Department of Agriculture, Natural Resources Conservation Service), 2019) and the site received only natural precipitation. The trial consisted of a two-year experimental design to test treatments applied in the fall (at the end of the sugarbeet growing season) for the potential to reduce inoculum the following season. In

all years, treatments were applied to four-row 3 m by 18 m plots, replicated four times, and arranged in a randomized complete block design (RCBD). Field tests of fall-applied treatments were repeated over two years.

2.3.1.2 *Cercospora beticola* inoculation

In the first year of each two-year study, inoculations were made using a tractor-mounted field sprayer to apply a *C. beticola* spore solution (approximately $1x10^3$ spores/mL) at 140 L/ha. The conidial suspension was produced from dried CLS-symptomatic sugarbeet leaves collected the previous season, rehydrated and agitated in water, and filtered from leaf particulates (Eujayl et al., 2022). Symptomatic leaves were naturally- and artificially-infested with *C. beticola*, resulting in a representative mixture of local isolates. Inoculum was applied July 9, July 23, and July 12 in 2019, 2020, and 2021, respectively. Sugarbeets were grown to at least the 10-12 leaf growth stage prior to inoculation. Initial lesions were observed approximately 7-10 days after inoculation and severe CLS was typically observed by early-September each year, reaching a KWS (Kleinwanzlebener Saatzucht, 1970) CLS severity rating of 8-9 (on a 0-10 scale, see 2.5). To accurately assess impacts of treatments on overwintered inoculum from the first year, a susceptible sugarbeet variety was planted, with a 3-m buffer surrounding all plots (Table 2.1), and not inoculated or treated in the second year of each two-year study; the two-year sugarbeet rotation was used only as a research tool and does not represent recommended industry practices.

2.3.1.3 Treatments

From 2019-20, the following fall treatments were evaluated: 1) non-treated control, 2) plow with a 3-m tandem disc set to invert soil 15-cm (6 inches) immediately post-harvest, 3) heat treatment using a custom designed 3.25-m wide propane-fueled, tractor- mounted shield burner initially designed for weed control (Multi- Trail Enterprises LLC; Figures A2.1A-D) calibrated to

heat foliage to 649-871°C at 1.6 kmph (1 mph) prior to defoliation, and 4) desiccant (saflufenacil; Sharpen 0.07 L/ha) applied seven days pre-harvest (McNaughton et al., 2015). The desiccant was applied with a CO2-powered backpack sprayer equipped with four 8004XR nozzles (76-cm spacing; TeeJet Technologies) calibrated at 140 L/ha. Methylated seed oil (1% v/v) surfactant and ammonium sulfate (2037 g/L) adjuvant were added to promote uptake and efficacy of the desiccant. The temperature of the heat treatment was measured prior to the study using several K thermocouple sensors connected to a S220-T8 data logger (Huato Electric Co., Ltd.). The thermocouple sensors were positioned at foliage level, at the soil surface level, and less than 1.25 cm beneath the soil surface as the burner was driven over them at 1.6 kmph. Beneath the burner implement, temperature notably decreased at and below ground levels (data not shown); thus, all heated plots were treated prior to defoliation to achieve high target temperatures at the canopy level. From 2020-21, experiments were repeated with the addition of the heat treatment applied at 4.8 kmph (3 mph). In 2021-22, the 4.8 kmph heat treatment was repeated for a second time with the non- treated control; as consistent performance was observed in 2019-20 and 2020-21 trials, the 1.6 kmph heat and desiccant treatments were not included.

2.3.2 Inoculum reduction trials of spring-applied treatments

2.3.2.1 Trial information

Three experiments were conducted on a trial site near Renville, MN in 2019, 2020, and 2021 (Table 2.1). Soil types at this site ranged from Cordova-Rolfe complex clay to silt loams at 0 to 2 percent slopes to Normania loam at 1 to 3 percent slopes (USDA-NRCS, 2019). The season before treatment application, susceptible sugarbeets were grown and not treated with fungicide to achieve a KWS severity rating of 9 (at least 25% of leaf surface area impacted) by the end of September. Beets were defoliated in the fall and leaf residue was left on the soil surface to

overwinter until spring. Treatments tested in this experiment were applied in the following spring the same day as planting. In all years, all treatments were applied to 3 m by 3.4 m plots, replicated four times, and arranged in an RCBD.

Table 2.1. Field trial information for six studies on Cercospora leaf spot of sugarbeet conducted at the Saginaw Valley Research and Extension Center (SVREC) in Frankenmuth, MI and in Renville, MN from 2019-2022.

Location	#	Year	Planting date	Variety [z, y]	Plot width x length (m)	Row spacing (cm)	Buffer spacing (m) [x]	Harvest date	Inoculation date
SVREC 43.396543, -83.689057	1	2019	7-May	Crystal G333NT	3 x 18	76.2	3	24-Oct	9-Jul
		2020	17-Apr	Crystal G333NT	3 x 18	76.2	3	8-Oct	-
	2	2020	17-Apr	Crystal G932NT	3 x 18	76.2	3	16-Oct	23-Jul
		2021	7-May	Crystal G932NT	3 x 18	76.2	3	17-Sep	-
	3	2021	7-May	Crystal G932NT	3 x 18	76.2	3	3-Nov	12-Jul
		2022	29-Apr	Crystal G932NT	3 x 18	76.2	3	23-Sep	-
Renville 44.787496, -95.148788	1	2019	14-May	Beta 9230	3.4 x 3	55.9	1.5	-	-
	2	2020	12-May	Crystal RR018	3.4 x 3	55.9	1.5	-	-
	3	2021	12-May	Crystal M977	3.4 x 3	55.9	1.5	-	-

[z] Cercospora leaf spot susceptible varieties selected based on Michigan Sugarbeet Research and Education Advisory Council variety trial results (REACh (Michigan Sugarbeet Research and Education Advisory Council), 2018; REACh (Michigan Sugarbeet Research and Education Advisory Council), 2019) and Southern Minnesota Beet Sugar Cooperative variety evaluations (SMBSC (Southern Minnesota Beet Sugar Cooperative), 2018; SMBSC (Southern Minnesota Beet Sugar Cooperative), 2019; SMBSC (Southern Minnesota Beet Sugar Cooperative), 2020)
[y] Varieties were planted at rates of123,500 seeds/ha at all SVREC trials and at 269,230 seeds/ha at all Renville trials. In Renville, the high seeding rate was to account for potential emergence issues in plots that were not tilled (heat and control).
[x] SVREC trial buffers were planted to soybean (Trial 1 in 2019 and Trial 3 in 2021), corn (Trial 2 in 2020), or wheat (Trial 1 in 2020, Trial 2 in 2021, and Trial 3 in 2022). Renville trial buffers were planted to sugarbeets in all years.

2.3.2.2 Treatments

Three treatments similar to the fall applications were tested for inoculum reduction potential. Treatments included a 1) non- treated control, 2) tillage with a rotary tiller in the spring (prior to planting) to a depth of 10 cm to bury the residue and then raking by hand to create a firm seed bed for planting, and 3) propane burner application using a handheld Flame King Heavy

Duty Propane Torch Weed Burner (Pico Rivera CA 90660; Figure A2.1E) to the residue to target *C. beticola* survival over the winter. Sugarbeets were planted immediately after treatments were applied to the trial area.

2.3.3 Overwintering assessments for fall-applied treatments

2.3.3.1 Symptomatic leaf samples

To evaluate survival of the pathogen over time, leaf samples from each treatment were assessed for percent lesion sporulation and isolation at 0-, 45-, 90-, and 135-days post-harvest (DPH) in the 2019-20 and 2020-21 trials. In 2021-22, leaf samples were assessed at 0-, 35-, 70-, and 168-DPH. All leaf samples contained eight sugarbeet leaves with distinct CLS lesions (between 0.1 to 3% symptomatic leaf area) that were arbitrarily collected from the middle canopy in the center two rows of each plot at harvest following treatment application. Post-harvest leaf samples were placed in mesh bags (approximately 66 cm by 37 cm, mesh size 5 mm2) and placed in the field. Bags were slightly incorporated (less than or equal to 2.5 cm) into the soil for all treatments except the plow treatment where the bags were buried 15.2 cm to simulate tillage effect on leaf residue.

2.3.3.2 Leaf degradation

In 2019-20 and 2020-21, at-harvest leaf samples were stored in a cold room at 4°C for four days prior to destructive sampling. After collection from the field, post-harvest leaf samples were stored for three days at 4°C, rinsed with tap water over a 2.00-mm sieve to remove soil debris, and then left to air dry overnight between paper towels, for a total of four days after collection. In 2021-22, at-harvest leaf samples were stored at 4°C for five days prior to destructive sampling; post-harvest leaf samples were stored for four days at 4°C then rinsed and dried as described, for a total of five days after collection. At-harvest samples were collected directly from

sugarbeet plants prior to defoliation and did not require rinsing to remove soil residue.

In 2019, leaf samples were photographed four days after collection and later measured using ImageJ software (Schneider et al., 2012). The total leaf area was calculated for each replicate sample and then divided by the number of leaves in each sample to estimate the standardized leaf area for each plot. To more accurately measure leaf degradation in 2020 and 2021, leaf samples were weighed at harvest and four or five days after recovery from the field, respectively. Percent leaf degradation was calculated by subtracting final weight from initial weight and dividing by the initial weight at harvest.

2.3.3.3 *Cercospora beticola* viability: lesion sporulation

After collection and handling as described, all eight leaves from each sample were placed in moist chambers at room temperature (21-24°C) with ambient light conditions to induce sporulation of CLS lesions (Bublitz, 2019). Moist chambers were composed of 3.8- liter plastic resealable bags with a moist paper towel wetted with deionized water. Bags were inflated gently with ambient air. After three days, percent CLS lesion sporulation was measured by counting the number of sporulating lesions on each leaf using a dissecting binocular stereo microscope (7-10x magnification; Leica ZOOM 2000) and dividing by the total number of lesions assessed on each leaf.

2.3.3.4 *Cercospora beticola* viability: lesion isolation

Ten lesions were arbitrarily selected from across each sample of eight leaves and excised using a 5-mm diameter cork-borer. The lesions were surface disinfested using an 8.25% sodium hypochlorite solution for 30 seconds, triple rinsed with sterile deionized water, air-dried on a sterile paper, and placed on 1.5% water or rose bengal agar (Millipore Sigma) with 0.5g/L streptomycin and 0.25 g/L ampicillin to inhibit bacterial growth. Hyphal tip transfers (Brown,

1924) onto clarified V8 (CV8) agar (Miller, 1955) media amended with CaCO3 (0.8 g/L) with 0.5 g/L streptomycin and 0.25 g/L ampicillin were used to isolate pure cultures of *C. beticola* based on morphological characteristics. Hyphal tip transfers were done twice to ensure pure cultures of *C. beticola* (Forsyth et al., 1963). Percent CLS lesion viability was calculated by dividing the number of successful *C. beticola* isolations by the total number of lesions plated.

2.3.4 Early-season sentinel beet sampling for fall-applied treatments

2.3.4.1 Live spore trap maintenance and in-field exposure

In 2020, 2021, and 2022, highly CLS susceptible beets (germplasm F1042) were used to assess the efficacy of inoculum reduction strategies (USDA-ARS (United States Department of Agriculture, Agricultural Research Service), 2017). These highly susceptible sugarbeets were referred to as "sentinel beets" because they were used to monitor in-field spore presence (Bublitz et al., 2021). In the MSU Plant Science Research Greenhouse Complex, F1042 sugarbeet seeds were mass planted in SureMix growing media (Michigan Grower Products,Inc.). Four sugarbeet seedlings at the cotyledon growth stage were transplanted to each planting box (61.0 cm by 30.5 cm) and maintained in the same greenhouse. Plants were fertilized at planting with Osmocote Smart-Release Plant Food Flower & Vegetable (Scotts Miracle- Gro, N-P-K 14-14-14) and approximately once per month with Osmocote Smart-Release Plant Food Plus Outdoor & Indoor (Scotts Miracle-Gro, N-P-K 15-9-12 plus micro- and secondary nutrients) according to labeled rates. Plants also were watered two to three times a week depending on greenhouse conditions. Nontarget diseases and insect pests impacting sentinel beets, e.g., powdery mildew, thrips, and aphids, were monitored and managed by greenhouse staff; to reduce potential residual effects on *C. beticola*, Sulfur Plant Fungicide (sulfur 90%) was applied as necessary to manage powdery mildew (2-3 applications per year).

Once the sugarbeets reached the 10-14 leaf stage, one box of sentinel beets was placed in three of the four replicate field plots for each treatment. Boxes were left in the field for seven days before returning to the greenhouse, starting at 7 to 39 days after planting (DAP) of the field experiments and continuing until 60 to 95 DAP (Table A2.1). Sentinel beets were used as live spore traps to estimate early levels of viable inoculum in each plot. Of note, an insecticide treatment of Mainspring (cyantraniliprole 18.66%) at 236.6 ml/3.8 L (8 fl oz/100 gal) was applied to sentinel beets seven days prior to field exposure to reduce leaf miner and thrips infestation in the field. While in the field, wire cages (constructed from poultry netting) were secured onto the planting box to reduce animal feeding and disturbance. Sentinel plants were also manually watered twice weekly dependent on occurrence of natural rain events.

2.3.4.2 Live spore trap incubation and symptom observation

After seven days of exposure in the field, sentinel beets (one box of four beets collected from each of three replicate plots per treatment) were placed in a humidity chamber to provide favorable conditions for *C. beticola* infection. The chamber consisted of steel shelves lined and enclosed with plastic sheeting (4-mm thick clear poly) and was kept at a temperature of 27°C with humidifiers supplying a humidity greater than 95% (Bublitz et al., 2021). After three days in the humidity chamber, the boxes of beets were returned to the greenhouse for three weeks. Greenhouse temperatures varied between 20 to 30°C degrees and received natural light (Bublitz et al., 2021). Characteristic CLS lesions (Windels et al., 1998; Weiland and Koch, 2004) were identified and counted on each plant and the total number of lesions for each box of beets was recorded. Lesions were only counted if pseudostromata, a distinguishing characteristic of Cercospora leaf spots, were detected using a hand lens (3x to 6x magnification).

A non-inoculated control was incubated with field-exposed sentinel beets to monitor

secondary dispersal of conidia within the humidity chamber. Asymptomatic sentinel beets of the same age as the field-exposed beets were kept separately in the greenhouse before placing in the moist chamber. No to low (1-6 lesions) CLS symptoms were observed on the non-inoculated controls during the study.

2.3.5 Disease pressure assessments for fall- and spring-applied treatments

Disease severity data were collected from the middle two rows of each plot. The KWS (Kleinwanzlebener Saatzucht, 1970) CLS standard surface area rating scale was used for fall-applied treatment evaluations in 2020 and 2021 and all spring-applied treatment evaluations. The KWS scale ranges from 1 to 10, in which 1 = 1-5 spots/leaf (0.1% severity), 2 = 6-12 spots/leaf (0.35% severity), 3 = 13-25 spots/leaf (0.75% severity), 4 = 26-50 spots/leaf (1.5% severity), 5 = 51-75 spots/leaf (2.5% severity), 6 = 3% severity (proven economic damage), 7 = 6% severity, 8 = 12% severity, 9 = 25% severity, and 10 = 50% to 100% severity.

To more easily evaluate whole plant symptoms, the Agronomica (Battilani et al., 1990) CLS severity scale (0-5) was used in 2022 and standardized to a 0-10 scale, where 0 = healthy foliage, 1 = a single isolated spot on some leaves, 2 = 50% of outer leaves show one to a few spots ~20 per leaf, 3 = outer leaves ~50% foliage show 20-100 spots per leaf, 4 = nearly all outer leave are affected by several spots (still isolated), 5 = some (2-4) outer leaves show coalescence of spots to necrotic areas (first spots appear on the inner leaves), 6 = fully and almost grown leaves show several coalesced necrotic areas of 1-2 cm in diameter (that do not lead to large necrotic areas), 7 = some (2-4) outer leaves show relatively large necrotic areas (20-30% of the leaf area), 8 = for the first time some leaves (2-8) show 80-100% severity, 9 = the entire foliage is strongly affected, 10 = the original foliage is completely destroyed.

Disease observations were collected biweekly from mid-June until harvest in late-August

or early-September. Area under the disease progress curves were calculated using CLS severity ratings in the below equation:

$$A_k = \sum_{i=1}^{N_i-1} \frac{(y_i + y_{i+1})}{2}(t_{i+1} - t_i)$$

The rating time points in a sequence are considered (t_i) and the associated measures of the disease level (y_i); $y(0) = y_0$ is defined as the initial infection or the disease level at t = 0. $A(t_k)$, the AUDPC at $t= t_k$, is the total accumulated disease until $t= t_k$ (Madden et al., 2017).

2.3.6 Yield and sugar assessments for fall-applied treatments

In the second year of each trial, two center rows of each treatment plot were mechanically harvested and weighed to determine yield. Root subsamples (approximately 10 kg) were collected from the center two rows of each plot. Sugar analysis was conducted by Michigan Sugar Company (Bay City, MI) as described in Tedford et al. (2019) to assess percent sugar and recoverable white sugar per ton (RWST). Samples were sliced using a rasping circular saw to obtain 1 L of root pulp (brei), which was filtered and the juice extracted for sucrose yield and standard quality analysis (Carruthers & Oldfield, 1961). The polarimeter method was used to determine sucrose content (Halvorson et al., 1978). Methods reported by Last et al. (1976) were used to determine sucrose purity (clear juice purity, CJP) and brei impurity amino-N. Recoverable white sucrose per metric ton of fresh beets (RWS) was calculated as in Van Eerd et al. (2012) and converted to recoverable white sucrose per hectare (RWSH) using the following equation: RWSH (metric ton/hectare) = RWS (kg/ metric ton) × Total Yield (metric ton/hectare) ÷ 1000. As disease impacts were the primary focus in studies evaluating spring-applied treatments, yield and sugar were not measured.

2.3.7 Statistical analyses

For all experiments, treatment was evaluated as the fixed effect of interest and replicate was considered a random effect. Due to differences in experimental treatments and design, years and locations were analyzed separately. Analysis of variance (ANOVA) was conducted using SAS (Statistical Analysis System) v. 9.4 software package (SAS Institute, Inc. Cary, NC, United States) to determine treatment effects on percent *C. beticola* sporulation and isolation, standardized leaf area, percent leaf degradation, early-season lesion counts from sentinel beets, AUDPC, yield, percent sugar, RWS, and RWSH values. Sentinel beet lesion count data were normalized using the lognormal distribution option to best fit this data (Gbur et al., 2012). Statistical analyses (mixed model ANOVA) were conducted using the generalized linear mixed model (GLIMMIX) procedure (SAS Institute Inc., 2013) and evaluated at the a =0.05significance level. Fisher's protected least significance difference (LSD) was used for mean comparisons. LSD was calculated to compare treatment differences using letter separation option "mult" macro (Piepho, 2012).

2.4 Results

2.4.1 Leaf degradation after fall-applied treatments

No differences were detected in standardized leaf area for treatments tested 2019-20 (P = 0.0757; Table 2.2). However, significant differences in percent leaf degradation were detected in 90- ($P < 0.05$) and 135-DPH ($P < 0.01$) samples for treatments tested 2020-21, with observable differences in leaf tissue recovered (Figure A2.2).

In 2020-21, heat treatment at 4.8 kmph and desiccant treatments resulted in leaf degradation comparable to the control at all timepoints. Leaf degradation resulting from the plow treatment was significantly greater than degradation in the control at 90-DPH ($P< 0.05$) and was

comparable to the 1.6- kmph heat treatment at 90- and 135-DPH. However, differences between the plow and control treatments were not detectable by 135-DPH. In the repeated test conducted in 2021-22, the heat treatment at 4.8 kmph resulted in significantly reduced leaf degradation compared to the control at 0-DPH (Table 2.3) but was comparable to the control at all subsequent timepoints.

Table 2.2. Standardized leaf area and percentage sugarbeet leaf degradation for at-harvest and soil incorporated post-harvest samples[z,y] collected 0-, 45-, 90-, and 135-days post-harvest (DPH) from fall-applied field studies in 2019-2020 and 2020-2021.

Treatment	2019-2020				2020-2021					
	Standardized Leaf Area (cm^2) [x]				Leaf Degradation (%) [w, v]					
	0-DPH	45-DPH	90-DPH	135-DPH	0-DPH	45-DPH	90-DPH		135-DPH	
Control	43.8	58.6	11.8	53.1	12.9	58.5	72.6	bc	80.4	bc
Plow	44.6	51.8	19.7	50.3	19.1	62.4	82.6	a	86.2	ab
Heat (1.6 kmph)	33.2	42.8	19.0	28.1	7.8	63.6	81.9	ab	89.3	a
Desiccant	38.7	55.1	14.5	48.8	14.1	53.9	67.7	c	74.3	c
Heat (4.8 kmph)	-	-	-	-	20.3	68.2	71.1	c	86.2	ab
SE	*2.6*	*6.5*	*5.8*	*8.2*	*2.8*	*3.4*	*3.1*		*2.5*	
P-value [u]	*0.0757*	*0.3600*	*0.7275*	*0.2727*	*0.0625*	*0.0654*	*0.0177*	*	*0.0014*	**
LSD	-	-	-	-	-	-	*9.6*		*6.2*	

[z] Non-treated control, plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 1.6 kmph and 4.8 kmph prior to defoliation, and a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre-harvest.

[y] Measurements were collected from independent sets of leaves at each timepoint, not repeated measurements from the same sets of leaves over time.

[x] Average of the total standardized leaf area (total area divided by number of leaves) quantified using ImageJ (Schneider et al., 2012).

[w] Percent leaf degradation calculated using initial leaf weights at-harvest and final weights post-harvest [(Initial – Final)/Initial].

[v] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD ($\alpha = 0.05$).

[u] Asterisk designations correspond to p-value thresholds <0.05 *, <0.01 **.

Table 2.3. Percent sugarbeet leaf degradation for at-harvest and soil-incorporated post-harvest samples[z,y] collected 0-, 35-, 70-, and 168-days post- harvest (DPH) from fall-applied treatments in field studies in 2021-2022

Treatment	2021-2022
	Leaf Degradation (%) [x, w]

Table 2.3 (cont'd)

	0-DPH		35-DPH	70-DPH	168-DPH
Control	20.1	a	40.9	75.1	91.4
Heat (4.8 kmph)	12.9	b	37.1	63.7	90.9
SE	*1.7*		*7.8*	*5.5*	*1.0*
P-value [v]	*0.0211 **		*0.2731*	*0.0691*	*0.7468*
LSD	*5.2*		-	-	-

[z] Non-treated control and heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C 4.8 kmph prior to defoliation.
[y] Measurements were collected from independent sets of leaves at each timepoint, not repeated measurements from the same sets of leaves over time.
[x] Percent leaf degradation calculated using initial leaf weights at-harvest and final weights post-harvest [(Initial – Final)/Initial].
[w] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05).
[v] Asterisk designations correspond to p-value thresholds<0.05 *.

2.4.2 *Cercospora beticola* survival after fall-applied treatments

In the 2019-20 trial, significant treatment differences were detected in percentage of CLS lesion sporulation ($P< 0.0001$) and *C. beticola* isolation frequencies ($P< 0.05$) for 0-DPH samples (Table 2.4). Compared to the non-treated control, CLS lesion sporulation and *C. beticola* isolation were reduced in leaf samples from heat-treated plots by 99 and 91%, respectively. No significant differences were detected in sporulation or isolation frequencies of *C. beticola* from leaf samples evaluated at 45-, 90-, or 135-DPH in this trial ($P > 0.05$).

In the 2020-21 trial, significant reductions in percent lesion sporulation were observed at 0-DPH ($P< 0.0001$), 45-DPH ($P< 0.01$), and 90-DPH ($P< 0.05$) for leaf samples in heat treated plots compared to the control (Table 2.4). Percentage point reductions in sporulation were greater than 70% at-harvest, 20% at 45-DPH, and 5% at 90-DPH for 1.6- and 4.8-kmph heat treatments compared to the control. Percent sporulation was reduced to 0% for the 1.6- kmph heat treatment at each time point and 0.3% or lower for the 4.8-kmph treatment. No significant differences were observed in lesion sporulation for samples evaluated at 135-DPH ($P > 0.05$). No differences were detected in isolation frequencies of *C. beticola* from leaf samples evaluated at 0-, 45-, 90-, or 135-

DPH ($P > 0.05$).

In the 2021-22 trial, the 4.8-kmph treatment significantly reduced percent sporulation for at-harvest, 35-, and 70-DPH samples compared to the control ($P< 0.05$, Table 2.4). A 31% decrease in percent sporulation was seen for the heat-treated samples compared to the control at-harvest. There were no significant differences between percent sporulation for the 4.8- kmph heat treated and control samples at 168-DPH or percent isolation at any sampling time.

In both the 2019-20 and 2020-21 trials, lesion sporulation was significantly reduced in the desiccant treatment compared to the control at 0-DPH (Table 2.4). This reduction, however, was not consistent at remaining overwintering timepoints in either year. The plow treatment did not result in consistent reductions of lesion sporulation in 2019-20 samples but significantly reduced lesion sporulation in 2020-21 samples collected 45- and 90-DPH.

Table 2.4. Cercospora leaf spot lesion sporulation (Sp) and *C. beticola* isolation frequencies (Is) from soil-incorporated sugarbeet leaf samples collected from Michigan studies at post-harvest timepoints following fall-applied treatments in 2019-2020, 2020-2021, and 2021-2022.

Trial Year	Treatment [z]	0-DPH [y]				45-DPH		90-DPH		135-DPH	
		Sp [x, w] (%)		Is [v] (%)		Sp (%)	Is (%)	Sp (%)	Is (%)	Sp [u] (%)	Is (%)
2019-20	Control	78.1	a	38.3	a	17.6	0.0	1.2	0.0	0.3	0.0
	Plow	60.3	b	43.3	a	0.6	0.0	0.0	0.0	0.0	0.0
	Heat (1.6 kmph)	1.1	c	3.3	b	0.7	0.0	0.0	0.0	0.0	0.0
	Desiccant	59.8	b	38.3	a	13.2	0.0	0.6	0.0	0.1	0.0
	SE	*4.5*		*10.3*		*7.4*	*0.0*	*0.5*	*0.0*	*0.1*	*0.0*
	P-value [t]	*< 0.0001 ****		*0.0235 **		*0.2601*	*NS*	*0.1879*	*NS*	*0.158*	*NS*
	LSD	*13.4*		*25.9*		-	-	-	-	-	-
2020-21	Control	77.9	a	5.0		22.2	a 0.0	7.2	a	2.5	8.8	0.0
	Plow	66.4	a b	2.5		0.0	b 0.0	0.0	b	0.0	1.6	0.0
	Heat (1.6 kmph)	0.0	c	0.0		0.0	b 0.0	0.0	b	0.0	0.0	0.0
	Desiccant	50.1	b	7.5		32.2	a 0.0	7.5	a	0.0	0.9	0.0
	Heat (4.8 kmph)	0.0	c	0.0		0.0	b 0.0	0.3	b	0.0	0.3	0.0
	SE	*5.8*		*3.3*		*6.2*	*0.0*	*1.9*	*1.1*	*2.7*	*0.0*

Table 2.4 (cont'd)

	P-value	*< 0.0001 ******	*0.4802*	*0.0083 ** *	*NS*	*0.0267 ** *	*0.4449*	*0.2113*	*NS*
	LSD	*19.7*	-	*19.8*	-	*6.1*	-	-	-
		0-DPH		35-DPH		70-DPH		168-DPH	
2021-22	Control	90.8 a	7.5	10.1 a	0.0	2.9 a	0.0	0.0	0.0
	Heat (4.8 kmph)	62.8 b	10.0	0.6 b	7.5	0.0 b	0.0	0.0	0.0
	SE	*4.3*	*4.4*	*1.3*	*3.4*	*0.2*	*0.0*	*0.0*	*0.0*
	P-value	*0.0183 ** *	*0.3910*	*0.0202 ** *	*0.2152*	*0.0022 ** *	*NS*	*NS*	*NS*
	LSD	*19.0*	-	*6.6*	-	*0.9*	-	-	-

[z] Non-treated control, plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 1.6 kmph and 4.8 kmph prior to defoliation, and a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre- harvest.

[y] Days post-harvest (DPH).

[x] Percent lesion sporulation (Sp) determined following a 3-d incubation in a moist chamber at 21-23.9°C. Lesion sporulation assessed for 1,636 to 3,895 lesions per timepoint (across all treatments) in 2019. Lesion sporulation assessed for 1,020 to 1,600 lesions per timepoint in 2020. Lesion sporulation assessed for 312 to 548 lesions per timepoint in 2021.

[w] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05).

[v] Frequency of *C. beticola* isolation (Is) determined from morphological confirmation of *C. beticola* growth from 15 (2019) and 10 (2020 and 2021) representative lesions, plated on half-strength clarified V8 juice agar (Miller, 1955) amended with 0.5 g/L streptomycin and 0.25 g/L ampicillin. [u] Late-winter sporulation observations may be limited by unknown lesion maturity (e.g., number of prior in-season sporulation events).

[t] Asterisk designations correspond to p-value thresholds<0.05 *,<0.01 **,<0.001 ***; NS indicates no significant differences were detected as data were all zeroes.

2.4.3 Early-season inoculum assessments after fall-applied treatments

In 2020, following 2019 fall-applied treatments, reduced numbers of CLS lesions were observed on live spore traps (sentinel beets) in heat-treated plots from May 26-June 2 ($P< 0.05$) and June 2-9 ($P< 0.01$) (Table 2.5), up to 46 to 53 days after planting. The number of CLS lesions observed on sentinel beets in the plow and desiccant treatments were not significantly different from the control at any sampling timepoint.

In 2021, following 2020 fall-applied treatments, the 1.6-kmph heat treatment resulted in significantly fewer lesions on sentinel beets sampled June 15-22 ($P< 0.005$, Table 2.5), 39 to 46 days after planting. No significant differences were seen in treatments sampled June 1-8 ($P= 0.1128$). Inoculum levels in the plow and desiccant treatments were again not significantly

different from the control at any sampling timepoint.

In 2022, following 2021 fall-applied treatments, no significant differences were detected between the number of lesions for the 4.8- kmph heat treatment compared to the control (Table 2.5).

Table 2.5. Number of Cercospora leaf spot lesions observed on live spore traps (sentinel beets) placed in Michigan field studies in 2020, 2021, and 2022 (the year following fall-applied treatments).

Trial Year	Treatment [z]	Sentinel [y, x]								
		May 26 – June 2 [w]			*June 2 – June 9*			*July 14 – July 21*		
2019-20	Control	5.5	(284)	a	4.1	(60)	b	5.3	(199)	
	Plow	5.4	(337)	a	3.9	(51)	b	5.4	(219)	
	Heat (1.6 kmph)	4.0	(65)	b	2.5	(16)	c	4.7	(130)	
	Desiccant	6.1	(482)	a	5.3	(212)	a	5.7	(320)	
	SE	*0.4*			*0.3*			*0.2*		
	P-values [v]	*0.0323*	*		*0.0063*	**		*0.0653*		
	LSD	*1.3*			*1.1*			-		
		June 1 – June 8			*June 15 – June 22*			*June 29 – July 6*		
2020-21	Control	2.9	(19)		6.7	(888)	ab	7.3	(1514)	
	Plow	2.0	(9)		6.3	(628)	ab	7.2	(1397)	
	Heat (1.6 kmph)	1.2	(4)		4.9	(144)	c	7.0	(1189)	
	Desiccant	2.1	(15)		7.0	(1239)	a	7.1	(1222)	
	Heat (4.8 kmph)	0.5	(2)		5.9	(396)	b	7.3	(1498)	
	SE	*0.7*			*0.3*			*0.2*		
	P-values	*0.1128*			*0.0027*	**		*0.8198*		
	LSD	-			*0.8*			-		
		May 17 – May 24			*May 24 – May 31*			*June 15 – June 22*		
2021-22	Control	5.1	(166)		6.4	(614)		5.7	(358)	
	Heat (4.8 kmph)	4.3	(98)		5.6	(305)		5.8	(391)	
	SE	*0.4*			*0.2*			*0.4*		
	P-value	*0.1926*			*0.2238*			*0.7912*		
	LSD	-			-			-		

[z] Non-treated control, plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 1.6 kmph and 4.8 kmph prior to defoliation, and a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre- harvest.

[y] Means generated under the lognormal distribution option in the GLIMMIX procedure (SAS v 9.4) of total CLS lesions counted on sentinel beets (USDA germplasm F1042) after 1-week exposure in the field, 3 d incubation in a 25°C humidity chamber, and 2 weeks in a greenhouse. (Non-normally distributed mean estimates shown in parentheses).

[x] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a =

Table 2.5 (cont'd)
0.05).
[w] Data shown for sampling weeks: 1st, 2nd, and 7th (2020); 2nd, 3rd, and 4th (2021); 1st, 2nd, 4th (2022). No or low detections in weeks not shown due to low spore concentrations or other external insect or drought stress factors (Table A2.1).
[v] Asterisk designations correspond to p-value thresholds<0.05 *,<0.01**.

2.4.4 Early development of CLS after fall- applied and spring-applied treatments

Following fall-applied heat treatments, next-year CLS was monitored until moderate to severe disease severity levels were achieved in 2020 (8-10), 2021 (3-6), and 2022 (8-10). No fungicide treatments were applied to this trial, and disease naturally progressed beyond these assessments until high levels of CLS developed across all treatments. Analyses focused, therefore, on early-season disease development 39-139 days after planting. The fall heat treatment significantly reduced CLS pressure in 2020 and 2021 ($P< 0.05$) compared to the non-treated control (Figures 2.1A, B). Greater than 35% reductions in AUDPC were observed in fall heat-treated plots at 1.6 kmph in 2020. In 2021, greater than 25, 30, and 20% reductions were measured for the plow, 1.6-kmph, and 4.8-kmph heat treatment, respectively. Upon further investigation, following 1.6-kmph heat treatment visual reductions in CLS severity were sustained for six to eight weeks of the growing season (Figures 2.2A, B). No significant differences between treatment AUDPC were observed in 2022 (Figure 2.1C). CLS severities following the 4.8-kmph heat treatment, however, were reduced for approximately four weeks of the growing season (Figure 2.2C).

In studies of spring-applied heat treatments, same-year disease development was assessed until moderate to severe CLS levels were achieved in 2019 (4-8.5), 2020 (7-9), and 2021 (7-9). AUDPC was significantly lower for plots following spring (pre-plant) tillage and heat treatment application of infected leaf residue in 2019 ($P< 0.05$) and 2021 ($P< 0.0001$) (Figures 2.3A, C) but not in 2020 ($P = 0.0704$; Figure 2.3B); significant reductions corresponded to three to four weeks

of visual CLS severities less than control plots (Figures 2.4A, C). In 2020, visible differences in CLS severities were detected for about four weeks of the growing season, though less delineation between treatments was observed than in other years (Figure 2.4B).

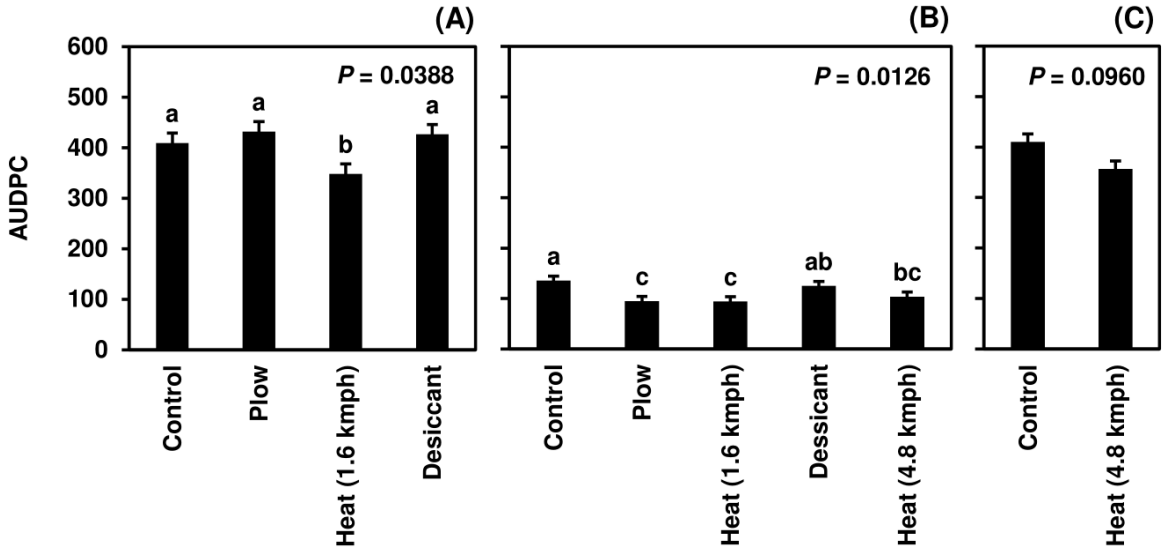

Figure 2.1. Mean area under the disease progress curve (AUDPC) values from Michigan field studies collected in (A) 2020, (B) 2021, and (C) 2022, the years following fall-applied treatments. Treatments included were a non-treated control, plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 1.6 kmph and 4.8 kmph prior to defoliation and a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre-harvest. AUDPC values were calculated according to Madden et al. (2017) using severity ratings collected at 5 timepoints; ratings were based on the KWS severity scale (0-10) in 2020 and 2021 and the Agronomica severity scale (standardized to 0-10) in 2022. Bars represent the means of four replicate plots and error bars represent standard errors. Bars with the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05). Analyses were conducted within each year.

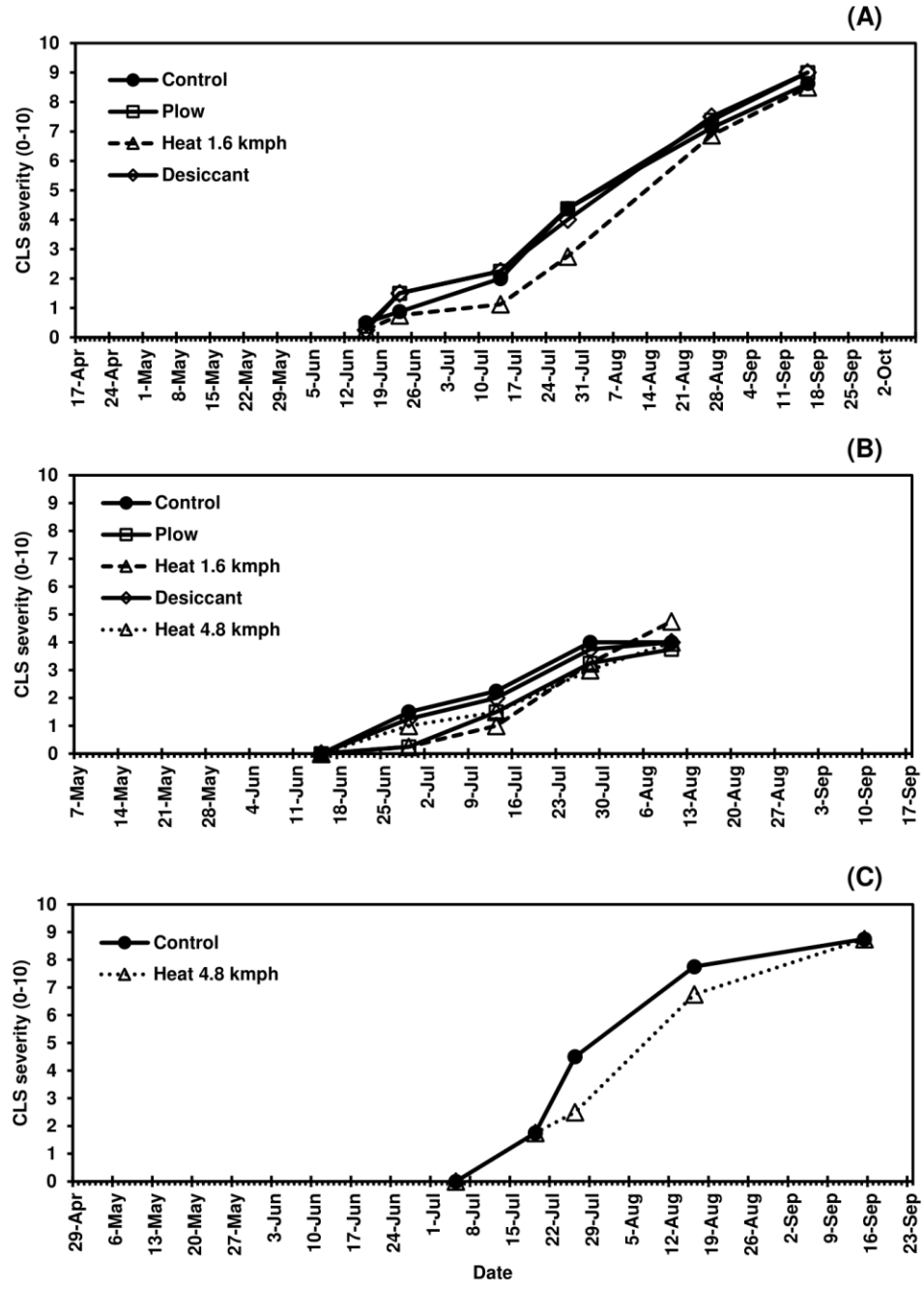

Figure 2.2. Mean Cercospora leaf spot severity progression on sugarbeet in **(A)** 2020, **(B)** 2021, and **(C)** 2022, following fall-applied treatments evaluated in Frankenmuth, MI. Treatments included a non-treated control, plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 1.6 kmph and 4.8 kmph prior to defoliation, and a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre-harvest. In 2020 and 2021, CLS ratings were based on the KWS severity scale (0-10) in 2020 and 2021 where 0 is 0.1% severity (1-5 spots per leaf) and 10 is 50% severity. In

Figure 2.2. (cont'd)
2022, the CLS ratings were based on the Agronomica (Battilani et al., 1990) severity scale standardized to 0-10 scale where 0 is completely healthy foliage and 10 is completely destroyed original foliage with respect to CLS. Each point represents a mean of four replicate field plots. Date axes start at the planting date and end at the harvest date for each year.

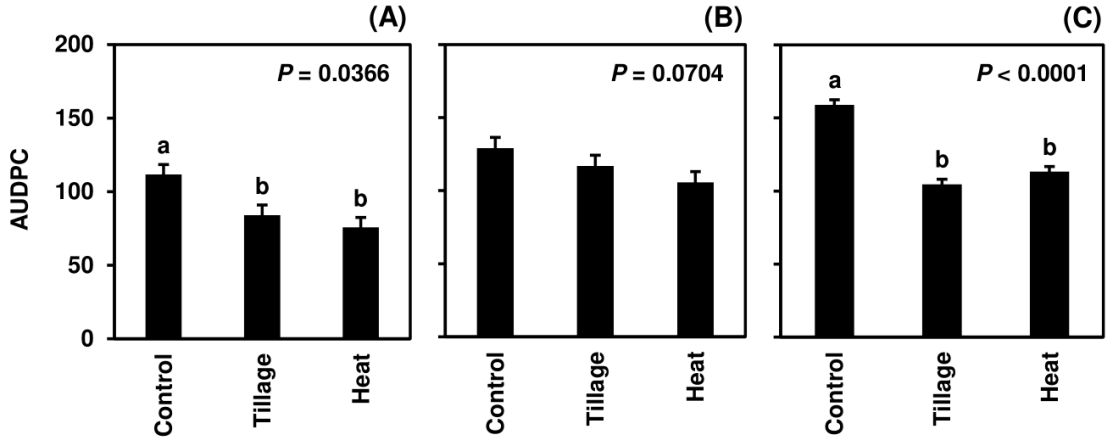

Figure 2.3. Mean area under the disease progress curve (AUDPC) values from Minnesota field studies in **(A)** 2019, **(B)** 2020, and **(C)** 2021. Treatments included a non-treated control, tillage with a rotary tiller in the spring (prior to planting) to a depth of 10 cm to bury the residue, and propane burner heat application using a handheld Flame King Heavy Duty Propane Torch Weed Burner (Pico Rivera CA 90660). AUDPC values were calculated according to Madden et al. (2017) using severity ratings collected at 8-9 timepoints; ratings represented scores assigned by two to four raters and were based on the KWS severity scale (0-10). Bars represent the means of four replicate plots and error bars represent standard errors. Bars with the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05). Analyses were conducted within each year.

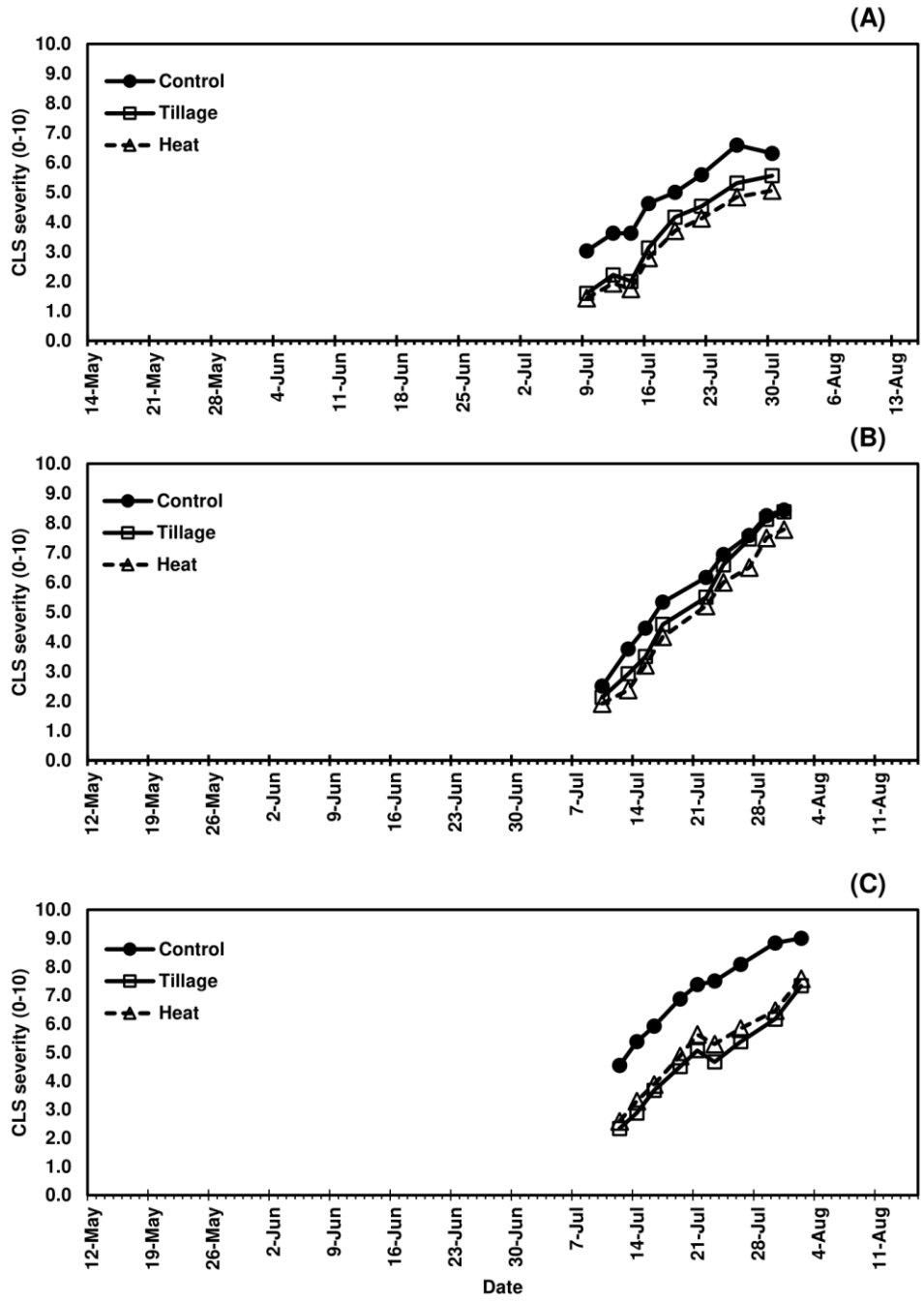

Figure 2.4. Mean Cercospora leaf spot severity progression in **(A)** 2019, **(B)** 2020, and **(C)** 2021, following spring-applied treatments evaluated in Renville, MN. Treatments included a non-treated control, tillage with a rotary tiller in the spring (prior to planting) to a depth of 10 cm to bury the residue, and heat treatment of residue using a propane-fueled using a handheld Flame King Heavy Duty Propane Torch Weed Burner (Pico Rivera CA 90660) immediately prior to planting. CLS ratings were based on the KWS severity scale (0-10) where 0 is 0.1% severity (1-5 spots per leaf) and 10 is 50% severity. Each point represents a mean of four replicate field plots. Date axes start at the planting date and end August 15 for each year as trials were not harvested.

2.4.5 Final yield and sugar after fall-applied treatments

No treatments yielded significantly differently from the control in any year ($P > 0.05$) (Table 2.6). There were no statistical differences detected in percent sugar, RWS, or RWSH for any treatments in the 2019-20, 2020-21, or 2021-22 trials. Overall, the heat treatment did not have any beneficial or deleterious effects on sugarbeet root or sugar yield at- or post-harvest (data not shown).

Table 2.6. Sugarbeet yield, percent sugar content, and recoverable white sugar from Michigan field studies collected in 2020 and 2021 (the year following fall-applied treatments).

Trial Year	Treatment [z]	Yield (t/ha)	At-harvest		
			Sugar (%)	RWS (kg/t) [y]	RWSH (t/ha) [x]
2019-20	Control	9.6	14.1	101.6	1.0
	Plow	9.7	14.9	109.1	1.1
	Heat (1.6 kmph)	7.7	14.3	103.9	0.8
	Desiccant	7.8	14.5	105.6	0.8
	SE	*0.7*	*0.2*	*1.9*	*0.1*
	P-values	*0.0908*	*0.1206*	*0.0797*	*0.0904*
	LSD	-	-	-	-
2020-21	Control	5.7	15.2	110.6	0.6
	Plow	5.7	14.8	107.3	0.6
	Heat (1.6 kmph)	7.2	14.9	108.1	0.8
	Desiccant	3.9	15.1	110.1	0.4
	Heat (4.8 kmph)	4.3	15.3	111.8	0.5
	SE	*1.0*	*0.2*	*1.7*	*0.1*
	P-values	*0.0783*	*0.3456*	*0.2598*	*0.1152*
	LSD	-	-	-	-
2021-22	Control	12.2	14.0	100.7	1.2
	Heat (4.8 kmph)	14.2	14.0	100.3	1.4
	SE	*4.5*	*0.2*	*1.8*	*0.5*
	P-values	*0.6358*	*0.9914*	*0.9154*	*0.6505*
	LSD	-	-	-	-

[z] Non-treated control, plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 1.6 kmph and 4.8 kmph prior to defoliation, and a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre-harvest.

[y] Kilograms recoverable white sugar per metric ton of fresh beets (RWS).

[x] Metric tons of recoverable white sugar per hectare (RWSH) calculated for each replicate using the following equation: RWSH (metric ton/hectare) = RWS (kg/metric ton) × Total Yield (metric ton/hectare) ÷ 1000; treatment means across four replicates are shown.

2.5 Discussion

Fall heat treatment of CLS-infested sugarbeet foliage (at temperatures 649-871°C) consistently reduced *C. beticola* sporulation in planta immediately following burner application. Reductions in isolations from leaves were not consistently observed, however, frequencies were low due to inhibition of *C. beticola* growth by competition of abundant soil microorganisms and potential antagonists. The sporulation results support previous reports that high temperatures were lethal to *C. beticola* (Pool and McKay, 1916). Following either fall- or spring-applied heat treatments, CLS levels in the subsequent sugarbeet crop were also reduced with consistent decreases in AUDPC. These observations indicate initial reductions in *C. beticola* viability and overwintering survival further impacted subsequent conidia production and in- field CLS severity. Burning for "thermosanitation" has been shown to reduce inoculum and incidence of plant pathogens in forestland, fruit crops, cotton, sugarcane, and grain crops (Hardison, 1976). In particular, burning stubble was shown to effectively control *Claviceps purpurea* (causal agent of ergot) and *Gloeotinia temulenta* (causal agent of blind seed disease) in grass seed crops (Hardison, 1980). It is important to note that the method tested in this study did not burn the leaves (Figure A2.1C) or yield large amounts of smoke, which are problems with burning for disease management. Moreover, temperatures at or above 121°C were found to reduce *Rhizoctonia oryzae-sativae* sclerotia survival *in vitro* (Lanoiselet et al., 2005). Together, these studies demonstrated that high temperatures for brief periods of time can affect fungal survival, reproduction, and subsequent disease development, which is likely to be a factor for *C. beticola* as well, even in leaf tissue.

Beyond the overwintering impacts, the fall-applied heat treatment at 1.6 kmph consistently resulted in significant reductions in early-season spore levels. No notable reductions were

achieved using the plow or desiccant treatments or using heat treatment at 4.8 kmph. The 4.8 kmph heat treatment did not significantly reduce early-season *C. beticola* levels, which indicates that a longer heat exposure, as would be provided by the 1.6 kmph treatment, may provide more effective and more consistent inoculum management. High temperatures over a certain period of time are typically needed to eliminate a particular pathogen, though this is temperature and pathogen dependent (Suárez- Estrella et al., 2003; Lanoiselet et al., 2005; Jung et al., 2009). Significant impacts on *C. beticola* were observed in the current study after an estimated less than 1 second exposure per plant (approx. 5-second exposure per meter) to temperatures of 649-871°C.

The results of this study also demonstrated the potential for both heat treatments, along with the already well-established plow treatments, to increase leaf degradation over the winter, which may also contribute to reductions in subsequent inoculum levels. Plowing has long been known to reduce CLS survival (Townsend, 1914). Buried residue has been shown to decompose faster than residue left on the soil surface (Nagel, 1938; Solel, 1970; Ruppel, 1986; Pereyra et al., 2004; Khan et al., 2008) with potential to reduce pathogen survival. Similarly, buried wheat residue decomposition to less than 2% after 24 months was associated with 50% reductions in colonization of *Fusarium graminearum* (syn. *Gibberella zeae*) inoculum, the disease-causing agent of Fusarium head blight (Pereyra et al., 2004). The current study demonstrated that the plow treatment (depth of 15 cm) increased sugarbeet leaf residue degradation numerically by 5 to 10 percentage points compared to the control at 90- and 135-DPH in 2020-21, with corresponding significant reductions in *C. beticola* sporulation observed up to 90- DPH. Notably, the fall heat treatment at 1.6 kmph resulted in comparable increases in residue degradation and reductions in *C. beticola* sporulation. Overall, significant impacts of tillage and heat treatment on sugarbeet leaf

residue (and *C. beticola* viability) were achieved after only 3 to 4 months. These findings support the potential use of heat treatment as a viable alternative to plowing, which is more compatible with current recommendations for limited tillage (Hao et al., 2001; Tzilivakis et al., 2005).

While effective at managing crop residues, studies also have shown that soil-disturbing management strategies, such as plowing and tillage, can have undesirable impacts on soil structure, nutrient levels, and microbial populations (Hungria et al., 2009; Miura et al., 2015; Xue et al., 2018). Further tillage consequences can include reduced soil fertility, loss of soil structure and porosity, as well as reduced soil organic matter and beneficial soil organisms (Rieke et al., 2022). Conventional tillage also may increase soil erosion, pesticide and nutrient runoff, and emission of greenhouse gases (Withers and Lord, 2002; Cerdan et al., 2010; Smith et al., 2016). Minimum tillage or no-till has been a recommended practice globally, especially in areas with limited water (Hao et al., 2001; Tzilivakis et al., 2005). In sugarbeet, root and sugar yields were not significantly altered from conventional tillage to no-till management systems and an estimated $111/ha could be saved through decreases in fuel, labor, and total machine costs under a no- till system (Afshar et al., 2019). While plow and tillage treatments have been shown to increase leaf degradation and reduce CLS pressure in numerous experiments (see above) including the current study, similarly effective alternative practices for beet residue management, such as heat treatment, would be valuable for the industry. The current study supports the need for additional management tools for conventional and organic systems with the fall- and spring-applied heat treatments showing greater promise as an integrated pest management strategy compared to tillage.

Despite consistent reductions in *C. beticola* sporulation over the winter following plow treatments, there were no observable differences in early-season *C. beticola* conidia levels

detected by sentinel beets and inconsistent impacts on AUDPC between trial years and locations. In Michigan, variability in efficacy of the plow treatment could be attributable to differences in winter conditions. Total precipitation from September to April was 190 mm more in 2019-20 (Figure A2.3A) than 2020-21 (Figure A2.3B); winter soil temperature during the same interval was slightly lower in 2020-21 compared to 2019-20 (Enviroweather, n.d). Differences in soils and environments are known to impact *C. beticola* survival. Changes in soil moisture, temperature, and microbial communities could all affect residue decomposition (Homet et al., 2021). Due to the variability in plow and tillage impacts observed here, the heat treatment may offer targeted and more reliable effects on *C. beticola* and subsequent CLS management, but this will need further testing in varied environments.

Compared to tillage treatments, heat treatment was expected to minimize effects in the soil profile (Hardison, 1976; Rahkonen et al., 1999). For example, minimal soil heating occurs during open grass fires and there is very little impact on the soil surface by flame treatment (Hardison, 1976). Furthermore, propane heat treatment at 100 kg/ha had little effect on microbial biomass in the upper soil profile where temperature increases of 4.0°C and 1.2°C were observed at 5- and 10-mm depths, respectively (Rahkonen et al., 1999). Thus, heat treatments present minimal threat to the soil microbial communities. In the current study, both heat treatments were briefly applied, with the fall treatment applied above the soil at canopy level and considerable reductions in temperature at and below the soil surface, further limiting the temperature impact on the soil structure and microbial communities. No negative impacts of the heat treatment were observed on sugarbeet yield or quality, however, further studies ofpotential impacts on microbial communities would be necessary. Notably, the heat treatment did not negatively affect sugar levels. Yields across all trial years in the Michigan tests were limited and below industry standards of

approximately 64 MT/ha (USDA-NASS (United States Department of Agriculture, National Agricultural Statistics Services), 2019), likely due to back-to-back planting of sugarbeets in two consecutive years (research tool only), minimal pre-season nutrient inputs, and no fungicide applications to manage diseases, including CLS, Rhizoctonia root and crown rot, and others present in the region. Current conclusions, therefore, focus on the potential of each practice to manage *C. beticola* survival in sugarbeet residues and the reductions in subsequent CLS pressure.

The cost of heat treatment is relatively inexpensive, compared to time and labor, for weed management (Crop watch, 2015; Flame Engineering Inc, n.d) or application costs for fungicide applications, as well as additional soil compaction if repeatedly entering the field for ground applications. At 1.6 kmph, the propane-fueled, tractor- mounted heat treatment used for the fall-applied field study used approximately $300/ha in propane (output of 465 L/ha), based on the ten-year average for U.S. residential propane prices of $0.63/L (ten-year minimum: $0.47; maximum: $0.97) (US DOE-EIA (United States Department of Energy - Energy Information Administration), 2022). In Michigan, sugarbeet growers can spend between $350-600/ha for one season of foliar disease management (7-8 fungicide applications each $50-75/ha), based on estimated prices of standard products and dependent on variety tolerance and chemical program used (personal communication, C. Guza, Michigan Sugar Company, 2022); similarly, in Minnesota, sugarbeet growers can spend $300-375/ha. The heat treatment alone was not sufficient for a commercially acceptable level of disease management in Michigan or Minnesota, but it could delay or reduce the number of fungicide sprays in the growing season. Observed reductions in CLS severity following spring and fall heat treatments were sustained over several weeks of the sugarbeet growing season (up to four to eight weeks after initial detection). These reductions suggest that integration of a heat treatment into an existing management program has potential to

replace up to two to four early-season fungicide applications (e.g., up to $300/ha in an 8-application foliar program in Michigan). Localized research on how heat treatment would fit into a larger CLS management program is needed before such technology could be adopted by beet growers.

Previous studies have demonstrated that propane-fueled flame management can be a useful strategy for weed and disease control for both organic and conventional production systems (Hardison, 1976; Hardison, 1980; Lanoiselet et al., 2005). Furthermore, heat treatments offer a potential integrated option where there are documented fungicide resistance issues, as heat exposure is a completely different mode of disease management. Pesticide use can lead to resistance development in weed and pathogen pests (OEPP/ EPPO, 1999; Leadbeater et al., 2019), and reduced sensitivity to multiple fungicide classes has been observed in widespread *C. beticola* populations. Considering the application and product costs associated with chemical control, heat treatments may be a beneficial tool to use in beet growing regions where CLS epidemics are frequent, severe, and where fungicide use or efficacy is limited due to resistance in *C. beticola* populations. Heat treatment could further be of interest where fungicide resistance and residue management are a concern for other Cercospora species, such as *C. zeae-maydis* (gray leaf spot of corn), *C. kikuchii* (leaf blight and purple seed stain of soybean), *C. sojina* (frogeye leaf spot of soybean), *C. arachidicola* (peanut), and *C. carotae* (leaf spot of carrot) (Farr et al., 1989).

Results from Michigan and Minnesota field trials (2019-22) consistently indicate the use of a foliar heat treatment at-harvest or pre-planting has the potential to significantly reduce inoculum and CLS levels for the following growing season. A heat treatment could be useful for regions annually impacted by CLS and might be used to reduce fungicide applications, as well as mitigate fungicide resistance development. The use of foliar heat treatment is a novel control

strategy for managing CLS as an additional tool in an integrated pest management program.

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APPENDIX A

Table A2.1. Early-season Cercospora leaf spot (CLS) lesion observations on live spore traps[z] (sugarbeet plants) in Michigan field studies sampled in 2020, 2021, and 2022 (the year following fall-applied treatments).

Year	Week	Placed in the field	Removed from the field	Sampling duration (days)	Days after planting
2020 [y]	**1**	**26-May**	**2-Jun**	**7**	**39-46**
2020	**2**	**2-Jun**	**9-Jun**	**7**	**46-53**
2020	3	16-Jun	23-Jun	7	60-67
2020	4	23-Jun	30-Jun	7	67-74
2020	5	30-Jun	7-Jul	7	74-81
2020	6	7-Jul	14-Jul	7	81-88
2020	**7**	**14-Jul**	**21-Jul**	**7**	**88-95**
2021	1	14-May	21-May	7	7-14
2021	**2**	**1-Jun**	**8-Jun**	**7**	**25-32**
2021	**3**	**15-Jun**	**22-Jun**	**7**	**39-46**
2021	**4**	**29-Jun**	**6-Jul**	**7**	**53-60**
2022	**1**	**17-May**	**24-May**	**7**	**18-25**
2022	**2**	**24-May**	**31-May**	**7**	**25-32**
2022	3	1-Jun	8-Jun	7	33-40
2022	**4**	**15-Jun**	**22-Jun**	**7**	**47-54**

[z] Live spore traps were placed in the center of each plot and consisted of four sentinel beets of USDA germplasm F1042 (USDA Agricultural Research Service, 2017) to detect viable *Cercospora beticola* airborne spores (Bublitz et al., 2021).

[y] Bolded weeks were included in the results and analyses. No or low detections in weeks not shown due to low spore concentrations or other external insect or drought stress factors.

Figure A2.1. **(A-B, D)** Custom designed 3.25-m wide propane-fueled, tractor-mounted shield burner (Multi-Trail Enterprises LLC) used for application of the fall heat treatments calibrated to heat foliage to 649-871°C. **(C)** Sugarbeet foliage immediately following 1.6 kmph heat treatment. **(E)** Handheld Flame King Heavy Duty Propane Torch Weed Burner (Pico Rivera CA 90660) used to apply spring heat treatments.

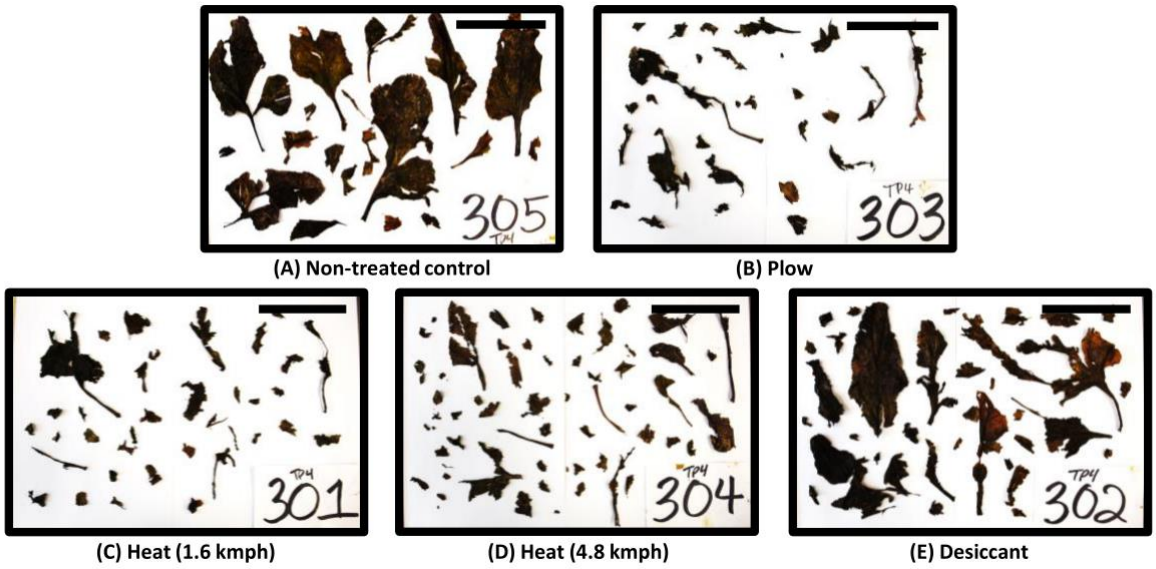

Figure A2.2. Sugarbeet leaf degradation in overwintered samples collected 135-days post-harvest (DPH) from one representative replicate of field plots treated with the following: **(A)** non-treated control, **(B)** plow with a 3-m tandem disc set to invert soil 15 cm. immediately post-harvest, heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at **(C)** 1.6 kmph and **(D)** 4.8 kmph prior to defoliation, and **(E)** a desiccant (Sharpen 0.07 L/ha, methylated seed oil 1% v/v, ammonium sulfate 2037 g/L) applied seven days pre-harvest (scale bar = 10 cm). Field plots were located at the Saginaw Valley Research and Extension Center in Frankenmuth, MI; all treatments were applied in the fall prior to or immediately following sugarbeet harvest in 2020 and leaf sampling continued through 135-DPH in 2021. Samples consisted of eight mid-canopy leaves which were collected at 0-DPH, weighed, and returned to the field in mesh bags to overwinter at representative soil depths for 45-, 90-, and 135-DPH.

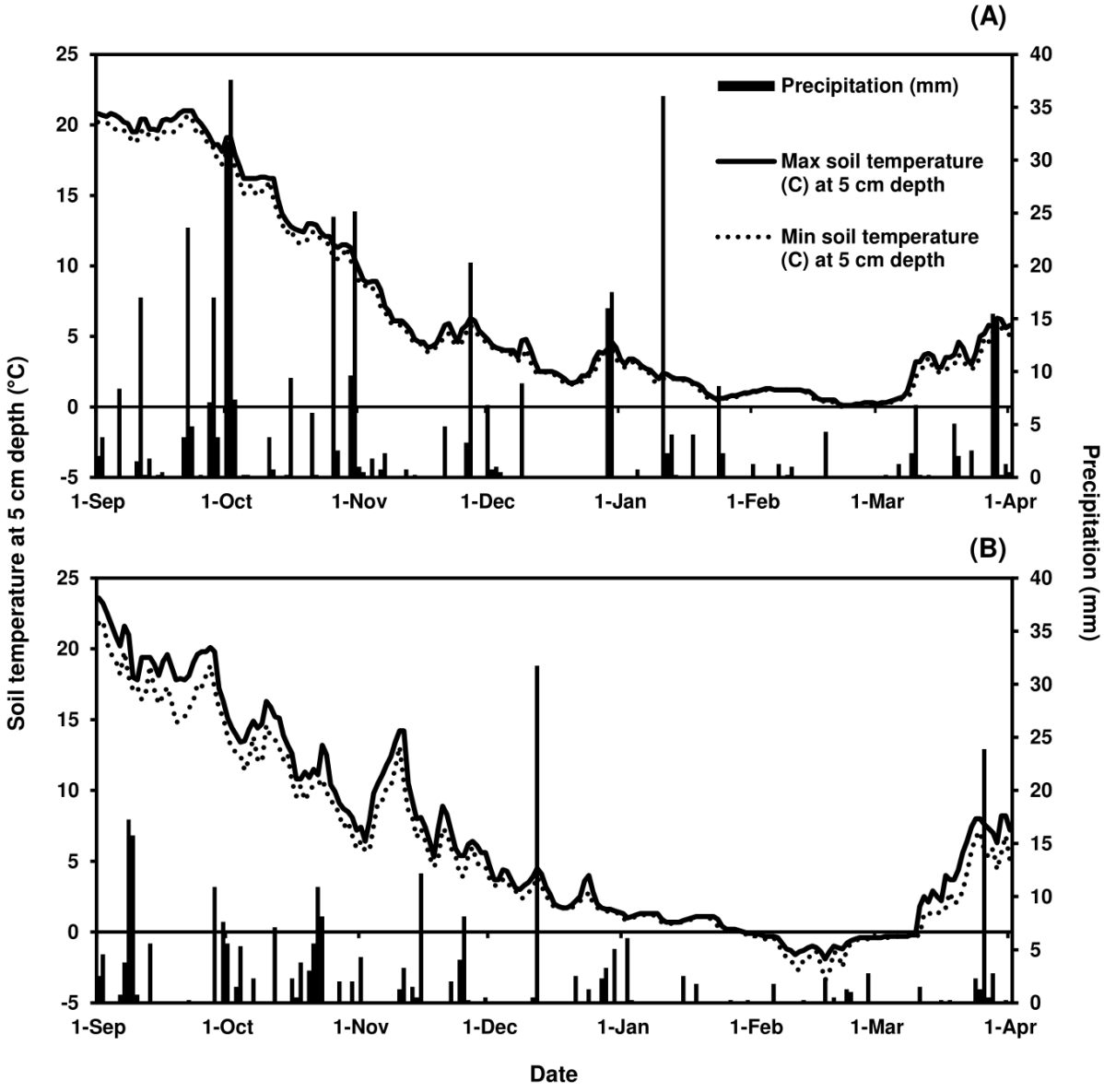

Figure A2.3. Daily maximum (solid line) and minimum (dotted line) soil temperatures at a depth of 5 cm and total precipitation (solid bars) measured from **(A)** September 1, 2019 to April 1, 2020 and **(B)** September 1, 2020 to April 1, 2021 at the Saginaw Valley Research and Extension Center in Frankenmuth, Michigan. Data were collected from the Richville/Frankenmuth weather station supported by Michigan State University and the Enviroweather project.

APPENDIX B

Cover crop and humic substance inhibition of *Cercospora beticola* as a cultural management strategy for controlling Cercospora leaf spot on sugarbeet

B1 Introduction

Additional tools for integrated pest management will suppress CLS pressure and improve the longevity of fungicide active ingredients. Cultural management strategies, such as planting of cover crops, have been considered for pathogen and disease management (Gao et al. 2022; Maglione et al. 2022). Cover crops have the potential to manage soil-borne diseases, while improving soil structure and enhancing the soil microbial community (Khan et al., 2008). Cover crop integration can disrupt the life cycle of the CLS pathogen by targeting *C. beticola* survival on infected leaf debris. Spread of *C. beticola* could be reduced by cover crops as they act as a physical barrier to inhibit movement of spores both pre- and post-termination. Dense cover crops could impede water- and wind-dispersed spores originating from the soil surface. In addition to disease management, rye cover crops have resulted in positive impacts sugarbeet yield and quality (Wilson and Smith, 1992).

Soil characteristics, such as pH, could have an important impact on crop residue and *C. beticola* inoculum. Altering the pH of the environment where *C. beticola* survives may be an effective alternative management strategy for control CLS on sugarbeet. While *C. beticola* was able to grow at wide range of pH, *in vitro* growth of *C. beticola* mycelia had been reduced at high pH levels above 8 and optimal growth was seen at a pH of 6.4 (Verma & Agnihotri, 1972). Higher pH levels also have been reported to enhance the competitiveness of *C. beticola* by suppressing the growth of fungal saprophytes like *Botrytis cinerea* and *Neocamarosporium betae* (Berl.) Ariyawansa & K.D. Hyde (formerly known as *Phoma beta*e), potentially aiding survival

of *C. beticola* overwintered (Jayawardana & Hanson, unpublished). Further investigation into impact of pH on *C. beticola* survival in leaf residue could identify alternative avenues for managing CLS pressure.

The direct effect of cover crops as a management tactic for CLS is unknown. Cover crops may produce compounds that are toxic to *C. beticola* or enhance the plant's defense mechanisms (Gao et al. 2022; Maglione et al. 2022). Research to determine the direct effect of cover crops on *C. beticola* needs to be explored, particularly in the context of Michigan's climate and soil conditions. Investigating the interactions between various cover crops and *C. beticola* could be used for disease management in sugarbeets. This study aims to determine the impact of different cover crops and pH on *C. beticola* growth and CLS on sugarbeet in Michigan. This research will provide valuable insights for sustainable disease management strategies for CLS on sugarbeet.

B2 Methods

B2.1 2022 field trial

The field trial testing the second year of the 4.8-kmph heat treatment included additional alternative management treatments aimed at reducing *C. beticola* survival over the winter. A rye cover crop and two rates of factory lime were evaluated for their ability to reduce *C. beticola* inoculum survival over the winter compared to a non-treated control. Trial details and data collection information as described in the 2021-22 Michigan field study section (2.3 Methods).

B2.2 *In vitro* germling-induced inhibition of *C. beticola*

Initial experiments were conducted by JaeJun Park under direct supervision by the Willbur Potato and Sugar Beet Pathology Program through the Michigan State University High School Honors Science Program. The following methods were similar to those tested during the

high school project and the results to follow were a continuation of this work by conducting a second iteration of the experiments.

Cover crop seeds were surface disinfested, germinated, and plated adjacent to *C. beticola* isolates to assess inhibitory properties of five cover crop seed types including rye, crimson clover, radish, mustard, and oat compared to a no seed control. 'Wheeler' rye (*Secale cereale*), crimson clover (*Trifolium incarnatum*), yellow mustard (*Sinapis alba*), common oat (*Avena sativa*), and oilseed radish seeds (*Raphanus sativus*) (Johnny's Selected Seeds, Winslow, ME) were surface disinfested using a one-minute immersion in 70% ethanol, followed by a 30-minute soak in an 80% bleach (8.25% sodium hypochlorite) solution. Seeds were rinsed twice with sterile deionized water and placed in a sterile paper towel lined, 100mm x 100mm square Petri dish. Once the seeds germinated, five seeds were positioned 3 cm from the edge of the Petri dish in a straight line on sugarbeet leaf extract agar (SBLEA; Forsyth et al., 1963) and soil extract agar plates (Leslie and Summerell, 2006). The antibiotics, streptomycin at 0.5 μg/ml and ampicillin at 0.25 μg/ml, were added to both media types in the second iteration of this experiment due to bacterial contamination from the seed. A 5-mm plug from a pure culture of *C. beticola* isolate, 'Blum 1-2' or 'RangeA', was placed on the opposite side of the media plate, 3 cm from the edge (Figure B2.1). This experiment tested four replicates of each seed, media, and isolate type including a no seed control. All plates were placed on a light bank (daylight 6500K) at room temperature (21-24°C) with 18 hours of light and 6 hours of dark for the duration of the study. Isolate radial growth on the seed-bearing and seedless sides were measured using a digital caliper at one-week and two-weeks after initiation of the experiment. Percent growth of *C. beticola* was calculated for each seed treatment, as well as the control plates without seeds, by dividing the seed side radius by the no-seed side radius.

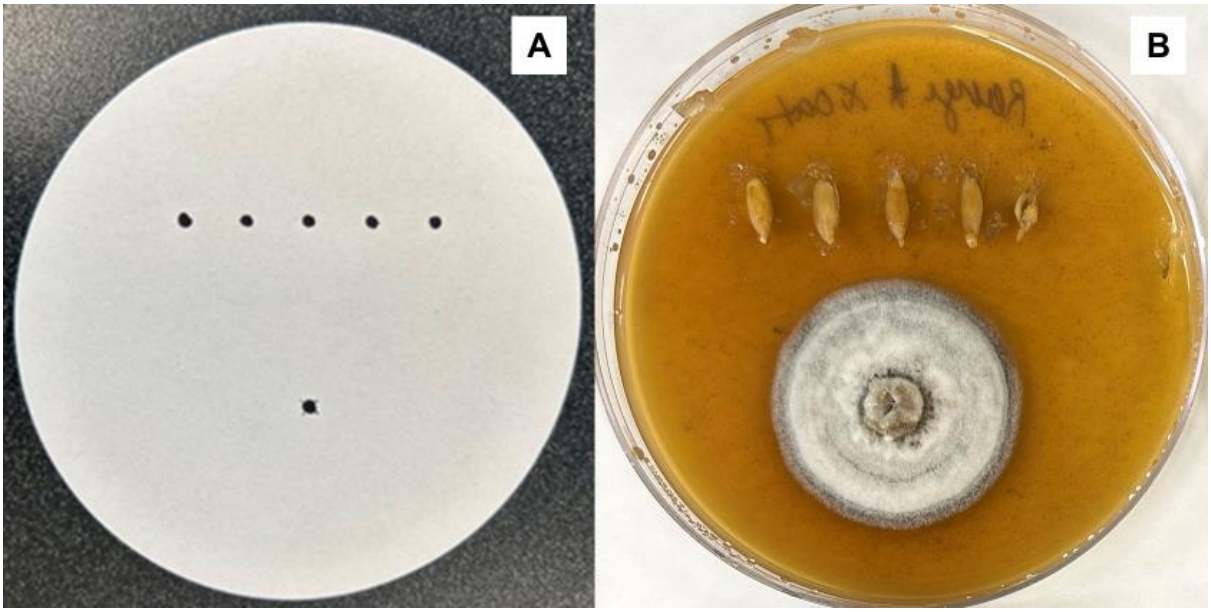

Figure B2.1. A) Template for the placement of cover crop seeds and *C. beticola* isolate in Petri dishes. B) Petri dish with SBLEA media with antibiotics containing the 'Range A' *C. beticola* isolate and oats seeds two weeks after placement.

B2.3 *In vitro* soil amendment-induced inhibition of *C. beticola*

Soil extract agar and SBLEA plates were amended with Fulmax (3% fulvic acid; JH Biotech inc. Ventura, CA) and Humax (12% humic acid; JH Biotech inc. Ventura, CA) to test impact on *C. beticola* growth compared to non-amended media. SBLEA and soil extract agar media were amended based on the recommended foliar (1.2-2.3 L/ha for Humax and 1-2 L/ha for Fulmax) and soil application rates (2.3-4.7 L/ha for Humax and 3-5 L/ha for Fulmax), respectively. Solutions of 1.7% and 4.2% (v/v) for Humax and a 5.0 and 3.0% (v/v) for Fulmax were created with soil extract agar and SBLEA, respectively. A 5-mm plug of Range A and Blum 1-2 isolates of *C. beticola* was placed on each amended agar plate in addition to non-amended agar with four replicates. The pH of the solutions was measured through MQuant pH indicator strips (Supelco, Bellefonte, PA) before and after the experiment. The diameter of isolate growth was measured using a digital caliper at one and two weeks after addition of isolates to seedling plates.

B2.4 2024 field trial

The field study was conducted at the Saginaw Valley Research and Extension Center (SVREC) in 2023 to 2024. Treatments were replicated four times in 3 m by 18 m plots using a randomized complete block design with 1.5 m wheat buffers. Cover crops treatments, including rye, radish, and a combination of oat and clover, were planted after sugarbeet harvest on Sept 18 with the aim to decrease *C. beticola* inoculum survival. The seeding rates for Wheeler rye (Moore Seed Farm, Elsie, MI), Defender oilseed radish (P. H. PETERSEN, Lundsgaard, Schleswig-Holstein, Germany), common oats (Johnny's Selected Seeds, Winslow, ME), and Crimson clover (Johnny's Selected Seeds, Winslow, ME) were 100, 35, 8, and 9 pounds per acre, respectively. Highly CLS-susceptible sentinel beets were placed biweekly into the plots to measure CLS pressure early in the season as described in section 2.3.4. The first sentinel beet observation from April 25 to May 2 was collected before the termination of the cover crops and wheat buffer. Subsequent sampling took place after chemical termination using Roundup (Bayer, Leverkusen, Germany) of the cover crops for May 14-21, May 28-June 4, May 11-18.

B3 Results

B3.1 2022 field trial

No significant differences in leaf degradation, *C. beticola* sporulation or viability, sentinel beet lesions, yield, percent sugar, RWSH, and RWS were observed for the Wheeler rye or factory lime treatments compared to the non-treated control (Table B2.1, B2.2, B2.3, B2.4). The Wheeler rye cover crop significantly reduced AUDPC the following season compared to the non-treated control (Figure B2.2).

Table B2.1. Percent sugarbeet leaf degradation for at-harvest and soil-incorporated post-harvest samples[z,y] collected 0-, 35-, 70-, and 168-days post- harvest (DPH) from fall-applied treatments in field studies in 2021-2022.

Treatment[z]	2021-2022 Leaf Degradation (%) [x, w]				
	0-DPH	35-DPH	70-DPH		168-DPH
Control	20.1	40.9	75.1	ab	91.4
Wheeler rye	19.5	30.3	65.5	b	91.1
Factory lime (6.7 MT/ha)	20.0	41.2	70.4	ab	89.9
Factory lime (13.5 MT/ha)	14.6	41.1	81.2	a	89.6
Heat (4.8 kmph)	12.9	37.1	63.7	b	90.9
SE	*2.3*	*11.0*	*5.8*		*1.0*
P-value [v]	*0.1365*	*0.8712*	*0.0486 **		*0.6214*
LSD	-	-	*12.2*		-

[z] Non-treated control, Wheeler rye cover crop planted immediately after harvest, two rates of factory lime applied to the soil surface immediately after harvest, and a heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C 4.8 kmph prior to defoliation.
[y] Measurements were collected from independent sets of leaves at each timepoint, not repeated measurements from the same sets of leaves over time.
[x] Percent leaf degradation calculated using initial leaf weights at-harvest and final weights post-harvest [(Initial – Final)/Initial].
[w] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05).
[v] Asterisk designations correspond to p-value thresholds<0.05 *.

Table B2.2. Cercospora leaf spot lesion sporulation (Sp) and *C. beticola* isolation frequencies (Is) from soil-incorporated sugarbeet leaf samples collected from Michigan studies at post-harvest timepoints following fall-applied treatments in 2021-2022.

Trial Year	Treatment [z]	0-DPH [y]			35-DPH		70-DPH		168-DPH	
		Sp [x, w] (%)		Is [v] (%)	Sp (%)	Is (%)	Sp (%)	Is (%)	Sp [u] (%)	Is (%)
2021-22	Control	90.8	a	7.5	10.1	0.0	2.9	0.0	0.0	0.0
	Wheeler rye	89.2	a	2.5	10.3	5.0	4.6	0.0	0.0	0.0
	Factory lime (6.7 MT/ha)	91.4	a	0.0	8.4	0.0	1.3	0.0	0.0	0.0
	Factory lime (13.5 MT/ha)	85.5	a	7.5	15.9	0.0	4.4	0.0	0.0	0.0
	Heat (4.8 kmph)	62.8	b	10.0	0.6	7.5	0.0	0.0	0.0	0.0
	SE	*3.1*		*4.5*	*13.3*	*3.1*	*1.8*	*0.0*	*0.0*	*0.0*
	P-value	*< 0.0001 ****		*0.4213*	*0.0521*	*0.3784*	*0.3375*	*NS*	*NS*	*NS*
	LSD	*9.0*		-	-	-	-	-	-	-

[z] Non-treated control, Wheeler rye cover crop planted immediately after harvest, two rates of factory lime applied to the soil surface immediately after harvest, and heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 4.8 kmph prior to defoliation.
[y] Days post-harvest (DPH).

Table B2.2. (cont'd)

[x] Percent lesion sporulation (Sp) determined following a 3-d incubation in a moist chamber at 21-23.9°C. Lesion sporulation assessed for 312 to 548 lesions per timepoint in 2021.

[w] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05).

[v] Frequency of *C. beticola* isolation (Is) determined from morphological confirmation of *C. beticola* growth from 10 representative lesions, plated on half-strength clarified V8 juice agar (Miller, 1955) amended with 0.5 g/L streptomycin and 0.25 g/L ampicillin. [u] Late-winter sporulation observations may be limited by unknown lesion maturity (e.g., number of prior in-season sporulation events).

[t] Asterisk designations correspond to p-value thresholds<0.05 *,<0.01 **,<0.001 ***; NS indicates no significant differences were detected as data were all zeroes.

Table B2.3. Number of Cercospora leaf spot lesions observed on live spore traps (sentinel beets) placed in Michigan field studies in 2022 (the year following fall-applied treatments).

Trial Year	Treatment [z]	Sentinel [y, x]					
		May 17 – May 24[w]		*May 24 – May 31*		*June 15 – June 22*	
2021-22	Control	5.1	(166)	6.4	(614)	5.7	(358)
	Wheeler rye	4.3	(80)	5.6	(289)	5.2	(202)
	Factory lime (6.7 MT/ha)	4.5	(116)	5.7	(323)	5.2	(198)
	Factory lime (13.5 MT/ha)	4.9	(144)	5.8	(355)	5.8	(333)
	Heat (4.8 kmph)	4.3	(98)	5.6	(305)	5.8	(391)
	SE	*0.4*		*0.2*		*0.4*	
	P-value	*0.566*		*0.2481*		*0.7912*	
	LSD	-		-		-	

[z] Non-treated control, Wheeler rye cover crop planted immediately after harvest, two rates of factory lime applied to the soil surface immediately after harvest, and heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 4.8 kmph prior to defoliation.

[y] Means generated under the lognormal distribution option in the GLIMMIX procedure (SAS v 9.4) of total CLS lesions counted on sentinel beets (USDA germplasm F1042) after 1-week exposure in the field, 3 d incubation in a 25°C humidity chamber, and 2 weeks in a greenhouse. (Non-normally distributed mean estimates shown in parentheses).

[x] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05).

[w] Data shown for sampling weeks: 1st, 2nd, 4th (2022). No or low detections in weeks not shown due to low spore concentrations or other external insect or drought stress factors.

[v] Asterisk designations correspond to p-value thresholds<0.05 *,<0.01**.

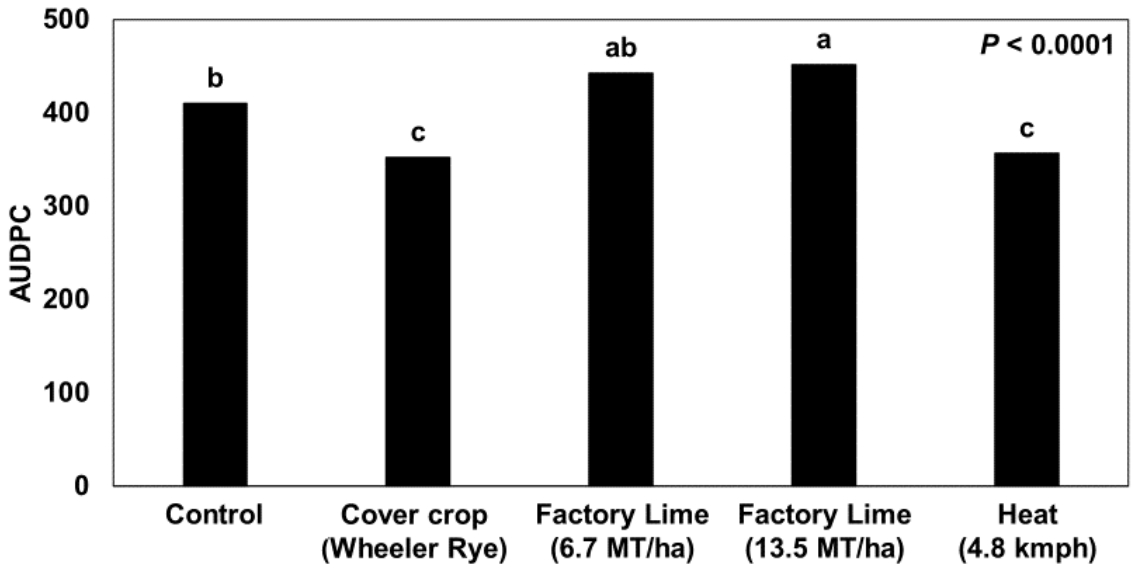

Figure B2.2. AUDPC for 2022 field trial treatments of a Wheeler rye cover crop, two factory lime rates, and the 4.8-kmph heat treatment compared to a non-treated control.

Table B2.4. Sugarbeet yield, percent sugar content, and recoverable white sugar from Michigan field studies collected in 2022 (the year following fall-applied treatments).

Trial Year	Treatment [z]	Yield (t/ha)	At-harvest		
			Sugar (%)	RWS (kg/t) [y]	RWSH (t/ha) [x]
2021-22	Control	12.2	14.0	100.7	1.2
	Wheeler rye	7.3	14.0	100.4	0.7
	Factory lime (6.7 MT/ha)	12.0	13.9	104.5	1.2
	Factory lime (13.5 MT/ha)	17.6	13.9	101.4	1.8
	Heat (4.8 kmph)	14.2	14.0	100.3	1.4
	SE	*4.0*	*0.2*	*1.6*	*0.4*
	P-values	*0.4928*	*0.2334*	*0.3343*	*0.4902*
	LSD	-	-	-	-

[z] Non-treated control, Wheeler rye cover crop planted immediately after harvest, two rates of factory lime applied to the soil surface immediately after harvest, and a heat treatment using a propane-fueled burner (Multi-Trail Enterprises LLC) calibrated to heat foliage to 649-871°C at 4.8 kmph prior to defoliation.
[y] Kilograms recoverable white sugar per metric ton of fresh beets (RWS).
[x] Metric tons of recoverable white sugar per hectare (RWSH) calculated for each replicate using the following equation: RWSH (metric ton/hectare) = RWS (kg/metric ton) × Total Yield (metric ton/hectare) ÷ 1000; treatment means across four replicates are shown.

B3.2 *In vitro* germling-induced inhibition of *C. beticola*

The Crimson clover reduced the growth of *C. beticola* on SBLEA after two weeks in the absence of antibiotics ($P < 0.01$; Table B2.5). Crimson clover and Defender oilseed radish did

not impact *C. beticola* growth when media was amended with antibiotics (Table B2.6). Wheeler rye seedlings did not significantly impact *C. beticola* growth when antibiotics were not introduced into media (Table B2.7). Wheeler rye seeds reduced *C. beticola* growth when antibiotics were added to both media types at two weeks ($P < 0.01$; Table B2.8). Yellow mustard did not significantly change the growth of *C. beticola* on either media type with antibiotics (Table B2.9). Common oat resulted in significantly increased growth of *C. beticola* on soil extract agar with antibiotics after one week but not at two weeks or on SBLEA ($P < 0.05$; Table B2.10).

Table B2.5. Percent growth of *C. beticola* at 1 week and 2 weeks after placement adjacent to germinated seed of Crimson clover and Defender oilseed radish on SBLEA and soil extract agar (EA) without antibiotics.

Treatment	**Percent growth (%)**			
	SBLEA		Soil EA	
	Week 1	Week 2	Week 1	Week 2
Control	98.1	98.1 a	-	-
Crimson clover	97.3	76.3 b	-	-
Defender oilseed radish	100.5	95.8 a	-	-
SE	*4.2*	*3.0*	-	-
P-value	*0.8619*	*0.0069* **	-	-
LSD	-	*11.6*	-	-

Table B2.6. Percent growth of *C. beticola* at 1 week and 2 weeks after placement adjacent to germinated seed of Crimson clover and Defender oilseed radish on SBLEA and soil extract agar (EA) amended with antibiotics streptomycin (0.5 ug/ml) and ampicillin (0.25 ug/ml).

Treatment	**Percent growth (%)**			
	SBLEA		Soil EA	
	Week 1	Week 2	Week 1	Week 2
Control	91.3	100.1	91.1	107.5
Crimson clover	92.6	103.3	94.7	115.0
Defender oilseed radish	95.8	102.3	102.8	112.0
SE	*2.1*	*1.9*	*7.7*	*6.4*
P-value	*0.41*	*0.5087*	*0.6428*	*0.7619*
LSD	-	-	-	-

Table B2.7. Percent growth of *C. beticola* at 1 week and 2 weeks after placement adjacent to germinated seed of Wheeler rye on SBLEA and soil extract agar (EA) without antibiotics.

Treatment	**Percent growth (%)**			
	SBLEA		Soil EA	
	Week 1	Week 2	Week 1	Week 2

Table B2.7 (cont'd)

Control	**99.1**	**100.5**	-	**85.1**
Wheeler rye	100.2	87.8	-	95.8
SE	*3.3*	*2.9*	-	*4.6*
P-value	*0.8515*	*0.1863*	-	*0.3355*
LSD	-	-	-	-

Table B2.8. Percent growth of *C. beticola* at 1 week and 2 weeks after placement adjacent to germinated seed of Wheeler rye on SBLEA and soil extract agar (EA) amended with antibiotics streptomycin (0.5 ug/ml) and ampicillin (0.25 ug/ml).

Treatment	**Percent growth (%)**			
	SBLEA		Soil EA	
	Week 1	Week 2	Week 1	Week 2
Control	95.2	103.7 a	113.7	99.1 a
Wheeler rye	98.1	81.4 b	89.6	88.2 b
SE	*5.6*	*3.1*	*4.3*	*3.0*
P-value	*0.793*	*0.002 **	*0.0778*	*0.0071 **
LSD	-	*7.0*	-	*5.2*

Table B2.9. Percent growth of *C. beticola* at 1 week and 2 weeks after placement adjacent to germinated seed of yellow mustard on SBLEA and soil extract agar (EA) amended with antibiotics streptomycin (0.5 ug/ml) and ampicillin (0.25 ug/ml).

Treatment	**Percent growth (%)**			
	SBLEA		Soil EA	
	Week 1	Week 2	Week 1	Week 2
Control	95.5	100.5	99.9	105.7
Yellow mustard	103.9	102.7	96.5	112.4
SE	*6.6*	*3.1*	*7.7*	*5.2*
P-value	*0.3779*	*0.4626*	*0.7993*	*0.2217*
LSD	-	-	-	-

Table B2.10. Percent growth of *C. beticola* at 1 week and 2 weeks after placement adjacent to germinated seed of common oat on SBLEA and soil extract agar (EA) amended with antibiotics streptomycin (0.5 ug/ml) and ampicillin (0.25 ug/ml).

Treatment	**Percent growth (%)**			
	SBLEA		Soil EA	
	Week 1	Week 2	Week 1	Week 2
Control	96.3	103.7	84.6 b	110.4
Common oat	92.3	104.4	122.5 a	155.8
SE	*1.6*	*3.1*	*4.4*	*22.3*
P-value	*0.1682*	*0.893*	*0.0177 **	*0.2183*
LSD	-	-	*25.4*	-

B3.3 *In vitro* soil amendment-induced inhibition of *C. beticola*

Fulmax altered the pH of both media types compared to the control (Table B2.5). Both Fulmax and Humax reduced the growth of *C. beticola* after one and two weeks ($P < 0.01$; Figure B2.3). After two weeks, the impact of Humax on *C. beticola* growth was significantly higher than Fulmax ($P < 0.001$).

Table B2.11. pH of each media type, SBLEA and soil extract agar (Soil EA) non-amended and amended with Fulmax and Humax.

Media	Amendment	pH
SBLEA	Control	8
	Fulmax	7
	Humax	8
Soil EA	Control	6.5
	Fulmax	8
	Humax	6.5

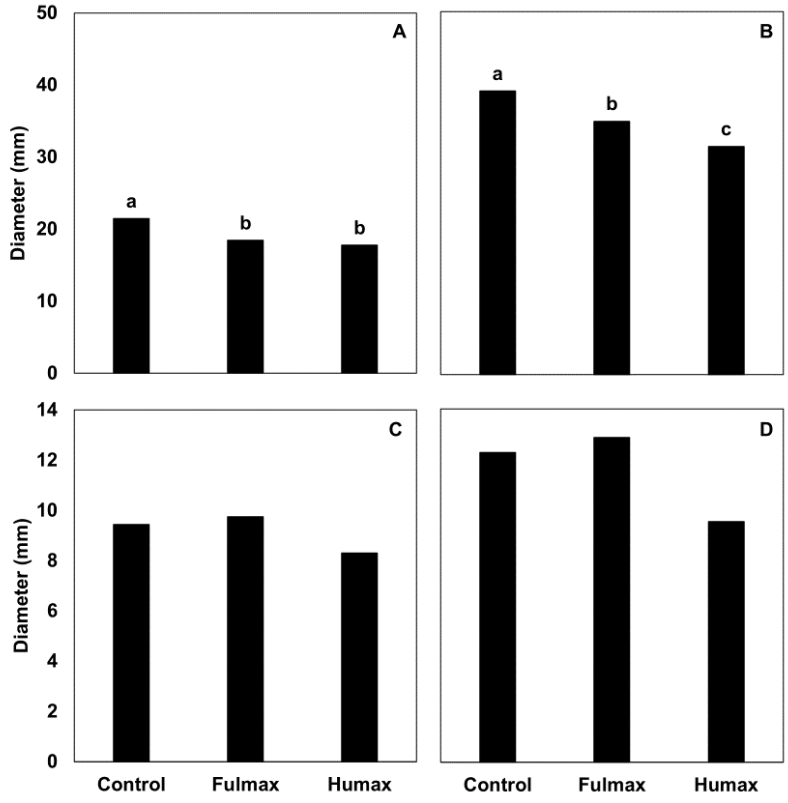

Figure B2.3. Diameter of *C. beticola* growth after A) one ($P < 0.01$) and B) two weeks ($P < 0.001$) after introduction to SBLEA media and after C) one and D) two weeks after *C. beticola* introductions to soil extract agar with a non-amended control, Fulmax, and Humax. Fulmax and Humax rates were determined based on labeled rates for foliar application.

B3.4 2024 field trial

No significant difference between the CLS ratings on sentinel beets was observed for treatments in this study (Figure B2.3).

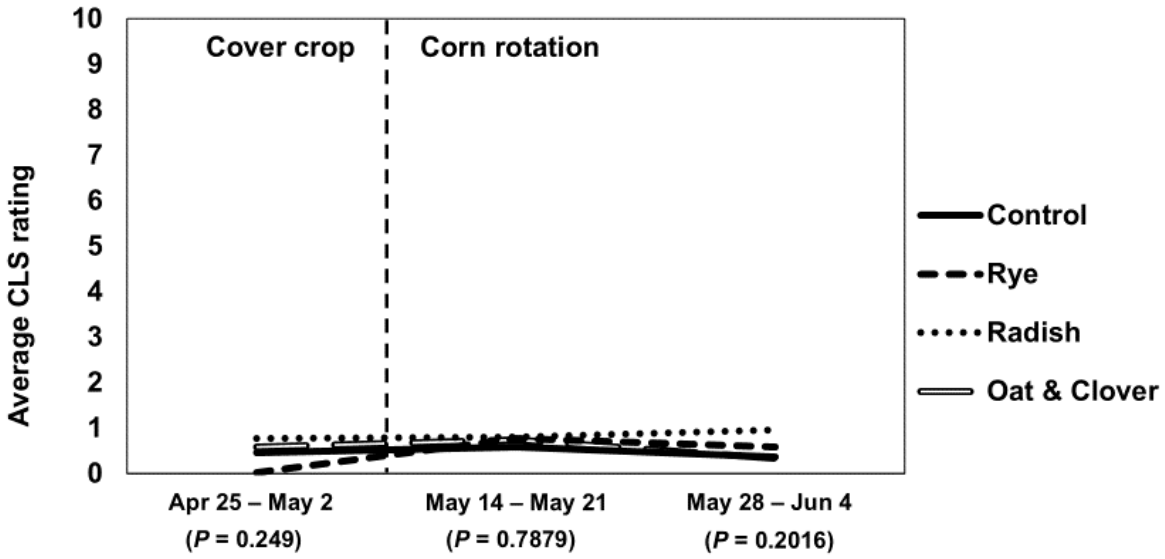

Figure B2.4. Average CLS bi-weekly rating on sentinel beets for the Wheeler rye, Defender oilseed radish, and the common oats and Crimson clover treatment compared to a non-treated control.

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CHAPTER 3: EFFECTS OF PYRACLOSTROBIN ROTATIONS AND TANK-MIXTURES ON *CERCOSPORA BETICOLA* SENSITIVITY AND CERCOSPORA LEAF SPOT CONTROL IN SUGARBEET

A version of this chapter has been published in *Crop Protection*: Hernandez, A. P., Ruth, S., Bloomingdale, C., Hanson, L. E., and Willbur J. F. 2024. Effects of pyraclostrobin rotations and tank-mixtures on *Cercospora beticola* sensitivity and Cercospora leaf spot control in sugarbeet. Crop Prot. 182:1-10 doi: 10.1016/j.cropro.2024.106710.

3.1 Abstract

Sugarbeet production supplies approximately 50–60% of domestic sugar in the United States. One of the most economically important foliar diseases of sugarbeet is Cercospora leaf spot (CLS), caused by *Cercospora beticola*. Fungicide resistance is a major concern due to the frequent applications needed to control multiple infection cycles each season and the reliance on single-site fungicides. Reduced *C. beticola* sensitivity to multiple fungicide groups, including the quinone outside inhibitors (QoIs) has been observed. The QoI fungicide Headline (23.6% pyraclostrobin) was exceptionally effective in controlling CLS until high levels of reduced sensitivity stabilized in *C. beticola* populations. This study aimed to test application programs to minimize *C. beticola* pyraclostrobin resistance development and maximize CLS management. Rotation (high- and low-risk) and tank-mixture pro- grams integrating pyraclostrobin (Headline™) with mancozeb (a multi-site fungicide) treatments were evaluated in 2019 and 2020 field studies. For all Headline programs, distributions of *C. beticola* pyraclostrobin sensitivities were not significantly different from each other but differed from the non-treated control ($P < 0.05$). In 2020, a significant increase in the percentage of isolates with half-maximal effective pyraclostrobin concentrations ≥5 μg/ml was noted in all Headline treatments ($P < 0.05$), even where a single application of Headline alone was used. Furthermore, no additional CLS control, yield, or sugar benefits from Headline were observed when compared to grower standard programs without Headline ($P > 0.05$). In conclusion, pyraclostrobin-based products used in chemical rotations or tank-mixtures were not more beneficial than lower-risk programs despite their historical CLS control efficacy.

3.2 Introduction

Sugarbeet is one of the major sucrose production crops (Draycott, 2006). Michigan

produced over 3.6 million metric tons of sugarbeet in 2022, which placed it 4th in the nation and accounted for 12.4% of total production in the United States (USDA-NASS, 2022). Both yield and sugar production can be affected by numerous biological agents, such as weeds, insects, and diseases (Draycott, 2006). Among the latter, Cercospora leaf spot (CLS), caused by *Cercospora beticola* Sacc., is one of the most economically important foliar diseases in many sugarbeet growing regions (Weiland and Koch, 2004; Jacobsen and Franc, 2009; Khan et al., 2009). Management of CLS relies heavily on timely fungicide applications throughout the growing season (Khan et al., 2007; Secor et al., 2010). Additionally, *C. beticola* is classified as a medium-risk pathogen for fungicide resistance development (FRAC pathogen risk list, 2019) due to repeated cycles of yearly proliferation, considerable genetic diversity (Vaghefi et al., 2016, 2017a, 2017b), and the noteworthy number of fungicide applications each season (average of 6–8 in areas of high CLS pressure; personal communication, C. Guza, Michigan Sugar Company). A major concern in sugarbeet production is the development of insensitive *C. beticola* populations to different classes of fungicides (Bolton et al., 2012, 2013; Davidson et al., 2006; Weiland and Halloin, 2001).

Continuous use of fungicide active ingredients with a single mode of action to control disease imposes selection pressure on the target pathogen population (Eckert and Ogawa, 1988). While the use of single-target active ingredients has greatly increased product specificity, this practice has increased the probability of the development of fungicide insensitivity in some fungal pathogens (Stevenson et al., 2019). Over the years, fungicide groups that have been commonly used and registered for CLS management on sugarbeet include single-site mode of action fungicides such as methyl benzimidazole carbamates (MBC, FRAC group 1), quinone outside inhibitors (QoI, FRAC group 11), and demethylation inhibitors (DMI, FRAC group 3), as well as

those with muti-site contact activity such as organotin (FRAC group 30), inorganic copper (FRAC group M01), and dithiocarbamate (FRAC group M03) fungicide classes (Rangel et al., 2020). To limit fungicide resistance development, rotating different active ingredients with varying modes of action, limiting the number of applications in one growing season, using broad spectrum tank-mix partners, utilizing labeled rates, and employing IPM strategies are recommended (Corkley et al., 2022; van den Bosch et al., 2014a, 2014b).

QoI fungicides are important for management of diseases caused by many plant-pathogenic fungi, including *C. beticola* (Gisi et al., 2000; Kirk et al., 2012; Rosenzweig et al., 2008b). QoI fungicides disrupt mitochondrial respiration by binding to the *cytochrome bc1* complex. QoIs such as pyraclostrobin are at high-risk for pathogen resistance development (FRAC code list, 2022) due to their site-specific mode of action. An experimental use permit (EUP) was granted by the Environmental Protection Agency (EPA) in 1998 and commercial registration was implemented in 2002 for the use of pyraclostrobin (Headline SC, EPA Reg. Number: 7969–289, BASF Corporation) in the control of CLS on sugarbeets. In 1999 and 2000, a study by Khan and Smith (2005) reported consistent control of CLS and high sucrose yield in programs with pyraclostrobin alone. Additionally, studies by Karadimos et al. (2005) and Secor et al. (2010) support the efficacy of QoI fungicides in controlling *C. beticola*. Shortly after registration, however, reduced sensitivity was detected in 2004 (Secor et al., 2010) followed by rapid development of resistance to QoIs (Kirk et al., 2012) which is shown to be stable in the US populations of *C. beticola* (Rangel et al., 2020). Reduced pathogen sensitivities may be associated with potential loss of control using field rates of QoI products (Kirk et al., 2012).

Cercospora beticola isolates that harbor genes associated with fungicide resistance have exhibited atypically wide ranges of *in vitro* pyraclostrobin sensitivities (Bolton et al., 2013).

Therefore, field studies are needed to understand the impacts of genetic resistance on in-field control of CLS. The most common mutation associated with genetic resistance to QoIs is the G143A amino acid shift in the *cytochrome b* gene and has been shown to confer a high level of resistance in some pathogens (Ma and Michailides, 2005), such as pistachio *Alternaria alternata* isolates with EC_{50} values greater than 100 μg/ml for azoxystrobin (Ma et al., 2003). In 2012, Rosenzweig et al. (2015) detected the G143A resistance-associated mutation in over 90% of *C. beticola* isolates from across Michigan. Similarly, Rangel et al. (2020) found that the incidence of G143A was above 90% for *C. beticola* isolates in the Red River Valley region in 2016 and in subsequent years tested through 2019. The mutation was widespread across counties and townships in Michigan sugarbeet production areas under fungicide management programs to control CLS (Kirk et al., 2012; Rosenzweig et al., 2015). However, reported *in vitro* sensitivities for QoI-resistant *C. beticola* were consistently variable for pyraclostrobin and influence on field control remains unclear.

Based on the historically superior efficacy of pyraclostrobin over other fungicides for CLS control, strategies to minimize *C. beticola* resistance development and maximize disease control and yield were of research interest. The current study tested the use of pyraclostrobin in various fungicide application programs and evaluated the resulting sensitivity of *C. beticola* populations and CLS control. Findings from field and *in vitro* studies provide critical evidence to advise the use of QoI fungicides for CLS management in sugarbeet.

3.3 Materials and methods

3.3.1 Field trials and treatment information

The field trials were established in 2019 and 2020 at the Saginaw Valley Research and Extension Center (SVREC) in Frankenmuth, MI. The location has a Tappan-Londo loam soil,

slopes of 0–3 percent (USDA-NRCS, 2019), and is not irrigated. In both years of study, the CLS-susceptible sugarbeet variety C-G333NT (REACh, 2018; REACh, 2019) was planted at a rate of 123,500 seeds/ha. In 2019 and 2020, fields were planted on April 24 or April 7 and harvested on September 16 or September 18, respectively. The sugarbeet plots were inoculated on July 9, 2019 and July 23, 2020 with a 1×10^3 *C. beticola* conidia/ml suspension using a tractor-mounted sprayer calibrated at 140 L/ha (Eujayl et al., 2022; Hernandez et al., 2023; Ruppel and Gaskill, 1971). For both years the field trial consisted of four treatments corresponding to fungicide application programs that were replicated four times and organized in a randomized complete block design (RCBD). Each plot consisted of four rows of sugarbeets with dimensions of 3 m by 18 m.

Rotation and tank-mixed treatments were evaluated where Headline (23.6% pyraclostrobin, BASF Corporation; Table 3.1) was applied either first (Treatment 2; designated "high-risk") or second (Treatment 3; "low- risk") in a chemical rotation alternating with the multi-site contact fungicide Manzate Max (37% mancozeb, United Phosphorus, Inc.; Table 3.1), or where Headline was tank-mixed with Manzate Max at each application (Treatment 4; "mixture") (Tables 3.2 and 3.3). Treatments were compared to a non-treated control (Treatment 1) where no fungicides were applied and a grower standard (Treatment 5) where an industry standard fungicide program was applied and no QoI fungicides were included. Fungicide applications were made with a CO2 powered backpack sprayer equipped with four TJ 8004XR nozzles (76-cm spacing), calibrated at 140 L/ha (15 gal/A) in 2019 and 187 L/ha (20 gal/A) in 2020.

Table 3.1. Fungicide product information for programs tested in sugarbeet field studies in Frankenmuth, MI in 2019 and 2020.

Product	**Active ingredient(s)**	**FRAC code**	**FRAC group**	**Mode of action**	**Rate** [a]
Headline	pyraclostrobin (23.6%)	11	Quinone outside inhibitors (QoI)	Respiration	876.9 ml

Table 3.1 (cont'd)

Manzate Max	mancozeb (37%)	M03	Dithiocarbamates and relatives	Multi-site activity	3.7 L
Inspire XT	difenoconazole (22.8%) + propiconazole (22.8%)	3	Demethylation inhibitors (DMI)	Sterol biosynthesis in membranes	511.5 ml
Super Tin	triphenyltin hydroxide (40%)	C6	Organo tin compounds	Respiration	584.6 ml

[a] All rates, unless otherwise specified, are listed as a measure of product per hectare. The adjuvant MasterLock™ was added to all tank mixes at a rate of 0.25 % v/v.

Table 3.2. Fungicide application programs tested in a Cercospora leaf spot sugarbeet field study in Frankenmuth, MI in 2019.

Timing [a]	**Treatment 2 [b] "High-risk"**	**Treatment 3 "Low-risk"**	**Treatment 4 "Mixture"**	**Treatment 5 Grower standard**
A	Headline	Manzate Max	Headline + Manzate Max	Inspire XT + Manzate Max
B	Manzate Max	Manzate Max	Manzate Max	Super Tin + Manzate Max
C	Headline	Headline	Headline + Manzate Max	Inspire XT + Manzate Max
D	Manzate Max	Manzate Max	Manzate Max	Super Tin + Manzate Max
E	Headline	Headline	Headline + Manzate Max	Manzate Max
F	Manzate Max	Manzate Max	Manzate Max	Manzate Max
No. QoI applications	3	2	3 (tank-mixed)	0

[a] Timing of application dates: A=26 Jun, B=8 Jul, C=22 Jul, D=31 Jul, E=14 Aug, F=23 Aug.
[b] Treatment 1 was a non-treated control where no fungicides were applied.

Table 3.3. Fungicide application programs tested in a Cercospora leaf spot sugarbeet field study in Frankenmuth, MI in 2020.

Timing [a]	**Treatment 2 [b] "High-risk"**	**Treatment 3 "Low-risk"**	**Treatment 4 "Mixture"**	**Treatment 5 Grower standard**
A	Headline	Manzate Max	Headline + Manzate Max	Manzate Max
B	Manzate Max	Manzate Max	Manzate Max	Inspire XT + Manzate Max
C	Headline	Headline	Headline + Manzate Max	Super Tin + Manzate Max
--- Badge SC --- [c]				
D	Manzate Max	Manzate Max	Manzate Max	Inspire XT + Manzate Max
No. QoI applications	2	1	2 (tank-mixed)	0

[a] Timing of application dates: A=29 Jun, B=13 Jul, C=21 Jul, D=20 Aug.
[b] Treatment 1 was a non-treated control where no fungicides were applied.
[c] Due to COVID-19 related complications, a blanket maintenance treatment of Badge SC (2.3 L/ha) was applied to the entire trial on August 7, 2020.

3.3.2 *In vitro* and mutation-based assessment of *Cercospora beticola* fungicide sensitivity

3.3.2.1 Isolation of *Cercospora beticola* from sugarbeet leaves

Cercospora beticola isolations were performed on infected leaf material from the following treatments for fungicide sensitivity analysis: non-treated control (Treatment 1), high-risk (Treatment 2), low-risk (Treatment 3), and mixture (Treatment 4). The grower standard (Treatment 5) was considered only for disease and yield and was not included in the fungicide sensitivity analysis. Ten to 15 CLS symptomatic leaves were collected from each plot on July 30 (after three treatment applications) in 2019 and mid-September (after all treatment applications, at-harvest) in 2019 and 2020. Characteristic CLS lesions (Jacobsen and Franc, 2009) were inspected for sporulation under a dissecting binocular stereo microscope (Leica ZOOM 2000, Wetzlar, Germany) and, if necessary, leaves were placed in plastic resealable bags with a moist paper towel to induce lesion sporulation (7-10× magnification). Initial isolations were performed on a subset of 10–15 lesions from each leaf sample (corresponding to 10–15 isolates per field treatment replicate). Due to laboratory limitations during 2019, up to 7–15 isolates for 2–4 replicates were tested for mid- and end-of season samples. Under a dissecting binocular stereo microscope, Cercospora-like conidia (Jacobsen and Franc, 2009) were gently dislodged using 15 µl of sterile deionized water and gently scraping with a sterile pipet tip. The spore suspension was transferred to a 1.5% (15 g/L) water agar media plate amended with 0.25 mg/ml of ampicillin and 0.5 mg/ml of streptomycin after autoclave sterilization of media, and a single streak was applied across the plate using a sterile glass cell spreader. Hyphal tip transfer (Brown, 1924) was conducted on single-spore isolates, morphologically characterized as likely *C. beticola* (Jacobsen and Franc, 2009), and transferred to half-strength clarified V8 juice (CV8) (Miller, 1955) agar plates amended with 0.25 mg/ml of ampicillin and 0.5 mg/ml of streptomycin. Pure cultures were

maintained for at least 2 weeks at ambient room temperature of 21 ± 2 °C in dark conditions to promote complete colonization of the 100-mm Petri dish. Putative *C. beticola* isolates were then tested for *in vitro* pyraclostrobin sensitivity; difenoconazole sensitivity also was assessed to check for background influences on isolate responses, e.g., potential movement or dispersal between field plots.

3.3.2.2 Spiral plate gradient assessment of *Cercospora beticola* fungicide sensitivity

Fungicide sensitivity of *C. beticola* was determined for the isolates recovered from the control, high-risk, low-risk, and mixture treatments for each year. A total of 117 and 196 isolates were tested in 2019 and 2020, respectively. In 2019, seven to 12 isolates per treatment collected mid-season and eight to 32 isolates collected per treatment end-of- season were screened. In 2020, 46 to 51 isolates per treatment collected end-of-season were screened. Reference *C. beticola* isolates 'Blum 1–2' (QoI-resistant) and 'Range A' (QoI-sensitive) from the United States Department of Agriculture-ARS Sugar Beet Research Unit (SBRU) fungal collection were also included as controls. Blum 1–2 was obtained from symptomatic sugarbeet leaf lesions in 2017 in Saginaw County, MI. 'Range A' was collected from a symptomatic sugarbeet leaf in 2008 in Ingham County, MI.

The spiral gradient dilution method (Förster et al., 2004) was used to determine the effective concentrations for inhibiting isolate growth by 50% (EC_{50}). Technical standards (Sigma-Aldrich, St. Louis, MO) for the active ingredients, pyraclostrobin (387.8 g/mol) and difenoconazole (406.26 g/mol), were dissolved in acetone at a concentration of 10,000 µg/ml to create a stock solution of each fungicide. To ensure consistent diffusion of active ingredients through the media, 40 ml of half-strength CV8 agar was added to each 150-mm plate to ensure a 3-mm agar depth. The total fungicide stock solution of 54.3 µl was added to the agar using an

Eddy Jet 2 spiral plater (Neutec Group Inc, Farmingdale, NY). In this study, the exponential mode of application was used, achieving a radial concentration gradient. Amended plates were incubated for 24 h at ambient light and temperature (21 ± 2 °C) conditions, resulting in a diffuse concentration gradient across the agar and evaporation of the acetone (Förster et al., 2004). The lower and upper detection limits for pyraclostrobin and difenoconazole sensitivity testing were 0.62–128.23 µg/ml and 0.60–126.30 µg/ml, respectively. The fungicide stock solution concentration was adjusted to achieve desired detection limits. Mycelial suspensions of each isolate were collected from at least 14-day-old-cultures. For each fully colonized isolate plate, 750 µl of sterile deionized water was added and a sterile glass cell spreader used to gently scrape and dislodge half of the culture into the suspension. Large clumps of mycelia were separated using the glass rod and the liquid was aspirated using a sterile pipet tip into a sterile and labeled 1.5 mL microcentrifuge tube. Then 15 µl of the mycelial suspension was added and streaked onto the spiral gradient plate from edge to center (150-mm agar plates) using an SGE spiral plate template (Spiral Biotech, Norwood, MA) to ensure consistent distribution of the suspensions. Eight isolates were tested per spiral plate. Each isolate x active ingredient combination was replicated twice, including two replicate acetone-amended control plates. Spiral plates were incubated at ambient light and temperature conditions for two weeks.

After two weeks, measurements were taken to determine the width of the isolate growth on the control plate 15 mm from the edge of plate. The 50% inhibition point was identified where the growth on the fungicide-amended plate was half the width of the isolate growth on the control plate; this point was recorded as distance from the center of the plate (in mm) using the SGE template for both replicates (Förster et al., 2004). These measurements along with the following additional parameters, active ingredient molecular weight, stock concentration (10, 000 µl/ml),

agar height (3 mm), and length of incubation (1 day), were then entered into the ECXv4.0 (v4.0; Torres, 2021) R package (Torres-Londoño et al., 2016) to calculate the concentration at which the growth of each isolate was inhibited by 50% (EC_{50}). Mean EC_{50} values were calculated by averaging the EC_{50} values of the two replicate spiral plates. For mean calculations, isolates with EC_{50} values above or below the detection limit were set to the upper or lower limit (35% of isolates tested).

3.3.2.3 Detection of *Cercospora beticola* G143A mutation associated with QoI resistance

QoI resistance is associated with the G143A point mutation present in the fungal mitochondrial *cytochrome b* (*cytb*) gene of *C. beticola* isolates (Bolton et al., 2012; Stevenson et al., 2019). DNA was extracted from one quarter of the pure culture isolate growth using a DNeasy Plant Mini Kit (Qiagen, Germantown, MD). Testing was conducted using a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay to detect a point mutation in the *C. beticola* genome associated with fungicide resistance (Rosenzweig et al., 2015). The PCR assay targeted the *C. beticola cytb* gene (325-bp amplicon) for amplifi- cation using the previously described primer pair cytbFu 5′-ACAAAGCACCTAGAACATTGG-3′ and cytbRu 5′ GAAACTCCTAAAGGATTACCTGAACC-3′ (Malandrakis et al., 2011). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR reactions consisted of 2.0 µl of genomic DNA template, 12.5 µl GoTaq Green Master 2x Mix (Promega Co., Madison, WI), and 1 µl of 10 µM of each primer in a 25-µl reaction volume. Amplifications were performed using the following parameters: 95 ◦C for 5 min followed by 35 cycles of 94 ◦C for 30 s, 52 ◦C for 30 s, 72 ◦C for 45 s, with a final 10-min extension at 72 ◦C (MiniAmp Plus thermocycler, Applied Biosystems, Waltham, MA). PCR products were separated on 1.5% (wt/vol) agarose gel in 0.5 × Tris-borate-EDTA (5.4 g Tris-base, 2.75 g boric acid, 2 ml 0.5 M

EDTA, 1000 ml H2O) stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) by electrophoresis and visualized using a Safe Imager 2.0 Blue Light Transilluminator (Invitrogen, Carlsbad, CA).

Following the amplification of the *cytb* amplicon, 10-µl volume of the PCR reaction was used for an overnight digestion. A digestion reaction consisted of 2 µl of CutSmart Buffer (New England Biolabs Inc., Ipswich, MA), 10 µl of PCR product, and 2 µl of *Fnu4HI* (New England Biolabs, Ipswich, MA) in a 20-µl reaction volume incubated at 37 ◦C for at least 1 h to overnight followed by 65 ◦C for 30 min. Restriction fragments were resolved on 2.5% agarose gels in 0.5 × TBE by electrophoresis and stained with SYBR Safe DNA Gel Stain. This point mutation changes the sequence 5′-GGTGC-3′ to 5′-GCTGC-3′, which is cleaved by the restriction endonuclease *Fnu4HI* and confers resistance to QoI fungicides (Ma and Michailides, 2005). The base pair change detected using the PCR-RFLP assay was confirmed in 16 representative isolates using Sanger sequencing through the MSU Genomics Core (Figs. S3.1A and B). For these 16 isolates, the Basic Local Alignment Search Tool (BLASTN) also was used to compare sequence similarity to known sequences in the National Center for Biotechnology Information (NCBI) database to confirm *C. beticola* identities (Bethesda, 1988).

3.3.3 Area under the disease progress curve and final midpoint calculations

CLS severity was evaluated using the KWS (Kleinwanzlebener Saatzucht, 1970) standard leaf surface area rating scale described by Shane and Teng (1992). Visual plot-level, mid-canopy severity ratings were recorded from the middle two rows of each plot. The KWS scale ranges from 1 to 10, in which 1 = 1–5 spots/leaf (0.1% severity), 5 = 51–75 spots/leaf (2.5% severity), and 10 = 50%–100% severity. In each year, biweekly disease ratings were collected from June until harvest in September. CLS severity observations were used to calculate the area under the

disease progress curves (AUDPC). AUDPC is calculated by the trapezoidal method that quantifies the average disease intensity between each pair of adjacent time points in relation to time in days (Madden et al., 2017). The final CLS rating of each plot was converted from the KWS scale to a midpoint value. The midpoint conversion was calculated based on the KWS scale severity ratings and percent leaf area affected.

3.3.4 At- and post-harvest yield and sugar assessments

The center two rows of each plot were mechanically harvested and weighed to determine yield. Root subsamples (approximately 10 kg) were collected from the center two rows of each plot. Sugar analysis was conducted by Michigan Sugar Company (Bay City, MI) as described in Tedford et al. (2019) and Hernandez et al. (2023) to assess percent sugar and recoverable white sugar per ton (RWST). Recoverable white sucrose per metric ton of fresh beets (RWS) was calculated as in Van Eerd et al. (2012) and converted to recoverable white sucrose per hectare (RWSH) using the following equation: RWSH (metric ton/hectare) = RWS (kg/metric ton) × total yield (metric ton/hectare)/1000.

3.3.5 Statistical analysis

In the data analysis of all experiments, the fungicide program was considered as the fixed effect and block was considered a random effect. EC_{50} and violin plot analyses were performed using R Statistical Software (v4.1.1; R Core Team, 2021). EC_{50} values were calculated using the 'shiny' (v1.7.1; Chang et al., 2021) and 'ECXv4.0' (v4.0; Torres, 2021) packages. Violin plots were created using 'ggplot2' (v3.4.2; Wickham, 2016). Analysis of variance (ANOVA) statistical tests, to determine treatment effect on EC_{50} threshold percentages, as well as Kruskal-Wallis rank sum (Kruskal and Wallis, 1952) and Wilcoxon signed-rank (Steel, 1959) tests to assess changes in the distributions of isolate EC_{50} values based on all treatments, were conducted in SAS (Statistical

Analysis System) v. 9.4 software package (SAS Institute, Inc. Cary, NC, United States). The Kruskal-Wallis test was used to detect differences between the EC_{50} distributions of all treatments and the Wilcoxon test was used to compare each treatment combination. Statistical analyses for ANOVA were conducted using the generalized linear mixed model (GLIMMIX) and nonparametric (npar1way) procedures (SAS Institute Inc, 2013) evaluated at the $\alpha = 0.05$ significance level. Fisher's protected least significance difference (LSD) was used for mean comparisons. LSD was calculated to compare treatment differences using letter separation option "mult" macro (Piepho, 2012).

3.4 Results

3.4.1 *Cercospora beticola* pyraclostrobin EC_{50} distributions

Of the 16 representative isolates submitted for sequencing of the *cytb* gene fragments, 14 with high quality sequences were highly similar (96.7–100%) to *C. beticola* (GenBank Accession No. LC427677.1 and MF327259.1) and *C. kikuchii* (GenBank Accession No. KX374079.1) partial mitochondrial *cytochrome b* gene in the NCBI database. Two isolates, 111-9-20 and 416-13-7, had lower quality sequences and were 94.3% and 92.5% similar to *C. beticola* partial mitochondrial *cytochrome b* gene in the NCBI database (GenBank Accession No. LC427677.1); no significant similarity was found between the two isolates and *C. apii* or *C. apiicola* in the NCBI database. Based on these results, the following *in vitro* assays were determined to represent the *C. beticola* populations impacting sugarbeet field studies in this region.

In 2019, the Kruskal-Wallis test indicated that there were no significant differences between the *C. beticola* isolate EC_{50} distributions for the treatments when collected mid-season or end-of-season ($P > 0.05$; Table 3.4). No consistent trends were observed in the *C. beticola* isolate pyraclostrobin EC_{50} distributions visualized in violin and boxplots (Fig. 3.1A and B). For the mid-

season timepoint, the mean pyraclostrobin EC_{50} values (± standard error) for each treatment was 50.0 ± 18.0 µg/ ml for the non-treated control, 52.1 ± 11.7 µg/ml for the high-risk, 58.6 ± 14.5 µg/ml for the low-risk, and 62.8 ± 14.4 µg/ml for the mixture treatment. The mean pyraclostrobin EC_{50} values at the end-of-season were as follows, 55.0 ± 20.2 µg/ml for control, 54.9 ± 7.7 µg/ml for high-risk, 47.3 ± 12.9 µg/ml for low-risk, and 38.6 ± 9.3 µg/ml for the mixture.

In 2020, the Kruskal-Wallis test detected differences between the EC_{50} distributions of treatments in 2020 end-of-season samples. The Wilcoxon tests indicated significant differences between all treatments compared to the non-treated control at the end-of-season ($P < 0.05$; Table 3.4). The *C. beticola* EC_{50} isolate distribution from the non-treated control also was visibly different from all other treatments, with greater "bell" shape at lower concentrations (Fig. 3.1C). The mean of the pyraclostrobin EC_{50} values (± standard error) for the control, high-risk, low-risk, and the mixture treatments for the 2020 end-of-season time- point were 26.3 ± 4.6, 55.9 ± 7.5, 54.1 ± 6.4, and 49.6 ± 6.4 µg/ml, respectively.

Based on the Kruskal-Wallis test, there were no significant difference between the distribution of difenoconazole EC_{50} values for the mid-season 2019, end-of-season 2019, or end-of-season 2020 sampling timepoints (Table S3.1). Distributions were visually similar for the difenoconazole EC_{50} values of *C. beticola* isolates in each treatment for all sampling timepoints (Fig. S3.2). The mean difenoconazole EC_{50} values for each treatment (± standard error) were 12.0 ± 3.5 for the control, 15.4 ± 4.9 for the high-risk, 13.9 ± 3.4 for the low-risk, and 11.3 ± 2.6 for the mixture treatment.

Table 3.4. Nonparametric statistical tests including the Kruskal-Wallis rank sum and Wilcoxon signed-rank tests with corresponding X^2 value and Z score, respectively, for evaluating differences in *Cercospora beticola* pyraclostrobin EC_{50} distributions for each treatment in 2020.

Statistical test	Treatments [a, b]	X^2 *or* Z	df	$P > x^2$
Kruskal-Wallis	All mid-season 2019	0.6728	3	0.8796
	All end-of-season 2019	2.2918	3	0.5141

Table 3.4 (cont'd)

	All 2020	13.1577	3	0.0043	** [c]
Wilcoxon	2020 Control*High-risk	3.1078	1	0.0025	**
	2020 Control*Low-risk	-2.9530	1	0.0039	**
	2020 Control*Mixture	-2.5624	1	0.0119	*
	2020 High-risk*Low-risk	0.4093	1	0.6832	
	2020 High-risk*Mixture	0.9369	1	0.3512	
	2020 Low-risk*Mixture	-0.4523	1	0.6520	

[a] Treatments included: non-treated control, high-risk where pyraclostrobin was applied first and then alternated with mancozeb, low-risk where mancozeb was applied first and then alternated with pyraclostrobin, and a mixture where pyraclostrobin and mancozeb were applied as a tank-mixture.
[b] By end-of-season in 2020, Headline (pyraclostrobin) was applied at 876.9 ml/ha twice in the high-risk treatment, once in low-risk, and twice tank-mixed with Manzate Max (mancozeb) at 3.7 L/ha in the mixture.
[c] Asterisk designations correspond to p-value thresholds <0.05 * and <0.01 **

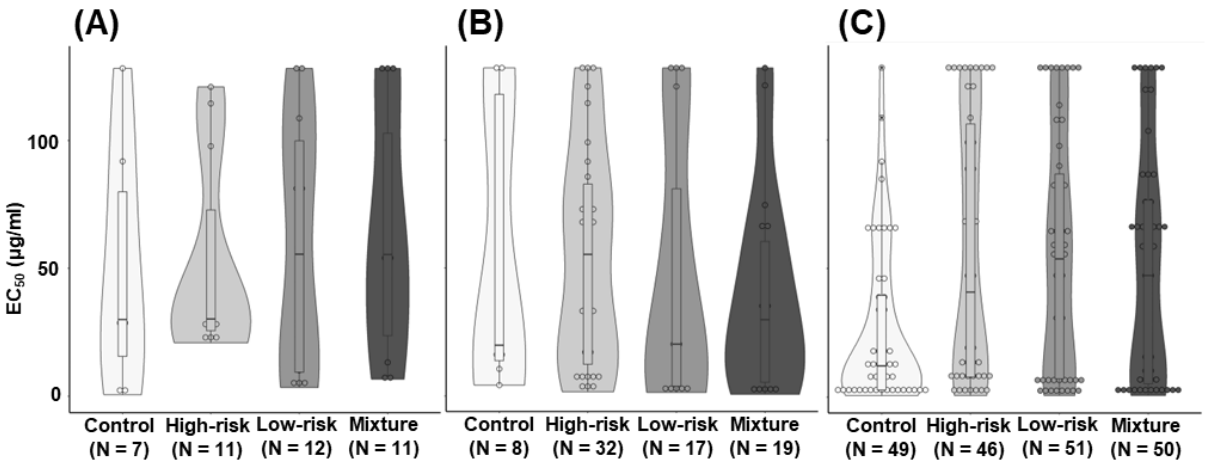

Figure 3.1. Pyraclostrobin EC_{50} values (µg/ml) shown in violin plots inset with boxplots to represent *C. beticola* population distribution in non-treated control, high-risk, low-risk, and mixture treatments at each collection timepoint A) mid-season 2019 B) end-of-season 2019, and C) end-of-season 2020. The boxplot includes the minimum, first quartile, median, third quartile, and maximum values for each treatment timepoint. Number of isolates collected per treatment (N) denoted below treatment labels. At mid-season in 2019, the pyraclostrobin-containing fungicide, Headline (876.9 ml/ha), was applied twice in the high-risk, once in low-risk, and twice tank-mixed with Manzate Max (3.7 L/ha) in the mixture; by end-of-season, one additional Headline application was applied to each treatment as described. By end-of-season in 2020, Headline (876.9 ml/ha) was applied twice in the high-risk treatment, once in low-risk, and twice tank-mixed in the mixture. Due to low and delayed CLS pressure in 2020, *C. beticola* isolates were only collected at one timepoint near harvest.

3.4.2 *Cercospora beticola* pyraclostrobin EC_{50} threshold analysis

In 2019, no differences were detected in mid- or end-of-season isolate pyraclostrobin sensitivities based on 1, 5, and 25 µg/ml EC_{50} value thresholds ($P > 0.05$; Fig. 3.2). In 2020,

however, significant differences were detected in the percentage of isolates with pyraclostrobin EC_{50} values above 5 µg/ml for the end-of-season timepoint ($P < 0.05$), where the non-treated control resulted in a significantly reduced resistance frequency compared to other treatments (Fig. 3.3).

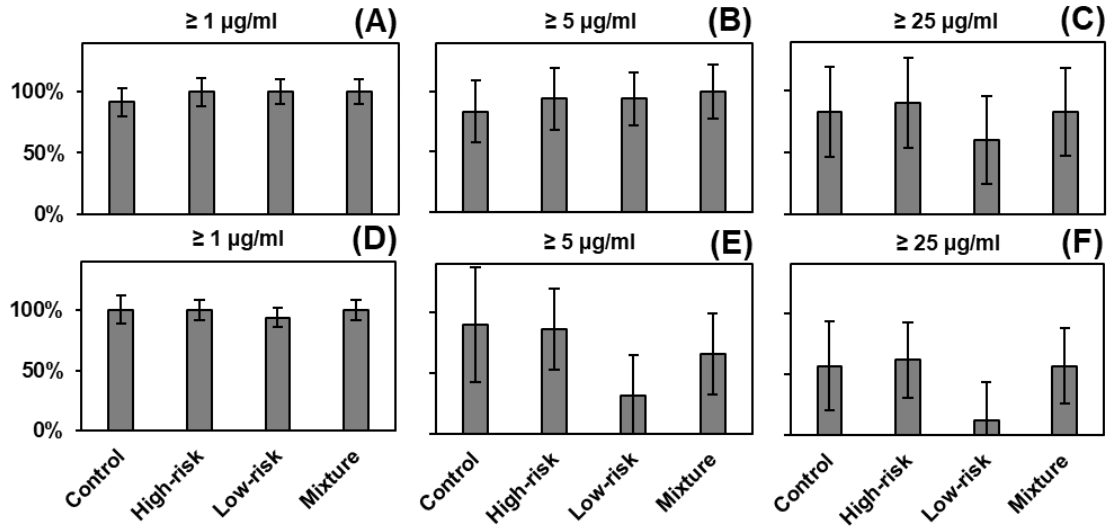

Figure 3.2. Percentages of *Cercospora beticola* isolates exhibiting pyraclostrobin EC_{50} sensitivities greater than or equal to thresholds of 1, 5, and 25 µg/ml for the mid-season (A-C) and end-of-season (D-F) isolates collected in 2019. Bars represent mean frequencies for each treatment and error bars represent the 95% confidence intervals. No significant differences between means of each treatment were detected in 2019 ($P > 0.05$). At mid-season in 2019, the pyraclostrobin-containing fungicide Headline (876.9 ml/ha) was applied twice in the high-risk, once in low-risk, and twice tank-mixed with Manzate Max (3.7 L/ha) in the mixture; by end-of-season, one additional Headline application was applied to each treatment as described.

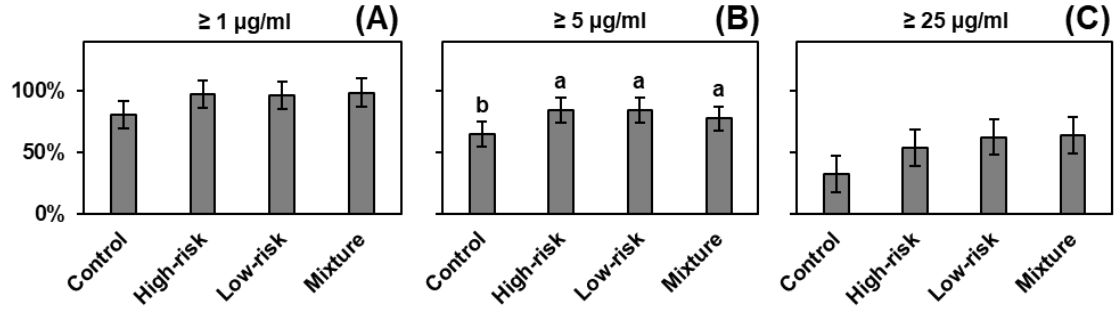

Figure 3.3. Percentages of *Cercospora beticola* isolates exhibiting pyraclostrobin EC_{50} sensitivities greater than or equal to thresholds of 1, 5, and 25 µg/ml for isolates collected end-of-

Figure 3.3. (cont'd)
season in 2020. Bars represent mean frequencies for each treatment and error bars represent the 95% confidence intervals. Significant differences between means of each treatment were detected for the 5 µg/ml threshold ($P < 0.01$). Bars with the same letter were not significantly different based on Fisher's Protected LSD ($\alpha = 0.05$). By end-of-season in 2020, the pyraclostrobin-containing fungicide, Headline (876.9 ml/ha) was applied twice in the high-risk treatment, once in low-risk, and twice tank-mixed in the mixture. Due to low and delayed CLS pressure in 2020, *C. beticola* isolates were only collected at one timepoint near harvest.

3.4.3 *Cercospora beticola* G143A mutation detection

Across 2019 and 2020 sampling, a total of 237 *C. beticola* isolates (90 in 2019, 145 in 2020, and two reference isolates) were screened for the G143A mutation associated with QoI resistance. Sequences associated with QoI-susceptible and -resistant banding patterns were confirmed via sequencing of 16 representative isolates (Figs. S3.1A and 1B). During these studies, only one wild-type isolate was identified; in addition to the known sensitive 'Range A' reference isolate, one wild-type isolate was collected from a non-treated control plot in 2020. Both wild-type isolates were sensitive to all tested *in vitro* concentrations of pyraclostrobin, with mean EC_{50} values below the lower detection limit (0.62 µg/ml). The G143A mutation was confirmed in the 'Blum 1–2' reference isolate, which exhibited *in vitro* pyraclostrobin insensitivity, with a mean EC_{50} above the upper detection limit (128.2 µg/ ml). For 178 field isolates where the G143A mutation was detected, corresponding EC_{50} values ranged widely from below (<0.6 µg/ml) to above (>128.2 µg/ml) the assay detection limits. The mean, median, and mode EC_{50} value for the *C. beticola* isolates with the mutation were 50.6 (±3.4 SE), 45.7, and 128.2 µg/ml, respectively. Due to high frequencies of the G143A mutation in these samples, no field treatment differences were detected based on mutation occurrences.

3.4.4 Cercospora leaf spot severity, yield, and sugar

In 2019, all treatments resulted in significantly reduced AUDPC values compared to the non-treated control ($P < 0.001$; Table 3.5). The final midpoint severity rating for the control was

significantly higher (65%) than all other treatments ($P < 0.0001$). The high-risk and low-risk treatments resulted in final midpoint severity ratings of 17.5–20.1% and were not significantly different from one another. The grower standard and mixture treatments resulted in final midpoint severity ratings of 5.2–5.6% and were not significantly different from each other. However, the mixture and grower standard treatments showed significantly reduced AUDPC compared to the high-risk treatment.

In 2020, CLS pressure was delayed and not as severe as in 2019; however, the AUDPC in the control was significantly higher compared to all other treatments ($P < 0.0001$), corresponding with a final midpoint rating of 18.0%. The high-risk and low-risk treatments resulted in final midpoint severity ratings of 3.0–3.6% and were not significantly different. The low-risk AUDPC value was not significantly different from the mixture treatment, which resulted in a final midpoint severity of 1.8%. The mixture resulted in a significantly lower mean AUDPC than the high-risk treatment and was comparable to the grower standard.

In 2019, the high-risk, low-risk, mixture, and the grower standard treatments resulted in significantly greater yields (43.5–47.5 t/ha) than the non-treated control, which yielded 30.4 t/ha ($P < 0.0001$; Table 3.6). Similarly, all treatments resulted in significantly greater percent sugar ($P < 0.0001$), recoverable white sugar ($P < 0.05$), and recoverable white sugar per hectare ($P < 0.001$) at harvest. For percent sugar, the high- and low-risk treatments were comparable (17.3–17.9%) and the mixture and grower standard were comparable (18.4–19.0%), and all were greater than the control (15.2%). Treatments resulted in RWS ranging from 113.9 to 132.7 kg/t, whereas the non-treated control yielded 97.3 kg/t; of note, the low-risk treatment was not significantly different from the non-treated control. While again greater than the control, the high- and low-risk treatments resulted in comparable RWSH at 5.1–5.2 t/ha, and the mixture and grower standard

were comparable at 5.6–6.3 t/ha. While treatment differences were observed for CLS pressure in 2020, no significant differences in yield or sugar metrics were detected ($P > 0.05$).

Table 3.5. Area under the disease progress curve (AUDPC) and average midpoint severity information for the CLS final rating for each treatment in 2019 and 2020 trials. Corresponding standard error (SE), p-value, and least significant difference (LSD) values shown.

Trial Year	Treatment	AUDPC [z]		Final rating midpoint (%)	
2019	Control	424.5	a	65.4	a
	High-risk	304.4	b	20.1	b
	Low-risk	284.0	bc	17.5	b
	Mixture	234.0	c	5.6	b
	Grower Standard	227.1	c	5.2	b
	SE	*20.0*		*5.1*	
	P-value [y]	*0.0002*	***	*< 0.0001*	***
	LSD	*64.8*		*17.2*	
2020	Control	141.1	a	18.0	a
	High-risk	85.9	b	3.6	b
	Low-risk	69.8	bc	3.0	b
	Mixture	49.3	cd	1.8	b
	Grower Standard	32.1	d	1.0	b
	SE	*9.1*		*2.9*	
	P-value	*< 0.0001*	***	*0.0085*	**
	LSD	*28.9*		*9.2*	

[z] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (a = 0.05).
[y] Asterisk designations correspond to p-value thresholds <0.05 *, <0.01 **, <0.001 ***.

Table 3.6. Yield, percent sugar, recoverable white sugar (RWS), and recoverable white sugar per hectare (RWSH) for each treatment in 2019 and 2020 trials. Corresponding standard error (SE), p-value, and least significant difference (LSD) values shown.

Trial Year	Treatment	Yield (t/ha) [z]		Sugar (%)		RWS (kg/t)		RWSH (t/ha)	
2019	Control	30.4	b	15.2	d	97.3	b	3.0	c
	High-risk	43.5	a	17.3	c	118.6	a	5.2	b
	Low-risk	44.5	a	17.9	bc	113.9	ab	5.1	b
	Mixture	46.7	a	18.4	ab	119.4	a	5.6	ab
	Grower Standard	47.5	a	19.0	a	132.7	a	6.3	a
	SE	*2.2*		*0.4*		*8.4*		*0.5*	
	P-value [y]	*< 0.0001*	***	*< 0.0001*	***	*0.0414*	*	*0.0002*	***
	LSD	*5.1*		*1.1*		*21.1*		*1.0*	
2020	Control	37.1		17.9		114.8		4.3	
	High-risk	36.6		17.6		112.4		4.1	
	Low-risk	37.4		17.7		113.3		4.2	

Table 3.6 (cont'd)

Mixture	42.8	17.7	113.3	4.8
Grower Standard	42.0	17.6	112.9	4.7
SE	*4.0*	*0.2*	*1.8*	*0.5*
P-value	*0.5559*	*0.8931*	*0.8859*	*0.6676*
LSD	- [x]	-	-	-

[z] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (α = 0.05).
[y] Asterisk designations correspond to p-value thresholds <0.05 *, <0.01 **, <0.001 ***.
[x] LSD values were not generated for non-significant effects, designated with a hyphen (-).

3.5 Discussion

While *C. beticola* QoI sensitivity in field populations significantly decreased following applications of a pyraclostrobin-containing fungicide, the frequency of resistance was not affected by the use of alter- nation or tank-mixture programs in the current study. Similarly, in other fungal pathogens, a study by Rosenzweig et al. (2008a) found that *Alternaria solani* mean EC_{50} values of isolates from plots treated with 0–6 apps of QoI active ingredients were generally similar in alternations or tank mixtures of the active ingredients azoxystrobin. A study by Cul- breath et al. (2002) also found that mixtures and alternating applications of chlorothalonil, a low-resistance risk fungicide, and benomyl, a high-resistance risk fungicide, did not reduce the rate of fungicide resistance development in populations of *Cercospora arachidicola* and *Cercosporidium personatum*. The number of fungicide applications in-season and between sampling timepoints may impact detectable differences in fungicide sensitivity. Though no notable changes in *C. beticola* populations between the mid- and end-of-season timepoints were observed in the first year of experiments, additional sampling timepoints and collection of greater numbers of isolates per timepoint should be considered in future studies.

Other studies, similar to the current, found *C. beticola* fungicide sensitivity was comparable across all treatment programs with only significant differences compared to the non-treated control. Dovas et al. (1976) determined that all treatments of benomyl alone, in alternation

with triphenyltin hydroxide, and both in tank-mixture, increased the relative portion of benomyl-resistant *C. beticola* strains, which is consistent with current findings for pyraclostrobin. However, in the Dovas et al. (1976) study, the rate of increase for resistant *C. beticola* strains was different between treatments with the highest rate of in- crease associated with the benomyl alone treatment, followed by the tank mixture treatment, and lastly the alternated treatment (Dovas et al., 1976); the rate of resistance development, however, was not measured in the current study. In a study by Karaoglanidis et al. (2001), application programs with flutriafol alternated or tank-mixed with maneb were tested and similarly found that repetitive applications of flutriafol at any rate selected for highly resistant *C. beticola* isolates. These findings were similar to the current study where one to three applications of pyraclostrobin were sufficient to cause increased QoI-resistance in *C. beticola* field populations. Overall, use of high-risk fungicides in foliar programs has potential to select for resistant strains across treatment alternations, tank-mix partners, product rates, or number of applications.

Regarding CLS control in sugarbeet, the current study found that the mixture application provided better control when pyraclostrobin was applied, followed by the low-risk alternation treatments, and lastly the high-risk rotation. Treatment programs that included the application of Headline alone had reduced efficacy, resulting in significantly higher CLS severity across both years and reduced sugar content in 2019, and indicated that field-level resistance likely resulted in reduced disease control. Yield and sugar quality were not impacted in the second year of this study, likely because of the lower CLS pressure, as evidenced by end- of-season severity levels. Similarly, mixtures and alternating applications of benomyl (FRAC group 1) provided more effective control of early and late leaf spot diseases of peanut than benomyl alone (Culbreath et al., 2002). Conversely, control of early blight on potato was not improved by alternations or tank-

mixtures of the active ingredients azoxystrobin (high-risk, FRAC group 11) and chlorothalonil (low-risk) (Rosenzweig et al., 2008a). In another scenario, application of flutriafol alone at the recommended dose resulted in significantly improved control of CLS compared to flutriafol applications alternated with tank-mixed fentin-acetate and maneb (Karaoglanidis et al., 2001), which differs from the results presented for pyraclostrobin. No pyraclostrobin alone treatments were tested in the current study, however, findings were in agreement with those in the benomyl studies where tank-mixture applications performed better than alternating treatments of a high-risk product.

The G143A mutation typically confers a high level of resistance to QoI products in other pathogens (Campbell et al., 2021; Ma et al., 2003), however, *C. beticola* isolates with the G143A mutation have been shown to exhibit pyraclostrobin EC_{50} values ranging from 0.92 to 72.2 μg/ml using a conidial germination assay (Bolton et al., 2013). The current study supports these findings, confirming that isolates of *C. beticola* with the G143A mutation exhibited a wide range of EC_{50} values spanning the upper and lower limits of the mycelial growth assay (0.6–128.2 μg/ml). In this study, wild-type isolates of *C. beticola* without the G143A mutation did not grow across the gradient of spiral plate concentrations and resulted in EC_{50} values below our testing limits (<0.6 μg/ml). Bolton et al. (2013) identified *C. beticola* isolates without the G143A mutation exhibiting EC_{50} values less than or equal to 0.006 μg/ml. In future studies, additional mutations of interest associated with QoI resistance could be evaluated for their ability to influence fungicides sensitivity to pyraclostrobin.

The F129L and G137R mutation with amino acid change from phenylalanine to leucine and glycine to arginine, respectively, also have been associated with reduced sensitivity to QoI fungicides (Stevenson et al., 2019). However, both generally confer a lower level of resistance

than the G143A mutation in most fungi (Ma and Michailides, 2005; Stevenson et al., 2019). The sample set of 16 *C. beticola* isolates used for the sequencing confirmation of the G143A mutation did not contain these mutations (data not shown, codons 126 to 194 were of sufficient sequence quality for observation), however, other regions may be more descriptive of the sensitivity observed in this pathosystem. Of note, the two reference *C. beticola* isolates included in the current study exhibited the typical QoI-resistant and -susceptible genotypes and phenotypes, as reported in previous work (Rosenzweig et al., 2015) and would be beneficial to include for comparison in future studies. In some fungi, a variation in the proportion of mitochondria containing the G143A mutation, i.e., heteroplasmy, can influence QoI sensitivity (Vielba-Fernandez et al., 2018; Mosquera et al., 2019; Villani and Cox, 2014). The sequencing done in the current study did not show more than one sequence, but cloning or other methods may be required in some systems to identify heteroplasmy (Vielba-Fernandez et al., 2018; Mosquera et al., 2019). Similarly, no mixed banding patterns were observed in the present study, similar to what Rosenzweig et al. (2015) reported for some PCR-RFLP testing from lesions. Rosenzweig et al. (2015) reported a requirement for at least 20% of sequences with a different codon before the test would show a mixed banding pattern, which might give some indication of limited heteroplasmy, if present. Other studies have indicated that in some fungi, QoI resistance may involve more than one gene (Villani and Cox, 2014). Something similar may be occurring in *C. beticola* and requires further investigation.

In Greece, the presence of benomyl-resistant *C. beticola* strains remained constant after three consecutive years following discontinued use of benomyl, which was attributed to equal fitness of the resistant and sensitive benomyl isolates (Dovas et al., 1976). Based on *in vitro* testing of *C. beticola* isolates from sugarbeet, resistance to pyraclostrobin was relatively stable

from 2003 to 2008 in Minnesota and North Dakota (Secor et al., 2010), suggesting there may not be a fitness cost between resistant and susceptible isolates. Similar stability had been observed with the G143A mutation in other fungi (Ma and Michailides, 2005). In Michigan, the G143A mutation was widely detected in approximately 94% of *C. beticola* isolates collected from sugarbeets in 2012 (Rosenzweig et al., 2015) and again approximately 99–100% of those tested in the current study from 2019 to 2020. Similarly, recent studies in the Red River Valley detected the G143A mutation in >90% of *C. beticola* isolates between 2016 and 2019 (Rangel et al., 2020). While these studies suggest QoI-resistance remains stable from year to year, latent early-season populations may be exposed to strobilurin fungicides used for soilborne disease control (Pooran-DeSouza et al., 2021; Sharma et al., 2021) e.g., azoxystrobin, masking potential seasonal differences. Additional studies on the fitness and seasonal fluctuations of QoI resistance in *C. beticola* would better support current observations.

In the current study, pyraclostrobin sensitivities were determined without blocking the alternative oxidase pathway with salicylhydroxamic acid (SHAM). In other studies, SHAM has been included in fungicide-amended media for *C. beticola* pyraclostrobin sensitivity testing (Bolton et al., 2013) and testing of other Cercospora spp. (Bradley and Pedersen, 2011). However, the inhibition of the alternative oxidation pathway has been shown not to significantly impact *in vitro* pyraclostrobin sensitivity results for other Cercospora species such as *Cercospora kikuchii* (Price et al., 2015). Malandrakis et al. (2006) re- ported that the biochemical mechanism of alternative oxidase was not responsible for the reduced sensitivity to pyraclostrobin in G143A mutants of *C. beticola*, though a follow up study found that the addition of SHAM to amended media increased sensitivity to pyraclostrobin for most *C. beticola* isolates (Malandrakis et al., 2011). Based on these studies, our findings would have been unaffected or shown sensitivities

increased with the addition of SHAM. Regardless, the high frequencies of the G143A mutation in Michigan *C. beticola* populations were not well-supported by the range of pyraclostrobin EC_{50} values observed. Historically, fungicide resistance development has been a long- documented challenge for CLS management in sugarbeet. Methyl benzimidazole carbamate (MBC) was initially used for control of CLS on sugarbeet in the early 1970s (Rangel et al., 2020). In 1973 and 1974, economic loss due to MBC resistance was first reported in Europe and the United States (Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974). Following rapid MBC-resistance development and field efficacy failures, organotin products were primarily and extensively used in the United States throughout the 1980s for CLS management without an effective rotation partner (Windels et al., 1998). As a result, decreased sensitivity to organotin products were first detected in 1994 in the United States (Bugbee, 1995) and lead to an epidemic in 1998. In 1999, an emergency exemption to control CLS on sugarbeet was implemented for the use of an unregistered triazole, which continued through 2004. Tetraconazole was then registered for management CLS of sugarbeets in 2005, stimulating the development of the many DMI active ingredients available today (Secor et al., 2010). Some decreases were detected in sensitivity to triazoles for *C. beticola* populations in Greece during 1995 (Karaoglanidis et al., 2000) and in the United States during the 2000s (Secor et al., 2010; Bolton et al., 2012; Rosenzweig et al., 2020). QoI resistance was first reported in the United States which could be related to the difference in chemical programs compared to Europe. Considering the resistance trends of chemical products used in foliar disease management, continued monitoring of shifts in insensitivity of other high-risk active ingredients could inform future resistance management studies utilizing alternation and tank-mix partners to prolong fungicide sensitivity and use.

This study was conducted to evaluate the use of pyraclostrobin in alternated or tank-mixed

fungicide application programs. Experiments tested the potential to maximize CLS control using pyraclostrobin while minimizing *C. beticola* resistance development. Repeated field studies did not provide sufficient evidence to support the use of any number of pyraclostrobin applications for CLS management. Other available active ingredients were successful in reducing CLS severity on sugarbeet with lower risk for *C. beticola* fungicide resistance development. The varied fungicide response in fungi with the G143A mutation needs further investigation. Differences in population distribution of EC_{50} values were observed and percentages of more highly resistant isolates were greater for all treatments compared to the control in the second year of testing. Compared to a grower standard program without pyraclostrobin, the use of the QoI-containing fungicide Headline did not provide additional control and did not impact yield or sugar parameters. Overall, due to the high risk of resistance development and lack of yield benefits, this study advises against the use of pyraclostrobin in rotation or as a tank-mix partner in CLS management programs.

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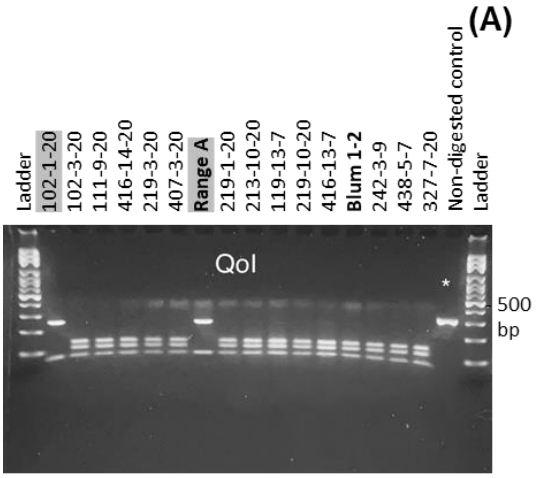
APPENDIX

Table S3.1. Nonparametric statistical tests including the Kruskal-Wallis rank sum and Wilcoxon signed-rank tests with corresponding X^2 value and Z score, respectively, for evaluating differences in *Cercospora beticola* difenoconazole EC_{50} distributions for each treatment in 2020.

Statistical test	Treatments a, b	X^2 or Z	df	*P* > x2
Kruskal-Wallis	All mid-season 2019	1.0380	3	0.7921
	All end-of-season 2019	5.7374	3	0.1251
	All 2020	0.4559	3	0.9285

[a] Treatments included: non-treated control, high-risk where pyraclostrobin was applied first and then alternated with mancozeb, low-risk where mancozeb was applied first and then alternated with pyraclostrobin, and a mixture where pyraclostrobin and mancozeb were applied as a tank-mixture.

[b] By end-of-season in 2020, Headline (pyraclostrobin) was applied at 876.9 ml/ha twice in the high-risk treatment, once in low-risk, and twice tank-mixed with Manzate Max (mancozeb) at 3.7 L/ha in the mixture.



(B)

Isolate ID	DNA Sequences (5' to 3')
Range A	AATGTCTTTATGAGGTGCAACTGTTATTAC
102-1-20	AATGTCTTTATGAGGTGCAACTGTTATTAC
102-3-20	AATGTCTTTATGAGCTGCAACTGTTATTAC
111-9-20	AATGCTTTTATGAGCTGCAACTGTTATTAC
119-13-7	AATGTCTTTATGAGCTGCAACTGTTATTAC
213-10-2	AATGTCTTTATGAGCTGCAACTGTTATTAC
219-1-20	AATGTCTTTATGAGCTGCAACTGTTATTAC
219-3-20	AATGCTTTTATGAGCTGCAACTGTTATTAC
219-10-2	AATGTCTTTATGAGCTGCAACTGTTATTAC
242-3-9	AATGTCTTTATGAGCTGCAACTGTTATTAC
327-7-20	AATGTCTTTATGAGCTGCAACTGTTATTAC
407-3-20	AATGTCTTTATGAGCTGCAACTGTTATTAC
416-13-7	AATGTCTTTAAGAACTGC_CCTGTTATTAC
416-14-2	AATGTATTTATGAGCTGCAACTGTTATTAC
438-5-7	AATGTCTTTATGAGCTGCAACTGTTATTAC
Blum 1-2	AATGTCTTTATGAGCTGCAACTGTTATTAC

Figure S3.1. A) Gel electrophoresis (2.5% agarose) of polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) products for 16 *Cercospora beticola* isolates, confirming *Fnu4HI*-digested *cytochrome b* gene fragment banding patterns associated with QoI-sensitivity (2 band) and QoI-resistance (3 bands), including 14 isolates selected from across

Figure S3.1. (cont'd)
treatments and replicates in 2019 and 2020 field studies and two reference isolates, one sensitive 'Range A' and one resistant 'Blum 1-2' (shown in bold). Results were run alongside a non-digested control fragment (indicated with an asterisk, 1 band, ~325 bp) and sample lanes flanked by a 1 kb ladder. Products were purified and Sanger sequencing conducted at the Michigan State University Research Technology Support Facility Genomics Core (East Lansing, MI) to confirm B) presence of the G143A mutation (DNA sequence mutation: GGT-GCT; amino acid mutation: glycine to alanine) associated with QoI resistance in *C. beticola* (Rosenzweig et al. 2015).

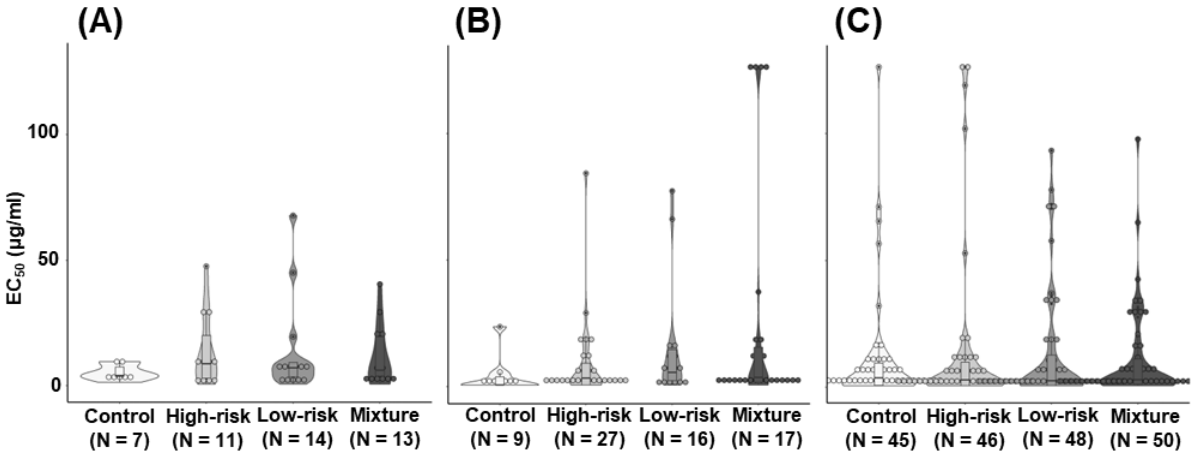


Figure S3.2. Difenoconazole EC_{50} values (µg/ml) shown in violin plots inset with boxplots to represent *C. beticola* population distribution in non-treated control, high-risk, low-risk, and mixture treatments at each collection timepoint A) mid-season 2019 B) end-of-season 2019, and C) end-of-season 2020. The boxplot includes the minimum, first quartile, median, third quartile, and maximum values for each treatment timepoint. Number of isolates collected per treatment (N) denoted below treatment labels. No applications of difenoconazole were applied in any tested treatment in either year; therefore, difenoconazole responses were used to screen for background influences on isolate sensitivity, e.g., potential movement or dispersal between field plots. Due to low and delayed CLS pressure in 2020, *C. beticola* isolates were only collected at one timepoint near harvest.

CHAPTER 4: CHARACTERIZATION OF FUNGICIDE RESISTANCE IN *CERCOSPORA BETICOLA* POPULATIONS FROM SUGARBEET (*BETA VULGARIS*) IN MICHIGAN

4.1 Abstract

BACKGROUND: Sugarbeets produce over half of domestic sugar, and Michigan is the fourth highest U.S. producer. *Cercospora beticola* causes one of the most impactful foliar diseases in sugarbeet growing regions, Cercospora leaf spot (CLS). Management of CLS relies heavily on timely and repeated fungicide applications, and fungicide resistance has been observed for several fungicide groups in *C. beticola*. In 2021 and 2022, *in vitro* fungicide sensitivity was tested for five demethylation inhibitors (DMI; difenoconazole, fenbuconazole, mefentrifluconazole, prothioconazole, tetraconazole), a quinone outside inhibitor (QoI; pyraclostrobin), a methyl benzimidazole carbamate (MBC; thiophanate-methyl), and triphenyltin hydroxide.

RESULTS: Cross resistance was detected between most DMIs with strongest correlations between difenoconazole and mefentrifluconazole ($r = 0.58$, $P < 0.0001$) and fenbuconazole and prothioconazole or tetraconazole ($r = 0.51$ or 0.54, $P < 0.0001$). Fungicide application data reiterated that *C. beticola* sensitivity to thiophanate-methyl and prothioconazole was affected by the number of MBC and DMI applications, respectively ($P < 0.05$). Mutations associated with resistance to QoI, MBC, and DMI fungicides also were evaluated using restriction fragment length polymorphisms (PCR-RFLP). While the E198A mutation was significantly associated with *C. beticola* responses to thiophanate-methyl ($P < 0.0001$), G143A and Glu169 were not as strongly associated with pyraclostrobin or prothioconazole and tetraconazole responses, respectively.

CONCLUSION: Overall, *in vitro* insensitivity (EC_{50} greater than k-means determined thresholds of sensitive groups) were observed for all tested active ingredients, however, elevated frequencies were observed for thiophanate-methyl (72-86%), tetraconazole (28-56%), and prothioconazole (78-92%), and pyraclostrobin (9-33%). Identifying mutations more explanatory of resistance to

DMI and QoI fungicides in *C. beticola* populations would improve timely monitoring efforts.

4.2 Introduction

The United States produced over 29 million metric tons of sugarbeet in 2022, which accounted for over 50% of domestic sugar production (USDA-NASS 2022). Michigan contributed 12.4% to the total sugarbeet production in the U.S. for 2022 (USDA-NASS 2022). Production is impacted by several factors, including disease. Among these, Cercospora leaf spot (CLS), caused by *Cercospora beticola* Sacc., is a highly destructive foliar disease of sugarbeet that can cause substantial yield losses and decreases in sugar extraction (Lamey et al. 1987; Shane and Teg 1992; Weiland and Koch 2004; Jacobsen and Franc 2009; Khan et al. 2009). Management of CLS requires integrated strategies including the use of resistant sugarbeet cultivars, rotation with nonhost crops, and timely fungicide applications (Jacobsen and Franc 2009; Windels et al. 1998; Khan et al. 2007; Jacobsen 2010). *Cercospora beticola* is classified as having a medium-risk for fungicide resistance development (FRAC 2019) due to its polycyclic life cycle, high genetic diversity (Vaghefi et al. 2016, 2017a, 2017b), and the significant number of seasonal fungicide applications (Khan and Smith 2005; Karaoglanidis and Ioannidis 2010; Hernandez et al. 2023).

Fungicide resistance has been widespread in *C. beticola*. Methyl benzimidazole carbamate (MBC, FRAC group 1) resistance and resulting economic losses were reported in Europe and the U.S. in 1973 and 1974, respectively (Georgopoulos and Dovas 1973; Ruppel and Scott 1974). Organotin (FRAC group 30) insensitivity was first detected in 1978 in Europe (Giannopolitis and Chrysayi-Tokousbalides 1980) and 1994 in the United States (Bugbee 1995). Reduced sensitivity to pyraclostrobin, a quinone outside inhibitor (QoI, FRAC group 11), was subsequently detected in 2004 (Secor et al. 2010) followed by rapid development of a stable resistance in U.S. *C.*

beticola populations (Kirk et al. 2012; Rangel et al. 2020). Furthermore, decreases in *C. beticola* tetraconazole sensitivity, a demethylation inhibitor (DMI, FRAC group 3), were reported in Greece during 1995 (Karaoglandis et al. 2000) and in the U.S. during the 2000s (Secor et al. 2010; Rosenzweig et al. 2020).

Currently in the U.S., fungicide groups commonly used for CLS management on sugarbeet include MBC, DMI, QoI, organotin, and multi-site contact (FRAC group M01 and M03) fungicides (Rangel et al. 2020). Succinate dehydrogenase inhibitors (FRAC group 7) are not commonly used for control of CLS on sugarbeet due to limited intrinsic activity in *C. beticola* populations (Pethybridge et al. 2020) and limited registrations. Excluding organotin products, the main chemical classes used in the U.S. are also registered for use in Canada (OMAFRA 2021). Resistance to MBC and QoI fungicides has been observed in Ontario *C. beticola* populations, and use of these groups is not advised in the region (LeBoeuf et al. 2012; Trueman et al. 2013). Rotating modes of action, using tank-mix partners, limiting the number of applications, following the labeled rate for treatment, and utilizing integrated pest management (IPM) strategies are recommended to limit fungicide resistance development (Corkley et al. 2022; van den Bosch et al. 2014). Despite these practices, continued screening is necessary to monitor potential shifts in *C. beticola* sensitivity.

Rapid screening methods are desirable for timely reporting (Olita et al. 2024), thus, genetic mutations associated with resistances have been described for some fungicides. A single base pair mutation in the beta tubulin gene (E198A) was shown to correlate with *C. beticola* resistance to the methyl benzimidazole carbamate (Davidson et al. 2006). For QoIs, the G143A substitution in the *cytochrome b* (cytb) gene has been associated with complete resistance in many fungal pathogens, including species of *Alternaria, Mycosphaerella*, and *Venturia* (Ma and Michailides

2005). Reduced sensitivity to a DMI (epoxiconazole) was associated with a silent mutation (Glu169) located in the *C. beticola* 14-C alpha-demethylase gene (CbCyp51; Nikou et al. 2009). Use of molecular testing can lead to high-throughput techniques to monitor the frequency of highly explanatory mutations could improve the efficiency of seasonal resistance screening efforts (Yin et al. 2023).

Continuous monitoring of fungicide resistance development in *C. beticola* populations is critical to guide effective management recommendations. This study evaluated the *in vitro* sensitivity of Michigan *C. beticola* strains to four different fungicide groups and eight active ingredients across 2021 and 2022. Across years, isolates from nine counties were screened and mutations associated with *C. beticola* QoI, DMI, and MBC resistance were investigated. Relationships within and between *C. beticola* responses to these fungicide groups and the utility of corresponding PCR-RFLP assays were determined.

4.3 Materials and methods

4.3.1 Sugarbeet leaf sample collection and *Cercospora beticola* isolation

CLS-symptomatic leaves were collected from mid-July through the end of October. Across nine counties in east-central Michigan, 29 and 30 field locations were sampled in 2021 and 2022, respectively (Figure 4.1). With each sample, submitters included information about the date of collection, field location, previous crop, fungicide applications, and CLS severity. From each field site, approximately eight CLS lesions from 8-16 leaves were collected at each sampling timepoint.

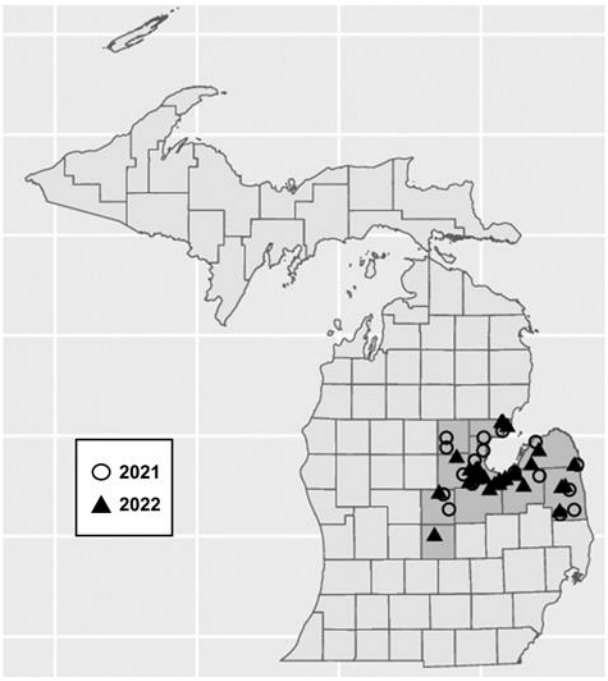


Figure 4.1. Michigan counties sampled for sugarbeet leaves symptomatic with Cercospora leaf spot in 2021 to 2022 (highlighted in gray): Arenac, Bay, Clinton, Gratiot, Huron, Midland, Saginaw, Sanilac, and Tuscola. Field locations indicated with filled circles for 2021 and filled triangles for 2022. Map generated using 'ggplot' package (Wickham 2016) in R Statistical Software (v4.1.1; R Core Team 2021) using methods from McCoy (2021).

Initial isolations were performed as described by Hernandez et al. (2024); in brief, *Cercospora*-like spore suspensions were collected from characteristic CLS lesions on infected sugarbeet leaf material and spread onto 1.5% water agar (15 g L^{-1}) amended with ampicillin (0.25 mg ml^{-1}) and streptomycin (0.5 mg ml^{-1}) to obtain mono-conidial colonies. Single-spore isolates morphologically characterized as likely *C. beticola* (Jacobsen and Franc 2009) were maintained on half-strength clarified V8 juice (CV8) agar (Miller 1955) amended with ampicillin (0.25 mg ml^{-1}) and streptomycin (0.5 mg ml^{-1}). Pure cultures were incubated for at least six weeks at ambient room temperature (21±2°C) in 24 h dark conditions to allow complete colonization of the 100-mm Petri dish.

4.3.2 Spiral plate gradient assessment of *Cercospora beticola* fungicide sensitivity

Fungicide sensitivity was determined for 78 *C. beticola* isolates in 2021 and 304 to 347 *C.*

beticola isolates in 2022, depending on the active ingredient. The spiral gradient dilution method (Förster et al. 2004) was used to determine the effective concentrations for inhibiting isolate growth by 50% (EC_{50}). Technical standards for difenoconazole, fenbuconazole, mefentrifluconazole, prothioconazole, pyraclostrobin, tetraconazole, thiophanate-methyl, and triphenyltin hydroxide were dissolved in acetone to obtain stock solutions (Table 4.1). To ensure consistent diffusion of active ingredients through the media, ½ CV8 agar (40 ml) was added to each 150-mm plate to ensure a 3-mm agar depth. The agar was amended with fungicide stock solution (54.3 µl) using an Eddy Jet 2 spiral plater (Neutec Group Inc, Farmingdale, NY). An exponential mode of application was used, achieving a radial concentration gradient. Amended plates were incubated for 48 hours at ambient (21±2°C) conditions, resulting in a diffuse concentration gradient across the agar and evaporation of the acetone (Förster et al. 2004). For pyraclostrobin screening, the alternative oxidase inhibitor salicylic hydroxamic acid (SHAM) was tested but not included in these studies due to significant inhibition of mycelial growth ($P < 0.0001$) and little explanatory benefit for the relationships between *in vitro* and genetic sensitivity information (data not shown). The lower and upper concentration limits varied for each active ingredient tested in this study (Table 4.1).

Table 4.1. Molecular weight, stock solution concentration, and lower and upper concentration limits tested for each active ingredient in 2021 and 2022.

Active ingredient	Molecular weight (g/mol)	2021			2022		
		Stock solution (µg ml^{-1})[†]	Lower limit (µg ml^{-1})	Upper limit (µg ml^{-1})	Stock solution (µg ml^{-1})	Lower limit (µg ml^{-1})	Upper limit (µg ml^{-1})
Difenoconazole	406.3	2,000	0.16	17.60	2,000	0.16	17.60
Fenbuconazole	336.8	2,000	0.16	17.87	-[‡]	-	-
Mefentrifluconazole	397.8	2,000	0.16	17.63	2,000	0.16	17.63
Prothioconazole	344.3	2,000	0.16	17.84	10,000	0.79	89.22
Pyraclostrobin	387.8	10,000	0.79	88.37	10,000	0.79	88.37
Tetraconazole	372.1	2,000	0.16	17.74	10,000	0.79	88.69

Table 4.1 (cont'd)

Thiophanate-methyl	342.4	2,000	0.16	17.85	10,000	0.79	89.26
Triphenyltin hydroxide	368.0	2,000	0.16	17.76	2,000	0.16	17.76

† Stock solution concentrations were adjusted for each standard to achieve desired lower and upper limits for the assay.
‡ Due to testing limitations, and similarity of sensitivity responses to other demethylation inhibitors, fenbuconazole was not tested in 2022.

Mycelial suspensions were collected from at least six-week-old cultures of each isolate as described by Hernandez et al. (2024). Briefly, sterile deionized water (750 µl) was added to each fully colonized plate, a sterile glass cell spreader was used to gently dislodge half of the aerial mycelia into suspension, and the resulting suspension was aspirated into a labeled sterile polypropylene test tube. Fifteen µl of the mycelial suspension was streaked onto a spiral gradient plate from edge to center (150-mm agar plates) using an SGE spiral plate template (Spiral Biotech, Norwood, MA) to ensure consistent distribution of the suspensions. Eight isolates were tested per spiral plate. Each isolate × active ingredient combination was replicated twice, including two replicates on acetone-amended control plates. Spiral plates were incubated at ambient light and room temperature conditions for two weeks.

After two weeks, isolate growth on the control and fungicide amended plates were assessed. The width of growth was measured with digital calipers 15 mm from the outer rim of each control plate and averaged for the two replicates. The 50% inhibition point was determined on fungicide-amended plates as the shortest distance from the center of the plate (in mm) where growth was half that of the control plates (Förster et al. 2004), this point was recorded using the SGE template for each replicate. These measurements along with the active ingredient molecular weight, stock concentration, agar height (3 mm), and length of incubation (2 days), were then entered into the ECXv4.0 (v4.0; Torres-Londoño et al. 2016; Torres 2021) R package to calculate the concentration at which the growth of each isolate was inhibited by 50% (EC_{50}). For mean

calculations, isolates with EC_{50} values above or below the detection limit were set to the upper or lower limit (13.8% of isolates tested, excluding thiophanate-methyl which was at either the upper or lower detection limit for all isolates tested).

4.3.3 Detection of *Cercospora beticola* mutations associated with fungicide resistance

DNA was extracted directly from CLS lesion tissue, or mono-conidial cultures when needed for confirmation, using a crude extraction method modified from Liber et al. (2022). Briefly, after conidia were dislodged from the lesion for *C. beticola* isolation, the entire lesion was excised using a sterile 5-mm cork borer and transferred to a 0.2-ml tube containing 20 µl of extraction solution (100 mM Tris, 250 mM KCl, 10 mM disodium EDTA, adjusted to pH 9.5–10). Tubes were incubated at 95°C for 10 minutes, then 60 µl of 3% bovine serum albumin was added, and resulting DNA extracts were stored at -20°C until PCR testing. DNA was subjected to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays (Yin et al. 2023; Obuya et al. 2008; Malandrakis et al. 2011, 2012; Rosenzweig et al. 2015). Assays targeted characterized *C. beticola* gene regions and resistance associated mutations using primers and restriction enzyme digests as described in Table 4.2. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR reactions consisted of genomic DNA template (2.0 µl), GoTaq Green Master 2x Mix (Promega Co., Madison, WI) (12.5 µl), and 10 µM of each primer (1 µl) in a 25-µl reaction volume. Amplifications were performed using the following parameters: 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 52°C for 30 s, 72°C for 1 min, with a final 10-min extension at 72°C (MiniAmp Plus thermocycler, Applied Biosystems, Waltham, MA). Following amplification, a 10-µl volume of the PCR reaction was reserved for an overnight digestion. Each digestion reaction consisted of CutSmart Buffer (New England Biolabs Inc., Ipswich. MA) (5 µl), PCR product (5 µl), and BstUI (0.15 µl) for E198A, BsmAI (0.15 µl)

for G143A, or Fnu4HI (0.3 µl) for Glu169 (New England Biolabs, Ipswich, MA). A final reaction volume of 50-µl was achieved with the addition of nuclease free water, and the reaction was incubated overnight at the designated temperatures (Table 4.2). Restriction fragments were resolved on 2.5% agarose gels in 0.5× Tris-borate-EDTA (5.4 g Tris-base, 2.75 g boric acid, 2 ml 0.5 M EDTA, 1,000 ml H2O) by electrophoresis and stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA). Gels were visualized using the Safe Imager 2.0 Blue Light Transilluminator (Invitrogen, Carlsbad, CA).

Reference *C. beticola* isolates 'Blum 1-2' and 'Range A' from the United States Department of Agriculture-Agricultural Research Service Sugar Beet Research Unit (SBRU) fungal collection were also included. Blum 1-2 was obtained from symptomatic sugarbeet leaf lesions from 2017 in Saginaw County, MI. Range A was collected from a symptomatic sugarbeet leaf in 2008 from Ingham County, MI. The base pair changes detected using PCR-RFLP assays were confirmed in six representative isolates for DMI- and MBC-associated mutations (Figures S4.1 and S4.2) and 16 representative isolates for QoI-associated mutations (Hernandez et al. 2024). Sanger sequencing services were provided by the MSU Research Technology Support Facility Genomics Core located in East Lansing, MI.

Table 4.2. Information for point mutations tested, including primers, restriction enzymes, and digest temperatures used for each fungicide group in the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays for *Cercospora beticola* from sugarbeet.

FRAC group/ Common name	Mutation †	Restriction enzyme / digest temperature (°C)	Sequence change	Primers ‡
1 / MBC	E198A	BstUI / 60	5′-GCG-3′ to 5′-GAG-3 (Obuya et al. 2008; Malandrakis et al. 2012)	CbBtrF 5′-ATTCTCCGTCATGCCATCTC-3′ CbBtrR 5′-GAAACGCAGACAGGTTGTCA-3′ (Rosenzweig et al. 2015)
3 / DMI	Glu169	BsmAI / 55	5′-GAG-3′ to 5′-GAA-3′	CYP51RT-F 5'-AACTCCAAATTGATGGAGCA-3' CYP51RT-R 5'-CGGCTAGCAGTGTAAATGGT-3' (Nikou et al. 2009)

Table 4.2 (cont'd)

11 / QoI	G143A	Fnu4H / 37	5′-GGTGC-3′ to 5′-GCTGC-3′	cytbFu 5′-ACAAAGCACCTAGAACATTGG-3′ cytbRu 5′-GAAACTCCTAAAGGATTACCTGAACC-3′ (Malandrakis et al. 2012)

† Location of mutations: beta-tubulin gene (E198A), 14-C alpha-demethylase gene (Glu169), and the mitochondrial cytochrome b gene (G143A).
‡ Amplicon lengths were 500, 200, and 325 bp for the MBC, DMI, and QoI target regions, respectively.

4.3.4 Statistical analysis

All statistical analyses were advised by the MSU Statistical Consulting Center and performed in R Statistical Software (v4.1.1; R Core Team 2021). All tests were evaluated at the α=0.05 significance threshold. The 'lmertest' R package (Kuznetsova et al. 2017) was used for all simple linear (lm), linear mixed (lmer), and generalized linear mixed (glm, glmer) model analyses. The 'Emmeans' and 'multcomp' packages (Lenth 2023; Hothorn et al. 2008) were used to generate mean comparisons with a Bonferroni adjustment. For across year analyses, data for prothio- and tetraconazole were adjusted to consistent upper and lower detection limits.

Using linear mixed models (lmer), the log transformed EC_{50} values or reduced sensitivity frequencies were evaluated with fixed effects of active ingredient, year, and year*active ingredient and random effects of field nested in year and sample nested in field nested in year. To determine EC_{50} thresholds for reduced sensitivity to each active ingredient, k-means cluster analysis was conducted on the residuals of the linear model of EC_{50} on year and field (to eliminate the effect of grouping due to year or field) using the 'kmeans' function in R (Rangel et al. 2024; Talas et al. 2024). The optimal number of clusters for each analysis was determined using the 'elbow' method. Reduced sensitivity frequencies were calculated as the percentage of isolates with EC_{50} values greater than the discriminatory sensitivity threshold, which was calculated as the maximum EC_{50} value of the most sensitive cluster plus the corresponding interquartile range to account for statistical dispersion (Table S4.1; Rangel et al. 2024). Calculated sensitivity

thresholds were 5.11 µg ml^{-1} for difenoconazole, 2.01 µg ml^{-1} for fenbuconazole, 3.26 µg ml^{-1} for mefentrifluconazole, 9.56 µg ml^{-1} for prothioconazole, 6.91 µg ml^{-1} for tetraconazole, 2.55 µg ml^{-1} for triphenyltin hydroxide, and 19.51 µg ml^{-1} for pyraclostrobin. For thiophanate-methyl, isolates with EC_{50} values > 5 µg ml^{-1} were considered to exhibit reduced sensitivity according to previous studies (Ma and Michailides 2005; Trivellas 1988). A Kendall correlation analysis of exact EC_{50} values was conducted within and between fungicide groups; correlation coefficients were generated using the SAS (Statistical Analysis System) v. 9.4 software (SAS Institute, Inc. Cary, NC, United States) 'proc corr' procedure with the 'kendall' option and a heat map created using the 'ggplot' package (Wickham 2016).

For 2022 chemical records, simple linear regression (lm) was used to evaluate the relationship between the mean isolate EC_{50} values or resistance frequencies by field and the number of applications for each fungicide group alone or divided by the total number of applications. Linear mixed models (lmer) were used to evaluate the relationship between mutations and isolate EC_{50} values for each of the associated active ingredients. For the E198A mutation, EC_{50} values were considered the response variable, and fixed effects included binary mutation results, year, and year*mutation interaction and the random effect was field nested in year. For the Glu169 mutation, log-transformed EC_{50} values were evaluated with added fixed effects of active ingredient and all two-way interactions and added random effect of sample nested in field nested in year. The G143A substitution was detected in all tested isolates, therefore no additional analysis was conducted.

4.4 Results

4.4.1 *Cercospora beticola* sensitivity across fungicide groups and shifts between years

Population distributions for *C. beticola* fungicide sensitivity varied considerably across

tested DMI, QoI, and organotin active ingredients (Figure 4.2 and Figure S4.4). Linear mixed models indicated that *C. beticola* log-transformed EC_{50} values and resistance frequencies were significantly impacted by active ingredient ($P < 0.0001$) and year*active ingredient ($P < 0.01$). In 2021, mean EC_{50} values for difenoconazole, fenbuconazole, and triphenyltin hydroxide were statistically similar and significantly lower than the remaining active ingredients tested. In 2022, *C. beticola* isolates exhibited the greatest sensitivities to difenoconazole and triphenyltin hydroxide (mean EC_{50} = 0.6-1.0 µg ml^{-1}). In both 2021 and 2022, EC_{50} means for pyraclostrobin were greater than all other active ingredients (14.5-10.5 µg ml^{-1}) but were not statistically different from prothioconazole. Though 70.1% of *C. beticola* isolates were classified as sensitive to mefentrifluconazole, mean EC_{50} values were significantly greater than difenoconazole in 2021 (0.6-1.8 µg ml^{-1}). For tetraconazole in both years, mean EC_{50} values were significantly higher (3.7-6.4 µg ml^{-1}) compared to mefentrifluconazole but lower than prothioconazole.

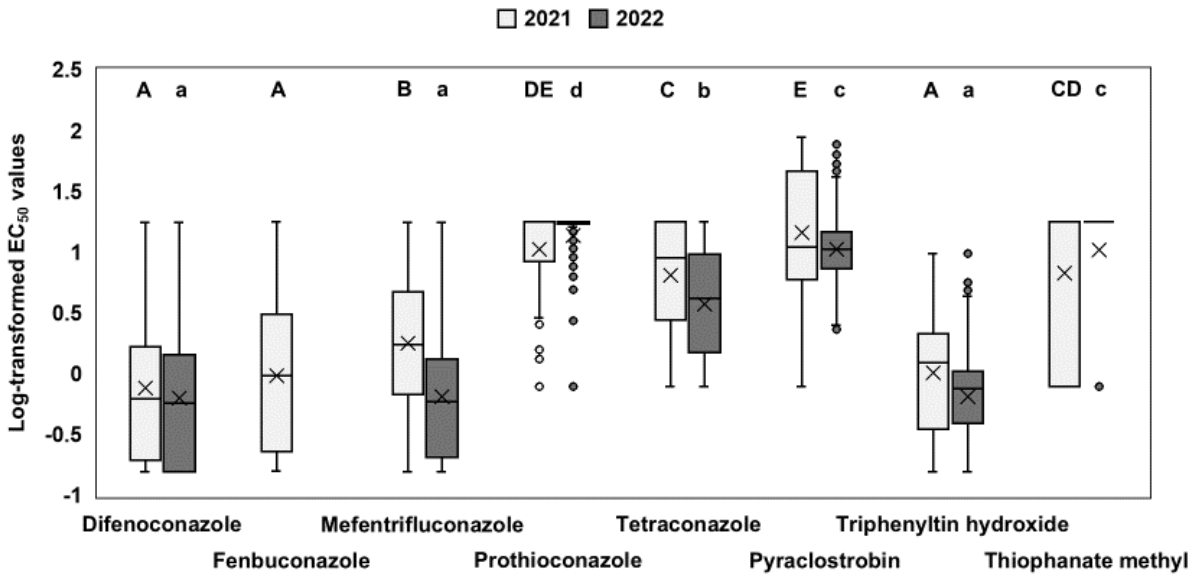


Figure 4.2. Box plots of the log-transformed EC_{50} values for each fungicide active ingredient tested for *Cercospora beticola* isolates collected from Michigan in 2021 (n = 78 isolates) and 2022 (n = 304-347) ($P < 0.0001$). The box represents the interquartile interval where 50% of the data points are found. The line that divides the box is the median and "X" represents the mean. The lines that extend vertically show variability outside of the interquartile interval. Outliers are

Figure 4.2. (cont'd)
shown as open circles. Active ingredient effects were evaluated using linear mixed model analysis separately for each year and letter separations were assigned based on the pairwise comparison *P*-values adjusted for multiple testing by the Bonferroni method (α=0.05).

Based on the frequencies of reduced sensitivity in *C. beticola* isolates in 2021 and 2022 (Table 4.3), significant decreases were detected between years for mefentrifluconazole ($P = 0.0046$), pyraclostrobin ($P < 0.0001$), tetraconazole ($P = 0.0010$), and triphenyltin hydroxide ($P = 0.0023$). In these instances, approximately 11-28% reductions in *C. beticola* resistance to select fungicides were observed in 2022 compared to 2021 levels. There were no significant differences between the 2021 and 2022 *C. beticola* reduced sensitivity frequencies for difenoconazole ($P > 0.05$). The frequency of *C. beticola* isolates with *in vitro* resistance to prothioconazole and thiophanate-methyl increased significantly (+13-14%) from 2021 to 2022 ($P < 0.05$).

Table 4.3. Frequency of isolates with reduced sensitivity of *C. beticola* isolates determined by *in vitro* spiral gradient fungicide screening in 2021 and 2022.

Active ingredient	2021 (%) †		2022 (%)		Δ (%)
	Mean ‡	SE	Mean	SE	
Difenoconazole	7.1	2.8	3.3	0.9	-3.8
Fenbuconazole	27.4	6.5	-	-	-
Mefentrifluconazole	29.9 b	6.7	12.7 a	2.3	-17.2
Prothioconazole	78.4 a	5.7	91.7 b	1.8	13.3
Tetraconazole	56.1 b	7.7	28.3 a	3.6	-27.8
Pyraclostrobin	33.2 b	7.1	8.6 a	1.7	-24.6
Thiophanate-methyl §	71.9 a	6.6	86.1 b	3.3	14.2
Triphenyltin hydroxide	11.3 b	3.8	2.9 a	0.8	-10.5

† Reduced sensitivity frequencies were calculated as the percentage of isolates with EC_{50} values greater than 5 μg ml^{-1} for thiophanate-methyl and the k-means sensitivity threshold for each active ingredient: difenoconazole (5.11 μg ml^{-1}), mefentrifluconazole (3.26 μg ml^{-1}), tetraconazole (6.91 μg ml^{-1}), prothioconazole (9.56 μg ml^{-1}), pyraclostrobin (19.51 μg ml^{-1}), and triphenyltin hydroxide (2.55 μg ml^{-1}). EC_{50} values between years were adjusted to the same detection limits for analysis.
‡ Year effects were investigated separately by active ingredient. Letter separations within the same row were based on linear mixed model analyses and multiple testing by the Bonferroni method (α=0.05).
§ For thiophate-methyl, 77 and 152 isolates were tested in 2021 and 2022, respectively; for all other active ingredients, 78 and 304-347 isolates were tested, respectively.

According to 2021 and 2022 sampling, *C. beticola* sensitivity varied by county (Figure S4.3). Over 40% of isolates sampled from Saginaw, Tuscola, and Sanilac Counties exhibited reduced sensitivity for tetraconazole. More than 80% of isolates from Huron, Bay, and Sanilac

Counties possessed reduced sensitivity to thiophanate-methyl. Greater than 10% of isolates in Midland and Sanilac Counties exhibited reduced sensitivity to triphenyltin hydroxide. Compared to other counties sampled, *C. beticola* populations in Midland, Sanilac, and Huron Counties remained relatively sensitive to difenoconazole, with less than 5% of isolates exhibiting reduced sensitivities.

4.4.2 Effects of fungicide application on in-season shifts in *C. beticola* fungicide sensitivity

Of the active ingredients included in this study, difenoconazole, mefentrifluconazole, prothioconazole, thiophanate-methyl, and triphenyltin hydroxide were the most commonly applied with at least one treatment reported in nearly 50-80% of fields sampled in 2022 (Table S4.2). Comparatively, pyraclostrobin and tetraconazole were applied to fewer sampled fields at 34 and 9% receiving at least one application, respectively.

Based on a k-means generated EC_{50} value thresholds, the number of DMI applications was significantly associated with the frequency of *in vitro* prothioconazole-resistant *C. beticola* isolates in 2022 ($P < 0.01$) (Table S4.3); when visualized, insensitive isolates increased by 14% when comparing between one and four DMI applications (Figure 4.3A and 4.3B). Difenoconazole and mefentrifluconazole responses showed no significant impact for number of DMI fungicide treatments ($P > 0.05$) (Table S4.3), with consistently 3-17% of *C. beticola* isolates categorized with reduced sensitivity across one to four applications (Figure 4.3A and 4.3B). The frequency of isolates categorized as resistant to pyraclostrobin (Figure 4.4A) and triphenyltin hydroxide (Figure 4.4B and 4.4D) were not significantly related to the number of tin or QoI applications, respectively ($P > 0.05$) (Table S4.3). When considering mean EC_{50} values, only *C. beticola* thiophanate-methyl responses were significantly impacted by the number of MBC fungicide applications ($P < 0.05$) (Table S4.3); frequency of *in vitro* resistant *C. beticola* isolates increased

by approximately 9% when comparing fields with zero versus one MBC application (Figure 4.4C).

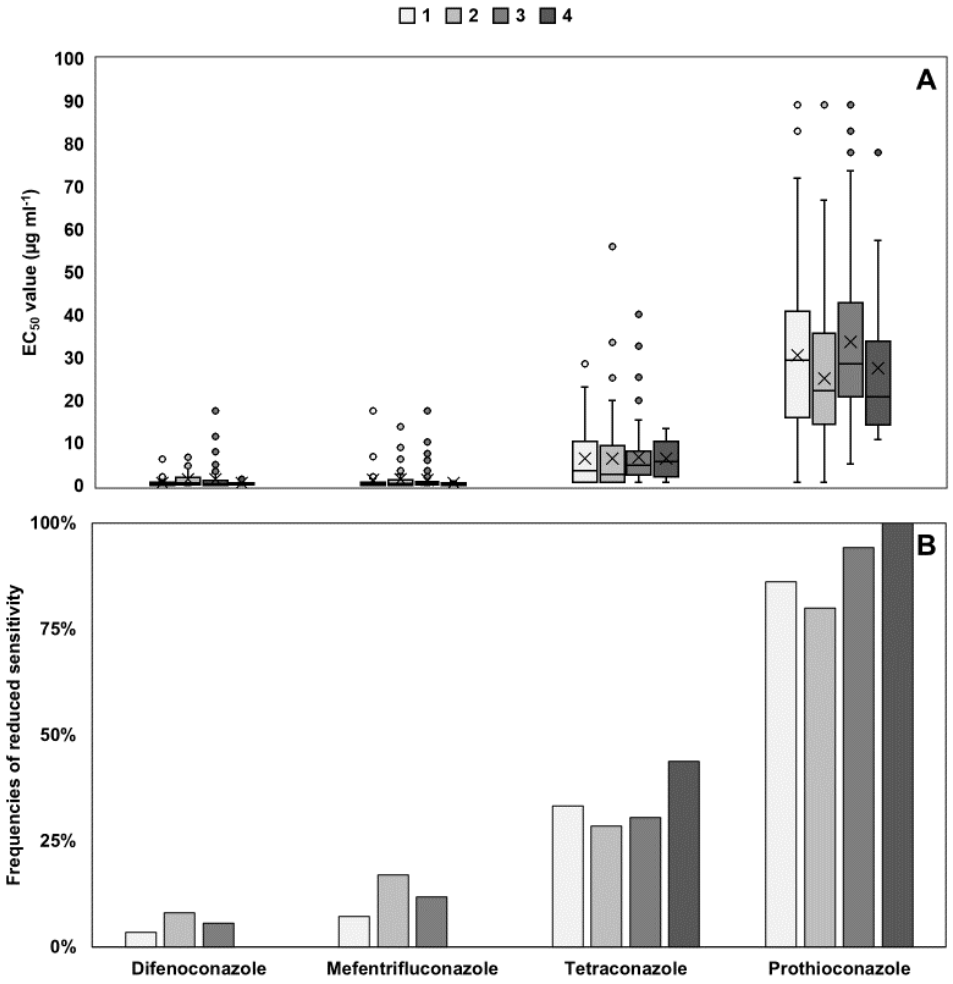


Figure 4.3. (A) Boxplots of *Cercospora beticola* isolate EC_{50} values for difenoconazole, mefentrifluconazole, tetraconazole, and prothioconazole arranged by number of DMI fungicide applications. The box represents the interquartile interval where 50% of the data points are found. The line that divides the box is the median and the "X" represents the mean. The lines that extend vertically show variability outside of the interquartile interval. Outliers are shown as open circles. **(B)** Frequency of isolates with EC_{50} values above the reduced sensitivity thresholds for difenoconazole (5.11 µg ml^{-1}), mefentrifluconazole (3.26 µg ml^{-1}), tetraconazole (6.91 µg ml^{-1}), and prothioconazole (9.56 µg ml^{-1}) arranged by number of DMI fungicide applications; thresholds were determined using maximum EC_{50} values and interquartile ranges of the most sensitive isolate groups according to k-means cluster analyses (Rangel et al. 2024; Talas et al. 2024). According to the simple linear regression, the resistance frequencies for tetraconazole and prothioconazole was significantly impacted by the number of DMI applications ($P < 0.01$), but not for difenoconazole and mefentrifluconazole ($P > 0.05$); no other tested interactions were significant.

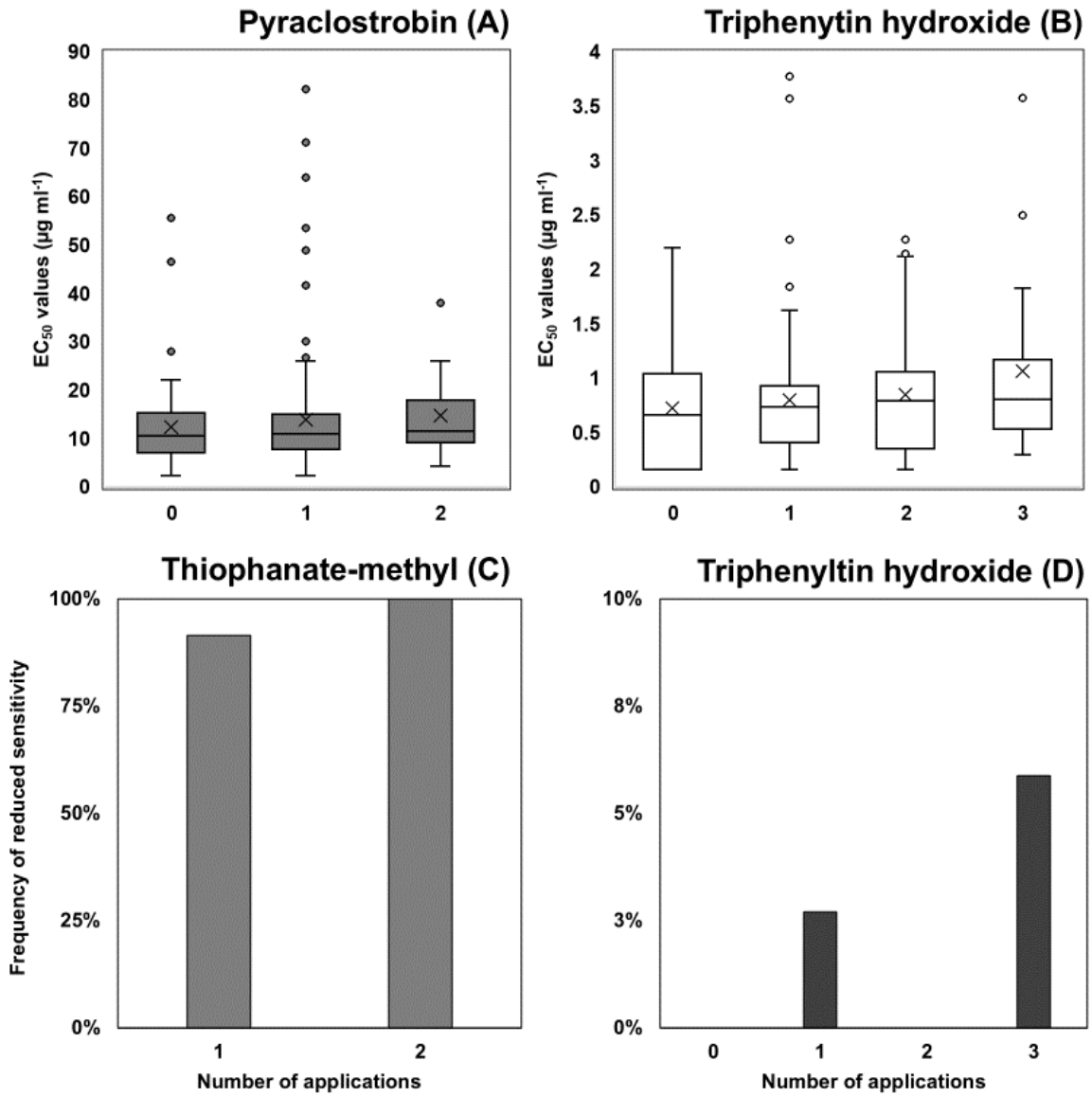


Figure 4.4. Boxplots of *Cercospora beticola* isolate EC_{50} values for **(A)** pyraclostrobin and **(B)** triphenyltin hydroxide across the number of fungicide group applications. The box represents the interquartile interval where 50% of the data points are found. The line that divides the box is the median and the "X" represents the mean. The lines that extend vertically show variability outside of the interquartile interval. Outliers are shown as open circles. Frequency of isolates with EC_{50} values above 5 µg ml^{-1} for **(C)** thiophanate-methyl and 2.55 µg ml^{-1} for **(D)** triphenyltin hydroxide across the number of fungicide group applications. According to simple linear regression, thiophanate-methyl EC_{50} values were significantly impacted by the number of MBC applications ($P < 0.05$); no other interactions were significant.

4.4.3 Cross and multiple resistances detected by correlation analyses between active ingredients

Cercospora beticola EC_{50} values for mefentrifluconazole and difenoconazole were the most highly and significantly correlated of all active ingredient combinations ($r = 0.58$, $P < 0.0001$) (Figure 4.5). Prothioconazole and tetraconazole ($r = 0.21$) and fenbuconazole and tetraconazole or prothioconazole ($r = 0.54$ or 0.51) were also highly correlated with one another ($P < 0.0001$). Within the DMI compounds, *C. beticola* difenoconazole responses were not strongly or significantly correlated with prothioconazole responses. Similarly, mefentrifluconazole response was not strongly or significantly correlated with prothioconazole or fenbuconazole responses. Between mode of action groups, moderate ($0.25 < r < 0.50$) yet significant correlations were noted between triphenyltin hydroxide and fenbuconazole or tetraconazole ($r = 0.26$ or 0.30, $P < 0.01$) and thiophanate-methyl and prothioconazole ($r = 0.38$, $P < 0.0001$).

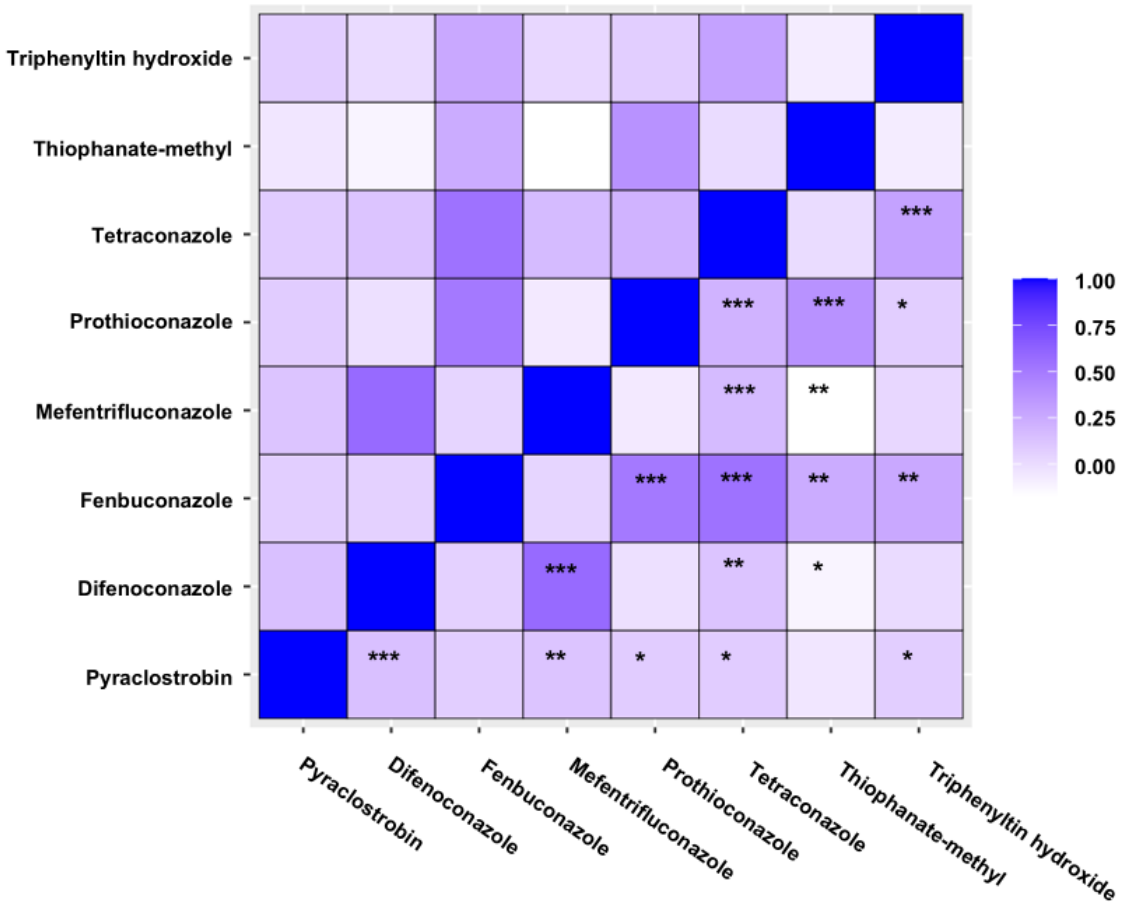


Figure 4.5. Heat map showing the Kendall correlation coefficients for *in vitro* fungicide sensitivity to difenoconazole, fenbuconazole, mefentrifluconazole, prothioconazole, pyraclostrobin, tetraconazole, and triphenyltin hydroxide for *Cercospora beticola* isolates from Michigan. Color intensity corresponds to the strength of correlation coefficients. Significance is denoted by the number of asterisks in the bottom right half of the heat map and corresponds to *P*-value thresholds <0.05 *, <0.01 **, and <0.0001 ***.

4.4.4 Prevalence and impact of PCR-RFLP detected mutations associated with *C. beticola* fungicide sensitivity

A total of 78 and 373 *C. beticola* isolates were screened for Glu169, E198A, and G143A mutations in 2021 and 2022, respectively. The frequency of *C. beticola* isolates with the Glu169 mutation associated with DMI resistance was 21% in 2021 and 13% in 2022. For the E198A mutation associated with MBC resistance, approximately 68% of isolates had the mutation in

2021 compared to 74% in 2022. Across both years of the study, all 451 *C. beticola* isolates screened possessed the G143A mutation associated with QoI resistance.

Based on linear mixed model analyses, the presence of the Glu169 mutation had a significant impact on EC_{50} responses for DMI fungicides ($P < 0.01$) with significant interactions between mutation and active ingredient ($P < 0.0001$). Difenoconazole and mefentrifluconazole EC_{50} values significantly increased when the Glu169 mutation was present (Figure 4.6). Conversely, tetraconazole EC_{50} values were significantly reduced when the Glu169 was present, and fenbuconazole and prothioconazole responses were not significantly different between wild-type and mutant isolates. Simple linear regression analysis indicated that benzimidazole EC_{50} responses were significantly impacted by year ($P < 0.05$), mutation ($P < 0.0001$), and their interaction ($P < 0.001$). Wild-type *C. beticola* isolates lacking the E198A mutation possessed significantly lower EC_{50} values (means of 0.79-2.40 µg ml^{-1}) compared to mutant isolates (17.54-17.74 µg ml^{-1}) in 2021 and 2022 (Figure S4.5).

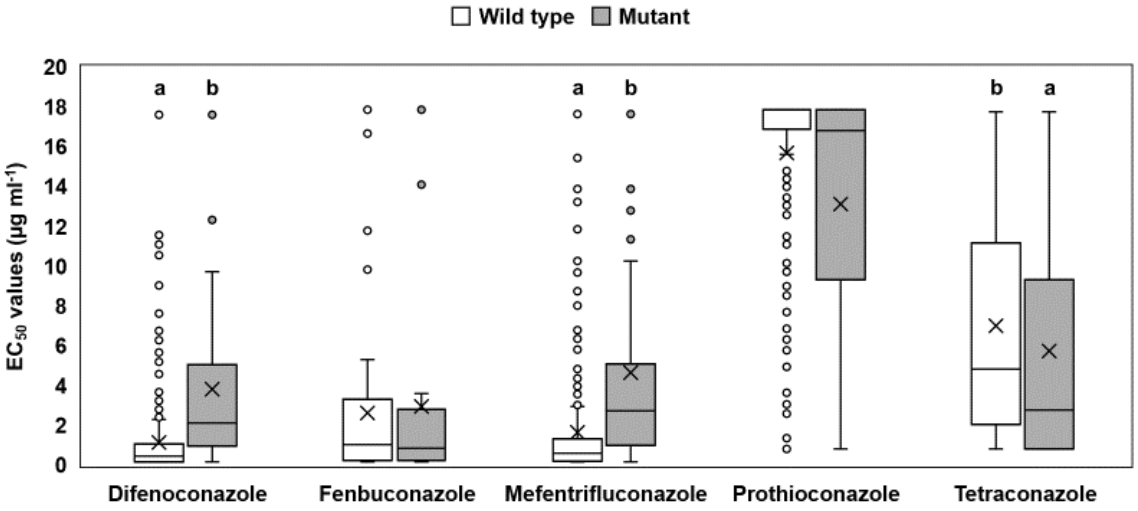


Figure 4.6. Box plots of *Cercospora beticola* EC_{50} values with (mutant) and without (wild type) the Glu169 mutation associated with DMI resistance for each active ingredient tested in 2021 and 2022. The results of the linear mixed model analysis indicated that the presence or absence of

Figure 4.6. (cont'd)
mutation significantly impacted difenoconazole, mefentrifluconazole, and tetraconazole ($P < 0.001$); no other active ingredients had significant interactions. The box represents the interquartile interval where 50% of the data points are found. The line that divides the box is the median and the "X" represents the mean. The lines that extend vertically show variability outside of the interquartile interval. Outliers are shown as open circles. Significant differences are indicated by letters assigned using pairwise comparison with *P*-values adjusted for multiple testing by the Bonferroni method (α=0.05). EC_{50} values between years were adjusted to the same detection limits.

4.5 Discussion

Michigan *C. beticola* isolates collected from commercial sugarbeet fields were most sensitive to the fungicides difenoconazole, fenbuconazole, and triphenyltin hydroxide and least sensitive to prothioconazole and pyraclostrobin in 2021 and 2022. Monitoring *in vitro* sensitivity of *C. beticola* isolates is critical because of potential impacts on CLS control. When exposed to field rates of Eminent (tetraconazole 11.6%), *C. beticola* isolates with high tetraconazole EC_{50} values (> 1 µg ml^{-1}) caused elevated CLS compared to isolates with lower EC_{50} values (Bolton et al. 2012a). Similarly, even limited applications of Headline (pyraclostrobin 23.6%) were found to increase pyraclostrobin insensitivity in *C. beticola* isolates and reduce CLS control (Hernandez et al. 2024); past and current use of pyraclostrobin-containing products could account for the insensitivity and genetic QoI resistance observed in the current study. Observed differences between active ingredients likely result from a variety of factors such as modes of action, product formulations, resistance mechanisms, and fungicide use, which has been shown in other fungal pathogens (Trkulja et al. 2015; Yang et al. 2019; Khan et al. 2008) and in the current study. Observed county-level differences and localized *C. beticola* dispersal (Yang et al. 2019) further support continued screening to inform local practices. As demonstrated by regional trials, integrated use of currently recommended products effectively manages CLS (Bloomingdale and Willbur 2021; Bloomingdale and Willbur 2022; Bloomingdale and Willbur 2023).

Cross resistance between DMI active ingredients was detected in Michigan isolates, as has

been reported in studies of *C. beticola* and other fungal plant pathogens (Avenot et al. 2016; Ishii et al. 2021; Köller et al. 1997; Karaoglanidis and Thanassoulopoulos 2003; Thomas et al. 2012; Hsiang et al. 1997). However, not all DMI responses were significantly correlated, which also is consistent with previous research findings that cross resistance can vary for different DMI active ingredients (Leroux et al. 2000; Köller and Wubben 1989; Hildebrand et al. 1988). This variability could be due to differences between disruptions in demethylation and specific resistance mechanisms (Karaoglanidis and Ioannidis 2010; Kendall et al. 1993) or uptake or movement (Erwin 1973; Lucas et al. 2015). Interestingly, some significant relationships were observed between triphenyltin hydroxide and other mode of action groups; this is supported by reports of individual *C. beticola* isolates with multiple resistances to unspecified DMI active ingredients and organotin fungicides (Rosenzweig et al. 2020). Correlations between different modes of action could be impacted by their target site activities or use pattern in fungicide programs. Certain fungicides may also influence these relationships, such as MBC resistance, shown to be associated with increased resistance to other fungicides (Köller and Wilcox 2001). These findings further support integrated sugarbeet CLS management recommendations to rotate fungicide modes of action and limit the repeated use of active ingredients within groups (Franc et al. 2001).

Multiple DMI applications have been found to reduce *C. beticola* sensitivity (Bolton et al. 2012a), which is consistent with the current study observations for prothioconazole and tetraconazole. Similarly, increases were observed in thiophanate-methyl EC_{50} values following MBC applications. Previous research in Serbia observed high frequencies of MBC-resistant *C. beticola* isolates before (ranging from 93.3-98.6%) and after benzimidazole applications (100%) tested in 2010 to 2011 (Trkulja et al. 2015); however, lower MBC resistance frequencies in

Michigan may have contributed to a higher capacity for change. In contrast, the high G143A-associated pyraclostrobin resistance observed in Michigan samples (98-100%) may have reduced any detection of potential QoI application impacts. These results support chemical rotation and tank-mixing, also evidenced by current recommendations to include a multi-site fungicide with fungicides like the ones studied here. For example, Yang et al. (2019) reported that increased application of a multi-site fungicide (mancozeb) led to reduced resistance to difenoconazole in *Alternaria alternata*. The reported frequent use of multi-site tank-mix partners (nearly 100% of applications in the current study), may have limited the measurable impacts of application number on at least some of the active ingredients tested. A more direct study would be beneficial to definitively evaluate the relationships between fungicide sensitivity and field applications.

While significant shifts in resistance frequencies for four of seven active ingredients were observed, directional increases or decreases were inconsistent. Previously, decreases in *C. beticola* sensitivity to triphenyltin hydroxide, difenoconazole, fenbuconazole, prothioconazole, and tetraconazole were observed in the Great Lakes region from 2014 to 2017 (Rosenzweig et al. 2020). In the Red River Valley, populations of *C. beticola* demonstrated variable shifts in sensitivities for triphenyltin hydroxide with both increases and decreases observed over time (Rangel et al. 2020), in part related to fungicide applications. In Minnesota and North Dakota, Bolton et al. (2012a) further reported increasing frequencies of *C. beticola* prothioconazole and tetraconazole insensitivity whereas difenoconazole responses remained comparable from year to year, which was consistent with findings in the current study. Previous shifts in Michigan *C. beticola* thiophanate-methyl insensitivity, from 20% to 80% over 11 years (Weiland and Halloin 2001), have been relatively sustained according to frequencies in the current study (71-85%). This is consistent with other reports, where MBC resistance remained at a high frequency even when

the fungicide was not used (Smith 1988). For other fungicides, potential fitness costs (Beyer et al. 2011; Standish et al. 2019) combined with integrated resistance management, may contribute to the decreased or slowed progression of insensitivity. Additional years of *C. beticola* monitoring are necessary to comprehensively understand meaningful and sustained population shifts.

The current study further evaluated mutations associated with *C. beticola* resistance to DMI, MBC, and QoI active ingredients for their explanatory value of *in vitro* responses. Previous studies identified *C. beticola* stains with the E198A mutation to have higher EC_{50} values (≥ 60 μg ml^{-1}) for methyl-benzimidazole carbamate compared to wild-type isolates (< 5 μg ml^{-1}; Davidson et al. 2006). This was consistent with highly significant associations observed in the current study where E198A mutants resulted in dramatically greater thiophanate-methyl EC_{50} values (≥ 89.5 μg ml^{-1}) than wild-type isolates (≤ 0.79 μg ml^{-1}). Compared to previous E198A detections of over 90% of *C. beticola* isolates sampled from Michigan in 2012 (Rosenzweig et al. 2015), the presence of this mutation remains prevalent in local populations at approximately 70% of isolates in 2021 and 2022. In contrast, for QoI resistance, all but one *C. beticola* isolate collected in this study (total 451 tested) had the G143A mutation but a wide range of EC_{50} values was observed. High levels of resistance have been associated with this mutation in other systems, such as *Alternaria alternata* isolates sensitivities to azoxystrobin ($EC_{50} > 100$ μg ml^{-1}) from pistachio (Ma et al. 2003). However, *C. beticola* isolates with the G143A mutation have exhibited wide ranges (0.92 – 100 μg ml^{-1}) of *in vitro* pyraclostrobin sensitivities in previous studies (Kirk et al. 2012; Bolton et al. 2013) consistent with the current study. Similarly high frequencies of G143A mutation (> 90%) have been reported in *C. beticola* isolates across Michigan in 2012 (Rosenzweig et al. 2015) and in the Red River Valley in 2016 through 2019 (Rangel et al. 2020). Overall, the G143A mutation continues to be widespread in Michigan sugarbeet production areas

fungicide resistance remains a concern for CLS management (Kirk et al. 2012; Rosenzweig et al. 2015).

The Glu169 mutation was not consistently associated with any particular levels of insensitivity for tested DMI active ingredients. While *C. beticola* isolates with this mutation were more resistant to *in vitro* difenoconazole and mefentrifluconazole than wild type isolates, the opposite relationship was observed for tetraconazole, and no detectable impact of the mutation was observed for the remaining triazoles. DMI sensitivity can be driven by target site mutations, overexpression of the target gene, increases in efflux activity of DMIs, as well as the presence of multiple copies of the target gene (Leroux et al. 2007; Ziogas and Malandrakis 2015). One or more of these alternate mechanisms could account for why the Glu169 mutation was ineffective in explaining *in vitro* sensitivities for all triazoles used in the current study. Overexpression of CbCyp51 was found to play a role in *C. beticola* tetraconazole resistant isolates (EC_{50} > 1 µg ml^{-1}) in one study (Bolton et al. 2012b), which could be a factor influencing sensitivity in the current study. Spanner et al. (2021) and Muellender et al. (2021) have since identified multiple mutations in the CbCyp51 gene including E170, I387M, Y464S, L144F, and I309T combined with L144F, as well as on chromosome 1, 4, and 9, associated with *C. beticola* tetraconazole or difenoconazole and epoxiconazole resistance, but their impact on other DMI fungicides is not known. Future studies may be beneficial to assess the importance of additional mutations in determining DMI sensitivity for Michigan *C. beticola* populations and impacts on response to other members of the DMI fungicide class.

4.6 Conclusion

This study presents prevalence and distribution information for *C. beticola* fungicide sensitivity from 2021 and 2022 in Michigan. Regional monitoring of fungicide resistance

development is important to inform effective management decisions. Evidence was presented to support impacts of in-season fungicide applications on *C. beticola* isolate sensitivity to thiophanate-methyl and prothioconazole and supports that potential cross resistance between DMI products should be considered. This study determined the E198A mutation may be useful in monitoring thiophanate-methyl resistance, while the G143A and Glu169 mutations were not sufficiently explanatory for pyraclostrobin or all tested DMI active ingredients, respectively. Further research would be needed to identify mutations or other factors more closely associated with DMI- and QoI-resistance in *C. beticola*. Highly descriptive genetic markers remain of interest for rapid detection and monitoring of *C. beticola* resistance to better advise CLS management and fungicide recommendations.

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APPENDIX

Table S4.1. Summary statistics for k-means clusters of DMI EC_{50} values (μg ml^{-1}). The optimal number of clusters was determined using the elbow method for each active ingredient. Cluster represents the cluster number and N corresponds to the number of isolates in the cluster. For each active ingredient cluster, mean, minimum (min), maximum (max), Q1 (quartile 1), median, Q3 (Quartile 3), and IQR (Interquartile range = Q3-Q1). The maximum plus interquartile range (Max + IQR) for the most sensitive cluster was used for a discriminatory sensitivity threshold. The most sensitive cluster is indicated in bold.

Difenoconazole									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	237	0.65	0.16	7.82	0.16	0.39	0.79	0.63	8.45
2	**45**	**0.59**	**0.16**	**4.55**	**0.16**	**0.35**	**0.72**	**0.56**	**5.11**
3	13	12.00	6.33	17.60	9.02	11.60	17.60	8.58	26.18
4	102	2.59	0.16	11.10	0.92	1.99	3.64	2.72	13.82
Fenbuconazole									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	18	1.90	0.16	4.57	0.16	1.69	3.77	3.61	8.18
2	6	13.50	9.81	17.90	10.30	12.90	16.90	6.60	24.50
3	16	2.02	0.40	5.28	1.04	1.78	2.55	1.51	6.79
4	6	0.81	0.16	2.47	0.25	0.55	0.87	0.62	3.09
5	12	1.13	0.16	4.57	0.16	0.57	1.53	1.37	5.94
6	2	17.30	16.70	17.90	17.00	17.30	17.60	0.60	18.50
7	**18**	**0.49**	**0.16**	**1.67**	**0.20**	**0.30**	**0.54**	**0.34**	**2.01**
Mefentrifluconazole									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	**25**	**0.65**	**0.16**	**2.66**	**0.21**	**0.46**	**0.81**	**0.60**	**3.26**
2	71	1.78	0.16	9.97	0.63	1.10	2.55	1.92	11.89
3	85	0.66	0.16	5.06	0.16	0.41	0.75	0.60	5.66
4	15	13.00	8.93	17.60	10.40	13.20	14.70	4.30	21.90
5	165	0.64	0.16	2.80	0.16	0.49	0.83	0.67	3.47
6	36	5.51	2.91	14.20	4.10	4.81	6.06	1.96	16.16
7	9	16.60	13.90	17.60	15.40	17.60	17.60	2.20	19.80
Prothioconazole									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	68	16.10	9.73	17.80	14.40	17.80	17.80	3.40	21.20
2	234	17.80	15.60	17.80	17.80	17.80	17.80	0.00	17.80
3	42	11.90	6.32	17.80	8.61	11.20	14.80	6.19	23.99
4	**38**	**2.29**	**0.79**	**6.87**	**0.79**	**0.79**	**3.48**	**2.69**	**9.56**
Pyraclostrobin									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	161	11.8	6.34	20.1	10.1	11.3	13.4	3.3	23.4
2	22	80.1	64.1	88.4	73.2	82.4	88.4	15.2	103.6
3	59	21.2	15.4	31.8	18	19.7	23.2	5.2	37
4	17	48.1	38.1	60	44.6	49	51.2	6.6	66.6
5	**166**	**6.29**	**0.786**	**16.1**	**4.59**	**6.33**	**8**	**3.41**	**19.51**
Tetraconazole									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	62	12.30	8.55	17.70	10.10	11.60	13.50	3.40	21.10
2	**81**	**1.45**	**0.79**	**6.04**	**0.79**	**0.79**	**1.66**	**0.87**	**6.91**
3	147	2.81	0.79	9.09	0.79	2.68	4.11	3.32	12.41
4	62	17.20	12.90	17.70	17.40	17.70	17.70	0.30	18.00

Table S4.1. (cont'd)

5	70	7.27	4.22	14.70	5.93	6.75	8.13	2.20	16.90
Triphenyltin hydroxide									
Cluster	N	Mean	Min	Max	Q1	Median	Q3	IQR	Max + IQR
1	89	0.67	0.16	4.62	0.16	0.52	0.87	0.72	5.34
2	**67**	**0.87**	**0.16**	**2.12**	**0.62**	**0.83**	**1.05**	**0.43**	**2.55**
3	79	0.94	0.16	3.77	0.53	0.77	1.24	0.71	4.48
4	11	1.34	0.16	4.25	0.37	0.78	1.76	1.40	5.65
5	52	1.35	0.16	3.84	0.76	1.05	1.83	1.07	4.91
6	20	3.32	0.46	9.81	1.59	3.46	4.49	2.90	12.71
7	60	0.70	0.16	3.10	0.28	0.48	0.88	0.60	3.70
8	7	1.22	0.16	2.55	0.40	1.26	1.88	1.49	4.04
9	22	1.04	0.16	5.10	0.45	0.71	1.16	0.72	5.82
10	3	8.86	6.10	10.70	7.96	9.81	10.20	2.24	12.94

Table S4.2. Fungicide application data obtained from 29 Michigan commercial sugarbeet fields in 2022. The percentages of fields with at least one application and the range of the number of applications recorded within a field location are listed below for each active ingredient.

Active ingredient †	Percentage of fields with at least one application	Number of applications
Triphenyltin hydroxide	81%	0-3
Prothioconazole	66%	0-3
Difenoconazole	66%	0-1
Mefentrifluconazole	56%	0-1
Thiophanate-methyl	47%	0-1
Trifloxystrobin	41%	0-1
Pyraclostrobin	34%	0-1
Fluxapyroxad	31%	0-1
Flutriafol	13%	0-2
Tetraconazole	9%	0-2
Fluopyram	9%	0-1
Copper	6%	0-1

† Multi-site ethylene bis-dithiocarbamate (EBDC; FRAC M3) tank-mix partners, such as mancozeb, were reported for nearly 100% of field applications.

Table S4.3. Complete results of the simple linear regression analyses conducted to evaluate the relationship between the number of field applications by fungicide group and isolate sensitivities to corresponding active ingredients in 2022 from 29 field locations. Responses were pooled by field location as mean isolate EC_{50} values or as frequencies of resistant isolates based on k-means designated sensitivity thresholds. Positive coefficient estimates indicate that isolate insensitivities increased as number of corresponding fungicide group applications increased.

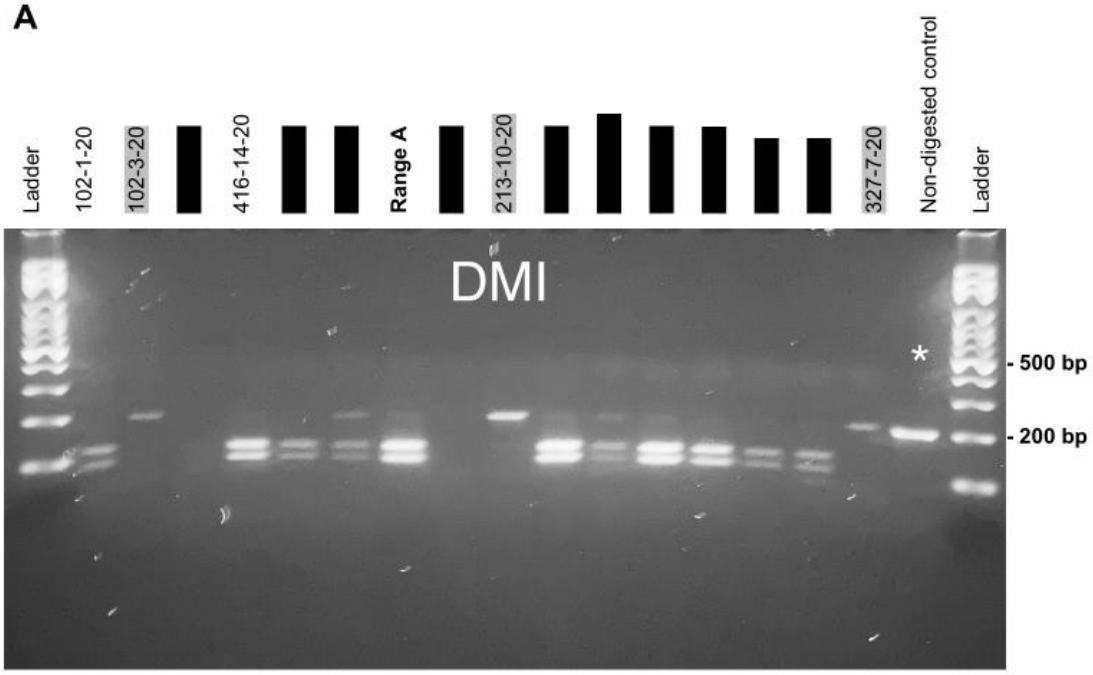
Appl. # †	Active ingredient	Sensitivity value [(exact EC_{50} or percent frequencies above a sensitivity threshold ($\mu g\ ml^{-1}$)]	Mean estimate	Standard error	*P* - value
QoI	Pyraclostrobin	EC_{50}	1.37	1.39	0.3250
DMI	Difenoconazole	EC_{50}	0.09	0.21	0.6536
DMI	Mefentrifluconazole	EC_{50}	-0.17	0.23	0.4690

Table S4.3 (cont'd)

DMI	Tetraconazole	EC_{50}	0.08	0.63	0.8980	
DMI	Prothioconazole	EC_{50}	1.69	1.70	0.3230	
Organotin	Triphenyltin hydroxide	EC_{50}	0.09	0.05	0.1030	
MBC	**Thiophanate-methyl**	**EC_{50}**	**8.15**	**3.94**	**0.0409**	*** ‡**
QoI	Pyraclostrobin	19.51	0.28	0.36	0.438	
DMI	Difenoconazole	5.11	-0.13	0.38	0.72714	
DMI	Mefentrifluconazole	3.26	-0.15	0.27	0.5787	
DMI	Tetraconazole	6.91	-0.08	0.18	0.654	
DMI	**Prothioconazole**	**9.56**	**0.64**	**0.28**	**0.0231**	*****
Organotin	Triphenyltin hydroxide	2.55	0.38	0.70	0.591276	
MBC	Thiophanate-methyl	5.00	1.96	1.10	0.074	
QoI/Total	Pyraclostrobin	EC_{50}	-0.04	6.13	0.9940	
DMI/Total	Difenoconazole	EC_{50}	-0.23	1.83	0.8990	
DMI/Total	Mefentrifluconazole	EC_{50}	0.18	1.89	0.9250	
DMI/Total	Tetraconazole	EC_{50}	-6.93	5.20	0.1836	
DMI/Total	Prothioconazole	EC_{50}	-25.15	13.82	0.0705	
Organotin/Total	Triphenyltin hydroxide	EC_{50}	0.24	0.29	0.4150	
MBC/Total	**Thiophanate-methyl**	**EC_{50}**	**49.85**	**24.91**	**0.0478**	*****
QoI/Total	Pyraclostrobin	19.51	0.34	1.57	0.83	
DMI/Total	Difenoconazole	5.11	-4.34	4.16	0.297	
DMI/Total	Mefentrifluconazole	3.26	0.31	2.14	0.8858	
DMI/Total	Tetraconazole	6.91	-1.23	1.59	0.441	
DMI/Total	Prothioconazole	9.56	-2.87	1.84	0.11849	
Organotin/Total	Triphenyltin hydroxide	2.55	1.70	3.82	0.656303	
MBC/Total	Thiophanate-methyl	5.00	11.99	6.85	0.08	

† Numbers of applications and numbers of applications/total fungicide applications were considered for each group.
‡ Asterisk designations correspond to *P*-value thresholds <0.05 * and <0.01 **. Bolded entries were significant at $\alpha = 0.05$.

A

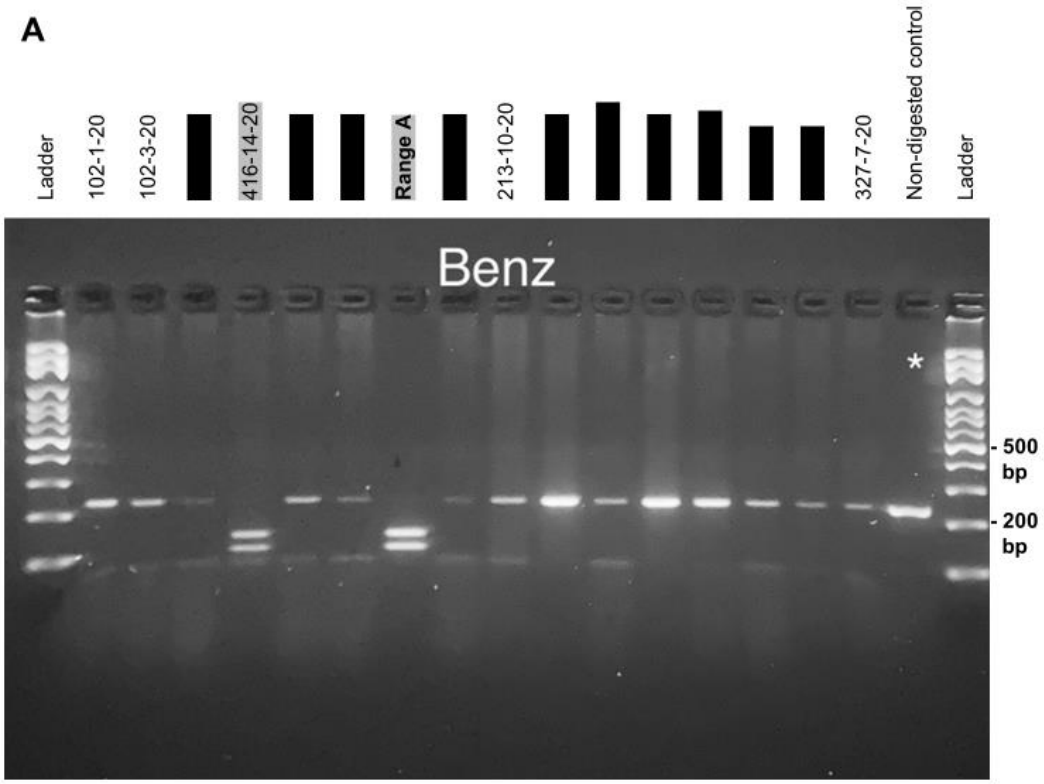


B

Isolate ID	DNA Sequences (5' to 3')
102-1-20	TTGATCATCACCGAAGAGACGCGCCAATTCTT
102-3-20	TTGATCATCACCGAAGAAACGCGCCAATTCTT
416-14-20	TTGATCATCACCGAAGAGACGCGCCAATTCTT
Range A	TTGATCATCACCGAAGAGACGCGCCAATTCTT
213-10-20	TTGATCATCACCGAAGAAACGCGCCAATTCTT
327-7-20	TTGATCATCACCGAAGAAACGCGCCAATTCTT

Figure S4.1. (A) Gel electrophoresis (2.5% agarose) of polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) products for six *Cercospora beticola* isolates, confirming BsmAI-digested *cytochrome b* gene fragment banding patterns associated with DMI-sensitivity (1 band) and DMI-resistance (2 bands), including five isolates selected from 2019 and 2020 research fields at the Michigan State University Saginaw Valley Research and Extension Center (Hernandez et al. 2024) and one reference isolate from the USDA-ARS Sugar Beet Research Unit (East Lansing, MI), resistant 'Range A' (shown in bold). Results were run alongside a non-digested control fragment (indicated with an asterisk, 1 band, ~200 bp) and sample lanes flanked by a 1 kb ladder (Axygen, Union City, CA). Products were purified and Sanger sequencing conducted at the Michigan State University Research Technology Support Facility Genomics Core (East Lansing, MI) to confirm **(B)** presence of the Glu169 mutation (DNA sequence mutation: GA**A**-GA**G**; amino acid remains glutamic acid) associated with DMI resistance in *C. beticola* (Nikou et al. 2009). Wild-type nucleotides shaded gray, and mutations associated with resistance highlighted yellow.

A



B

Isolate ID	DNA Sequences (5' to 3')
102-1-20	CGAGAACTCCGACGAGACCTTCTGTATCG
102-3-20	CGAGAACTCCGACGAGACCTTCTGTATCG
416-14-20	CGAGAACTCCGACGCGACCTTCTGTATCG
Range A	CGAGAACTCCGACGCGACCTTCTGTATCG
213-10-20	CGAGAACTCCGACGAGACCTTCTGTATCG
327-7-20	CGAGAACTCCGACGAGACCTTCTGTATCG

Figure S4.2. (A) Gel electrophoresis (2.5% agarose) of polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) products for 16 *Cercospora beticola* isolates, confirming BstUI-digested *cytochrome b* gene fragment banding patterns associated with MBC-sensitivity (2 band) and MBC-resistance (1 bands), including five isolates selected from 2019 and 2020 research fields at the Michigan State University Saginaw Valley Research and Extension Center (Hernandez et al. 2024) and one reference isolate from the USDA-ARS Sugar Beet Research Unit (East Lansing, MI), sensitive 'Range A' (shown in bold). Results were run alongside a non-digested control fragment (indicated with an asterisk, 1 band, ~225 bp) and sample lanes flanked by a 1 kb ladder (Axygen, Union City, CA). Products were purified and Sanger sequencing conducted at the Michigan State University Research Technology Support Facility Genomics Core (East Lansing, MI) to confirm **(B)** presence of the E198A mutation (DNA sequence mutation: G**A**G-G**C**G; amino acid mutation: glutamic acid to alanine) associated with MBC resistance in *C. beticola* (Obuya et al. 2008). Wild-type nucleotides shaded gray, and mutations associated with resistance highlighted yellow.

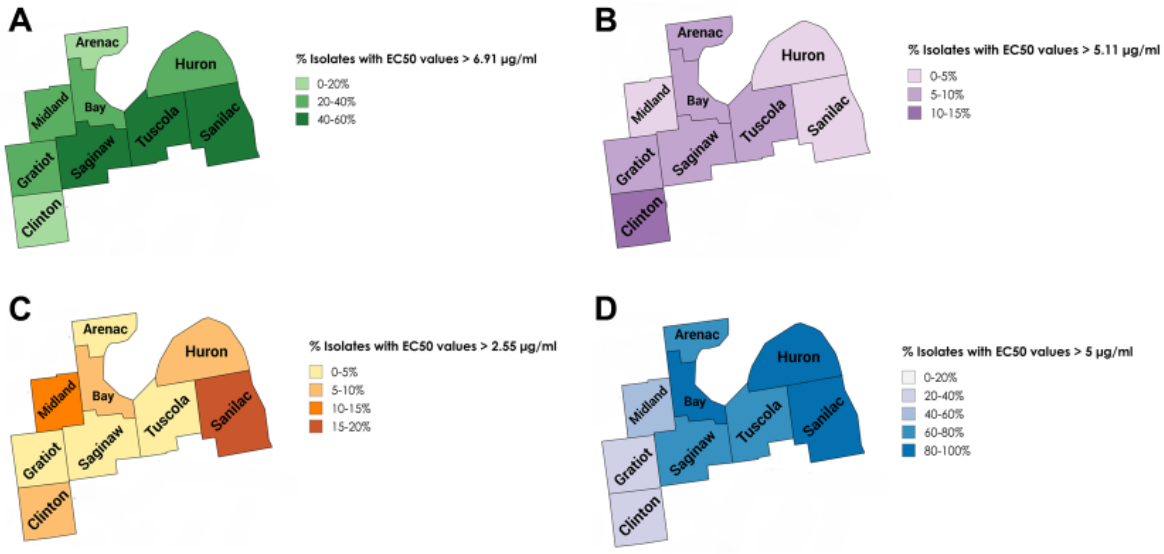


Figure S4.3. County-level percentages of *Cercospora beticola* isolates with reduced sensitivity based on EC_{50} k-means established thresholds to (A) tetraconazole, (B) difenoconazole, (C) triphenyltin hydroxide, and > 5 µg ml^{-1} (D) thiophanate-methyl (Secor et al. 2010; Bolton et al. 2012b). Isolates were pooled across both sampling years 2021 (29 field locations) and 2022 (30 field locations). Michigan sugarbeet growing counties included in this study were Arenac (n = 12 isolates), Bay (n =124), Clinton (n = 18), Gratiot (n = 24), Huron (n = 77), Midland (n = 4), Saginaw (n = 33), Sanilac (n = 40), and Tuscola (n = 41).

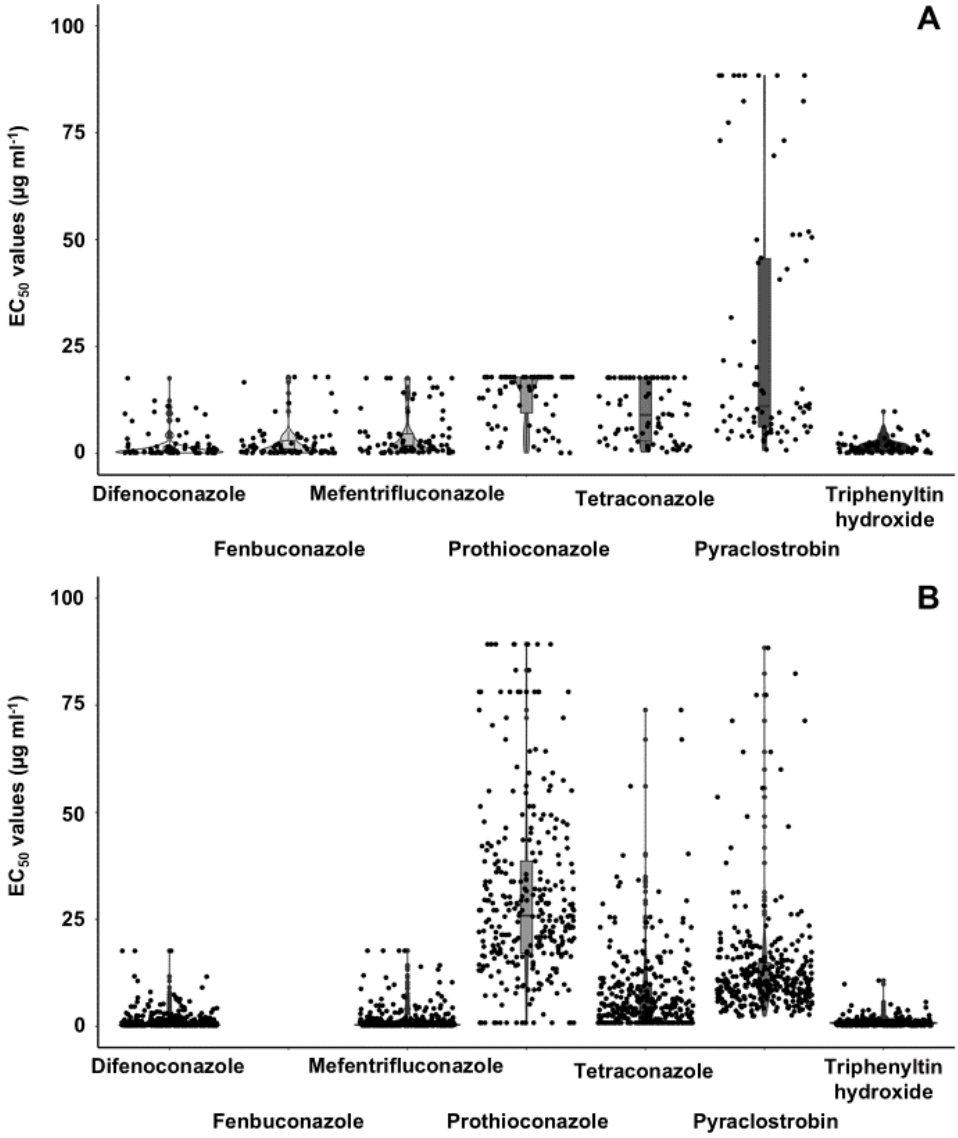


Figure S4.4. Violin plots with datapoint overlay of *C. beticola* EC_{50} values for each active ingredient tested on isolates collected in 2021 **(A)** and 2022 **(B)**. Isolates were collected from 29 and 30 commercial sugarbeet fields in Michigan for 2021 (n = 78 isolates) and 2022 (n = 304-347), respectively. The box represents the interquartile interval where 50% of the data points are found and the line that divides the box is the median. The upper and lower detection limits were not adjusted to be the same across years.

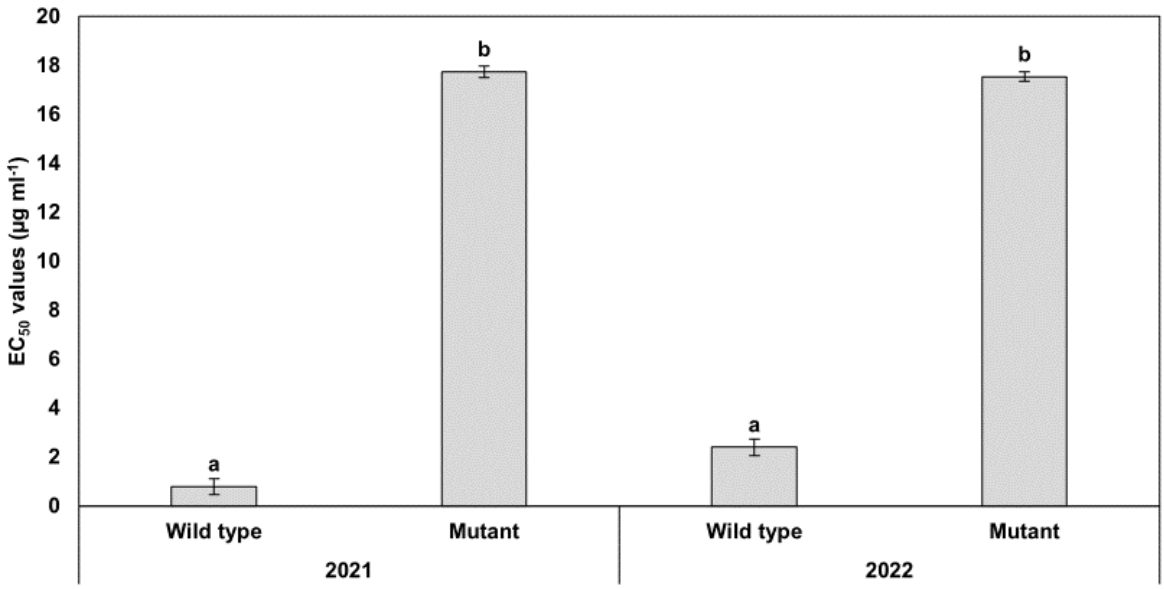


Figure S4.5. Mean thiophanate-methyl EC_{50} values of *C. beticola* isolates with (mutant) and without (wild type) the E198A mutation associated with benzimidazole resistance for 2021 and 2022. The simple linear model analysis indicated that thiophanate-methyl EC_{50} values were significantly impacted by the presence of the E198A mutation and year ($P < 0.001$). Bars represent means ± standard error. Mean comparisons denoted by letters generated based on the pairwise comparison *P*-values adjusted for multiple testing by the Bonferroni method (α=0.05).

CHAPTER 5: EARLY-SEASON PREDICTIONS OF AERIAL SPORES TO ENHANCE INFECTION MODEL EFFICACY FOR CERCOSPORA LEAF SPOT MANAGEMENT IN SUGARBEET

5.1 Abstract

Cercospora beticola causes one of the most destructive foliar diseases of sugarbeet in many growing regions. Management of Cercospora leaf spot (CLS) relies heavily on timely and repeated fungicide applications. Current treatment initiation is often supported by models predicting conditions favorable for infection; however, these models lack information of *C. beticola* presence and abundance. Burkard volumetric mechanical samplers and highly CLS-susceptible sentinel beets (biological samplers) were used to assess early-season aerial *C. beticola* conidia from sugarbeet fields in Michigan and in Ontario, Canada from 2019-2022. In initial correlation and logistic regression analyses (n=449), duration of leaf wetness, air temperature, and wind speed were found to predict the risk of elevated *Cercospora* spore concentrations with 67.9% accuracy. In 2022 and 2023, a select model and a limited set of action thresholds, in addition to the BEETcast model, were tested for fungicide application timing. When CLS pressure was high, extending the interval between applications showed reduced management of CLS ($P < 0.001$), sugar percentage, and RWS ($P < 0.05$) compared to the grower standard. Model-based programs integrating canopy closure information resulted in CLS, yield, and sugar metrics comparable to the grower standard despite one less fungicide application. In additional training analysis (n=402), an ensemble model included leaf wetness, air temperature, relative humidity, and wind speed variables with a testing accuracy of 73.2% (n=101). Based on model development, refinement, and validation, assessment of elevated early-season *C. beticola* presence and abundance has potential to improve application timing and efficacy for preventative CLS management.

5.2 Introduction

Cercospora beticola is an important fungal pathogen of sugarbeet (Jacobsen and Franc

2009; Khan et al. 2009; Weiland and Koch 2004), which causes the foliar disease Cercospora leaf spot (CLS). The pathogen overwinters largely as pseudostromata on infected leaf residue (Pool and McKay 1916). Under favorable conditions in temperate climates, conidia are released in the spring, germinate, and penetrate leaves through the stomates often using appressoria (Rathaiah 1977; Steinkamp et al. 1979). This disease is polycyclic with the ability for many iterations of asexual spore production (McKay and Pool 1918; Nagel 1945; Vereijssen et al. 2007). The dark pseudostromata, found in the center of mature CLS lesions, produce silver, needlelike conidia (Franc 2010; Groenewald et al. 2013; Jacobsen and Franc 2009; Weiland and Koch 2004). Conidia of *C. beticola* are produced most readily at temperatures from 15 to 23°C with relative humidities (RH) greater than 60% and are inhibited at temperatures less than 10°C or above 38°C (Jacobsen and Franc 2009). Conidia of *C. beticola* are dispersed by wind, water splash, running water, and insects (Carlson 1967; McKay and Pool 1918; Meredith 1967).

Historically, CLS has been primarily managed by tillage, crop rotation, in-season fungicide applications, and selection of resistant varieties (Asher and Hanson 2006; Jacobsen 2010; Jacobsen and Franc 2009). Field isolation of at least 33 to 91.4 meters away from any previous year sugarbeet fields has been shown to reduce CLS severity (Khan et al. 2008; McKay and Pool 1918). Inoculum can be reduced through crop rotation and tillage to help manage CLS (Khan et al. 2008; McKay and Pool 1918), though the use of tillage practices has been declining due to soil health initiatives. While newer resistant varieties have shown reduced severity, CLS symptoms can still develop and other disease or agronomic characteristics may be less desirable, which necessitates integrated management and ongoing development for sustainable use (REACh 2023). Despite these strategies, significant outbreaks of CLS have been observed over the years.

One of the most critical aspects of CLS management has been maintaining timely

fungicide applications for the growing season (Khan et al. 2008) and fungicide efficacy is dependent in part on initial timing of infection. For example, propiconazole, a demethylation inhibitor (DMI; FRAC Group 3) was not as effective once CLS lesions were present compared to application prior to lesion detection, however, a pre-mix of benzovindiflupyr or pydiflumetofen, succinate dehydrogenase inhibitors (SDHI; FRAC Group 7), and difenoconazole was effective at a threshold of one CLS lesion per leaf (Pethybridge et al. 2020). While the efficacy of foliar programs may be dependent on fungicide active ingredient, generally those initiated either prior to infection or at early leaf spot detection (<1 spot per leaf) are most effective (Liebe et al. 2023; Pundhir and Mukhopadhyay 1987). Due to several factors, including small lesion diameters, dense sugarbeet canopies, and considerable size of commercial fields, these first spots may be difficult to detect in time to accurately inform initiation of fungicide programs.

Disease forecasting tools can help to initiate effective management decisions in high-risk conditions and limit applications in low-risk conditions. Studies of weather-related factors impacting infection, sporulation, disease intensity, and potential loss have led to the development of several forecasting systems for CLS in sugarbeet (Khan et al. 2007; Pitblado and Nichols 2005; Shane and Teng 1992; Smith and Ruppel 1973; Wallin and Loonan 1971; Windels 2010; Windels et al. 1998). However, there have been increasing concerns about the models available (MSC, personal communication). BEETcast, the current model used in Michigan and Ontario, Canada (Pitblado and Nichols 2005), is hosted by Weather INnovations and uses temperature and leaf wetness data to estimate daily severity values (DSV) of 0 to 4; action thresholds based on cumulative DSVs are determined based on varietal resistance and regional CLS pressure (www.michiganbeets.com). Timing of the first and second fungicide application is critical for effective management and prevent pathogen establishment early in the season (Liebe et al. 2023).

In 2018, the model-based thresholds were met two weeks after the first lesions were reported in Michigan (Willbur et al. 2018), which is not ideal for the most effective management. Similarly, in Canada, *Cercospora* conidia were first observed before the BEETcast threshold of 50 DSVs was reached in 2014 and 2015 (Tedford et al. 2018), and CLS symptoms were seen before a 50 DSV threshold was reached in 2019 (Tedford et al. 2018). Recurrent false-negative predictions are problematic and lead to costly CLS spread, possible defoliation, and sucrose losses.

Some growing regions refer to the North Dakota Agricultural Weather Network (NDAWN) model (Jones and Windels 1991; Shane and Teng 1984, 1985; Windels et al. 1998). The NDAWN model integrates in-field disease monitoring and prediction of infection events based on meteorological conditions. Disease monitoring starts at canopy closure (~90% of plants from adjoining rows touching) and continues weekly thereafter. Daily infection values (DIVs), which range from 0 to 7, are calculated from 48 hours of temperature and rainfall information to determine whether conditions are favorable for *C. beticola* infection (Windels et al. 1998). The NDAWN model is dependent on early-season detection and monitors weather favorable for infection. Current models do not consider spore production and dispersal. Development of models or model factors to accurately predict inoculum presence and abundance would complement models such as NDAWN, BEETcast, and others that predict *C. beticola* infection events (El Jarroudi et al. 2001; Vereijssen et al. 2007). Spore-based models would support in-field monitoring, provide remote, site-specific inoculum information, and enhance the use of other decision support tools for CLS management.

CLS severity is associated with *C. beticola* conidia concentrations (Khan et al. 2009; Pundhir and Mukhopadhyay 1987; Tedford et al. 2018). In Michigan, evidence of infectious *C. beticola* spores has been found as early as April, with consistent early-season detections in 2017-

2018 (Bublitz et al. 2021). Tedford et al. (2018) reported similar findings of airborne conidia present in early May in Ontario, Canada. Early detections of *C. beticola* spores in fields previously planted to beet confirm these as a primary inoculum source in North Central and Northeastern regions. This further provides evidence for successful overwintering of *C. beticola*, which may be present on leaf debris in the soil (Khan et al. 2008; McKay and Pool 1918; Pool and McKay 1916). The current recommendations to plant at least 100 m from previous fields may not be entirely effective, especially when beet crops are densely located in particular areas of a state or region.

Currently used disease forecasting models for CLS focus on predicting infection or disease development conditions to predict the proper timing of fungicide applications to manage CLS on sugarbeet (Windels 2010). Weekly mean conidia concentration was found to be significantly associated with disease severity (Khan et al. 2009). Airborne spores of *C. beticola* play an important role in primary infection, secondary infection, and in disease development. In Michigan, the impact of environmental variables on *C. beticola* spore abundance was assessed using live beet spore traps (Bublitz et al. 2021). Patterns of airborne *Cercospora* conidia concentrations have been assessed using a mechanical spore trap from April to July in Ontario and late-June to September in Minnesota and North Dakota (Khan et al. 2009; Tedford et al. 2018). Additional predictors of *C. beticola* inoculum dispersal prior to symptom development may provide useful information to improve disease management.

Accurate predictions of the probable initial infection timing, based on a better understanding of *C. beticola* spore presence and abundance in relation to weather conditions and already established knowledge pertaining to CLS development, is critical. This study evaluates weather variables for their importance in predicting aerial spore concentrations. These

relationships were then used to create a logistic model predicting periods of elevated spore levels. A simple model was selected for implementation in field validation studies to support the current BEETcast model in the timing of fungicide application for CLS management.

5.3 Materials and methods

5.3.1 Mechanical spore collection for model development

Aerial spores were collected in sugarbeet fields during 7-day intervals using a Burkard volumetric spore sampler (Stedman 1978; Tedford et al. 2018). This study collected aerial spores during May to July from Michigan in 2019, 2020, 2021 and 2022 and Ontario, Canada in 2019 and 2021 (Table 5.1) with an aim to understand early spore presence. Spore sampling terminated each year once visible CLS symptoms were detected to avoid capturing spores from secondary infection. A total of 503 days of aerial spore observations were collected across eight site years. The vacuum in the Burkard spore trap aspirated air at 10 L min^{-1} (0.01 cubic meter min^{-1}) onto transparent adhesive tape (3M Scotch, 19 mm x 25.4 mm) wrapped around the drum to sample airborne particles (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK). The Burkard drum rotated at 2-mm per hour and sampled continuously for 7 days. Spore collection generally started on Tuesdays and sampling began at 11:00 AM due to peak conidia concentrations observed from 12:00 PM to 6:00 PM (Tedford et al. 2018). For each week of collection, the Burkard spore tape containing seven days of airborne particles was divided into 48 mm sections for each day (24-hour period). The collection tape was observed under a compound microscope at 100x magnification, and the number *Cercospora*-like spores (Jacobsen and Franc 2009; Ruppel 1986) were counted in 3-mm sections horizontally across each daily collection tape (Figure S5.1). Conidia were identified as likely *C. beticola* if they had the following characteristics: multiseptate, tapered, solitary, straight or slightly curved, and between 2.5-4.0 µm

wide and 50-200 µm long (Groenewald et al. 2013; Weiland and Koch 2004). The entire tape was counted for all locations from 2019 to 2020; in 2021 and 2022, two of six 3-mm sections were counted for each day and multiplied by three to estimate the total spores per day. The concentration of spores m^{-3} air was calculated by dividing the total daily spore count by the total aspirated air volume [0.6 m^3/hour × 24 hours] (Aylor and Sutton 1992; Khan et al. 2009).

Table 5.1. Mechanical and biological spore trap sites across years and locations including information of nearest weather station, previous crop, soil type, and start and end dates of collection periods.

Location[a]	Approx. weather station distance (km)	Year	Soil type[b]	Collection	
				Start date	End date
Ridgetown, ON, CAN	≤ 1	2019	Clay loam	May 15	July 9
East Lansing, MI, USA	≤ 1	2019	Loam	May 7	July 2
Frankentrost, MI, USA	≤ 9	2019	Loam	May 7	July 8
Frankenmuth, MI, USA	≤ 1	2020	Loam	June 2	July 27
Frankenmuth, MI, USA	≤ 1	2021	Loam	May 4	July 12
Dresden, ON, CAN	≤ 10	2021	Clay loam	April 28	July 13
RCUG[c], ON, CAN	≤ 1	2021	Clay loam	May 5	July 13
Frankenmuth, MI, USA	≤ 1	2022	Loam	May 17	July 11

[a] All locations were adjacent to current or previous year sugarbeet fields.
[b] Soil types determined from the United States Department of Agriculture Natural Resources Conservation Service Web Soil Survey (USDA-NRCS 2019).
[c] Ridgetown Campus of the University of Guelph

5.3.2 Biological spore collection for model development

To monitor and confirm *C. beticola* spore presence, highly susceptible sugarbeets (germplasm F1042; Campbell 2015) were grown in the greenhouse and placed in the field near spore traps (Bublitz et al. 2021). These sugarbeets were referred to as “sentinel beets”. In the MSU Plant Science Research Greenhouse Complex, four sugarbeet seedlings were transplanted at the cotyledon growth stage to each planting box (61.0 cm by 30.5 cm) with SureMix growing media (Michigan Grower Products, Inc.) and managed as described in Hernandez et al. (2023). The sentinel beets, two boxes containing four beets each, were placed in the field and collected after seven days corresponding to the weekly Burkard spore tape collections. The beets were placed in a humid chamber for 3 days with humidity greater than 95% to supply favorable

conditions for *C. beticola* infection (Bublitz et al. 2021; Pool and McKay 1916). After a subsequent 3 weeks in the greenhouse with temperatures between 20 to 30°C degrees and natural light, characteristic CLS lesions (Weiland and Koch 2004; Windels et al. 1998) were counted and totaled for each box of beets. Visible pseudostromata identified using a hand lens (3x to 6x magnification) were required for lesions to be counted in this study, which are a distinguishing characteristic of Cercospora leaf spots (Franc and Jacobsen 2009). Each year 2 to 3 lesions for each time point were confirmed to be *C. beticola* by isolation into a mono-conidial isolate culture (Hansen and Smith 1932; Leslie and Summerell 2006). A non-inoculated control was included to monitor secondary dispersal of conidia within the humidity chamber as described in Hernandez et al. (2023). No or low (1-6 lesions) CLS symptoms were observed on the non-inoculated controls.

5.3.3 Environmental variables for model development

Data for environmental variables were collected using local Michigan State University Enviroweather stations (Enviroweather 2024) for Michigan data provided by the Michigan Automated Weather Network (MAWN) and Enviro-weather Program and from weather stations operated by Weather Innovations LLC (WIN 2022) for Ontario as described by Tedford et al. (2018). Hourly average data were used to calculate weather variables over a 24-hour period from 11:00 AM to 10:59 AM (Table 5.2). The soil temperature was taken at a depth of 10 centimeters for Michigan and 30 cm for Ontario. The leaf wetness sensor was a standard mount sensor that measures the percentage of the hour that the leaf was wet. The air temperature and relative humidity were taken at a height of 1.5 feet. Wind speed measurements were taken at a height of 3 meters.

Table 5.2. Response and predictor variable descriptions calculated from daily spore counts measured by Burkard samplers and hourly temperature, relative humidity, precipitation, wind speed, and leaf wetness data over 24-hour periods from 11AM to 11AM.

Variable	Description
SporeCount	The total number of daily Cercospora spores
Spore10[b]	Binary variable for the total number of daily Cercospora spores (>10 = 1; ≤10 =0)
Spore35	Binary variable for the total number of daily Cercospora spores (>35 = 1; ≤35 = 0)
Spore50	Binary variable for the total number of daily Cercospora spores (>50 = 1; ≤50 = 0)
Spore100	Binary variable for the total number of daily Cercospora spores (>100 = 1; ≤100 = 0)
MaxTemp	Maximum daily air temperature (°C)
MinTemp	Minimum daily air temperature (°C)
AvgTemp	Average daily air temperature (°C)
Precip	Total daily precipitation (mm)
AvgRH	Average daily relative humidity (%)
MaxWS	Maximum daily wind speed (km/hr)
MaxLW	Maximum daily leaf wetness (%)
AvgLW	Average daily leaf wetness (%)
AvgSoilTemp	Average soil temperature (°C)
DurLW	Duration in number of hours for leaf wetness above 0%
DurTemp	Duration in number of hours for air temperature above 15°C
DurRH	Duration in number of hours for relative humidity above 80%
MinTemp20C[a]	Binary variable for minimum air temperature (> 20°C = 1; ≤20°C = 0)
MinTemp15C	Binary variable for minimum air temperature (>15°C = 1; ≤15°C = 0)
AvgTemp25C	Binary variable for average air temperature (>25°C = 1; ≤25°C = 0)
AvgTemp20C	Binary variable for average air temperature (>20°C = 1; ≤20°C = 0)
AvgTemp15C	Binary variable for average air temperature (>15°C = 1; ≤15°C = 0)
MaxTemp25C	Binary variable for maximum air temperature (>25°C = 1; ≤25°C = 0)
MaxTemp20C	Binary variable for maximum air temperature (>20°C = 1; ≤20°C = 0)
MaxTemp15C	Binary variable for maximum air temperature (>15°C = 1; ≤15°C = 0)
AvgRH90	Binary variable for average relative humidity (>90% = 1; ≤90% = 0)
AvgRH85	Binary variable for average relative humidity (>85% = 1; ≤85% = 0)
AvgRH80	Binary variable for average relative humidity (>80% = 1; ≤80% = 0)

[a] Binary variable based on temperature and humidity thresholds were chosen based on relevant literature and environmental factors that are associated with *C. beticola* conidia (Forsyth et al. 1963; Jacobsen and Franc, 2009; McKay and Pool 1918; Pool and McKay 1916).

[b] The spore 10, 35, and 50 thresholds were selected based on the mean and median for the number of total daily spores over the entire data set. The spore 100 threshold was selected to test the instances when aerial spores were extremely elevated. The Spore 10, 35, 50, and 100 thresholds convert to 0.7, 2.4, 3.5, and 7.0 spores per cubic meter per day.

5.3.4 Correlation and logistic regression analyses for model development

All statistical analyses were conducted in SAS (Statistical Analysis System v 9.4; SAS Institute, Cary, NC). A total of 423 daily observations across seven site-years were included in

the 2021 model; 2022 spore data was not used in this model. Due to the persistence of low levels of conidia throughout the sampling periods, binary spore thresholds were used to investigate elevated spore counts of 10, 35, 50, and 100 spores per day. In 2020, the use of moving averages and sums was evaluated for their ability to account for the changes in the concentration of aerial spores. In 2020, moving averages and sums for 2 and 7 days were generated using the SAS 'expand' procedure for the following variables: MaxTemp, MinTemp, AvgTemp, Precip, AvgRH, and MaxWS (Willbur et al. 2018).

All correlation analyses were conducted in SAS using the 'corr' procedures. A Pearson correlation analysis was used to evaluate correlations between weather variables, sentinel beet spot counts, and aerial spore abundance. A Kendall correlation was conducted to detect correlations between weather variable and binary spore thresholds. A simple linear model analysis was used to test for the presence of a significant relationship between weekly spore counts and sentinel beet spot counts using the 'glimmix' procedure.

Model selection was based on correlated weather predictors and logistic modeling was used to predict elevated spore counts. Forward, backward, score and stepwise regression analyses were conducted to assess the accuracy of the model variables separately and together for each binary spore threshold using SAS 'logistic' procedure. Model accuracy metrics including goodness of fit, sensitivity, and specificity were generated using a max-rescaled R2 'rsq' (Allison 1999), Hosmer-Lemeshow test 'lackfit' (Allison 1999, Hosmer and Lemeshow 2000), and classification table 'ctable' (Hosmer and Lemeshow 2000) analyses in 'proc logistic'.

As described by Willbur et al. (2018), goodness of fit was evaluated to indicate the accuracy of predicted elevated spores compared to the observed elevated spores using the max-rescaled R^2 statistic and Hosmer-Lemeshow test. Sensitivity, the model's ability to correctly

predict positive outcomes, and specificity, the model's ability to correctly predict negative outcomes, were assessed using the classification table. The action threshold, where the probability at which sensitivity and specificity converged, was determined using the classification table and models were evaluated for percentage correctness, as well as sensitivity, specificity, false positives, and false negatives at this threshold. Variable collinearity was assessed using a weighted regression procedure (Allison 1999) within 'proc reg' to avoid redundancy in environmental factors. Tolerance and variation inflation factors (VIF) were included in these analyses using the 'TOL' and 'VIF' options, respectively. Variables were considered to be collinear at tolerance values < 0.40 and VIF values > 3.0 and one of the two variables was subsequently removed. The most accurate and simple model was selected in 2021 to predict elevated spore numbers with a threshold of 35 spores and was tested for its ability to predict 2022 observed spores.

5.3.5 Model validation field studies

Validation studies were conducted in 2022 and 2023 to test the utility of the 2021 model to assist in fungicide application timing for CLS management. In both years, trials were conducted in Frankenmuth, Michigan at the Michigan State University Saginaw Valley Research and Extension Center. The field treatments were arranged in a randomized complete block design with four replicates and plot sizes of 3 by 10.7 meters. The field trial sites had loam soil with 5.0% organic matter and a pH of 7.5. In 2022, three treatments were applied to a CLS-susceptible (C-G932NT) and CLS-resistant (C-G021) sugarbeet variety (American Crystal Sugar Company, MN), including a non-treated control, grower standard fungicide program, and a 2021 model-based fungicide application timing derived from the 35-spore threshold (Table 5.3). In 2023, six treatments were applied to a CLS-susceptible variety (SX-1278; Seedex Inc., ND),

including a non-treated control, two grower standard fungicide programs with calendar initiation and 2021 model-based application interval, and three 2021 model-based fungicide application initiation timings (Table 5.4). The 2022 and 2023 trials were planted on April 29 or May 12 and harvested September 23 or October 2, respectively. Grower standard initiation treatments were selected based on typical thresholds ranging from 40-50 cumulative DSVs in the BEETcast model for initiation of fungicide programs, dependent on varietal tolerance to CLS.

Table 5.3. Model validation treatment programs tested in 2022. After initiation, subsequent spray timings followed a 14-day interval for the susceptible (C-G932NT) and 28-day interval for the resistant variety (C-G021).

Trt	Variety	Program	Initiation Criteria [a]	Actual Initiation Date	# App.	App. Interval
1	C-G021	Non-treated control	-			
2	C-G021	Grower standard [c]	55 DSV	Jul 12	3	28-day
3	C-G021	Model 1	70% + DSV 3 or 4	Jul 8	3	28-day
4	C-G932NT	Non-treated control	-			
5	C-G932NT	Grower standard	50 DSV	Jul 8	5	14-day
6	C-G932NT	Model 1	70% + DSV 3 or 4	Jul 8	5	14-day

[a] Model 1was implemented to trigger at a 70% likelihood threshold for the presence of 35 or more *C. beticola* spores paired with a BEETcast DSV value of 3 or 4 on the same day. Cumulative BEETcast DSV information was used to initiate the grower standard programs.
[b] Grower standard program as follows for the susceptible variety Manzate Max (1.6 qt) ACDFG; Inspire XT (7 fl oz) CF; Super Tin (8 fl oz) D. Grower standard program as follows for the resistant variety Manzate Max (1.6 qt) ADG and BEH; Inspire XT (7 fl oz) DE; Super Tin (8 fl oz) GH. Application letters code for the following dates: A=8 Jul, B=12 Jul, C=19 July, D=2 Aug, E=9 Aug, F=16 Aug, G=30 Aug, and H=6 Sept.

Table 5.4. Model validation treatment programs tested in 2023. All models were based off of the Spore35 model using various BEETcast disease severity value and row closure initiation criteria. After initiation, subsequent spray timings followed a 14-day (calendar) or model-based interval.

Trt	Program	Initiation Criteria [a]	Actual Initiation Date	# App.	App. Interval
1	Non-treated control	-	-	-	-
2	Model 1	50% + DSV 3 or 4	Jun 30	6	14-day
3	Grower standard [b] + calendar interval	50 DSV	Jul 10	6	14-day
4	Grower standard + model interval	50 DSV	Jul 10	4	After 14 days, apply when 50% + DSV 3 or 4
5	Model 2	50% + DSV 3 or 4 + 40% row closure	Jul 19	5	14-day
6	Model 3	40% + DSV 3 or 4 + 50% row closure	Jul 25	5	14-day

Table 5.4. (cont'd)

[a] Model 1, 2, and 3 programs were initiated based on the Spore35 model threshold of 50% likelihood of 35 or more *C. beticola* spores paired with a BEETcast DSV value of 3 or 4 on the same day. Cumulative BEETcast DSV information was used to initiate the grower standard programs.

[b] Grower standard program as follows: Manzate Max (1.6 qt) ABCDE; Inspire XT (7 fl oz) BD; Badge (1.5 pt) F. Application letters code for the following dates: A=10 Jul, B= 25 Jul, C=8 Aug, D=22 Aug, E=5 Sept, and F=16 Aug.

Bi-weekly CLS disease severity ratings were taken on the mid-canopy of the center two rows of each plot and visually averaged. The KWS (Kleinwanzlebener Saatzucht 1970) standard surface area rating scale for CLS was used to measure disease pressure for 2022 and 2023 model field validation studies. The KWS scale ranges from 1 to 10 with 1 as the lowest disease rating (1-5 spots/leaf or 0.1% severity) and 10 as the highest rating (50% to 100% severity) as described by Shane and Teng (1992). Area under the disease progress curve (AUDPC) was calculated from disease rating taken from July to August. AUDPC was calculated as described by Madden et al. (2017).

The center two rows of each plot were mechanically harvested and weighed to determine yield. Root subsamples (approximately 10 kg) from the harvested beets were used to evaluate percent sugar and recoverable white sugar per ton (RWST) with the assistance of the Michigan Sugar Company tare lab (Bay City, MI) as described by Tedford et al. (2019) and Hernandez et al. (2023). Recoverable white sucrose per metric ton of fresh beets (RWS) was calculated (Van Eerd et al. 2012) and converted to recoverable white sucrose per hectare (RWSH) using the equation: RWSH (metric ton/hectare) = RWS (kg/ metric ton) × total yield (metric ton/hectare) / 1000.

5.3.6 Statistical analysis for model validation field studies

For the 2022 and 2023 field validation studies, treatment was evaluated as the fixed effect of interest and replicate was considered a random effect to evaluate model potential for managing CLS. The two years of field validation were analyzed separately due to differences

between treatments. Analysis of variance (ANOVA) was conducted using SAS v. 9.4 software to determine treatment effects on AUDPC, yield, percent sugar, RWS, and RWSH values. Statistical analyses (mixed model ANOVA) were conducted using the generalized linear mixed model (GLIMMIX) procedure and evaluated at the α=0.05 significance level. Fisher's protected least significance difference (LSD) was used for mean comparisons. LSD was calculated to compare treatment differences using letter separation option "mult" macro (Piepho 2012).

5.3.7 Final model refinement

For final model refinement, a total of 503 daily observations across eight site-years from 2019-2022 were used for model development and subsequent testing. Pearson and Kendall correlations between environmental variables and daily aerial conidia abundance were generated as previously described for the complete data set. The data set was randomly split into an 80% training subset and a 20% testing subset for final model refinement. The training data set was used to generate the logistic models as previously described and the testing data set was used to quantify model accuracy, i.e., agreement between actual and predicted observations were assessed. Predictor selection and model fit were evaluated using the previously described methods. A final ensemble model was created by averaging the top three best fit models (Shah et al. 2021).

5.4 Results

5.4.1 Sentinel beets and aerial spore concentrations

Each location and year showed variability in *Cercospora* conidia concentration per cubic meter (Figure 5.1 and 5.2). Over the sampling periods, daily *Cercospora*-like conidial counts ranged from 0 to >1,200 conidia, corresponding to daily concentrations from 0 to <25 conidia m^{-3} of air. On approximately 20% of the 477 collection days, zero *Cercospora*-like spores were

observed. Due to the steady presence of low concentrations of spores (approximately <2.5 conidia m^{-3}), binary predictor variables were generated from the daily data. The median and mean number of daily counts were 9 and 39, respectively; a binary 10-spore (0.7 spores m^{-3}) threshold was set based on the median, and 35- (2.4 spores m^{-3}) and 50-spore (3.5 spores m^{-3}) thresholds were based on the mean daily counts. A 100-spore (7.0 spores m^{-3}) threshold also was tested to target highly elevated aerial spore levels.

In Michigan, the total number of CLS lesions on sentinel beets were significantly correlated with the total aerial *Cercospora* spore counts for the corresponding week (r = 0.548; $P < 0.0012$). A simple linear regression analysis also determined that total *Cercospora*-like spores over one week were significantly related to the total CLS lesions on sentinel beets after exposure in the field for the same week (Figure S5.2). In Canada, sentinel beets were only used to confirm presence or absence of CLS symptoms; in 2019, no symptoms were confirmed, and the other Ontario site-years did not record number of CLS lesions but instead reported confirmation of CLS symptoms.

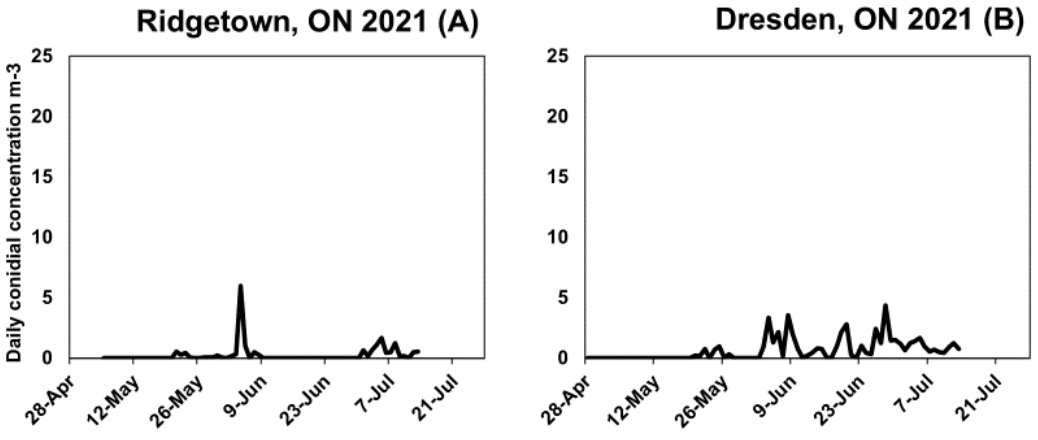


Figure 5.1. Daily *Cercospora*-like conidial concentrations per cubic meter collected from two locations in 2021 in Ontario including (A) Ridgetown and (B) Dresden. Conidia were also collected in Ridgetown in 2019 from 17 May to 9 Jul, however, daily concentrations were less

Figure 5.1. (cont'd)
than 1 conidia m^{-3} for the entire sample period (data not shown). Daily conidial counts of 10, 35, 50, and 100 convert to 0.7, 2.4, 3.5, and 7.0 spores per cubic meter per day.

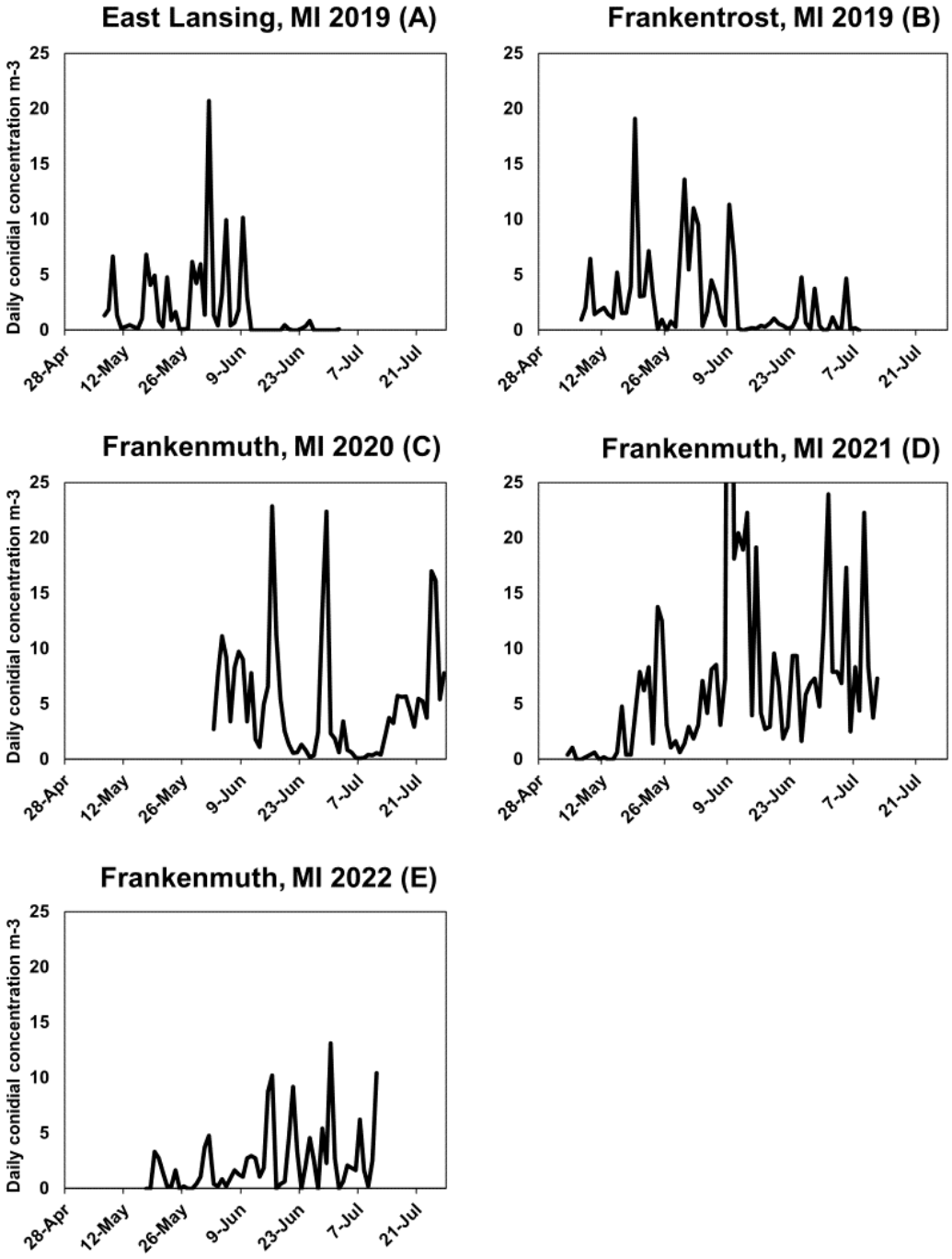


Figure 5.2. Daily *Cercospora*-like conidial concentrations per cubic meter collected from locations in Michigan across three years including (A) East Lansing and (B) Frankentrost in 2019, as well as Frankenmuth in (C) 2020, (D) 2021, and (E) 2022. In 2021, 1,278 spores were detected at the Frankenmuth site on 9 June.

5.4.2 Correlations between response and predictor variables

The moving sum and average variables were not as strongly correlated with spore counts and thresholds as the other variables being evaluated, therefore were not further investigated for this study. Average soil temperature was highly and significantly correlated with daily aerial *Cercospora*-like spores (r = 0.23; $P < 0.0001$; Figure S5.3). Additional temperature and leaf wetness variables also were generally correlated with daily spore counts ($P < 0.05$). All binary weather data was significantly correlated with daily *Cercospora*-like spores with 50% of variables considered to be highly significantly correlated (r > 0.15; $P < 0.0001$). Highly influential environmental variables included average air temperature above or below 15°C (60°F; r = 0.23), average air temperature above or below 20°C (68°F; r = 0.20), and maximum air temperature above or below 25°C (77°F; r = 0.16).

For the 10-spore threshold, 13% of environmental variables were highly significantly correlated (r > 0.15; Figure S5.4). Highly significant predictor variables for the 10-spore threshold included duration of hourly leaf wetness (r = 0.21) as well as minimum air temperature (r = 0.16). For the 35-spore threshold, 30% of variables tested were highly significantly correlated including the duration of hourly leaf wetness (r = 0.22), average soil temperature (r = 0.20), average air temperature (r = 0.16), as well as the binary variable average air temperature above (15°C) 60°F (r = 0.20). For the 50-spore threshold, average soil temperature (r = 0.19), minimum air temperature (r = 0.16), and duration of hourly leaf wetness were most significantly correlated (r = 0.20) and equated to 17% of variables were highly significantly correlated with spore absence and presence. For the 100-spore threshold, average soil temperature (r = 0.17) was the only statistically influential variable resulting in only 4% of variables considered to be highly significantly correlated.

5.4.3 Evaluation of fitness criteria for initial model selection

Including spore collection data from 2019-2021, the percentages of positive binary responses (i.e., *Cercospora*-like conidia detected) at the 10-, 35-, and 50-, and 100-thresholds were 46%, 28%, 23%, and 12%, respectively. The following selection criteria, relatively high max-rescaled R^2 (≥ 0.20), high c values (> 0.70) and percent correct ($> 65\%$), in addition to low AIC values (< 500) were considered for evaluation of model accuracy. The best-fit models developed from 2019 to 2021 data included those describing the 35-Spore threshold and incorporating predictor variables of duration of leaf wetness, average air or soil temperature, and relative humidity or maximum wind speed (DurLW, AvgTemp, AvgRH; DurLW, AvgSoilTemp, MaxWS; DurLW, AvgTemp, MaxWS) or the 50-Spore threshold incorporating duration of leaf wetness, minimum air temperature, and relative humidity (DurLW, MinTemp, AvgRH) (Table 5.5). After evaluation of collinearity, tolerance, variance inflation factors, and variable redundancy, a simple model was selected for field validation in 2022 and 2033. The selected model ($R^2 = 0.18$, $P < 0.0001$) (logit Equation 1):

(Eq. 1) Spore35 = 0.1132*DurLW + 0.1285*AvgTemp + 0.0369*MaxWS - 5.0814

predicted elevated daily conidial presence at a threshold of 35 spores (Spore35) based on number of hours with leaf wetness ≥25% from 11:00 AM to 10:59 AM (DurLW), average daily air temperature in Celsius from 11AM to 10AM (AvgTemp), and maximum daily wind speed in km/h (MaxWS).

Compared to actual conidial observations in 2022, the 2021 model (Equation 1) achieved 50.0% and 67.9% accuracy when predicting days with > 35 conidia using a 30% and 80% probability threshold, respectively (n = 54 daily counts) (Figure 5.3). Notably, the 2021 model was more accurate at predicting elevated spore levels earlier in the season achieving 53.1% and

78.1% accuracy from May 17 through June 17 in 2022 using a 30% and an 80% probability threshold, respectively (n = 30 observations).

Table 5.5. Final logistic model candidates for field validation, generated from seven site-years of data collected in 2019-2021. Information on max-rescaled R^2, Akaike's information criterion (AIC), percentage correct, sensitivity, specificity, predicted positive (Ppos), and predicted negative (Pneg) was reported for the probability level (Prob) where sensitivity and specificity percentages converged.

Threshold	Variables	Max-rescaled R^2	AIC	c	Prob (%)	Corr (%)	Sens (%)	Spec (%)	Ppos (%)	Pneg (%)
Spore10	MinTemp, MaxWS	0.09	588.6	0.64	44	56.5	53.4	58.8	49.5	62.5
Spore10	DurLW, AvgTemp, MaxWS	0.18	555.6	0.71	44	64.5	62.7	65.9	58.2	70
Spore35	DurLW, AvgTemp	0.17	468.6	0.73	26	65.2	68.1	64.1	40.7	84.7
Spore35	DurLW, AvgSoilTemp, MaxWS	0.18	437.8	0.71	30	63.4	66.4	62	43.2	81
Spore35	DurLW, AvgTemp, AvgRH	0.23	449.2	0.76	28	69.4	69.7	69.3	45.1	86.4
Spore35	DurLW, AvgTemp, MaxWS	0.18	467.3	0.73	28	66.7	66.4	66.9	42	84.6
Spore50	DurLW, AvgTemp	0.16	422.8	0.73	22	67.0	64.6	67.6	35.2	87.5
Spore50	DurLW, MinTemp, AvgRH	0.21	408.2	0.76	22	66.7	69.8	65.9	35.8	88.9
Spore100	MinTemp, AvgRH	0.09	299.6	0.68	12	61.6	56	62.3	15.7	91.9

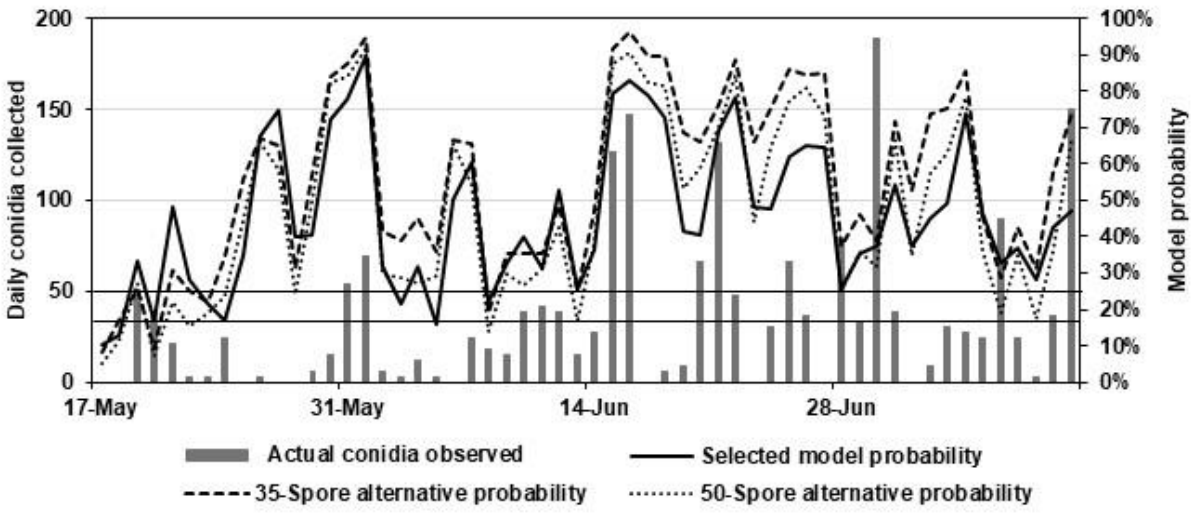


Figure 5.3. The actual abundance of daily aerial *Cercospora*-like conidia collected at 14.4 cubic meters per day in 2022 (n = 54) compared to the predicted probabilities that greater than 35 aerial conidia were present, calculated using the selected logistic model developed in 2021 (logit: Spore35 = 0.1132*DurLW + 0.1285*AvgTemp + 0.0369*MaxWS - 5.0814). Two alternative prediction models were used to predict the probability of the presence of greater than 35 (logit: Spore35 = 0.1548*DurLW + 0.1281*AvgTemp - 0.0436*AvgRH - 1.8905) and 50 spores (logit: Spore50 = 0.1428*DurLW + 0.1275*MinTemp - 0.0449*AvgRH - 1.3384). The probability was calculated as x divided by (1+x) where x is the exponential value of the model output value (Spore35 or Spore50). The solid black lines represent the 35- and 50-spore thresholds.

5.4.4 2022 field validation study

The model-based application timing triggered at the same time as the susceptible control treatment was initiated. Therefore, no significant differences in AUDPC were observed between treatment programs using the model-based timing and the grower standard control for the susceptible variety ($P > 0.05$; Figure 5.4 and Figure S5.5). Both the model-based, and the grower standard fungicide application programs resulted in significantly lower CLS pressure than the non-treated control ($P < 0.05$). No significant difference in AUDPC was detected between any of the treatments for the resistant variety ($P < 0.05$). No significant differences in yield or sugar were observed between treatments programs evaluated in this study ($P > 0.05$; Table 5.6).

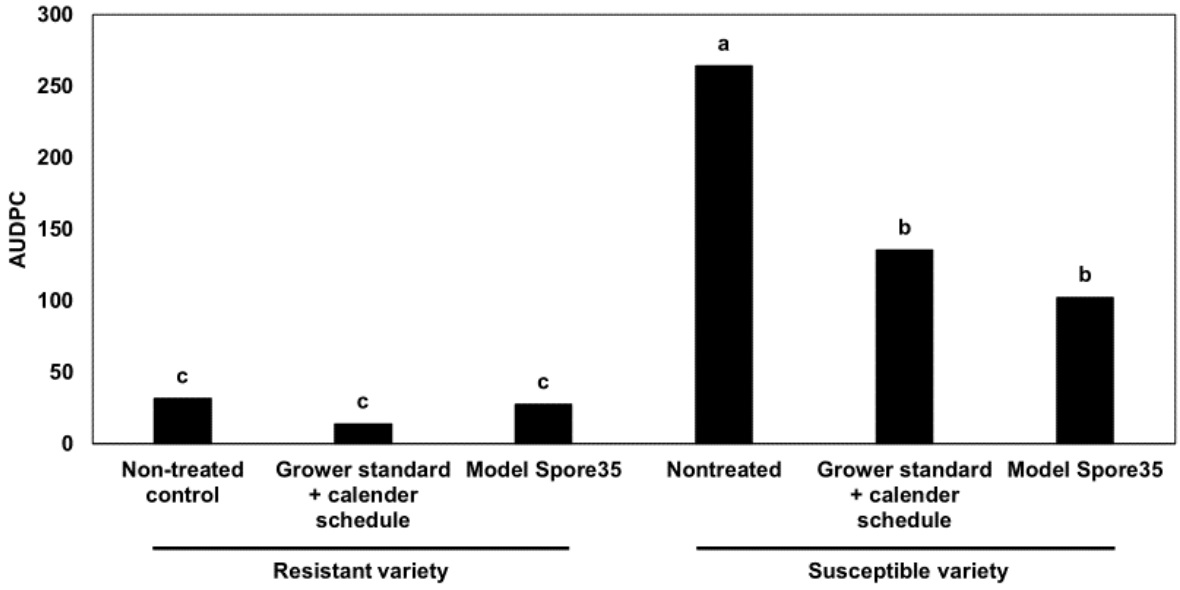


Figure 5.4. Area under the disease progress curve (AUDPC) of CLS severities assessed for a CLS-susceptible (C-G932NT) and CLS-resistant (C-G021) variety in the model validation trial in Frankenmuth, MI in 2022. Treatments were applied according to the Spore35 model initiation threshold (70% probability of elevated *C. beticola* conidial presence according to logit Eq. 1) or a grower standard program according to BEETcast cumulative daily severity values of 50 or 55. AUDPC values were calculated as described by Madden et al. (2017) using severity ratings collected at 4 timepoints from Jul 26 through Aug 15; ratings were based on the KWS severity scale (0-10). Bars represent the means of four replicate plots for each treatment and variety combination. Bars with the same letter were not significantly different based on Fisher's protected LSD (α= 0.05).

5.4.5 2023 field validation study

The treatments in this study did not result in any significant differences in yield. All treatments resulted in significantly reduced AUDPC compared to the non-treatment control ($P <$ 0.001; Figure 5.5 and Figure S5.6). The earliest model initiation (Model 1) resulted in significantly lower AUDPC compared to the grower standard with the extended model interval and the two delayed model initiations (Model 2 and 3) ($P < 0.001$). Numerically, Model 1 resulted in the lowest AUDPC, but was not significantly different from the grower standard with calendar interval. The grower standard with the extended model interval resulted in significantly higher CLS compared to the grower standard with the calendar interval ($P < 0.001$).

All spray programs resulted in significantly greater sugar content than the non-treated control ($P < 0.05$; Table 5.6). The percent sugar was greatest in the grower standard with the calendar spray interval but was not significantly different from treatments based on Model 1, 2, or 3. Percent sugar was significantly reduced in the grower standard program with extended model-based spray interval ($P < 0.05$; Table 5.6). The grower standard initiation program with model application intervals had a significantly higher AUDPC compared to the control, resulting in reduced sugar percentage and RWS ($P < 0.05$; Table 5.6). The model 2 and 3 application programs reduced the number of fungicide applications by one compared to the grower standard program (Table 5.4), while also maintaining comparable AUDPC (Figure 5.5), yield, percent sugar, RWS, and RWSH (Table 5.6).

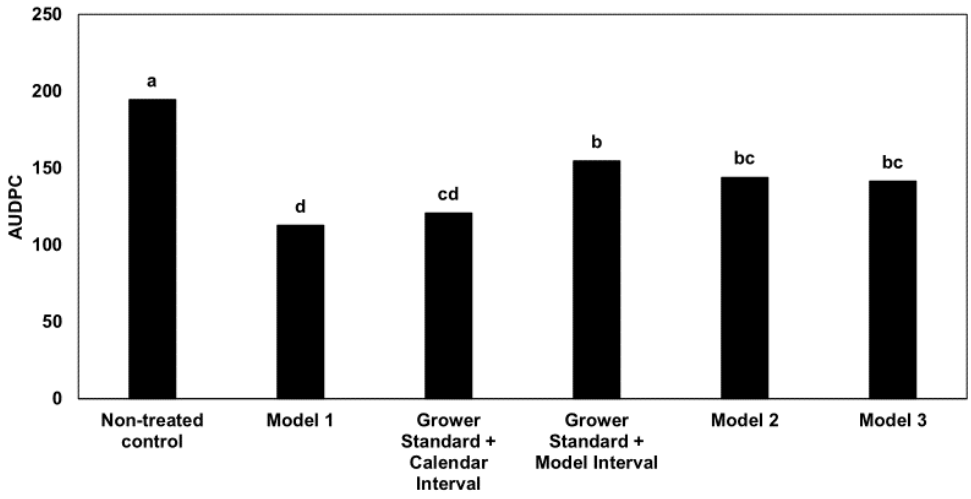


Figure 5.5. Area under the disease progress curve (AUDPC) of CLS severities assessed in a CLS-susceptible variety (SX-1278) the model validation trial in Frankenmuth, MI in 2023. Model 1, 2, and 3 programs were initiated according to probability and canopy thresholds (Model 1: 50% probability; Model 2: 50% probability + 40% row closure; Model 3: 40% probability + 50% row closure) and compared to a grower standard program initiated at BEETcast cumulative daily severity values (50) followed by a 14-day application interval. A grower standard initiation treatment (50 DSV) with a model predicted application interval was tested with a 50% probability. AUDPC values were calculated as described by Madden et al. (2017) using severity ratings collected at 5 timepoints from Jul 7 through Aug 5; ratings were based on the KWS severity scale (0-10). Bars represent the means of four replicate plots for each treatment. Bars with the same letter were not significantly different based on Fisher's protected LSD ($\alpha = 0.05$).

Table 5.6. Sugarbeet yield, percent sugar content, and recoverable white sugar for each grower standard or model-based fungicide treatment program evaluated in 2022 and 2023.

Year	Trt	Variety	Program	Yield (t/ha)	Sugar (%)		RWS[z] (kg/t)		RWSH[y] (t/ha)
2022	1	C-G021	Non-treated control	39.4	15.9		116.3		4.6
	2	C-G021	Grower standard	38.9	15.8		116.4		4.6
	3	C-G021	Model 1[w]	44.9	15.9		116.5		5.3
	4	C-G932NT	Non-treated control	34.4	15.8		114.8		3.9
	5	C-G932NT	Grower standard	35.3	15.7		113.6		4.1
	6	C-G932NT	Model 1	31.7	15.6		117.6		3.8
2023	1	SX-1278	Non-treated control	20.5	13.2	c[x]	90.6	c	1.9
	2	SX-1278	Model 1[v]	20.2	14.5	ab	102.2	ab	2.1
	3	SX-1278	Grower standard + calendar interval	22.8	15.3	a	108.8	a	2.5
	4	SX-1278	Grower standard + model interval	22.4	14.2	b	100.0	b	2.2
	5	SX-1278	Model 2	22.9	14.6	ab	104.2	ab	2.4
	6	SX-1278	Model 3	26.7	14.6	ab	104.7	ab	2.9

[z] Kilograms recoverable white sugar per metric ton of fresh beets (RWS). [y] Metric tons of recoverable white sugar per hectare (RWSH) calculated for each replicate using the following equation: RWSH (metric ton/hectare) = RWS (kg/metric ton) × Total Yield (metric ton/hectare) ÷ 1000; treatment means across four replicates are shown.
[x] Column values followed by the same letter were not significantly different based on Fisher's Protected LSD (α=0.05).
[w] 2022 Model 1 was implemented to trigger at a 70% likelihood threshold for the presence of 35 or more *C. beticola* spores paired with a BEETcast DSV value of 3 or 4 on the same day.
[v] 2023 Model 1, 2, and 3 programs were initiated based on the Spore35 model threshold of 40 or 50% likelihood of 35 or more *C. beticola* spores paired with a BEETcast DSV value of 3 or 4 on the same day and various row closure contingencies (Model 1: 50% + DSV 3 or 4; Model 2: 50% + DSV 3 or 4 + 40% row closure; Model 3: 40% + DSV 3 or 4 + 50% row closure).

5.4.6 Final model refinement

Environmental variables that were significantly correlated to aerial conidia numbers and conidia thresholds for all site-years were similar across the 2019-2021 and 2019-2022 data analyses. For 2019-2022, the percentage of positive binary responses (i.e., *Cercospora*-like conidia detected) for the 10-, 35-, and 50-, and 100-threshold was 48%, 29%, 22%, and 12%, respectively. Variables such as leaf wetness duration, were important factors influencing aerial conidia numbers (Figure 5.6 and 5.7).

From 2019-2022, the model training subset (80% dataset; n = 402 actual daily observations) had ranges of temperature from 0.7 to 35.1°C, relative humidity from 35.1 to 98.2%, and leaf wetness durations from 0 to 24 hours. Across selection criteria, the refined

model generated predicted observations using a 35-spore threshold and incorporated maximum leaf wetness, average relative humidity over 80%, and minimum temperature ($R^2 = 0.19$; $P < 0.0001$; Table 5.7) (logit Equation 2):

(Eq. 2) Spore35 = 0.0146*MaxLW + -1.7194*AvgRH80 + 0.118*MinTemp – 3.6515

An additional refined model of interest predicted observations using a 50-spore threshold and incorporated the same variables as logit Equation 2 ($R^2 = 0.15$; $P < 0.0001$; Table 5.7) (logit Equation 3):

(Eq. 3) Spore50 = 0.0123*MaxLW – 1.4549*AvgRH80 * P3+0.1167*MinTemp – 3.8485

For the testing data subset (20% dataset; n = 101 actual daily observations), the logit Equation 2 model accuracy was 69.3% for predictions of >35 conidia based on a probability or action threshold of 80% (Figure 5.8). The logit Equation 3 model was 66.3% accurate at prediction >50 conidia at an 80% probability threshold. The initial 2021 model (Equation 1) achieved an accuracy of 67.3% when predicting >35 conidia at an action threshold of 80%. At a 30% probability threshold, the 2021 model (Equation 1) maintained a 72.3% accuracy, while the Equation 2 and Equation 3 model accuracy was reduced to 44.6% and 69.3%. The ensemble model, which takes the average probability output of logit Equation 1, 2, and 3, has an accuracy of 66.3% and 64.4% respectively for an 80% and 30% action threshold. The 50% probability threshold was the most accurate with 73.2% correct predictions for the ensemble model.

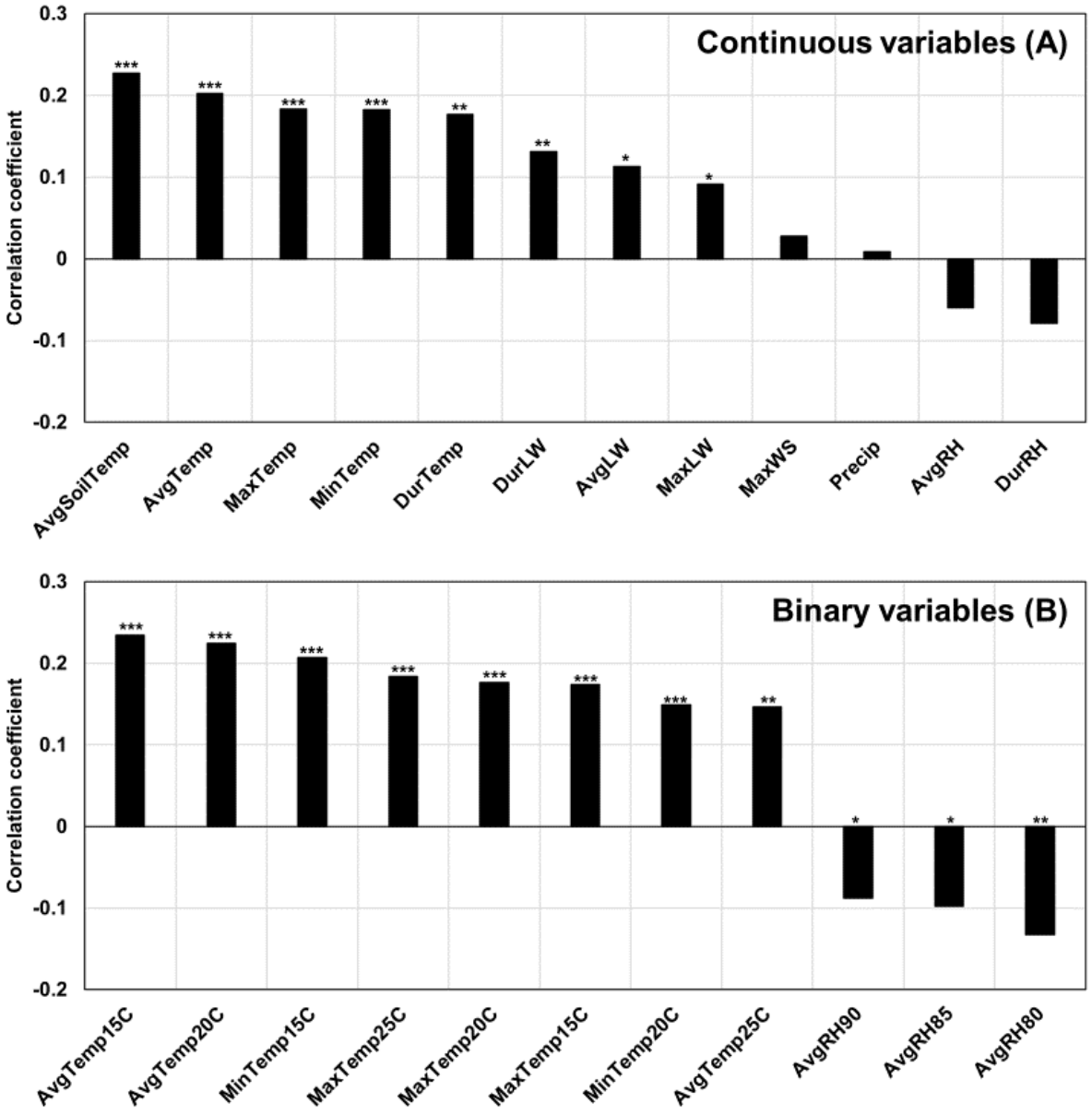


Figure 5.6. (A) Pearson and (B) Kendall correlations between numbers of daily aerial *Cercospora* spores and continuous or binary environmental predictor variables collected from sites in 2019 to 2022. Significance is indicated by *P*-value thresholds <0.05 *, <0.01 **, and <0.001 ***.

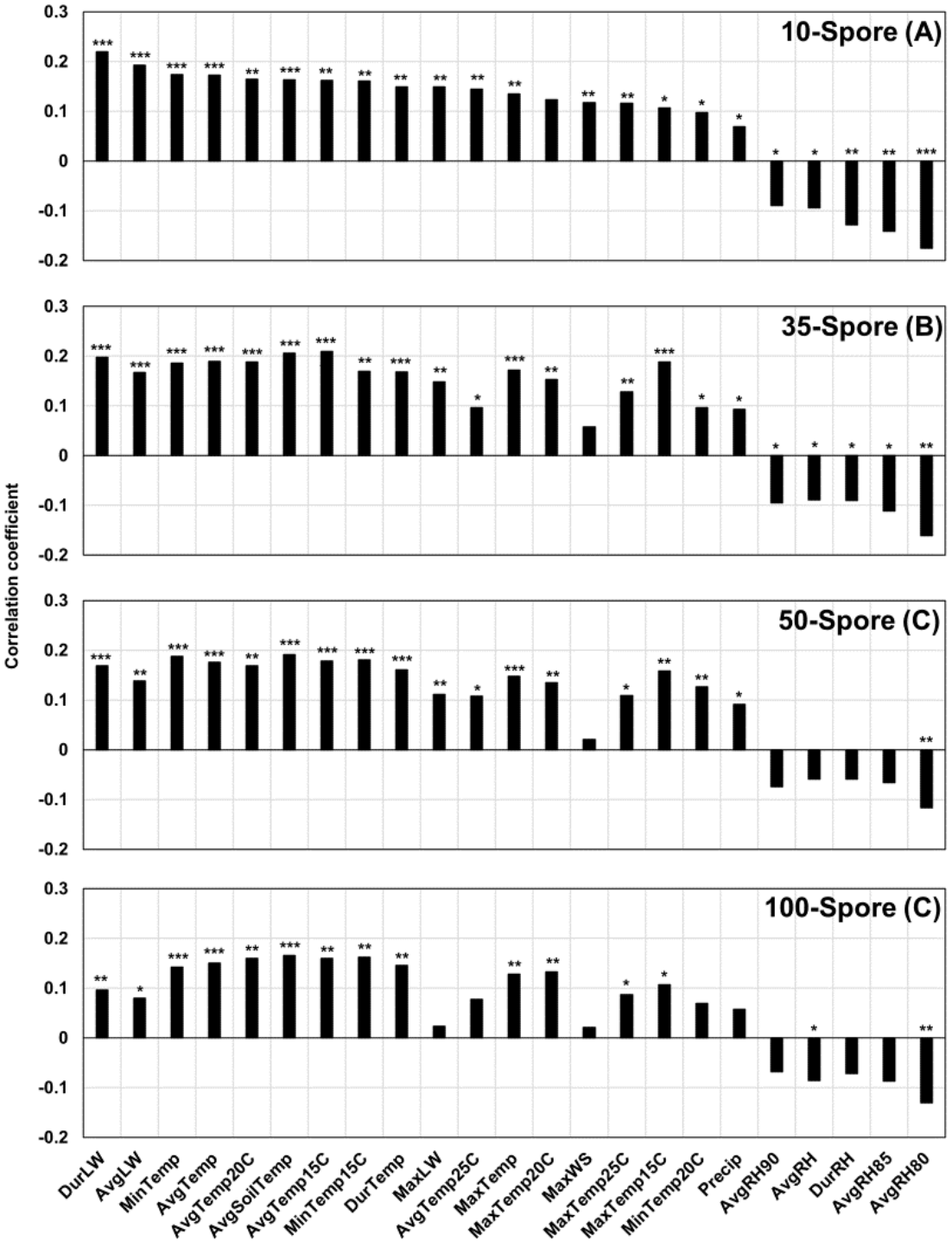


Figure 5.7. Kendall correlations for binary daily *Cercospora* spore thresholds of (A) 10, (B) 35, (C) 50, and (D) 100 with continuous and binary environmental predictor variables collected from sites in 2019 to 2022. Significance is indicated by *P*-value thresholds <0.05 *, <0.01 **, and <0.0001 ***.

Table 5.7. Refined logistic model candidates generated from an 80% training set (n = 402 actual daily observations) from eight site-years of data collected in 2019-2022. Information on max-rescaled R^2, Akaike's information criterion (AIC), percentage correct, sensitivity, specificity, predicted positive (Ppos), and predicted negative (Pneg) was reported for the probability level (Prob) where sensitivity and specificity percentages converged.

Threshold	Variables	Max-rescaled R^2	AIC	c	Prob (%)	Corr (%)	Sens (%)	Spec (%)	Ppos (%)	Pneg (%)
Spore35	DurLW, MaxTemp	0.13	435.1	0.70	28	63.7	61.1	64.6	38.8	81.9
Spore35	DurLW, AvgTemp	0.14	432.8	0.70	28	65.4	66.7	65	41.1	84.1
Spore35[a]	DurLW, AvgTemp, MaxWS	0.15	430.7	0.71	28	63.2	63	63.3	38.6	82.3
Spore35	MaxLW, AvgRH80, MinTemp	0.19	418.1	0.74	28	66.7	68.5	66	42.5	85.1
Spore50	DurLW, AvgTemp	0.12	384.5	0.69	22	65.7	61.4	66.8	32.5	86.9
Spore50	AvgRH80, MinTemp	0.11	385.0	0.69	22	63.9	63.9	63.9	31.5	87.2
Spore50	MaxLW, AvgRH80, MinTemp	0.15	376.2	0.72	22	64.9	63.9	65.2	32.3	87.4

[a] Model variables used in the 2021 model (Equation 1) generated to predict fungicide application timing in 2022 and 2023 field trials.

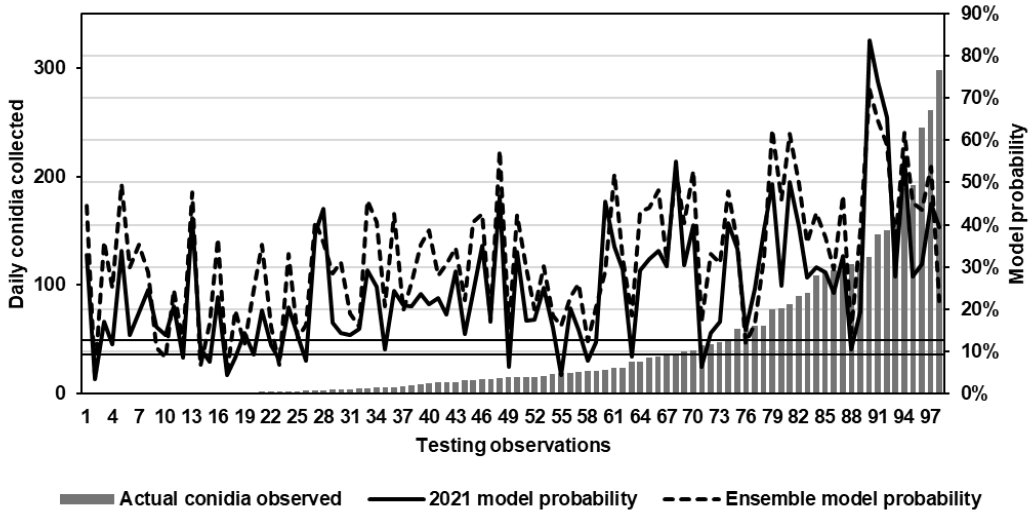


Figure 5.8. The 20% testing data from 2019-2022 of daily aerial *Cercospora*-like conidia collected at 14.4 cubic meters per day (n = 101 actual daily observations) compared to the predicted probabilities that greater than 35 aerial conidia were present from two models developed using the 80% training data. The ensemble logistic model predictions were calculated using the average probability of logit Equation 1, 2, and 3. The initial 2021 model was included

Figure 5.8. (cont'd)
according to the following logit: Spore35 = 0.0761*DurLW + 0.1258*AvgTemp + 0.0413*MaxWS - 4.8943 (Equation 1). The probability was calculated by taking x divided by (1+x) where x is the exponential value of the model output value (e.g., Spore35 or Spore50). The solid black lines represent the 35- and 50-spore thresholds.

5.5 Discussion

In the current study, peak spore concentrations were observed at variable times throughout the sampling period, occurring anytime from May to June and even sometimes in early July for various site locations. It should be noted that the majority of spore sampling occurred prior to CLS symptom development on sugarbeet, between late-April and mid- to late-July, so these are hypothesized to represent peak *initial* inoculum levels rather than those experienced following repeated polycyclic infection cycles. As noted in Minnesota and North Dakota, peak *full-season* levels of aerial conidia were observed in late August 2003 and in early September 2004 (Khan et al. 2009); however, conidia were not collected during this time for the current study due to the focus on early season predictions for initial spay program timing. Previously in Michigan, elevated periods of *C. beticola* conidia, as measured by sugarbeets as live spore traps, were observed consistently in mid-June for monitoring during April to the first week of July in 2017 and 2018 (Bublitz et al. 2021). Patterns of airborne *C. beticola* conidia concentration have been assessed using similar Burkard sample collection as the current study in Ontario, Canada and Minnesota and North Dakota (Khan et al. 2009; Tedford et al. 2018). As shown, weather variables impacting early-season spore levels may be helpful to determine risk of initial infections and inform the initiation of chemical programs.

Findings from the current study indicated that minimum air temperature was highly influential on *Cercospora* conidial thresholds of 10, 35, and 50. At the 35-spore threshold, the conidial risk in a 24-hour period was associated with mean temperature, which is supported by previous research where airborne *C. beticola* conidia concentration was highly influenced by

mean air temperature (Khan et al. 2009; Tedford et al. 2018). The current study also showed daily *Cercospora* spore numbers were highly correlated to the binary variables of temperatures above or below 15°C (60°F), 20°C (68°F), and 25°C (77°F). These thresholds coincide with previous work where *C. beticola* conidia were produced most readily at temperatures from 15 to 23°C and inhibited by temperatures <10°C and >38°C (Jacobsen and Franc 2009; Khan et al. 2009). Germination and growth are possible for *C. beticola* at temperatures as low as 9°C, although temperatures around 30°C were considered optimal (Pool and McKay 1916). Correspondingly, average soil temperature was highly correlated with *C. beticola* conidial presence and abundance. While soil temperature impacts have been less well-studied for *C. beticola* spore abundance, previous studies have shown that air temperature is an important factor in predicting soil temperature (Araghi et al. 2017; Bond-Lamberty et al. 2005; Kang et al. 2000; Paul et al. 2004). Air temperatures resulted in similar explanatory power as the soil temperatures in selected models with ambient air temperature sensors being typically more readily available for model implementation. Thus, temperature predictors explanatory of *C. beticola* sporulation appeared to be useful for airborne inoculum risk assessment.

A previous study showed that peak airborne *Cercospora* conidia concentration was significantly correlated with the average daily temperature when RH was greater than 87% (Khan et al. 2009). In the current study, average and duration of leaf wetness were highly associated with conidial thresholds of 10, 35, and 50. *Cercospora beticola* conidia production has long been associated with relative humidity greater than 60% (Pool and McKay 1916), and prolonged periods of relative humidity from 90 to 95% and available moisture on leaves are optimal for CLS development (Forsyth et al. 1963; Jacobsen and Franc 2009; Ruppel 1986; Shane and Teng 1983, 1984). Additionally, previous studies found that number of CLS lesions (Bublitz et al. 2021) and

number of aerial *Cercospora* conidia (Tedford et al. 2018) were significantly associated with relative humidity, similar to the current study. Interestingly, Bublitz et al. (2021) observed a positive relationship whereas the current study observed a negative correlation between conidia and relative humidity predictors. Previous studies have shown that relative humidity variables were able to predict duration of leaf wetness with a positive coefficient (Sentelhas et al. 2008). Alternatively, other studies have shown that relative humidity is not a direct substitute for leaf wetness duration when estimating disease progression (Lomas and Shashaua 1969; Thompson 1981). While relative humidity predictors were either not strongly or significantly correlated with observed airborne *Cercospora* levels, microclimate or soil-level moisture variability may be influencing this relationship with early-season inoculum.

Bublitz et al. (2021) reported that rainfall was significantly correlated (R = 0.31-0.56; $P < 0.01$) with number of CLS lesions for each year of testing. The current study and Tedford et al. (2018) reported a slightly less significant correlation for rainfall ($P < 0.05$) over multiple years and locations. Tedford et al. (2018) used similar mechanical spore sampling methods to the current study, while Bublitz et al. (2021) used sentinel beets to measure spore levels. Sentinel beet CLS lesion presence would be a product of *C. beticola* conidial concentrations but also may be impacted by in-field environmental conditions favoring germination and infection. The active impaction sampler used in the current study may favor the capture and identification of wind-dispersed rather than rain-dispersed conidia (Lawrence and Meredith 1970). Notably, the NDAWN model incorporates rainfall information for determining favorable conditions for *C. beticola* infection (Jones and Windels 1991; Shane and Teng 1984, 1985; Windels et al. 1998). While the most explanatory models selected in the current study contained temperature variables, consistent with both the BEETcast (Pitblado and Nichols 2005) and NDAWN models (Jones and

Windels 1991; Shane and Teng 1984, 1985; Windels et al. 1998), duration of leaf wetness was only similar to the use of leaf wetness in the BEETcast model. Differences in favorable conditions for *C. beticola* infection compared to spore presence were expected but overlap in these predictors could be considered in future studies for a comprehensive CLS risk assessment tool.

Wind has been considered a major component of *C. beticola* dispersal (Khan et al. 2008; Lawrence and Meredith 1970; Meredith 1967). In the current study, maximum wind speed was not initially identified in correlation analyses; however, during model selection, inclusion of wind speed was found to improve fitness indices. Conidia dispersal is an important dynamic of spore presence and the contribution of rain splashing (Carlson 1967), wind (Lawrence and Meredith 1970; McKay and Pool 1918), or arthropod movement (McKay and Pool 1918; Meredith 1967) has been a point of discussion in this field. Previous studies identified that wind- (Khan et al. 2008) and rain-dispersed (Carlson 1967) *C. beticola* spores were the primary source of inoculum within a field; and that conidia were readily dispersed over short distances (less than 100 m) (Lawrence and Meredith 1970; McKay and Pool 1918). Using mechanical spore samplers, Lawrence and Meredith (1970) found that aerial conidia were highly influenced by wind, while those who used live spore traps have reported that conidia dispersal was more highly influenced by rain than wind (Bublitz et al. 2021; Carlson 1967). While the Burkard sampling method has limited use during rain periods (Jackson and Bayliss 2011), live spore traps have a bias towards rain-dispersed spores (Carlson 1967; Lawrence and Meredith 1970). In the current study, sampling was conducted immediately adjacent to or in previous year CLS-infested fields; while wind speed generally resulted in positive correlations with aerial spore concentrations, the close proximity to inoculum sources may have limited the influence of wind in these studies. Further investigations of wind speed in relation to the proximity of inoculum sources could be beneficial

due to its role in local and long-distance dispersal.

While no molecular quantification of *C. beticola* conidia was conducted, regression results between mechanical Burkard and sentinel beet observations indicate a large portion (at least 50%) of *Cercospora*-like spores assessed were likely *C. beticola*. Sentinel beets have been useful in distinguishing between *C. beticola* conidia and conidia of other *Cercospora* species, which is difficult by morphology alone (Groenewald et al. 2013). Conidia of *C. apii* and *C. apiicola* are morphologically similar to or indistinguishable from *C. beticola* (Groenewald et al. 2006); if present in our samples, some model discrepancies may be attributed to the presence of other *Cercospora* species. This may result in an overprediction of spore presence but can be offset by adjusting the action thresholds used in model implementation. Previous research has also shown that the concentration of aerial conidia was correlated with CLS disease pressure (Khan et al. 2009; Tedford et al. 2018). Limitations in the passive sampling style of the live spore traps compared to the active impaction sampler (McCartney et al. 1997; Van den Heyden et al. 2021) as well as potential for imperfect infection conditions may account for some of the differences between the number of aerial *Cercospora* conidia and the number of CLS lesions on live spore traps. Further refinement of *C. beticola* airborne inoculum models may consider processing samples using quantitative PCR-based methods (Knight and Pethybridge 2020). However, benefits of the presented methods include validation of spore viability and virulence using CLS-susceptible sentinel beets, which would remain an important consideration for future studies.

In the current study using a CLS-susceptible variety, extended intervals between fungicide applications resulted in diminished control of CLS, as well as reduced sugar percentage and RWS; previous studies also found intervals of 21 days (Khan and Smith 2005) or initiation at 50 and 55 with 35 and 50 DSV intervals, respectively (Tedford et al. 2019) were less effective for

CLS control compared to a 14-day calendar schedule. In 2023, two model-based treatments resulted in CLS control and yield comparable to the grower standard with a slightly reduced number of fungicide applications (one less than the standard program). While model-based programs were expected to increase precision of fungicide initiation, the validation studies indicate the model supports accurate timing with the potential to limit unnecessary applications and increase grower profit. Application timing that accounts for spore abundance could further delay resistance development for single-site fungicides since it promotes preventive use; additional studies would be necessary to fully understand the impacts of this approach. Field studies indicated highly CLS-resistant sugarbeet varieties were not ideal to evaluate model performance, with low CLS severity limiting the ability to detect differences in fungicide application timings. Similarly, a field study by Lien et al. (2023) did not find differences in fungicide treatment efficacy when using a highly CLS-tolerant sugarbeet variety. Evaluation of CLS-resistant varieties may still be beneficial for testing dependent on favorable infection or disease environments, *C. beticola* populations or the presence of resistance breaking strains, and sustained variety performance.

Current model selection was limited to readily available predictor information through the MSU Enviroweather or WINnovations networks of on-site network of meteorological-grade weather stations. However, other practical, geographical, or weather information not considered could be useful in improving model accuracies. For example, inoculum pressure was assumed to be present in the design of current sampling approaches, however, field history would be a critical factor influencing the presence and abundance of *C. beticola* conidia. Duration of leaf wetness in the current study had a substantial impact on *Cercospora* conidia abundance, but similar duration variables for air temperature, relative humidity, wind speed, or other environmental variables

were not considered and may be useful for further model testing. Environmental variables to capture the microclimate at the leaf surface could be more explanatory for predicting conidial production and release (Huber and Gillespie 1992), though these factors are more difficult to incorporate in a commercial utilized model due to limited senor information. As explored by Huber and Gillespie (1992), additional variables not tested in this study that could be influencing spore abundance include factors like solar radiation, cloud cover, wet dry cycles, vapor pressure deficit, air turbulence, and soil properties, including soil moisture. Solar radiation was not considered in the current study though it was seen to be significantly correlated with lesion number in previous studies (Bublitz et al. 2021) and Canova (1959) reported decreased *C. beticola* germination following exposure to solar radiation. Future studies should consider validation of these models across diverse climatic conditions and may consider other regionally specific factors impacting the risk of *C. beticola* inoculum presence and abundance.

Overall, the models developed in the current study performed most accurately early in the growing season from mid-May to mid-June, supporting utility of these in complementing existing infection-based tools to improve timing and initiation of fungicides programs. Integrating information of the risk of spore presence in current decision support models would be especially beneficial for applying protectant fungicides. Application decisions considering even brief periods where weather is conducive for spore production, dispersal, and infection could better be used in preventative management than those only considering infection and first symptom development. Accurate timing of fungicide applications is critical for management with ideal applications initiated prior to infection and symptom development. Continued validation and refinement of the models developed in the current study across representative field sites and conditions will further identify robust models useful for informing CLS management in sugarbeet. Despite refinement

across eight-site years, models and selected variables have remained similar; therefore, current models are likely to be informative of early-season risk. Field validation studies demonstrated that models created in this study were able to reduce the number of fungicide applications while also maintaining similar management of CLS.

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APPENDIX

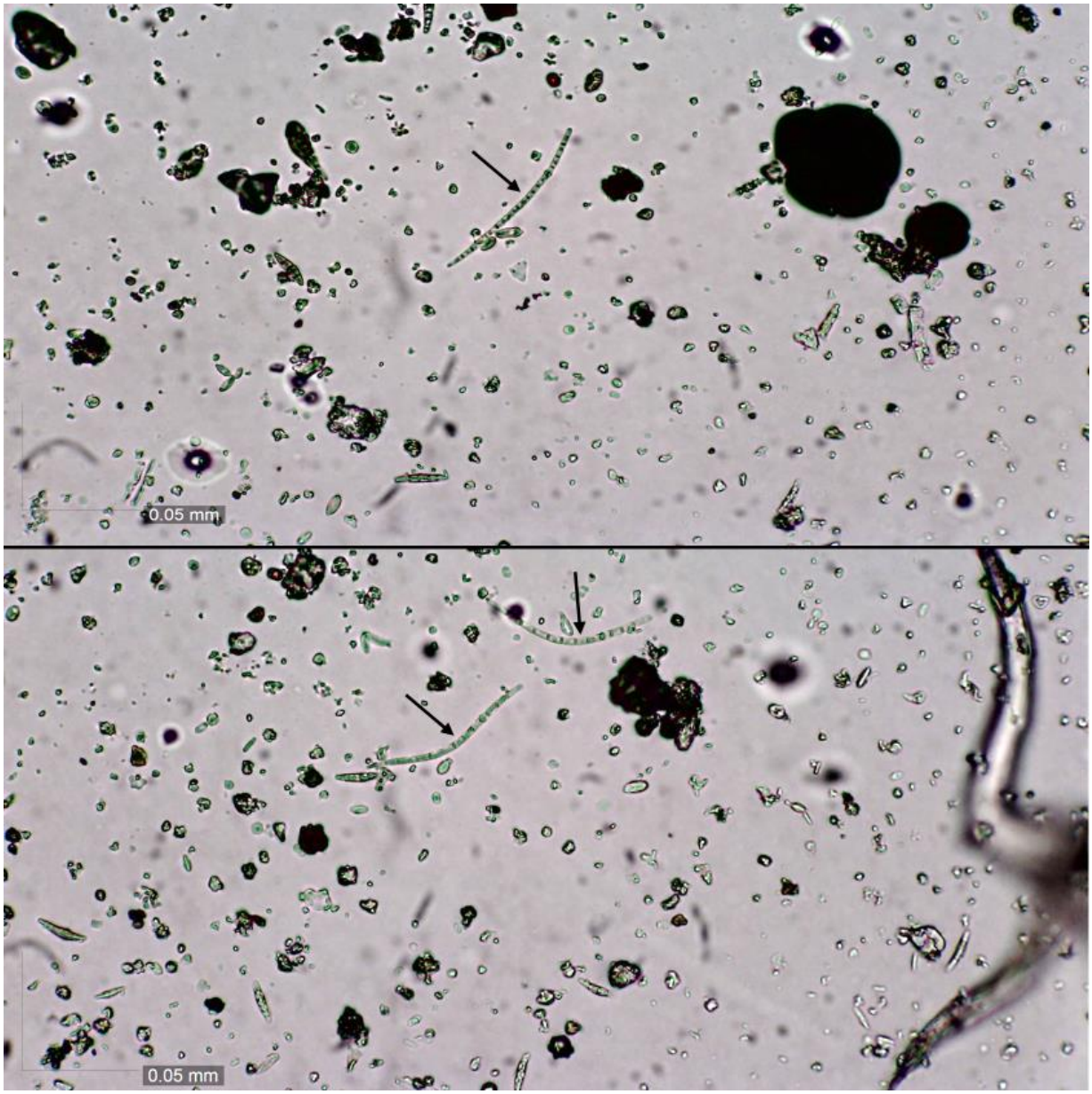

Figure S5.1. Examples of three spores collected via volumetric Burkard sampler tape and identified as *Cercospora*-like conidia (indicated by black arrows). Micrographs were captured using a compound microscope at 200x magnification and measured at 400x magnification. On average, the conidia were 3-4 µm wide and 78-88 µm long.

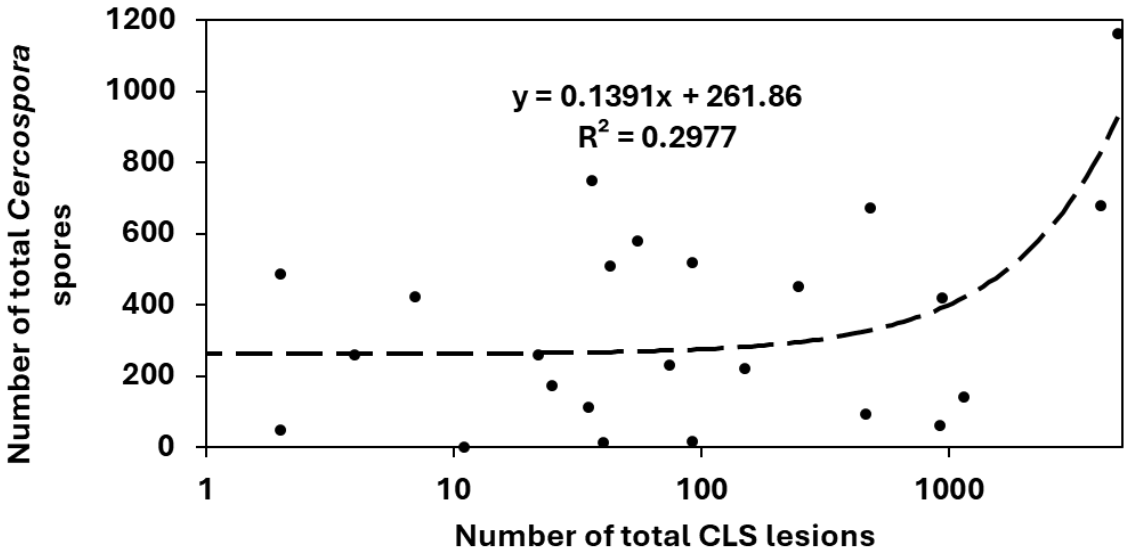

Figure S5.2. A scatter plot with dots representing weekly data points of *Cercospora*-like spores observed using a mechanical sampler and total number of characteristic Cercospora leaf spot (CLS) lesions observed using live "biological" sentinel plants from Michigan locations in 2019, 2020, 2021, and 2022 (n=31 weeks). Due to the range of lesions and substantial number of weekly observations where <1,000 lesions were detected on sentinel beets, a logarithmic scale is shown for the x-axis. A significant linear relationship was detected between CLS lesions on highly CLS-susceptible sentinel beets (USDA germplasm F1042) after a 7-day exposure in the field and the total number of *Cercospora*-like spores collected using a volumetric Burkard spore sampler over the same 7-day period ($P < 0.0012$).

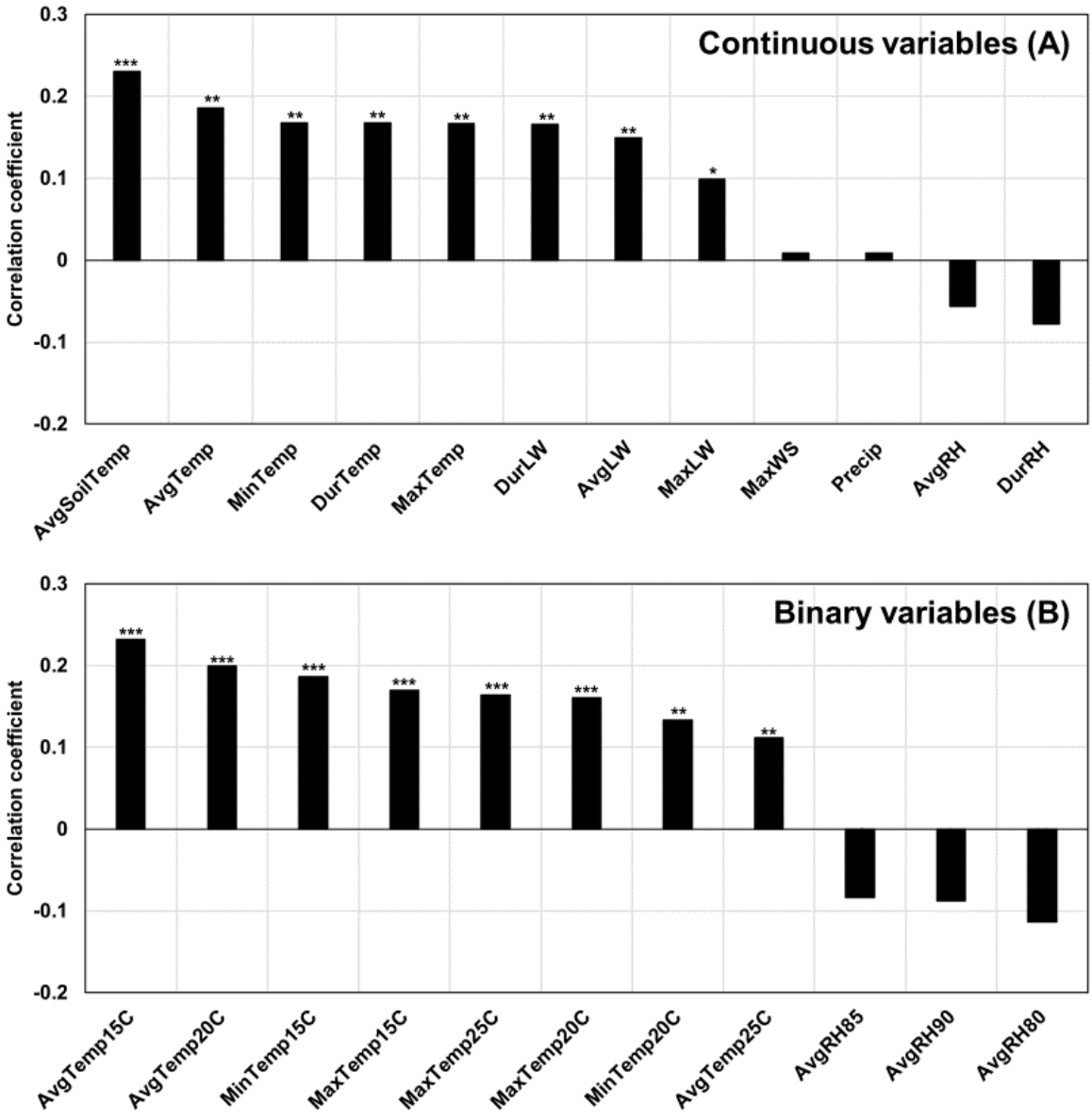

Figure S5.3. (A) Pearson and (B) Kendall correlations between numbers of daily aerial *Cercospora* spores and continuous or binary environmental predictor variables collected from sites in 2019 to 2021. Significance is indicated by *P*-value thresholds <0.05 *, <0.01 **, and <0.0001 ***.

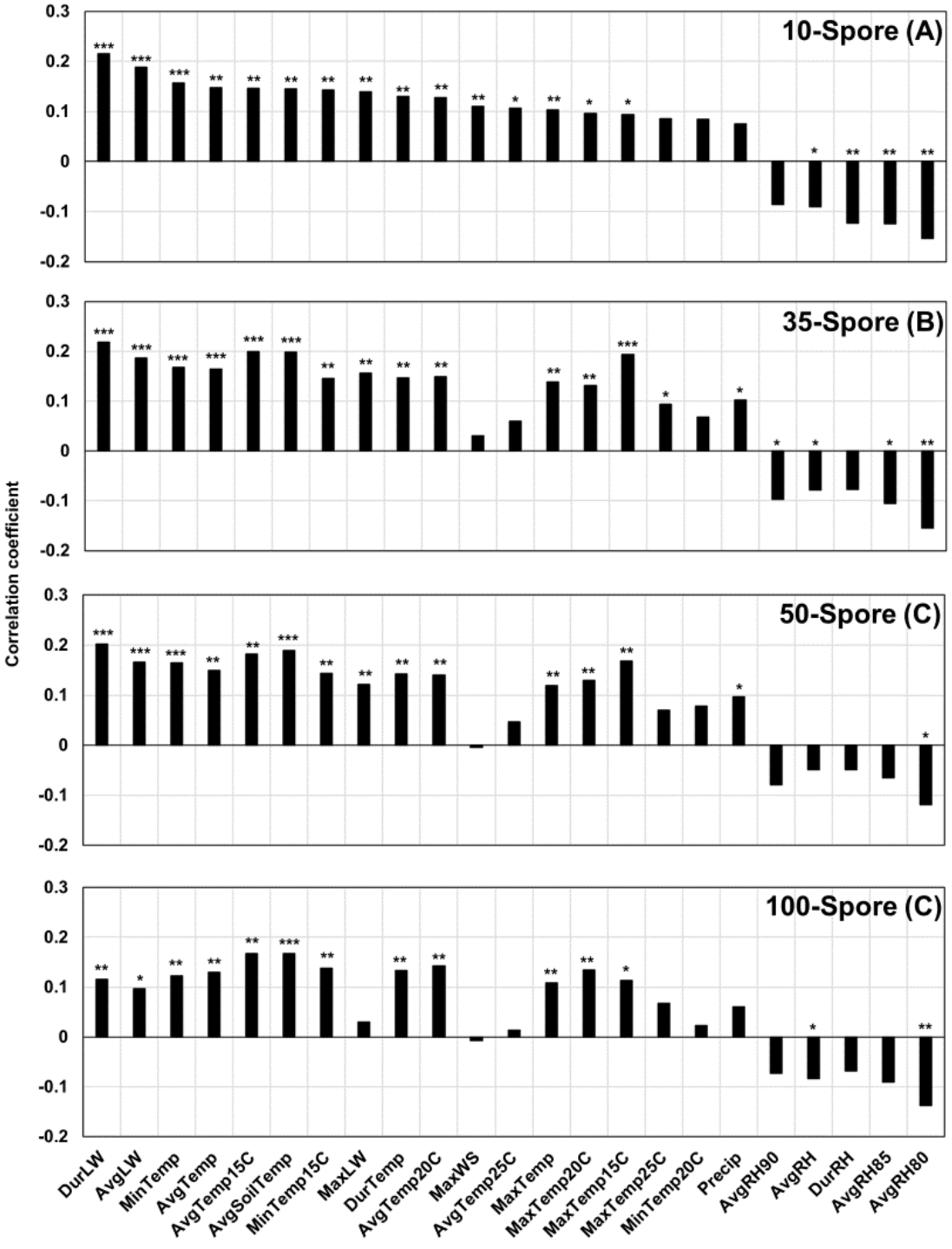

Figure S5.4. Kendall correlations for binary daily Cercospora spore thresholds of (A) 10, (B) 35, (C) 50, and (D) 100 with continuous and binary environmental predictor variables collected from sites in 2019 to 2021. Significance is indicated by *P*-value thresholds <0.05 *, <0.01 **, and <0.0001 ***.

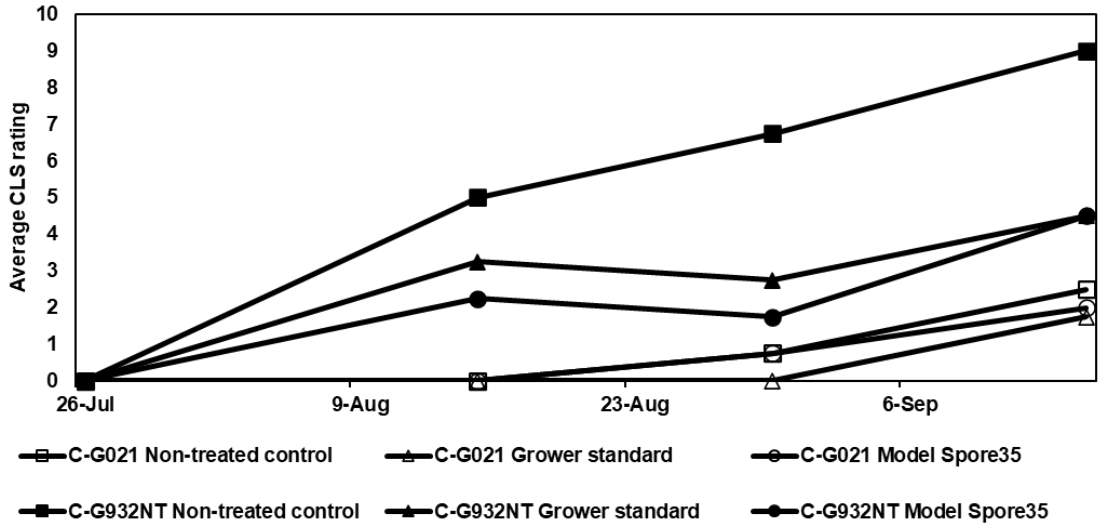

Figure S5.5. Mean Cercospora leaf spot (CLS) severity progression on a resistant (C-G021) and susceptible (C-G932NT) sugarbeet variety with different fungicide programs, including non-treated control, a grower standard, and the Spore35 model-based application timings, tested in field validation studies in Frankenmuth, MI in 2022. CLS ratings were based on mid-canopy averages using the KWS severity scale (1-10) in 2020 and 2021 where 1 is 0.1% severity (1-5 spots per leaf) and 10 is 50% severity. Each point represents a mean of four replicate field plots.

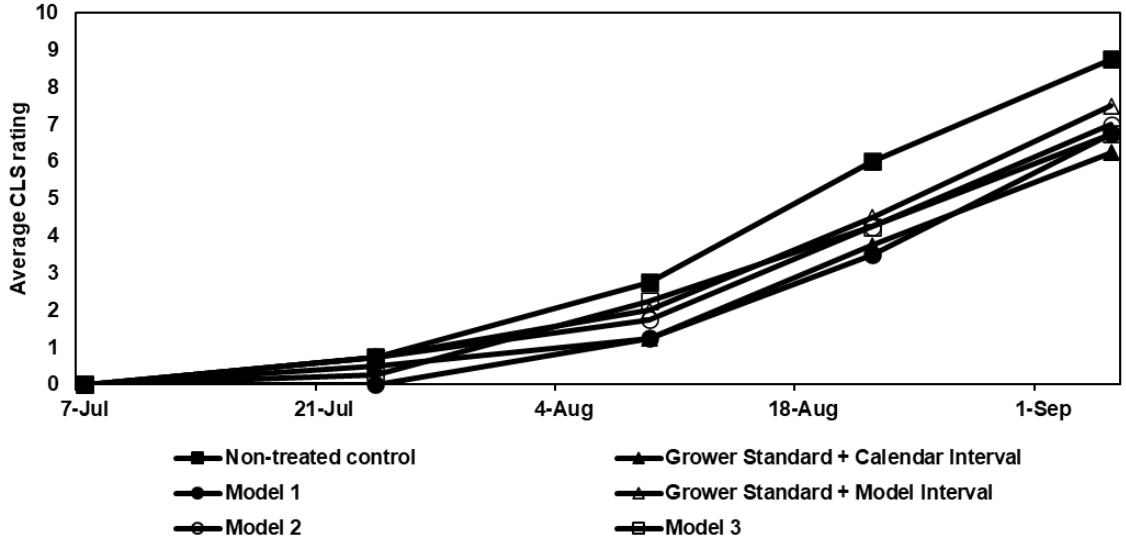

Figure S5.6. Mean Cercospora leaf spot (CLS) severity progression in a susceptible sugarbeet variety with different fungicide programs, including non-treated control, grower standard (using a model- or calendar-based application intervals), and the Spore35 model-based application initiation and interval timings (Model 1, 2, and 3), tested in field validation studies in Frankenmuth, MI in 2023. CLS ratings were based on mid-canopy averages using the KWS severity scale (0-10) in 2020 and 2021 where 1 is 0.1% severity (1-5 spots per leaf) and 10 is 50% severity. Each point represents a mean of four replicate field plots.

CHAPTER 6: CONCLUSIONS

Exploration into alternative cultural management options for CLS on sugarbeet is critical for a sustainable crop. In the wake of increased fungicide resistance in *C. beticola* (Secor et al. 2010a; Rangel et al. 2020), finding effective fungicide-alternative strategies to control CLS is important for growers and the sugarbeet industry. Additional management options are incredibly valuable to maintain adequate CLS control and profitable sugarbeet yields. In the field studies assessing heat treatments (Chapter 2), immediate reductions in *C. beticola* viability and next-season reductions for CLS inoculum indicate this tillage-alternative strategy could be used in CLS management. With the movement to minimal or conservation tillage systems, these residue management strategies may be especially useful in disease control. Moreover, heat treatment could be a viable option for management of other foliar pathogens on sugarbeet or other pathogens that infect the plant phyllosphere and survive on leaf debris left on the soil surface between cropping seasons (Cook et al. 1978). There is an increased risk for foliar disease pressure when residue is left unmanaged, especially on cereal crops (Bailey 1996; Bailey and Lazarovits 2003; Bockus and Shroyer 1998). Heat treatment may provide improved management for a number of wheat pathogens where infected plant reside is a concern, including *Pyrenophora tritici repentis* (Adee and Pfender 1989), *Zymoseptoria tritici* (Suffert and Sache 2011), and *Oculimacula yallundae* (Vera and Murray 2016). Heat treatment might be of interest for pathosystems that include other *Cercospora* species where fungicide resistance and residue management are considerations (Bradley et al. 2019). Application of a direct-flame heat treatment on sugarbeet leaves before harvest has a low chance of fire hazard compared to other cropping systems with flammable dry plant material during harvest. Therefore, using this tool for foliar pathogen management on sugarbeet has an advantage and crop residue properties should be

considered when testing this method in other systems.

Heat treatment and cover crop utilization in the field were investigated (Chapter 2). Cover crops might be useful to act as physical dispersal barriers to limit pathogen movement or recruit antagonistic microbial communities (Gao et al. 2022; Maglione et al. 2022). Many other cultural options for residue management, such as microbial antagonists and plant or microbial extracts have been tested on plant pathogens in sugarbeet (Derbalah et al. 2012; El-Tarabily 2004; Errakhi et al. 2007; Georgakopoulos et al. 2002; Ghazy et al. 2021; Kakvan et al. 2013; Kiewnick et al. 2001; Zachow et al. 2008) but remain to be tested on *C. beticola*. Additional cultural management strategies could be tested such as the utilization of alternative amendments or microbial antagonists to inhibit *C. beticola* growth (Derbalah et al. 2013; Sehsah et al. 2022; Ziedan and Farrag 2011). Products containing humic acid and fulvic acid, labeled for foliar or soil amendment applications, were tested *in vitro* in the current study (Chapter 2: Appendix B). Humic and fulvic acid were found to inhibit *C. beticola* growth. Fulvic acid impacted media pH while humic acid did not. Humic substances tested in the current study and additional factors (Derbalah et al. 2012; El-Tarabily 2004; Errakhi et al. 2007; Georgakopoulos et al. 2002; Ghazy et al. 2021; Kakvan et al. 2013; Kiewnick et al. 2001; Zachow et al. 2008) remain to be evaluated for sugarbeet disease control in the greenhouse or field.

As resistance to certain fungicides increases and becomes more prevalent in *C. beticola* isolates, efforts to reduce selection pressure and maintain efficacy of fungicide active ingredients must be further explored. While pyraclostrobin was highly effective at managing CLS when first approved for use on sugarbeet (Karadimos et al. 2005; Khan and Smith 2005; Secor et al. 2010a), fungicide resistance and efficacy has been a major concern (Kirk et al. 2012). Pyraclostrobin proved to provide no benefit in the current study (Chapter 3) in a program when rotated or tank-

mixed with mancozeb and increased the risk of resistance development in *C. beticola*. Results from this study (Chapter 3) indicate that the use of pyraclostrobin for CLS control on sugarbeet should be avoided. Evaluating the efficacy of fungicides and their respective influence on pathogen resistance continues to be important for the sugarbeet industry and the agricultural industry. Testing other novel and current fungicide active ingredients for their effect on resistance and yield metrics in various rotations provides support for application recommendations. Impact of application programs is important for active ingredients with a history of inconsistent CLS control due to *C. beticola* insensitivity, such as organotin fungicides (Bugbee 1995; Rangel et al. 2020; Secor et al. 2010a; Weiland and Smith 1999).

The sugarbeet foliar pathogen, *C. beticola*, showed various levels of sensitivity to different fungicide active ingredients and mode of action groups in the current study (Chapter 4), which is consistent with previous work (Rosenzweig et al. 2020). It is important to evaluate the effect of field application on fungicide resistance and assess the relationships between different active ingredients by monitoring cross and multiple resistance (Avenot et al. 2016; Bolton et al. 2012; Hsiang et al. 1997; Ishii et al. 2021; Karaoglanidis and Thanassoulopoulos 2003; Köller et al. 1997; Thomas et al. 2012; Trkulja et al. 2015). This information will support growers in making educated application decisions potentially limiting resistance development and maintaining high yields.

The E198A mutation on the *beta tubulin* gene was highly accurate in predicting resistance to thiophanate-methyl in the current study (Chapter 4), which is consistent with previous studies (Davidson et al. 2006). Results from the current study (Chapter 4) supported findings that isolates of *C. beticola* with the G143A mutation on the *cytochrome b* gene had a wide range of *in vitro* pyraclostrobin EC_{50} values (Bolton et al. 2013). The Glu169 mutation on the *C-14 alpha-*

demethylase gene was not sufficient to forecast resistance for all triazole active ingredients as seen in a previous study by Rangel et al. (2024). Finding mechanisms associated with pathogen fungicide resistance is useful for testing during the growing season (Briere et al. 2001). Mutations that can better characterize and describe *C. beticola* sensitivity in our region in important for in-season application decisions.

Multiple applications in a single season are a major reason that in-season testing is valuable, particularly for advising field specific applications throughout the season. Field specific sensitivity testing is recommended due to the differences between individual field-level samples in the current study (Chapter 4). Monitoring pathogen fungicide sensitivity is useful to provide field-specific recommendation for growers on the most effective products because product efficacy is impacted by pathogen sensitivity (Bolton et al. 2012). In addition, continuing yearly fungicide sensitivity surveys will help develop knowledge on the patterns of resistance in the Great Lakes growing region (Chapter 4; Rosenzweig et al. 2020). Expanding fungicide sensitivity observation to encompass the United States or worldwide growing regions improves our understanding of *C. beticola* populations (Chapter 4; Rangel et al. 2020; Rosenzweig et al. 2020; Secor et al. 2010a). Seasonal field application records, including information on fungicide timing and active ingredient, may help researchers, agronomists, and growers understand some of the driving forces behind fungicide resistance patterns overtime. Ideally this will be a continued practice in various sugarbeet growing regions to compare population dynamics and evaluate if differences in management practices have a direct impact on fungicide sensitivity for each region. Fungicide sensitivity and application data should continue to be monitored and recorded for pathogens with histories for fungicide resistance (Karaoglanidis and Ioannidis 2010; Rosenzweig et al. 2020; Secor et al. 2010b).

Current models used for fungicide application timing in the U.S. (e.g., BEETcast and NDAWN) are developed to predict the risk of infection and disease development but do not consider inoculum presence prior to symptom development (Jones and Windels 1991; Pitblado and Nichols 2005; Shane and Teng 1984; 1985; Windels et al. 1998). Disease epidemiology is dynamic with different environmental requirements for various life cycle stages including spore production, release, dispersal, adhesion and infection, as well as symptom development on a susceptible host (Carlson 1967; Forsyth et al. 1963; Jacobsen and Franc 2009; Khan et al. 2008; 2009; Lawrence and Meredith 1970; Meredith 1967; Pool and McKay 1916; Ruppel 1986; Shane and Teng 1983; 1984; Wolf and Verreet 2005). The presence of aerial spores for foliar pathogens is an important consideration to estimate the risk of disease occurrence and pressure (Khan et al. 2008; Lawrence and Meredith 1970; Meredith 1967). There was a benefit to incorporating the risk of elevated *C. beticola* conidia into current CLS prediction models (Chapter 5), which has been observed in other systems (Carisse et al. 2005; Gent et al. 2009; Thiessen et al. 2016). Early-season risk predictions were 78% accurate in the current study (Chapter 5). Two models generated during this study resulted in comparable CLS management and yield to a grower standard with one less fungicide application (Chapter 5).

While the efficacy of foliar programs may be dependent on fungicide active ingredient (Khan and Smith 2005), generally those initiated either prior to infection or at early leaf spot detection (<1 spot per leaf) are most effective (Liebe et al. 2023; Pundhir and Mukhopadhyay 1987). Fungicide applications early in the season before pathogen establishment in the field are critical for managing disease pressure for the growing season (Chapter 5). More accurate application timing can directly benefit growers by providing improved risk predictions which could more effectively reduce CLS pressure or limit unnecessary applications resulting in

increased profit margins (Chapter 5; Gadoury and MacHardy 1982; Khan et al. 2007; Vincelli and Lorbeer 1989). More research into the conditions for spore production and dispersal early in the season is important for understanding epidemiology for not only *C. beticola* but also other detrimental agricultural pathogens. Evaluating temporal and spatial spore dynamics for foliar pathogens can help growers predict periods or locations when disease pressure will be high (Gadoury and MacHardy 1982; Vincelli and Lorbeer 1989). For *C. beticola*, exploration into environmental variables, such as solar radiation, cloud cover, wet/dry cycles, vapor pressure deficit, air turbulence, and soil properties, including soil moisture, could improve model accuracy as explored by Huber and Gillespie (1992). Additional data sets and field studies could expand model capabilities.

The research conducted in this dissertation supplies critical additional information on *C. beticola*, a foliar pathogen of sugarbeet and causal agent of CLS. Overall, these studies can be used to improve management of CLS on sugarbeet. Expanding the body of research and knowledge on alternative management, fungicide sensitivity and efficacy, and aerial conidia dynamics is incredibly valuable for the sugar industry, growing operations, chemical companies, and pathology communities. The experiments and concepts of this research can be utilized and tested to benefit the management of other similar pathogens and cropping systems by providing additional control options for sustainable agricultural practices.

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