FITNESS AND MANAGEMENT OF SITE-SPECIFIC FUNGICIDE RESISTANT *CERCOSPORA BETICOLA* ISOLATES FROM SUGAR BEET

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Plant Pathology

November 2020

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Fitness and Management of Site-specific Fungicide Resistant *Cercospora beticola* Isolates from Sugar Beet

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ABSTRACT

Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola*, is one of the most destructive foliar diseases on sugar beet. It severely affects productivity and profitability of the sugar beet industry. CLS is managed by using resistant cultivars, rotating with non-host crops, and applying effective fungicides in a timely manner. In North Dakota and Minnesota, site-specific fungicides, such as quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs), had been widely and extensively applied to control *C. beticola* which has developed resistances to these fungicides. The mycelial growth, spore production, spore germination, and aggressiveness of QoI and/or DMI resistant isolates were compared to sensitive isolates in a laboratory and greenhouse study. Results indicated that the QoI and/or DMI resistant isolates had a relatively slower disease development on sugar beet leaves due to their fitness penalty in sporulation and mycelial growth but still caused high disease severities as sensitive isolates.

Fungicides were evaluated to manage the QoI and/or DMI resistant *C. beticola* isolates in a sensitivity study and a greenhouse and field study. Copper-based multisite activity fungicides were evaluated for controlling fungicide resistant *C. beticola*. The mean EC_{50} values for nine copper-based chemicals ranged from 1 to 10 ug/ml using a spore germination assay. In a greenhouse study, Fertileader (a copper-based fertilizer) caused leaf injury and was not evaluated, but the other tested chemicals provided significantly better control of CLS compared to the control check with Badge X2, Champion, Cuprofix, COCS and Ridomil having significantly small AUDPC. Newer site specific fungicides with different modes of action that have never been used for CLS management in North Dakota and Minnesota were also tested. The mean EC_{50} values were 4.9, 33.1, 99.4, and 481.6 ug/ml using mycelial growth assay and

5.7, 4.1, 9.2, and 4.2 ug/ml using spore germination assay for cyprodinil, fluazinam, pydiflumetofen, and Chlorothalonil, respectively. In a two-year field study, all the fungicides resulted in significantly better disease control, significantly higher beet tonnage, and recovered sucrose compared to the nontreated check. However, none of tested fungicide treatments performed better than the industry's standard fungicide, triphenyltin hydroxide.

ACKNOWLEDGMENTS

First of all, I would like to express my sincere gratitude to my major advisor, Dr. Mohamed Khan, for his considerable patience, guidance, and suggestions during my research years and in the preparation of this dissertation. Also, I extend thanks to my graduate committee members, Drs. Luis del Rio, Shaobin Zhong, and Juan M. Osorno, for their time, helpful suggestions and assistance to my research program, and valuable comments to improve my dissertation. Additionally, I would like to thank all faculty members, staff, and graduate students of the Plant Pathology Department at North Dakota State University for their kindness and support.

Special thanks to my colleague Mr. Peter Hakk for his consistent help in every aspect of my research work, Drs. Luis del Rio and Shaobin Zhong for their tremendous assistance in my laboratory work, Dr. Gary A. Secor and Ms. Viviana Rivera-Varas for providing *Cercospora beticola* isolates and technical help, and Drs. Gongjun Shi and Kishore Chittem for their detailed teachings in statistical data analysis and molecular techniques. I would not have been able to complete this research without the help from these people.

I would like to thank my parents, Mr. Donglai Liu and Mrs. Jiaping Yang, who allowed me to pursue my life's goal with their unconditional support and love. Special thanks to my aunt, Mrs. Donghong Liu, for her practical support and consistent encouragement.

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1. LITERATURE REVIEW

1.1. Sugar beet industry

Sugar, the common name for sucrose, is mainly obtained from sugar cane (*Saccharum officinarum*) and sugar beets (*Beta vulgaris* L.). Sugar cane has been grown in large quantities in tropical and subtropical regions for centuries, while the sugar beet is a relatively new crop developed in the 19th century for cool temperate regions. Sugar beet producing countries are usually located at latitudes between 30 and 60° N, such as Northern Europe, Asia, North America, and North Africa. Currently, sugar beets are grown in 50 countries (Draycott, 2006) and account for 14% of the world's sucrose production (FAOSTAT, 2017).

Sugar beets are classified in the family Chenopodiaceae and can be tracked back to wild sea beets distributed along all the coasts of Europe and Western Asia (De Rougemont, 1989). Sugar beets were cultivated from a fodder beet called the White Silesian Beet after the German chemist Andreas Margraff discovered crystallized sugar in beet juice in 1747. Due to the European Continental Blockade at the beginning of the 19th century, sugar beets became well known as a sugar crop and were rapidly planted to provide an alternative source of sucrose. By the second half of the 19th century, the sugar beet industry had been well established for sugar production with the advancement of cultivation, varieties, and industrial equipment in Europe (Cooke and Scott, 1993). In the United States, the sugar beet industry establishment was marked by the first successful sugar beet processing factory built in Alvarado, California in 1870 (Coons, 1949).

Today, the United States ranks as the world's 4th largest producer of sugar beets, preceded by the Russian Federation, France, and Germany (FAOSTAT, 2017). In 2019, 33 million tonnes of beets were harvested from approximately 450,000 ha from ten major sugar beet

producing states: Michigan, Minnesota, North Dakota, Colorado, Montana, Nebraska, Wyoming, California, Idaho, and Oregon (USDA-ERS, 2019). In North Dakota and Minnesota, the first sugar beet factory was built in 1926 in East Grand Forks. Now, there are seven sugar beet processing factories owned by three different cooperatives: the American Crystal Sugar Company (ACSC), the Minn-Dak Farmer Cooperative (MDFC), and the Southern Minnesota Beet Sugar Cooperative (SMBSC) (Shoptaugh, 1997). In 2019, these two states produced 12.8 million tonnes of sugar beets, contributing 51% to the total U.S. production (USDA-ERS, 2019). In terms of economic impact, the local sugar beet industry generates roughly $4.9 billion in total economic activities (Bangsund et al., 2012).

However, the sugar beet industry has always been challenged by weeds, insects, and diseases damaging the economic value of the sugar beets. In the Red River Valley of North Dakota and Minnesota, several pathogenic diseases restrict the productivity of sugar beet production, such as Rhizoctonia crown and root rot (*Rhizoctonia solani* Kühn), Fusarium yellows (*Fusarium oxysporum* f. sp. *betae* Snyder & Hansen), Fusarium yellowing decline (*Fusarium secorum*), Aphanomyces root rot (*Aphanomyces cochlioides* Drechsler), Rhizomania (beet necrotic yellow vein virus transmitted by *Polymyxa betae* Keskin), and Cercospora leaf spot (*Cercospora beticola* Saccardo).

1.2. Cercospora leaf spot

Cercospora leaf spot (CLS) is considered the most important sugar beet foliar disease due to its destructiveness and wide distribution around the world (Jacobsen and Franc, 2009; Skaracis et al., 2010; Weiland and Koch, 2004; Wolf and Verreet, 2002). The causal agent of CLS is a fungal pathogen *Cercospora beticola* that is assumed to originate from central Europe and Mediterranean (Groenewald et al., 2005). In a survey on the distribution and severity of CLS

conducted by Holtschulte (2000), the disease occurs with varying incidences in more than a third of the global sugar beet production areas and is particularly damaging in Greece, northern Italy, northern Spain, Austria, Japan, China, and parts of the United States. The leaf death caused by CLS reduces photo-synthetic capacity of sugar beets, followed by a continual regrowth of new leaves at the expense of stored reserves in the root, leading to reduced tonnage, reduced sucrose, and increased impurities (Shane and Teng, 1992). Yield losses can be over 50% if weather conditions are favorable to the disease (Jacobsen and Franc, 2009; Rossi et al., 2000b). More importantly, higher impurities resulting from CLS severely impact the profitability of sugar beet industry (Shoptauph, 1997; Smith and Martin, 1978). A 30% loss of recoverable sucrose is common under moderate disease pressure, and the pile storability is also compromised in the roots of CLS-affected sugar beets compared to the healthy ones (Lamey et al., 1996). In 1998, an outbreak of CLS in sugar beet fields in North Dakota and Minnesota caused estimated losses of $45 million to the American Crystal Sugar Company (Ellington et al., 2001). In 2016, CLS was considered the most important problem by growers, with an estimated loss of over $100 million due to an outbreak in these two states (Khan, 2018).

1.2.1. Symptoms

A single leaf spot caused by CLS is delimited, circular, and 2-5 mm in diameter when mature with a tan to light brown lesion center and dark-brown or reddish-purple margins (Asher and Hanson, 2006). The petioles and crowns of sugar beets are also affected (Giannopolitis, 1978), and possible infection might occur on sugar beet roots (Vereijssen et al., 2004). As the disease progresses, individual spots coalesce into large necrotic areas and eventually the entire leaf becomes necrotic. Furthermore, under humid, warm weather, pesudostromata produce conidiophore and conidia, giving the leaf spot a gray to steel-blue fuzzy appearance (Asher and

Hanson, 2006). An important diagnostic feature for CLS is that tiny black dots (pseudostromata) can be observed within the grayish-tan lesions with a hand lens (Windels et al., 2003).

1.2.2. The fungus *Cercospora beticola*

Cercospora beticola was first described as a species of the genus *Cercospora* by Saccardo (1876) in Italy and further characterized as a fungal pathogen causing leaf spot disease of sugar beet by Chupp (1953). *C. beticola* has a broad host range, including most species of the genus *Beta* as well as a number of species within different genera of *Chenopodiaceae*, *Amaranthus, Atriplex*, *Chenopodium*, *Cycloloma*, *Plantago* (Fransden, 1955), *Carthamus* (Lartey et al., 2005), *Malva*, *Spinacta*, *Limonium*, and *Apium* (Groenewald et al., 2006a).

C. beticola is a hemi-biotrophic fungus classified into the phylum Ascomycota, class Dothideomycetes, order Capnodiales, and family Mycosphaerellaceae. Hyphae are septate, hyaline to pale olivaceous brown, and vary from 2 to 4 um in diameter. For asexual stage, conidia are produced holoblastically from specialized stalks called conidiophores and seceded by the schizolytic process (Kirk et al., 1982). Conidiophores are unbranched, brown, moderately thick-walled, and typically measure 3-5.5 × 40-60 um with conspicuous conidial scars at geniculations and at the apex. Conidia are hyaline, filiform to acicular, straight to mildly curved with 3-28 septate, and typically 2-5 × 27-250 um (Geoenewald et al., 2005; Jacobsen and Franc, 2009). *C. beticola* is considered to be a heterothallic fungus, but a teleomorph is yet to be described. It has a bipolar mating system controlled by two alternate mating-type genes (*MAT 1-1-1* and *MAT 1-2*) (Groenewald et al., 2006b). Melton et al. (2012) reported that *C. beticola* isolates from sugar beet fields in the Red River Valley region of Minnesota and North Dakota were tested and found to have an equal distribution of these mating type genes, indicating that *C. beticola* has a potential sexual cycle in sugar beet fields.

Generally, the environmental conditions in the favor of disease development of *C. beticola* are daytime temperatures of 25 to 35°C, night temperatures above 16°C, and prolonged period of high humidity (RH) of 90-95% or free moisture on leaves (Lamey et al., 1996; McKay and Pool, 1918; Pool and McKay, 1916). Sporulation occurs between 10 and 35°C at a high RH (98–100%), with 30°C as an optimal temperature (Bleiholder and Weltzien, 1972). Conidia production is readily available from 15 to 23°C and relative humidity (RH) greater than 60%, but they are not produced at temperatures less than 10°C (Pool and McKay, 1916).

1.2.3. Disease cycle and infection process

CLS is a polycyclic disease caused by *C. beticola* capable of multiple disease cycles within one sugar beet growing season. *C. beticola* overwinters as pseudostromata in infected leaf residues on or directly below the soil surface (Khan et al., 2008). These pseudostromata can survive in the soil for 1-2 years (McKay and Pool, 1918; Nagel, 1938) and serve as an important primary inoculum source, especially in sugar beet fields without crop rotation. Other secondary sources are weed hosts (Vestal, 1933) and sugar beet seeds (McKay and Pool, 1918). Symptoms appear as necrotic lesions from 5 to 21 days after infection depending on the weather conditions (Windels et al., 1998). Then, numerous conidia are produced from necrotic lesions on infected leaves to cause secondary infections for another disease cycle. Conidia are locally disseminated by rain, rain-splash (Carlson, 1967; Pool and McKay, 1916), wind, irrigation water, insects, and mites (McKay and Pool, 1918). Wind has been considered the primary agent for dispersal of *C. beticola* conidia (Lawrence and Meredith, 1970). Completion of one disease cycle typically takes 12 days depending on field conditions (Rossi et al., 2000a; Weiland and Koch, 2004).

The initial infection process begins with *C. beticola* conidia landing on the leave surface. Conidia can germinate and then penetrate through stomata under these favorable conditions.

Then the fungal hyphae grow intercellularly into the apoplast and are present in the intercellular spaces, sometimes appressed or attached to host cell walls (Steinkamp et al., 1979). This intercellular phase is followed by an intracellular phase. However, it is still in debate whether *C. beticola* is an intercellular or intracellular fungus. Daub and Ehrenshaft (2000) hypothesized that more nutrients from damaged cell membranes are available in the leaf intercellular space for fungal growth and sporulation. On the other hand, the intracellular phase has been considered an essential part of disease development associated with cell death (Steinkamp et al., 1979).

During the infection process, multiple effectors secreted by the invading hyphae plays an important role in disease establishment and development (Ebert, 2018). Cercosporin and beticolin are well-known toxins produced from *C. beticola*. Cercosporin is a perylenequinone photo-sensitizing toxin with a non-host-specific toxicity (Balis and Payne, 1971; Yamazaki and Ogawa, 1972). Under light, this toxin can generate activated oxygen species such as singlet oxygen and superoxide, resulting in oxidative damages to lipids, proteins, and nucleic acids, eventually leading to cell death (Daub and Chung, 2007). Beticolins are a family of 20 nonpeptidic compounds. They are non-host-specific toxins causing a broad range of cytotoxic effects, such as ATP inhibition (Blein et al., 1988) and membrane depolarization (Gapillout et al., 1996).

1.3. Management of Cercospora leaf spot

In North Dakota and Minnesota, applying effective fungicides in a timely manner is the most important approach used by growers to control CLS in sugar beet fields. However, fungicides are no longer effective in the fields after *C. beticola* develops resistance, so an integrated strategy is recommended to manage CLS based on cultural practices, resistant

cultivars, and timely chemical applications (Lamey et al., 1996; Miller et al., 1994, Secor et al., 2010).

1.3.1. Cultural practices

C. beticola overwinters in infected leaf debris that serves as primary inoculums to initiate a new disease cycle in a new growing season. To reduce overwintering inoculum, cultural practices such as crop rotation and cultivation are recommended for growers. Crop rotation with non-host crops, such as small grains, corn, and beans, for 2 to 3 years significantly reduces the quantity of *C. beticola* inoculum in the field soils (Jacobsen and Franc, 2009; Nagel, 1938). It is also suggested that new sugar beet fields be separated from fields with *C. beticola*-infected leaf debris at a minimum distance of at least 100 yards in order to prevent early-season infections (McKay and Pool, 1918). Fall cultivation facilitates the degradation of crop residues by incorporation of leaf debris into the soils, reducing the survival period of *C. beticola* (Khan et al., 2008).

1.3.2. Resistant cultivars

Sugar beet is a relatively new crop, with a limited diversity of known disease resistance genes in the germplasm. CLS resistance was introgressed into sugar beets by backcrossing sugar beets with wild sea beets, *B. Vulgaris* spp. *Maritima* (Coon et al., 1955). The resultant off-springs have become breeding materials that are distributed around the world (Skaracis and Biancardi, 2000). CLS resistance in sugar beets is a quantitative trait and is assumed to be controlled by at least 4 to 5 major resistance genes (Smith and Gaskill, 1970). Quantitative Trait Locus (QTL) associated with the resistance against *C. beticola* were identified on all sugar beet chromosomes and exhibited partially dominant to additive gene action (Koch and Jung, 2000; Nilsson et al., 1999; Schäfer-Pregl et al., 1999; Setiawan et al., 2000). Recently,

research on the precise mapping of resistant QTLs for *C. beticola* in sugar beets found that four QTLs affect CLS resistance differently: the *qcr1* and the *qcr4* promoted resistance, while the *qcr2* and the *qcr3* appeared to confer susceptibility (Taguchi et al., 2011).

However, the resistant sugar beet cultivars are not immune to CLS because these cultivars can still be infected with CLS if climate conditions are favorable. Nevertheless, the use of resistance cultivars can help lower the infection rate or delay the infection process of *C. beticola* compared to susceptible cultivars (Rossi et al., 2000a), thus extending the time window for effective fungicides to be applied before infection occurs. Still, developing resistant cultivars that also have high sucrose yields is challenging (Saito, 1966). In the countries where disease severity is high, the effective control of CLS can only be achieved by combining planting resistant cultivars and timely fungicide applications together (Mechelke, 2000).

In North Dakota and Minnesota, only cultivars approved by sugar beet cooperatives can be planted by sugar beet growers. Cultivars are approved based on the average KWS rating for three years in field trials where the cultivars are evaluated annually for their response to CLS using the Klein Wanzlebener Saatzucht (KWS) rating scale from 1 to 9 (Jones and Windels, 1991). The sugar cooperatives ACSC and the MDFC approved cultivars with KWS ratings of 5.2 or less, while the SMBSC required more resistant cultivars with KWS ratings of 5.0 or less (Secor et al., 2010). Currently, CLS has become the most important problem in our growing area, so planting resistant cultivars is an indispensable part of integrating management strategies, which allows a wider application window for fungicide applications.

1.3.3. Fungicide use

Applying effective fungicides is the most common and efficient approach for controlling CLS. In North Dakota and Minnesota, several fungicides have been widely used by growers

since the1970s. According to the Fungicide Resistance Action Committee (FRAC), these fungicides are classified based on their mode of action (MOA) with a specific code: thiophanate-methyl [Methyl Benzimidazole Carbamates; FRAC group 1], tri-phenyltin hydroxide [Organo tin compounds; FRAC group 30], tetraconazole [DeMethylation Inhibitors (DMIs); FRAC group 3], pyraclostrobin [Quinone outside Inhibitors (QoIs); FRAC group 11], trifloxystrobin [QoIs; FRAC group 11], difenoconazole [DMIs; FRAC group 3], and prothioconazole [DMIs; FRAC group 3] (FRAC, 2020; Khan, 2018; Secor et al., 2010). These fungicides should be used individually in rotation programs at full labeled rates or in mixtures at 75-80% of the full labeled rates to prevent or delay the development of resistant populations of *C. beticola* (Muller et al., 2013).

However, fungicides must be applied in a timely manner because they are not effective after symptoms appear (Khan et al., 2007). On one hand, the first fungicide application should be scheduled at disease onset or when field conditions favor CLS infections, usually in late June or early July after row closure in North Dakota and Minnesota. On the other hand, subsequent applications need to be scheduled between July and mid-September based on the fixed-schedule or prediction model. A typical interval schedule is 14 days for most fungicides but can be reduced to 10-12 days if rainfall events prevail. A Cercospora leaf spot prediction model, developed by Shane and Teng (1983), can help local sugar beet growers to determine when to apply depending on careful field monitoring and the Cercospora Advisory System, which is based on field environmental conditions. Moreover, the number of fungicide applications needed to control CLS varies due to climatic conditions and disease pressure. Of the three sugar cooperatives, the most southernly SMBSC, with its warmer, wetter conditions, usually has the most severe CLS. Centrally located MDFC typically have moderate to high disease severity, and

the most northernly located ACSC usually records the least severe CLS. For example, in 2016, growers at the SMBSC averaged 6 fungicide applications, growers at the MDFC averaged 5 applications, and growers at the ACSC averaged 3 applications (Hakk et al., 2016).

1.3.4. Fungicide resistance

The most challenging aspect in the management of CLS is that the causal agent *C. beticola* can develop resistance against one or more fungicides, leading to fungicide failure in fields with a disease epidemic. *C. beticola* can produce a large amount of conidia during multiple disease cycles resulting in potential for mutation to resistance (FRAC, 2019). Additionally, infrequent, possible sexual cycle for *C. beticola* might contribute to the resistance against fungicides (Bolton et al., 2012b). Moreover, fungicides used to control CLS, such as benzimidazole, DMIs, and QoIs, are single-site MOA fungicides with a relatively higher risk for targeted pathogens to develop fungicide resistance compared to the multiple-site MOA (FRAC, 2019; McGrath, 2004). Furthermore, the repeated usage of the same MOA fungicides without effective alternating partners will also promote the emergence of fungicide resistance. In the past, *C. beticola* has developed resistance to several fungicide groups, such as thiophanate-methyl (Briere et al., 2001; Campbell et al., 1998, Georgopoulos and Dovas, 1973), tri-phenyltin hydroxide (Briere et al., 2001; Bugbee, 1996; Campbell et al., 1998), DMIs (Karaoglanidis et al., 2002, 2003; Secor et al., 2010), and QoIs (Bolton et al., 2013; Kirk et al., 2012). The sugar beet industry in North Dakota and Minnesota has witnessed three CLS epidemics in 1981, 1998, and 2016, all due to fungicide failures of benzimidazole, tri-phenyltin, and QoIs, respectively (Bugbee, 1981; Secor et al., 2010; Khan et al., 2018).

To manage fungicide resistance in the *C. beticola* population, the fungicidal sensitivity of *C. beticola* isolates from sugar beet production areas has been determined on a regular basis.

Each year in North Dakota and Minnesota, *C. beticola* field isolates are recovered from sugar beet leaves with CLS symptoms collected from the seven factory districts and tested for their sensitivity to one or more commonly used fungicides. For thiophanate-methyl and tri-phenyltin hydroxide, sensitivity is dependent on how many spores can germinate on the amended water agar (WA) with a discriminate dose of 5 ug/ml for thiophanate-methyl and 1 ug/ml for tri-phenyltin hydroxide (Secor and Rivera, 2012). For DMIs, sensitivity is determined by a radial growth procedure on a clarified V8 medium (CV8) amended with serial 10-fold dilutions of DMIs from 0.001 to 1.0 ug/ml (Secor and Rivera, 2012). For QoIs, a similar method by calculating the spore germination can be used, but a real-time PCR testing G143A point mutation of *C. beticola* isolates has been more widely used to determine resistance to QoIs (Birla et al., 2012).

1.4. Research justification

Sugar beet growers in North Dakota and Minnesota have relied on QoIs and DMIs to control CLS for more than ten years (Luecke and Dexter, 2003). However, both QoIs and DMIs are single-site fungicides with a high risk of selecting resistant pathogen strains, especially when applied in a repeated manner (McGrath, 2004). QoI-resistant *C. beticola* isolates are associated with a point mutation (G143A) in cytochrome b complex (Bolton et al., 2013), while DMI-resistant isolates are associated with the over-expression of the C-14 alpha-demethylase gene (*CYP51*) (Nikou et al., 2009). In 2016, there was a CLS epidemic in North Dakota, Minnesota and Michigan, which initially resulted from field failures of QoI fungicides. In 2017, the incidence of field isolates of *C. beticola* with G143A mutations remained the same as 2016, and isolates showed an increased resistance to DMIs (Secor et al., 2017). The usage of currently used fungicides (described in 3.3. Fungicide Use) for managing the existing QoI- and DMI-resistant

C. beticola population is limited because *C. beticola* has a history of having developed multiple fungicide resistances against these fungicides.

Chlorothalonil [chloronitriles; FRAC group M05] was one of the most widely used multiple-site fungicides that has been used in different patho-systems (Bounds and Hausbeck, 2007; FRAC, 2020; Holm et al., 2003). In 2017, the US Environmental Protection Agency (EPA) denied a special emergency use label by the sugar beet industry for the use of chlorothalonil to help manage *C. beticola* with resistance to different fungicide chemistries. Chlorothalonil was under review for re-registration and there was some concern about safety to humans when inhaled. One chemical company (Sipcam) is interested in labeling chlorothalonil for use on sugar beet. The efficacy of chlorothalonil on *C. beticola* needs to be evaluated so this product can become available to growers.

Moreover, EPA suggested that a request of newer, site specific fungicides that are considered safer will probably be viewed more favorably for an emergency use. As such, newer fungicides with different MOAs and that are deemed safer for people, wildlife and the environment need to be tested for their efficacy at controlling *C. beticola* resistant to several fungicides. Fungicides with some potential of controlling *C. beticola* on sugar beets included fluazinam [uncouplers of oxidative phosphorylation; FRAC group 29], cyprodinil [anilino-pyrimidines; FRAC group D01], and pydiflumetofen [Succinate-dehydrogenase inhibitors (SDHIs); FRAC group C2] (FRAC, 2020; Matheron and Porchas, 2004; McManus et al., 2007; Neves and Bradley, 2019; Sun et al., 2011).

In 2017, copper fungicides, registered for use in the USA, which did not provide control against sensitive populations of *C. beticola* (prior to 2016) was providing some level of control against the pathogen compared to the non-treated check and other fungicides such as Headline

(pyraclostrobin) that was once very effective (M.F.R. Khan, personal communication). There were several copper products on the market with different active ingredients including copper hydroxide, copper oxychloride, copper pentahydrate and basic copper sulfate. Products may have one or two forms of copper. It will be useful for growers to know the level of efficacy provided by the different copper products. In some countries in Europe, including the United Kingdom (U.K.), copper is not approved for use as a fungicide on sugar beet. However, some forms of copper can be used as a fertilizer. As such, it will be useful to know whether Fertileader® (soluble copper nitrate, 11.9% a.i.; Timac Agro) available as a fertilizer, provides control of *C. beticola*.

The fungal strains with a mutation conferring fungicide resistance may result in a lower fitness than the sensitive strains in the absence of the fungicide. Knowledge of fitness costs associated with fungicide resistance is useful to optimize the effectiveness and durability of fungicide management strategies (Mikaberidze and McDonald, 2015). The fitness of *C. beticola* strains seems to be negatively impacted by QoI or DMI resistance. A fitness study, conducted by Malandrakis et al. (2006), showed that QoI-resistant *C. beticola* isolates had a significant reduction in sporulation and pathogenicity compared to wild-type parental isolates. Also, DMI-resistant fungal strains are less fit than sensitive strains in virulence, spore production, and mycelium radial growth (Karaoglanidis et al., 2001; Moretti et al., 2003; Nikou et al., 2009), but similar in spore germination, incubation period, and germ tube length (Karaoglanidis et al., 2001; Moretti et al., 2003), and disease severity (Bolton et al., 2012a). No research has been published to determine the fitness of *C. beticola* resistant to both QoI and DMI fungicides.

1.5. Research objectives

This research was conducted to gain a better understanding of the biology of fungicide resistant *C. beticola* isolates from sugar beet fields and identify more effective fungicides with different MOAs for controlling this pathogen. The objectives of this research were

(i) To determine the fitness of *C. beticola* isolates resistant to site-specific fungicides;

(ii) To evaluate the sensitivity of site-specific resistant *C. beticola* isolates to copper-based chemicals and their efficacies at controlling CLS in a greenhouse study;

(iii) To evaluate the sensitivity of site-specific resistant *C. beticola* isolates to cyprodinil, fluazinam, pydiflumetofen, and chlorothalonil as well as their efficacies at controlling CLS in a field study.

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2. FITNESS TRAITS OF *CERCOSPORA BETICOLA* FIELD ISOLATES RESISTANT TO QOI, DMI (SITE-SPECIFIC FUNGICIDES) FUNGICIDES

2.1. Introduction

In North Dakota and Minnesota, sugar beet (*Beta vulgaris* L.) is an economically important crop but its sustainability is threatened by the foliar disease Cercospora leaf spot (CLS) (Bangsund et al., 2012; Weiland and Koch, 2004). Successful management of CLS is usually achieved by multiple fungicide applications from July through September during the peak growing season to protect the sugar beet crop (Secor et al., 2010). Sugar beet producers are advised to apply effective fungicides in a timely manner based on scouting, calendar-based schedule, and forecasting models (Shane and Teng, 1983; Windels et al., 1998). Fungicides approved for use on sugar beet crop belong to different chemical groups with a specific code assigned by Fungicide Resistance Action Committee (FRAC): thiophanate-methyl [Methyl Benzimidazole Carbamates; FRAC group 1], tri-phenyltin hydroxide [Organo tin compounds; FRAC group 30], tetraconazole [DeMethylation Inhibitors (DMIs); FRAC group 3], pyraclostrobin [Quinone outside Inhibitors (QoIs); FRAC group 11], trifloxystrobin [QoIs; FRAC group 11], difenoconazole [DMIs; FRAC group 3], and prothioconazole [DMIs; FRAC group 3] (FRAC, 2020; Secor et al., 2010).

The most widely and extensively used fungicides for CLS in North Dakota and Minnesota were QoIs and DMIs (Luecke and Dexter, 2003). Both QoI and DMI fungicides are specific inhibitors with a medium to high inherent risk for selecting resistant isolates of targeted fungal pathogen population (Mcgrath, 2004; FRAC, 2019). Moreover, CLS is a polycyclic disease, and the causal fungal pathogen *Cercospora beticola* can have multiple infection cycles to produce plenty of asexual conidia as well as possible sexual recombination (Bolton et al.,

2012b), resulting in the mutation potential of this pathogen for resistance development. The resistance to QoI fungicides in *C. beticola* isolates was reported from a field failure in Michigan due to the substitution of glycine with alanine at position 143 in the cytochrome b (*cytb*) gene (Bolton et al., 2013; Kirk et al., 2012). Reduced sensitivity to DMI fungicides in *C. beticola* isolates is associated with overexpression of the cytochrome P450 sterol C-14 alpha-demethylase (*cyp51*) gene (Bolton et al., 2012a).

The fitness of resistant strains in a population of fungi plays a crucial role in the development and stability of fungicide resistance (Cox et al., 2007; Dekker, 1976). In general, the fitness of fungal plant pathogens is the ability to compete with other strains and to survive under environmental conditions. Specifically, the fitness can be measured as several components during a pathogen's life cycle including spore production, spore dispersal, aggressiveness, mycelial growth, spore germination, and the ability to overwinter for long-term survival (Mikaberidze and McDonald, 2015). Fungicide resistance may provide a selective advantage for the resistant isolates compared to sensitive isolates but resistance-conferring mutations may affect negatively important physiological and biochemical processes (Anderson, 2005). Plant pathologists study fitness costs of fungal pathogens in mycelial growth, spore productivity, and germination ability in laboratory (in vitro) as well as pathogenicity and aggressiveness in greenhouse experiments (in vivo) (Antonovics and Alexander 1989; Mikaberidze and McDonald, 2015). The measurement of fitness costs in resistant fungal population will help us predict the resistance stability in order to determine whether growers can expect to reuse these fungicides after their withdrawal for years. Theoretically, this information can also help figure out the optimal proportion of high- and low- risk fungicides in the mixture (Mikaberidze and McDonald, 2015).

The results of an annual monitoring program sponsored by sugar beet growers in North Dakota and Minnesota indicated hat the sensitivity to both QoI and DMI fungicides of *C. beticola* isolates decreased over years (Secor et al., 2017). Furthermore, the insensitivity to QoI fungicides resulted in a CLS control failure in sugar beet fields, leading to an epidemic in 2016 (Khan, 2018). The objective of this research was to determine the fitness cost of *C. beticola* isolates resistant to QoIs, DMIs, and both QoI and DMI fungicides compared to isolates sensitive to both QoI and DMI fungicides.

2.2. Materials and methods

2.2.1. Selecting *C. beticola* isolates

We used 16 *C. beticola* isolates in this study (Appendix A.). Out of these, 4 QoI-resistant, 4 DMI-resistant, and 4 with both QoI and DMI resistance were recovered from sugar beet fields in Minnesota in 2017; and 4 isolates that were sensitive to both QoI and DMI (obtained from Dr. Gary Secor, North Dakota State University, Fargo, ND) were collected in 1998 and 2016 from sugar beet infected leaf samples submitted from growers in North Dakota and Minnesota. The recovered *C. beticola* isolates were tested for their sensitivity to 1) DMIs (difenoconazole, prothioconazole, and tetraconazole) using a radial growth procedure (Secor and Rivera, 2012) and 2) QoIs using a PCR-based molecular procedure to test the presence of G143A conferring the QoI resistance (Bolton et al., 2013). These isolates were grown on CV-8 media (15g Agar, 100 ml V8 juice, and 900 ml dH_2O) and tested for their sensitivities to QoIs and DMIs following the methodology described by Secor and Rivera (2012) and Bolton (2013). This research evaluated the fitness cost of each isolate group as mycelial growth, conidial production, and spore germination in laboratory study (in vitro) and as aggressiveness (amount of disease

caused by each isolate group) in greenhouse study (in vivo). Details of the tested isolates are given in Table 2.1.

2.2.2. In vitro fitness of *C. beticola* isolates with different fungicide resistance

The 16 *C. beticola* isolates were assessed for mycelial growth in vitro. Under laminar flow (Air science; Fort Myers, FL), agar plugs (5 mm in the diameter) were cut using a sterile scalpel from the leading edge of growth in 14-day-old *C. beticola* cultures and then inverted onto 100 × 15 mm petri dishes containing CV-8 media. The inoculated plates were incubated in the dark at room temperature (22 ± 2°C). After 14 days, when mycelial growth covered 2/3 of the petri dish, two perpendicular measurements of mycelial growth for each isolate were measured using a digital ruler. This trial was conducted twice each with three replicates.

The same 16 *C. beticola* isolates were evaluated for conidial production. First, sporulation plates were made by adding sterile distill water onto 14-day-old isolates, scraping the mycelia from the isolate surface, transferring the supernatant onto petri dishes of CV-8 media, and incubating under continuous fluorescent light at 22 ± 2°C (Secor and Rivera, 2012). After 3 to 4 days of incubation, conidial spores were abundantly induced. Five ml sterile distill water was added to the plates and a sterile glass rod was used to dislodge conidia from each sporulation plate. A 100 µl aliquot of the conidia suspension was applied to a hemocytometer (Hausser Scientific; Horsham, PA, USA), and the conidial concentration was counted using a compound microscope at × 400 magnification. Each isolate has three replicates and the experiment was conducted twice.

Conidial germination was determined for each of the 16 *C. beticola* isolates. For each isolate, the conidial concentration from a sporulation plate was determined using a hemocytometer as described above and then adjusted to 1×10^4 conidia/ml by adding sterile

distill water. A 120 ul aliquot of the conidia suspension was added onto 1.5% water agar media (15g Agar and 1000 ml dH_2O). The inoculated plates were incubated at 22 ± 2°C in the dark for 16 h to allow conidial germination. Then, 50 conidia per plate were examined for germination (considered when the germ tube length was at least twice as long as the conidium) under a dissecting microscope at × 50 magnification. In this trial, the percentage of germinated conidia for each isolate was calculated with three replicates in two repeats.

2.2.3. In vivo fitness of *C. beticola* isolates with different fungicide resistance

This research was conducted in a greenhouse facility (Agricultural Experiment Station at North Dakota State University; Fargo, ND), where environmental conditions were set with a 16-h photoperiod and at 22 ± 2 °C (Argus Control Systems Ltd.; British Columbia, Canada). A CLS-susceptible sugar beet cultivar (Niehaus and Moomjian, 2019), MA 504, was used, and three seeds were planted in each pot (10 × 10 × 12 cm) filled with peat mix (Sunshine mix 1, Sun Gro Horticulture Ltd.; Alberta, Canada). Each pot was watered as needed, thinned to have one vigorous seedling, and grown to be used at the 6-leaf stage.

The aggressiveness of the 16 *C. beticola* isolates previously used in in vitro experiments was evaluated under greenhouse conditions. The *C. beticola* isolates were separated into four groups according to their fungicide resistance status. Conidial suspension was made from the same group using a sterile glass rod to free conidia from each sporulation plate into sterile distill water. The conidial suspension was then mixed from the isolates of the same resistant group. The number of conidial spores was counted and adjusted to 4×10^4 conidia/ml using a hemocytometer. Inoculation was conducted using a Preval paint-spray gun (Preval Sprayer Division, Precision Valve Corporation, Yonkers, NY) to spray the conidial suspension onto five fully expanded leaves of each plant at the 6-leaf stage. On the same day, the plants inoculated

with the *C. beticola* isolates of the same resistance group were transferred together into confined humidity chambers (100 × 90 × 140 cm) to prevent interference from the other resistant groups. All the inoculated plants were incubated at 95 to 100% relative humidity with a 16-h photoperiod and average temperature of 28 ± 2°C. At 7 days after inoculation (DAI), the plants were moved back into the greenhouse and watered as needed at the base of each plant. CLS disease severity was evaluated visually at 7, 14, and 21 DAI by estimating the number of leaf spots on the five inoculated leaves (five subsamples). A disease scale from 1 to 10 was used as follows: 1 = 1-5 spots/leaf; 2 = 6-12 spots/leaf; 3 = 13-25 spots/leaf; 4 = 26-50 spots/leaf; 5 = 51-75 spots/leaf; 6 = 76-99 spots/leaf; 7 = 100-124 spots/leaf; 8 = 125-149 spots/leaf; 9 = 150-200 spots/leaf; and 10 = >200 spots/leaf (Jones and Windels, 1991). The experiment was conducted twice as a CRD with four replicates each.

2.2.4. Data analysis

In the in vitro study, the Levene's test was conducted to test the homogeneity of variance across the two data repeats of mycelial growth, spore production, and spore germination before they were combined for analysis of variance. For each fitness parameter, an analysis of variance (ANOVA) was performed among *C. beticola* isolates of four fungicide resistance status. The fitness parameters for the isolates with different fungicide resistance status were separated by the post hoc test of Fisher's Least Significant Difference (LSD) at $P = 0.05$. The calculation process was achieved using the general linear model procedure (Proc GLM) in the Statistical Analysis System (Version 9.3, SAS Institute Inc.; Cary, NC, USA).

In the in vivo study, the disease severity caused by *C. beticola* isolates of each fungicide resistant status was evaluated using the disease scale from 1 to 10 on the inoculated leaves at 7, 14, and 21 DAI. The disease rating data were analyzed by a non-parametric analysis using SAS

procedures of Proc Rank and Proc Mixed with LSMEANS. The relative effect of disease severity for each fungicide resistance status with its confidence interval was calculated using LD-CI macro in SAS (Shah and Madden, 2004). The area under the disease progress curve (AUDPC) was calculated using the SAS general linear models (Proc GLM) procedure, following the formula:

$$\text{AUDPC}= \sum_{i=1}^{n-1}[(y_i + y_{i+1})/2](t_{i+1} - t_i)$$

where y_i= disease severity at the ith observation, t_i= time (days) at the ith observation, and n = total number of observations. The homogeneity for variances between the two data repeats of AUDPC was tested via Levene's test before combining the data for an analysis of variance. Analysis of variance (ANONA) of AUDPC resulted from resistant status isolates was performed, the means of treatments were separated by Fisher's Least Significant Difference (LSD) calculated at P= 0.05 using the SAS general linear models (Proc GLM) procedure.

2.3. Results

2.3.1. In vitro fitness of *C. beticola* isolates with different fungicide resistance

The Levene's test was conducted for the homogeneity of variances, indicating that there were no significant differences between the two data repeats of mycelial growth (P = 0.19), spore production (P = 0.76), and spore germination (P = 0.30) for *C. beticola* isolates. Therefore, a combined analysis was performed on the two repeats of these datasets.

In the in vitro study, the fitness traits were evaluated as mycelial growth, spore germination, and spore production of *C. beticola* isolates with four fungicides resistance status: (1) both QoI- and DMI-sensitive, (2) QoI-resistant, (3) DMI-resistant, and (4) both QoI- and DMI-resistance isolates (Table 1). Two significant separations in mycelial growth between fungicide resistance groups were observed. The sensitive isolates had the most mycelial growth

but did not significantly differ from the QoI-resistant isolates. The isolates resistant to both QoI and DMI were similar to DMI-resistant isolates, sharing a significantly lower mycelial growth compared to the other two isolate groups. For spore production, the sensitive isolates produced the highest number of conida estimated at 48,000 spore/ml under microscopic views on a hemocytometer. Under the same conditions, the other isolate groups with QoI or/and DMI resistance had a range of counted spores between 14,000 and 19,000 spore/ml, which was significantly lower compared to the sensitive isolates. For spore germination, all the isolates had the germination percentage up to 98% without significant differences among different resistance groups.

2.3.2. In vivo fitness of *C. beticola* isolates with different fungicide resistance

The homogeneity of variance was tested with Levene's test between two repeats of AUDPC. No significant differences were found (P =0.81) so that the data repeats of AUDPC was combined for analysis of variance.

In the in vivo study, disease severities of CLS within each of *C. beticola* isolates of four fungicide resistant status were evaluated at 7, 14, and 21 DAI to assess development of CLS by calculating AUDPC (Table 2.2; Fig. 2.1). An increase in the CLS severity between 7 DAI and 14 DAI was seen much greater than that between 14 DAI and 21 DAI. On 7 DAI, the isolates resistant to DMI fungicides caused a significantly lower CLS severity compared to other isolate groups, therefore resulting in a lowest value of AUDPC. The sensitive isolates and the both QoI- and DMI- resistant isolates did not differ significantly and had the highest AUDPC values, followed by the QoI-resistant isolates with intermediate AUDPC values. On 21 DAI, all the isolate groups with different fungicide resistant status resulted in a high disease severity of CLS (see Figure 2.2).

Table 2.1: Mycelial growth, spore germination, and spore production of *Cercospora beticola* isolates in different fungicide resistace groups (in vitro).

***C. beticola* isolates**[a]	**Radial growth**[b] **(mm)**	**Spore production**[b] **(10^3 Spores/ml)**	**Spore germination**[b] **(%)**
QoI- and DMI-sensitive	63.9 a	68.6 a	98.8 a
QoI-resistant	63.8 a	26.4 b	98.6 a
DMI-resistant	60.7 b	32.5 b	98.8 a
QoI- and DMI-resistant	58.9 b	33.5 b	98.7 a
LSD (*P*=0.05)	1.8	7.8	0.5

[a]Four isolates were selected for each of four fungicide resistance groups and a total of 16 *C. beticola* isolates were evaluated in this study.
[b]Means of the treatment with the same letter were not significantly different at $P = 0.05$ using the post hoc test of Fisher's Least Significant Difference (LSD).

Table 2.2: Evaluation of disease severity and aggressiveness (areas under disease progress curve [AUDPC]) caused by *Cercospora beticola* isolates in different fungicide resistace groups in greenhouse study (in vivo).

	CLS Ratings (disease scale from 1 to 10)[b]			
***C. beticola* isolates**[a]	**7 DAI**[c]	**14 DAI**[c]	**21 DAI**[c]	**AUDPC**
QoI- and DMI-sensitive	4.8 a	8.7 a	9.1 a	123 a
QoI-resistant	4.1 ab	7.9 a	9 a	113 bc
DMI-resistant	3 b	8.1 a	8.5 a	107 c
QoI- and DMI-resistant	4.9 a	8 a	9.1 a	120 ab
LSD (*P*=0.05)[d]				10

[a]Four isolates were selected for each of four fungicide resistance groups to make a total of 16 *C. beticola* isolates that were evaluated in this study.
[b]A disease scale from 1 to 10 for CLS evaluation was used: 1 = 1-5 spots/leaf; 2 = 6-12 spots/leaf; 3 = 13-25 spots/leaf; 4 = 26-50 spots/leaf; 5 = 51-75 spots/leaf; 6 = 76-99 spots/leaf; 7 = 100-124 spots/leaf; 8 = 125-149 spots/leaf; 9 = 150-200 spots/leaf; and 10 = >200 spots/leaf.
[c]Means of disease scale between treatments with the same letter were not significantly different at $P = 0.05$ using a non-parametric analysis.
[d]Fisher's Least Significant Difference (LSD) was calculated at $P = 0.05$.

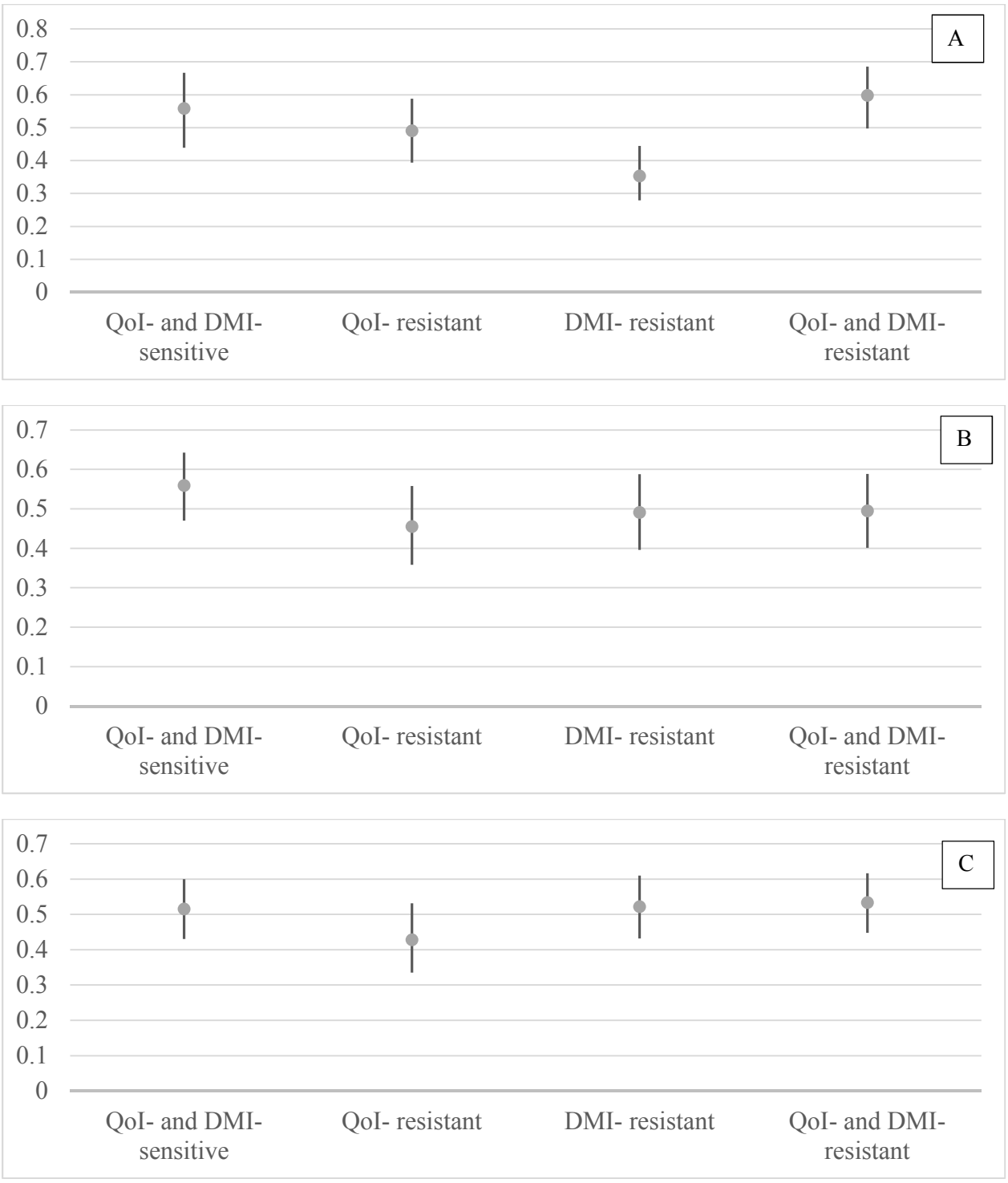

Figure 2.1: Relative effects of disease severity caused by *C. beticola* isolates in different fungicide resistace groups under the greenhouse conditions (in vivo). A: at 7 days after inoculation (DAI); B: at 14 DAI; C: at 21 DAI. For each fungicide resistant group of *C. beticola* isolates, the relative effect of the disease severity with 95% confidence intervals was calculated using a non-parametric analysis.

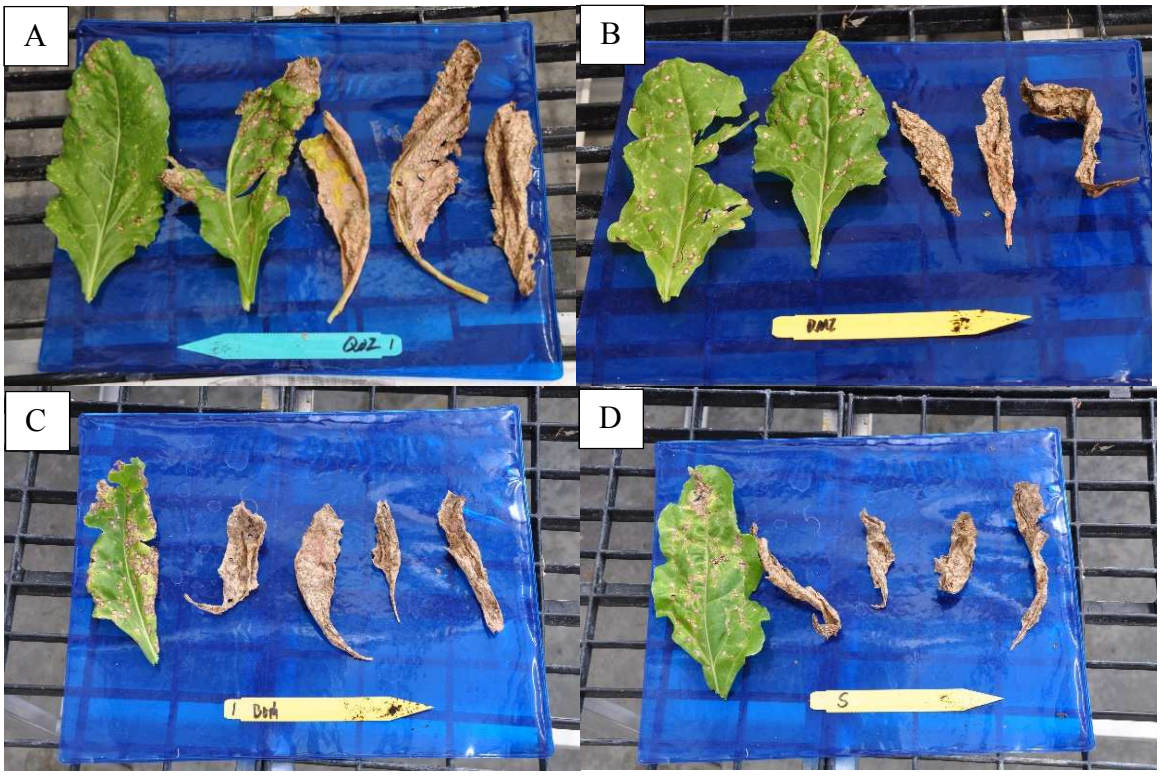

Figure 2.2: Cercospora leaf spot symptoms on sugar beet leaves at 21 days after inoculation with *Cercospora beticola* isolates A: caused by isolates resistant to QoIs; B: caused by isolates resistant to DMIs; C: caused by isolates resistant to both QoIs and DMIs; D; caused by isolates sensitive to both QoIs and DMIs.

Figure 2.3: Sugar beet fields in Minnesota state with severe Cercospora Leaf Spot (CLS) caused by *Cercospora beticola*.

2.4. Discussion

C. beticola has a high risk of developing fungicide resistance in sugar beet growing regions where warm and humid environmental conditions favor disease development on susceptible cultivars, which requires repeated fungicide applications for effective disease control. The extensive use of QoIs and DMIs for over two decades has resulted in resistant field isolates of *C. beticola* in Serbia (Trkulja et al., 2017), Greece (Karaoglanidis et al., 2000; Nikou et al., 2009), Canada (Truman et al., 2013), and the United States (Kirk et al., 2012; Secor et al., 2017). Field isolates with a dual resistance or multiple resistances were reported by Trkulja et al. (2017) and Secor (2017, 2020). In North Dakota and Minnesota, QoI fungicide failure and reduced efficacy of DMI fungicides in sugar beet fields resulted in a CLS epidemic in 2016 (Khan, 2018; Figure 2.3). An annual fungicide resistance monitor program, which tested CLS-infected leave samples from different factory district of sugar beet growing areas in North Dakota and Minnesota, indicate that QoI-resistant *C. beticola* isolates still remained at a high frequency (up to 90%) and the sensitivity of DMI-resistant isolates continued to decrease from 2016 to 2019 (Secor et al., 2019). This study was to assess the variation in fitness components of *C. beticola* isolates with different resistance status to QoIs and/or DMIs. This knowledge of parasitic fitness can assist growers with fungicide resistance management to combat the resistance for CLS disease control, because the resistant strains may suffer fitness penalties that could affect stability of resistance in the absence of fungicide selection pressure (Ishii, 2015).

In in vitro study, QoI-resistant *C. beticola* isolates possessing G143A mutation showed no significant differences in spore germination or mycelial growth but produced significantly lower number of conidial spores compared to the sensitive ones. The specific point mutation confering QoI resistance would exert effects on the fitness parameter in resistant strains. For

example, *Alternaria solani* field isolates with F129L point mutation conferring QoI resistance were less fit in spore germination but more aggressive to tomato plants (Pasche and Gudmestad, 2008). Malandrakis et al. (2017) studied laboratory QoI-resistant *C. beticola* isolates with reduced spore production and pathogenicity compared with wild-type isolates. However, these resistant isolates were not obtained from fields and the fitness cost was likely due to pleiotropic effects of accumulated mutations induced by ultraviolent mutagenesis (Karaoglanidis et al., 2011). Our in vivo study showed that QoI-resistant isolates, although having a slower disease progress, were still as aggressive as sensitive ones. In North Dakota and Minnesota, Secor et al. (2019) demonstrated that the percentage of QoI-resistant isolates with G143A mutation was still stable in field population three years after they were confirmed, when growers are told to avoid applying QoI fungicides to control CLS after field failures occurred to QoIs in 2016. Other research studies indicated that QoI resistance was relatively stable in *A. alternata* isolates (Vega and Dewdney, 2014) and that resistant strains recovered rapidly if QoI fungicide was applied again (Genet et al., 2006; Ishii et al., 2007). The phenomenon that QoI-resistant strains are still dominant in our sugar beet fields could be explained by two reasons: 1) a fitness penalty shown in our research was not enough to offer an advantage of sensitive strains over resistant isolates; 2) the reduced usage of QoI fungicides still play an important role in selecting resistant strains.

DMI-resistant isolates, compared to sensitive ones, were significantly less fit in mycelial growth (radial growth rate) and sporulation in vitro. Similarly, the fitness penalty associated with DMI resistance in *C. beticola* isolates were also found in other studies. Moretti et al. (2003) compared the fitness of DMI laboratory-induced mutants of *C. beticola* with wild-type strains and found that the radial growth rate and pathogenicity were negatively affected by the DMI resistance. Karaoglanidis et al. (2001) also reported that fluriafol-resistant isolates produced

fewer conidial spores than sensitive isolates. In contrast, the isolates tested in our study were recovered from fields and then confirmed to be resistant against three DMIs (difenoconazole, prothioconazole, and tetraconazole) with high EC_{50} values (>1 µg/ml). In in vivo study, DMI isolates had significantly lower disease severity at 7 DAI with the lowest AUDPC, although disease severity was still high at 21 DAI. Bolton et al. (2012c) conducted a greenhouse study showing that *C. beticola* isolates with high EC_{50} values (>1 µg/ml) for DMIs caused significantly more disease than the isolates with low EC_{50} values, but tetraconazole (DMI) applications still provided disease control compared to the non-treated check. Moreover, the cold winter lasting for five months in North Dakota and Minnesota may lead to a shift in the sensitivity of DMI-resistant strains. Arabiat (2015) and Karaoglanidis et al. (2002) reported the instability of DMI-resistant *C. beticola* strains after cold exposure, although the treatments of low temperature and exposure period were different.

All resistant groups of isolates tested in this study produced significantly fewer spores compared to the sensitive group. While the isolate group resistant to both DMIs and QoIs showed the similar fitness penalty in mycelial growth with DMI-resistant isolate group. It seemed that this reduced mycelial growth in the isolate group with the dual resistance was due to the DMI-resistance. This additive effect was also found in another study. Veloukas et al. (2014) reported that *Botrytis cinerea* field isolates with dual resistance to SDHIs and QoIs showed reduced fitness values compared to the sensitive isolates, while there was no penalty shown in the isolate group possessing only G143A mutation. Surprisingly, the isolate group with dual resistance resulted in a similar disease severity and AUDPC as the sensitive group in an in vivo study. This is an indication that the isolate group with the dual resistance might be as aggressive as sensitive strains. Due to an instability of DMI-resistance in *C. beticola* isolates, the frequency

of the dual resistance group might decrease over time. The annual report from Secor et al. (2016) indicated the percentage of the field isolates with multiple fungicide resistance.

In conclusion, compared to sensitive *C. beticola* isolates, the QoI and/or DMI resistant isolates had a relatively slower disease development on sugar beet leaves due to their fitness penalty in sporulation and mycelial growth. However, these resistant isolates still caused high disease severities as sensitive ones and are difficult to be controlled by QoI and DMI fungicides in fields. The *C. beticola* population resistant to site-specific fungicides, such as QoIs and DMIs, might persist in the field. A similar case occurs with another site-specific chemical benzimidazole, where the resistant *C. beticola* strains associated with two amino acid replacements can be rapidly selected if the fungicide was applied again (Karaoglanidis et al., 2003; Trkulja et al., 2013). DMI fungicides are slightly different in chemical structures and, therefore, individual DMI fungicide might still work on controlling CLS. As such, there is a need to consider using multi-site fungicides, such as copper-based chemicals, as management options for CLS control. More importantly, it is wise to use effective fungicides belonging to a different mode of action in alternation or mixture fungicide application programs to better CLS control as well as fungicide resistance management for sugar beet industry. Field sanitation, crop rotation with non-host crops, and planting resistant cultivars are always recommended for growers to suppress field population of various *C. beticola* strains. Also, efforts are ongoing by seed companies to develop cultivars with improved resistance to *C. beticola* without significant reduction in tonnage and sucrose concentration.

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3. RESPONSES TO COPPER-BASED CHEMICALS OF DIFFERENT FUNGICIDE-RESISTANT *CERCOSPORA BETICOLA* ISOLATES

3.1. Introduction

Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola* (Saccardo, 1876), is the most damaging foliar disease on sugar beets (*Beta vulgaris* L.) (Holtschulte, 2000). Typically, individual leaf spots are circular, tan to brown, and 3-5 mm in diameter with dark-brown or reddish-purple margins. The pathogen structure is characterized by black pseudostroma scattered within mature lesions. As individual spots coalesce later in the season, CLS-infected leaves become yellow and necrotic, causing a complete collapse of the leaves (Asher and Hanson, 2006). The resulting leaf death not only reduces the photo-synthetic capacity of leaves, but also results in a continual regrowth of new leaves at the expense of stored sucrose in roots. CLS negatively affects recoverable sucrose, yield tonnage, and pile storability, severely impacting the profitability of the sugar beet industry (Lamey et al., 1996; Shane and Teng, 1992). In 2016, a CLS outbreak caused an estimated loss of over $100 million to the sugar beet industry in North Dakota and Minnesota (Khan, 2018), where the U.S. sugar beet production is concentrated with $4.9 billion in total economic activities (Bangsund et al., 2012).

CLS management integrates cultural practices, resistant cultivars, and fungicide applications. Crop rotation with non-host crops for 3 years is recommended to reduce *C. beticola* inoculum in the affected fields (Jacobsen and Franc, 2009). In CLS-epidemic areas, growers are required to plant resistant cultivars that can slow down the infection process initiated by *C. beticola* (Rossi et al., 2000). More importantly, multiple applications of effective fungicides in a timely manner play an indispensable role in a successful CLS management (Khan, 2018). For sugar beet growers in North Dakota and Minnesota, two types of organic fungicides have been

extensively used to control CLS: 1) quinone outside inhibitors (QoIs) such as pyraclostrobin (Headline, 98% a.i.; BASF) and trifloxystrobin (Gem, 42.6% a.i.; Bayer) and 2) demethylation inhibitors (DMIs) such as difenoconazole (Inspire, 23.2% a.i.; Syngenta), tetraconazole (Eminent, 11.6% a.i.; Gowan Company), and prothioconazole (Proline, 99.4% a.i.; Bayer) (Carlson et al., 2010).

However, both QoIs and DMIs are single-site fungicides with a high risk of selecting resistant pathogen strains, especially when applied in a repeated manner (McGrath, 2004). QoI-resistant *C. beticola* isolates are associated with a point mutation (G143A) in cytochrome b complex (Bolton et al., 2013), while DMI-resistant isolates are associated with the over-expression of the C-14 alpha-demethylase gene (*CYP51*) (Nikou et al., 2009). The QoI-resistant *C. beticola* isolates have been reported from sugar beet growing areas of Ontario, Canada (Trueman et al., 2012), Michigan (Bolton et al., 2013), and Nebraska (Kirk et al., 2012) in the U. S.. The isolates with reduced sensitivity to DMIs were also reported in Greece (Karaoglanidis et al., 2002) and the U. S. (Bolton et al., 2012). In 2016, there was a CLS epidemic in North Dakota, Minnesota, and Michigan, initially caused by field failures of QoI fungicides. The majority of *C. beticola* field isolates were confirmed as resistant to QoIs, while some isolates were tested with reduced sensitivity to DMIs, and these resistant isolates were still dominant in 2017 (Secor et al., 2017).

There is an urgent need to find other fungicide options to manage these resistant populations of *C. beticola* in the fields because the pathogen has developed resistance against currently used fungicides, such as thiophanate-methyl (Campbell et al., 1998) and tri-phenyltin hydroxide (Bugbee, 1996). Although commonly used as bactericides (Scheck and Pscheidt, 1998; Strayer-Scherer et al., 2018), copper-based compounds can also be used as fungicides to

control foliar diseases on crops (Culbreath et al., 2001; Dorman et al., 2009). Also, as multiple-site fungicides, they have a low risk for development of resistant targeted pathogens (FRAC, 2020). Furthermore, several copper fungicide formulations using various concentrations and mixtures are currently available in the market, such as copper oxychloride, copper hydroxide, copper sulfate, and copper sulfate pentahydrate. It would be useful for growers to know the levels of efficacy of controlling *C. beticola* provided by various copper-based products. In addition, a copper fertilizer Fertileader Copper (soluble copper nitrate, 11.9% a.i.; Timac Ago, Reading, PA) was also included in this study because copper is not approved for use as a fungicide in some European countries, such as the United Kingdom (U.K.). Additionally, the presence of sulfur in basic copper sulfate compound plays an indirectly role in enhancing the fungicidal activity of coppers (Baldwin, 1950). We want to know whether the addition of a sulfur-based product, Microthiol (sulfur, 80% a.i.; United Phosphorus; King of Prussia, PA), into Cuprofix (basic copper sulfate, 37.5% a.i.; United Phosphorus; King of Prussia, PA) will have synergistic effects on controlling the fungal pathogen *C. beticola*.

The objectives of this research were to 1) determine the sensitivity to copper-based chemicals of QoI- and/or DMI-resistant *C. beticola* isolates collected from Minnesota sugar beet fields in 2017; 2) evaluate the efficacy of copper-based chemicals at controlling CLS on sugar beet plants inoculated with QoI- and DMI-resistant *C. beticola* isolates; 3) determine if a sulfur-based product in the mixture with a product of basic copper sulfur improves the CLS control in a greenhouse study.

3.2. Materials and methods

3.2.1. Fungal isolate collection

In 2017, CLS-infected leaves were collected from sugar beet fields at Foxhome, Minnesota, USA. For each field sample, *C. beticola* spores were collected with tween 20 solution (1L distill H_2O [dH_2O], 200 µl Tween 20, and 200 mg ampicillin) from five spots per leaf from five leaves (i.e. 5x5 = 25 spots in total). The spore suspension was transferred onto 1.5% water agar media (15g Agar and 1000 ml dH_2O) amended with ampicillin (0.2 g/L). This plate was used as a source of single spore subcultures on CV-8 media (15g Agar, 100 ml V8 juice, and 900 ml dH_2O) for subsequent testing. The recovered *C. beticola* isolates were tested for their sensitivity to 1) DMIs (difenoconazole, prothioconazole, and tetraconazole) using a radial growth procedure (Secor and Rivera, 2012); and 2) QoIs using a PCR-based molecular procedure to test the presence of G143A conferring the QoI resistance (Bolton et al., 2012). In this research, 13 QoI-resistant isolates, 15 DMI-resistant isolates, and 19 isolates resistant to both QoI and DMI (Appendix A.) were used to determine the sensitivity to nine commercially available copper-based products (Table 3.1).

3.2.2. Copper-based chemicals

Nine copper-based chemicals and a sulfur-based product used in this research are listed in Table 1. These products were marketed by various companies: Badge® SC and Badge® X2 (Isagro USA; Morrisville, NC), Champion™ (Nufarm Americans; Alsip, IL), Champ® Formula 2 (Nufarm Americans; Burr Ridge, IL), Cuprofix® Ultra 40 (United Phosphorus; King of Prussia, PA), C-O-C-S® (Loveland Products; Greeley, CO), Mastercop® (Makhteshim Agan of North American; Raleigh, NC), Ridomil Gold® Copper (Syngenta Crop Protection; Greensboro,

NC), Fertileader® Copper (Timac Agro USA; Reading, PA), and Microthiol® (United Phosphorus; King of Prussia, PA).

3.2.3. In vitro assessment of *C. beticola* sensitivity to copper-based chemicals

The sensitivities of those commercial copper-based products were determined by comparing spore germination of *C. beticola* conidia spores on 1.5% water agar amended with five concentrations at 0, 0.01, 0.1, 1, and 10 µg/ml of each of the nine copper-based chemicals. They were prepared by dissolving separately these nine commercially formulated copper-based products in sterile distilled water to obtain stock solutions of 100,000 µg a.i./ml. The serial concentrations of copper-based products were prepared by adding a series of dilutions of the stock solution into sterile molten water agar media at approximately 45 to 50°C.

Approximately 3 ml of tween water was added to each *C. beticola* culture, where conidia spores were induced following the methodology described by Secor and Rivera (2012) and the conidia were dislodged using a sterile glass rod. A 120 µl aliquot of the conidial suspension of each isolate was added to two plates of each fungicide concentration. The added suspension was evenly spread across each plate using a sterile glass rod. The plates were left unsealed with Parafilm in order to allow them to dry sufficiently and then they were incubated at room temperature (21 ± 2°C) in the dark for 16 h. After incubation, 50 conidia on each plate were examined with a stereo microscope at ×50 magnification. A conidium was considered germinated if the germ tube from the conidium had at least doubled in length.

A total of 47 *C. beticola* isolates among three resistant isolate groups (QoI-, DMI-, and both QoI- and DMI-resistance) were evaluated for their sensitivities to nine commercial copper-based products; isolate sensitivities to each of these copper-based products were measured by measuring the copper-based chemical concentration that inhibited conidial germination by 50%

(EC_{50}). To determine the EC_{50} value for each isolate, the number of conidia germinated on copper-amended media was converted into a percentage of germination reduction as below:

[100 − ((the number of germinated spores in amended media/ the number of germinated spores in non-amended media) × 100)]

The germination reduction data were linearly regressed against the $\log_{10}$ transformed concentrations of copper-based chemicals and the EC_{50} value was determined by interpolation of the 50% intercept (Russell, 2004). Each isolate was tested in a completely randomized design (CRD) with two replicates per copper-based product concentration. All experiments were conducted twice.

3.2.4. In vivo assessment of copper-based chemical efficacy at controlling resistant *C. beticola* isolates

This research was conducted in the greenhouse in the Agricultural Experiment Station at North Dakota State University, Fargo, ND, USA. The greenhouse conditions were maintained with a 16-h photoperiod and at 22 ± 2 °C (Argus Control Systems Ltd.; British Columbia, Canada). Three seeds of a CLS-susceptible sugar beet cultivar "MA504" (Niehaus and Moomjian, 2018) were planted in pots 10 × 10 × 12 cm (L x W x H) filled with peat mix (Sunshine mix 1, Sun Gro Horticulture Ltd.; Alberta, Canada). Each pot was watered as needed and thinned to have one healthy plant at the two-leaf stage.

Between 4 and 5 weeks (i.e. plants at about 6-leaf stage) after planting, fully expanded leaves of each sugar beet plants received copper-based chemical treatments from each copper-based product at label rates as shown in Table 1. An 18-cm band spray was applied using a spraying system (De Vries Manufacturing; Hollandaise, MN, USA) calibrated to deliver 187 L/ha at 138 kPa through a TeeJet 8001E nozzle.

At 24 h after copper-based chemical applications, the plants were inoculated with a conidial suspension collected from the nine isolates of 47 *C. beticola* isolates where three isolates were selected from each fungicide resistant isolate group. Conidial suspension was produced following the methodology by Secor and Rivera (2012); the number of conidia was calculated via hemocytometer (Hausser Scientific; Horsham, PA, USA) and adjusted to 4×10^4 conidia/ml. Three fully expanded leaves of each plant were inoculated with the conidial suspension using a Preval paint-spray gun (Preval Sprayer Division, Precision Valve Corporation, Yonkers, NY) (Figure 3.7). The same day after inoculation the plants were incubated at 95 to 100% relative humidity in chambers 100 × 90 × 140 cm (L x W x H) with a 16-h photoperiod and average temperature of 28 ± 2°C. At 7 days after inoculation (DAI), the plants were moved back into the greenhouse and watered as needed at the base of each plant. CLS symptom severity was evaluated visually every other day beginning 9 DAI and continuing until 17 DAI by estimating the number of leaf spots on the three inoculated leaves (three subsamples). A severity scale from 1 to 10 was used as follows: 1 = 1-5 spots/leaf; 2 = 6-12 spots/leaf; 3 = 13-25 spots/leaf; 4 = 26-50 spots/leaf; 5 = 51-75 spots/leaf; 6 = 76-99 spots/leaf; 7 = 100-124 spots/leaf; 8 = 125-149 spots/leaf; 9 = 150-200 spots/leaf; and 10 = >200 spots/leaf (Jones and Windels, 1991). This experiment was conducted twice as a CRD with four replicates.

3.2.5. Data analysis

In the in vitro study, the Levene's test was conducted to test the homogeneity of variance across the two repeats of the same experiments before the data were combined. For each isolate, the EC_{50} value was calculated for each copper-based chemical using the general linear model procedure (Proc GLM) in the Statistical Analysis System (Version 9.3, SAS Institute Inc.; Cary, NC, USA). A two-way analysis of variance (ANOVA) for EC_{50} values was performed to test the

effects of resistant isolate group and copper-based chemicals using SAS. Using the SAS general linear models (Proc GLM) procedure, the mean EC_{50} values for copper-based chemical treatments were separated by Fisher's Least Significant Difference (LSD) at *P*= 0.05, while the resistant isolate groups were compared with one another in contrast analysis.

In the in vivo study, the CLS symptom severity data for each treatment were collected from the inoculated leaves at 9, 11, 13, 15, and 17 DAI and transformed into the areas under the disease progress curve (AUDPC) using the SAS general linear models (Proc GLM) procedure, following the formula:

$$\text{AUDPC}= \sum_{i=1}^{n-1}[(y_i + y_{i+1})/2](t_{i+1} - t_i)$$

where y_i= disease severity at the ith observation, t_i= time (days) at the ith observation, and n = total number of observations. The homogeneity for variances in measurements between the two repeated experiments was tested by the Levene's test, and then the repeated measurements were combined. Analysis of variance (ANONA) for AUDPC was performed across copper-based products and means of AUDPC between the treatments were separated by Fisher's Least Significant Difference (LSD) at *P*= 0.05 using the SAS general linear models (Proc GLM) procedure.

3.3. Results

3.3.1. In-vitro sensitivity of QoI- and/or DMI-resistant *C. beticola* isolates to copper-based products

The Levene's test for the homogeneity of variance of estimated EC_{50} concentrations from the two repeated experiments was not significant (*P*>0.89) and therefore the two EC_{50} datasets were combined for analysis of variance. The main effect of resistant groups (QoI-, DMI-, and both QoI- and DMI- resistance) was not significant (P>0.16) and nor was the effect of interaction

of the resistant groups with copper-based products (P>0.85). However, significant differences in EC_{50} values were detected among nine copper products ($P < 0.001$).

Figure 3.1 shows the distribution of estimated EC_{50} concentrations for all isolates treated with nine copper-based products in the three respective QoI-, DMI- and both QoI- and DMI-resistant groups. Isolates resistant to QoI showed the most spread in EC_{50} with the interquartile range being 3.6741 followed by isolates resistant to DMI and to both QoI and DMI, with the interquartile range being 3.12859 and 2.9703, respectively. Figure 3.2 shows the estimated EC_{50} concentrations of 47 isolates treated by nine copper-based products. The separation of mean EC_{50} concentrations of 47 *C. beticola* isolates among nine copper-based products in inhibition of spore germination is given in Table 3.2. Four distinctive classes of these copper-based products emerged. The copper-based product that had the lowest EC_{50} concentration was Fertileader at 1.726 µg/ml). Badge X2, Champion and Mastercop did not differ significantly and shared the second lowest EC_{50} concentrations. Champion F2, Cuprofix, COCS and Ridomil did not differ significantly and formed the third lowest EC_{50} concentrations while Badge SC had the highest EC_{50} concentration at 7.523 µg/ml.

Figure 3.3 shows a dose-response curve between the copper-based chemical concentrations and the inhibited germination percentage of *C. beticola* spores. Generally, the tested copper-based chemical concentration at 10 µg/ml was the most effective in inhibiting *C. beticola* spore germination of the four concentrations. Figure 3.4 shows that after 16 hours of incubation at room temperatures without light, the conidial germination from *C. beticola* was inhibited on water agar (WA) media mediated with each of copper chemicals at 10 µg/ml under microscopic views, while spores can germinate well on WA media without any copper-based product as a control.

3.3.2. Efficacy of copper-based products in controlling fungicide resistant *C. beticola* in the greenhouse

The Levene's test for homogeneity of variance indicated that there was no significant difference in the AUDPC between two repeated experiments ($P > 0.48$). It was therefore that the calculated AUDPC from two repeated representments were combined for analysis of variance.

Under the greenhouse conditions, all copper-based products applied before *C. beticola* inoculation decreased the development of CLS severity, resulting in significantly lower AUDPC than the inoculated check without any copper-based chemical sprays (Figure 3.5 and 3.6). Among the copper-based products, the most effective copper-based product was Ridomil with the smallest AUDPC although it was not significantly different from Cuprofix, Champion, Badge X2 and COCS. Badge SC, Champ F2 and Mastercop did not differ significantly but they significantly limited the CLS severity and resulted in significantly smaller AUDPC compared with the control treatment. The AUDPC was not possible to be calculated for the copper-based fertilizer product – Fertileader. This was because it was phytotoxic to sugar beet foliage after treatments and made it impractical to determine disease severity on the treated leaves. Additionally, the sulphur-based product, Microthiol, had similar AUDPC to Badge SC, Champ F2, and Mastercop. However, Microthiol mixed with Cuprofix was seen most effective and resulted in smaller AUDPC than the single use of Cuprofix although they did significantly differ, suggesting that Microthiol can help enhance the efficacy of Cuprofix.

Table 3.1: Commercial products tested in sensitivity study to *C. beticola* isolates and applied on sugar beet to control CLS in a greenhouse study.

Copper Products	Formulation	Active Ingredient	Use	Label Rate[a]
Badge SC	Suspension Concentrate	17.6% Copper Oxychloride + 16.4% Copper Hydroxide	Fungicide/ Bactericide	955 ml a.i/ha
Badge X2	Dry Flowable	22.82% Copper Oxychloride + 21.49% Copper Hydroxide	Fungicide/ Bactericide	632 g a.i./ha
Champion	Dispersible Granule	46.1% Copper Hydroxide	Fungicide/ Bactericide	605 g a.i./ha
Champ Formula 2 (Champion F2)	Flowable	37.5% Copper Hydroxide	Fungicide/ Bactericide	877 ml a.i./ha
Cuprofix Ultra 40 (Cuprofix)	Dry Flowable	71% Basic Copper sulfate	Fungicide/ Bactericide	897 g a.i./ha
C-O-C-S (COCS)	Water-Dispersible Granules	73.49% Copper Oxychloride + 13.39% Basic Copper Sulfate	Fungicide/ Bactericide	1149 g a.i./ha
Mastercop	Soluble	21.46% Copper sulfate pentahydrate	Fungicide/ Bactericide	452 ml a.i./ha
Ridomil Gold Copper (Ridomil)	Wettable Powder	60% Copper Hydroxide + 5% Mefenoxam	Fungicide/ Bactericide	876 g a.i./ha
Fertileader Copper (Fertileader)	Soluble	11.9% Copper Nitrate	Fertilizer	104 ml a.i./ha
Microthiol	Micronized Wettable	80% Sulfur	Fungicide	4482 g a.i./ha

[a]The label rate for each cooper product registered for controlling *C. beticola* on sugar beet was used in the greenhouse study. Since Ridomil Gold Copper was not labeled for use on sugar beet and Fertileader was a fertilizer, the recommended rates were used in the greenhouse study.

Table 3.2: Mean effective concentration that inhibited spore germination by 50% (EC_{50}) of nine copper-based products with 47 *Cercospora beticola* isolates collected from sugar beet fields at Foxhome, Minnesota, USA in 2017.

Copper-based products	**Mean EC_{50} (µg/ml)[a]**	**EC50 range**
Badge SC	7.52 a	0.67-10
Badge X2	3.44 d	0.08-10
Champion	3.20 d	0.05-5.92
Champ F2	4.87 b	0.78-8.63
Cuprofix	4.79 b	0.08-10
COCS	4.52 bc	0.93-10
Mastercop	3.82 cd	0.02-10
Ridomil	3.97 bc	0.16-7.25
Fertileader	1.72 e	0.14-4.99

[a]EC_{50} concentrations that share a common letter were not significantly different at $P = 0.05$ using the Fisher's Least Significant Difference (LSD) post hoc test. LSD was calculated to be 0.88 at $P = 0.05$.

Table 3.3: Pair-wise comparison of copper EC_{50} values among *Cercospora beticola* isolates in different fungicide resistance groups.

***C. beticola* resistant isolates[a]**	**QoI-resistant**	**DMI-resistant**	**QoI- and DMI-resistant**
QoI-resistant		0.2528	0.5862
DMI-resistant	0.2528		0.5292
QoI- and DMI- resistant	0.5862	0.5292	

[a] Among 47 isolates of *C. beticola* collected from Minnesota fields, 13 isolates, 15 isolates, and 19 isolates were confirmed as QoI-resistant, DMI-resistant, and QoI- and DMI- resistant ones, respectively.

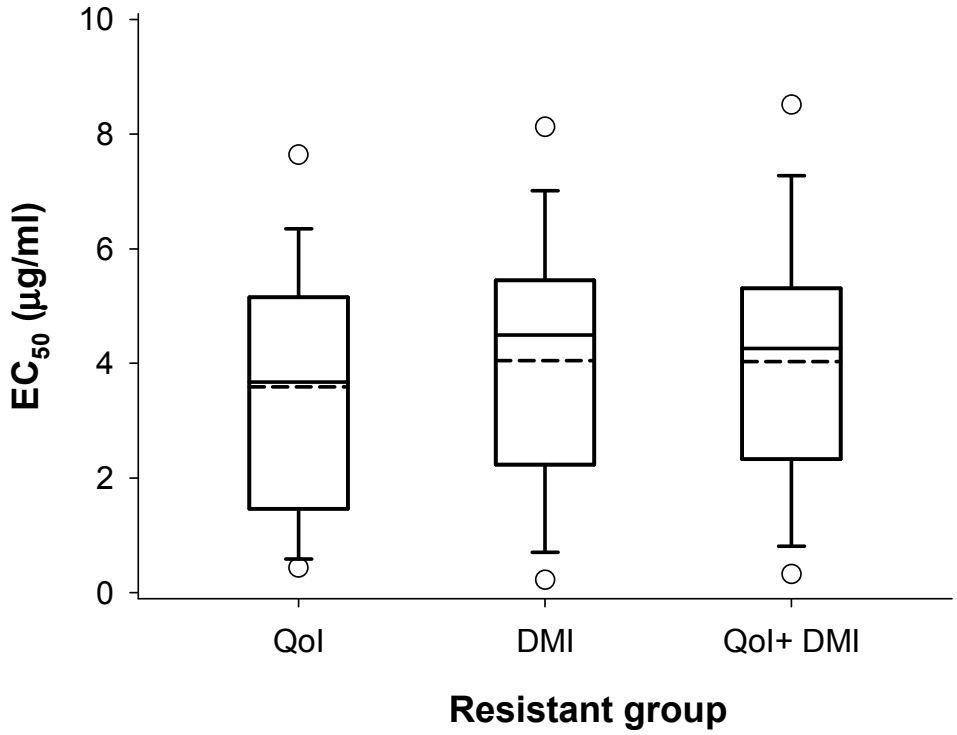

Figure 3.1: Distribution of estimated concentrations (µg/ml) of EC_{50} for nine copper-based products-treated isolates grouped into resistance to QoI, DMI and both QoI and DMI (QoI+DMI). Each boxplot shows the mean (broken line) and median (solid line) concentrations of EC_{50}. The lower and upper boundaries of the boxes indicate concentrations of EC_{50} for the 25^{th} and 75^{th} percentiles, while whisker bars below and above each box indicate concentrations of EC_{50} for the 5^{th} and 95^{th} percentiles. The black dots below and above each boxplot represent the minimum and maximum concentrations of EC_{50}, respectively.

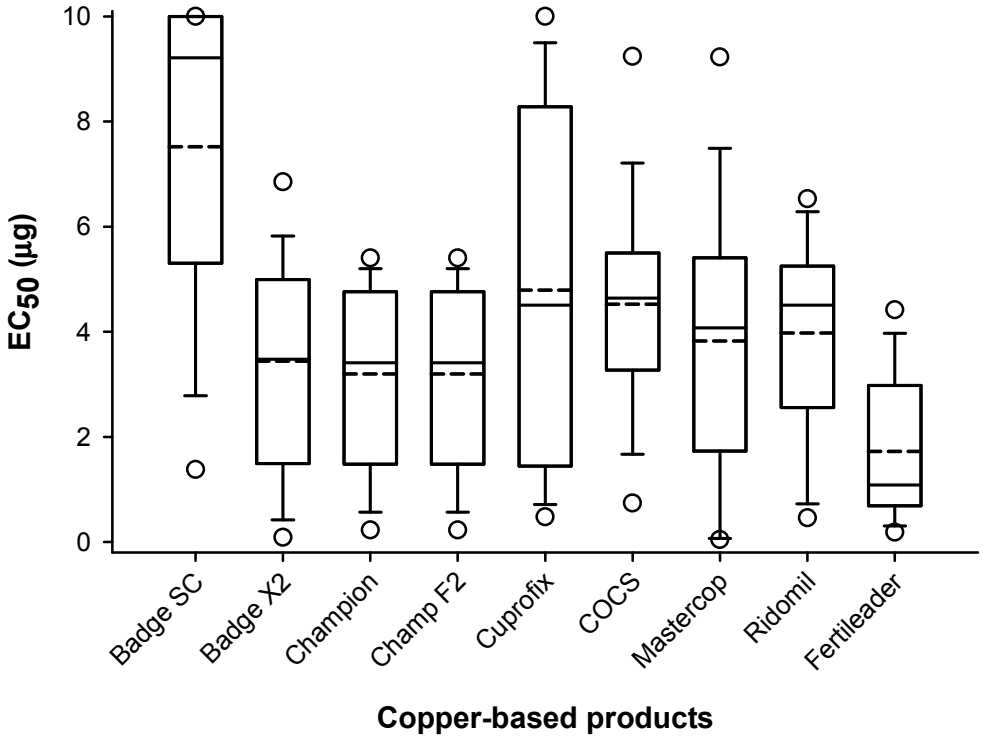

Figure 3.2: Distribution of estimated concentrations (µg/ml) of EC_{50} for all 47 isolates treated by nine copper-based products. Each boxplot shows the mean (broken line) and median (solid line) concentrations of EC_{50}. The lower and upper boundaries of the boxes indicate concentrations of EC_{50} for the 25th and 75th percentiles, while whisker bars below and above each box indicate concentrations of EC_{50} for the 5th and 95th percentiles. The black dots below and above each boxplot represent the minimum and maximum concentrations of EC_{50}, respectively.

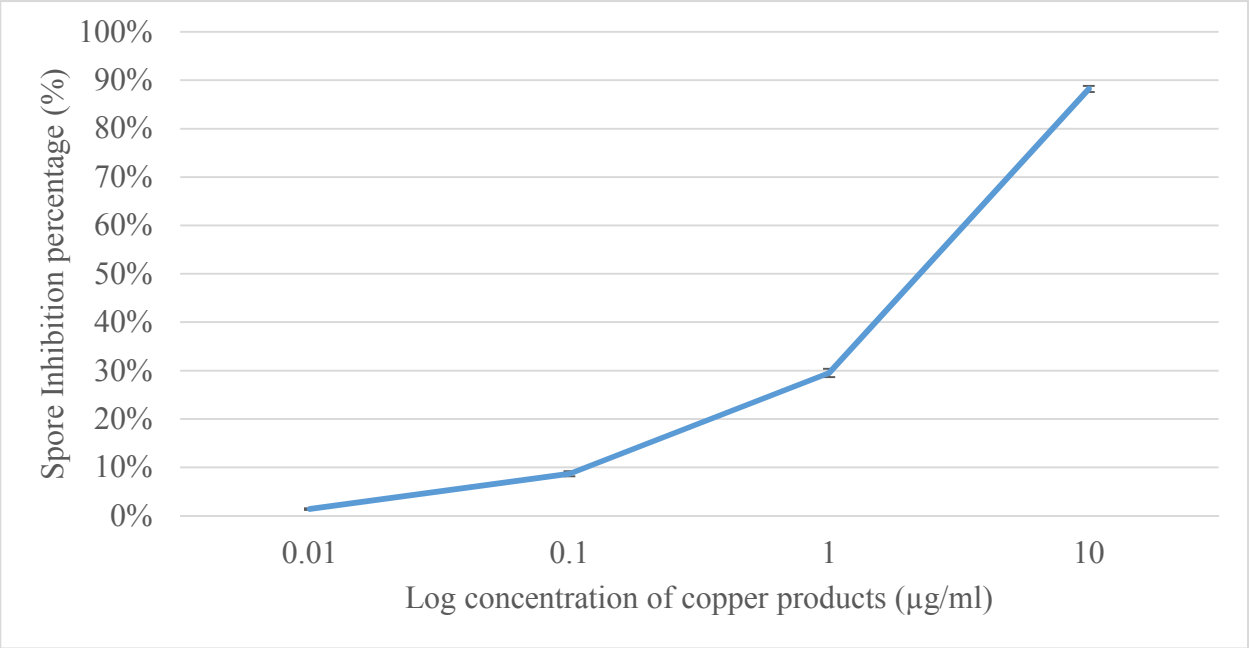

Figure 3.3: Dose-response curves for spore inhibition from 47 *C. beticola* isolates by nine copper-based products. Bars represent standard error of the means (SEM).

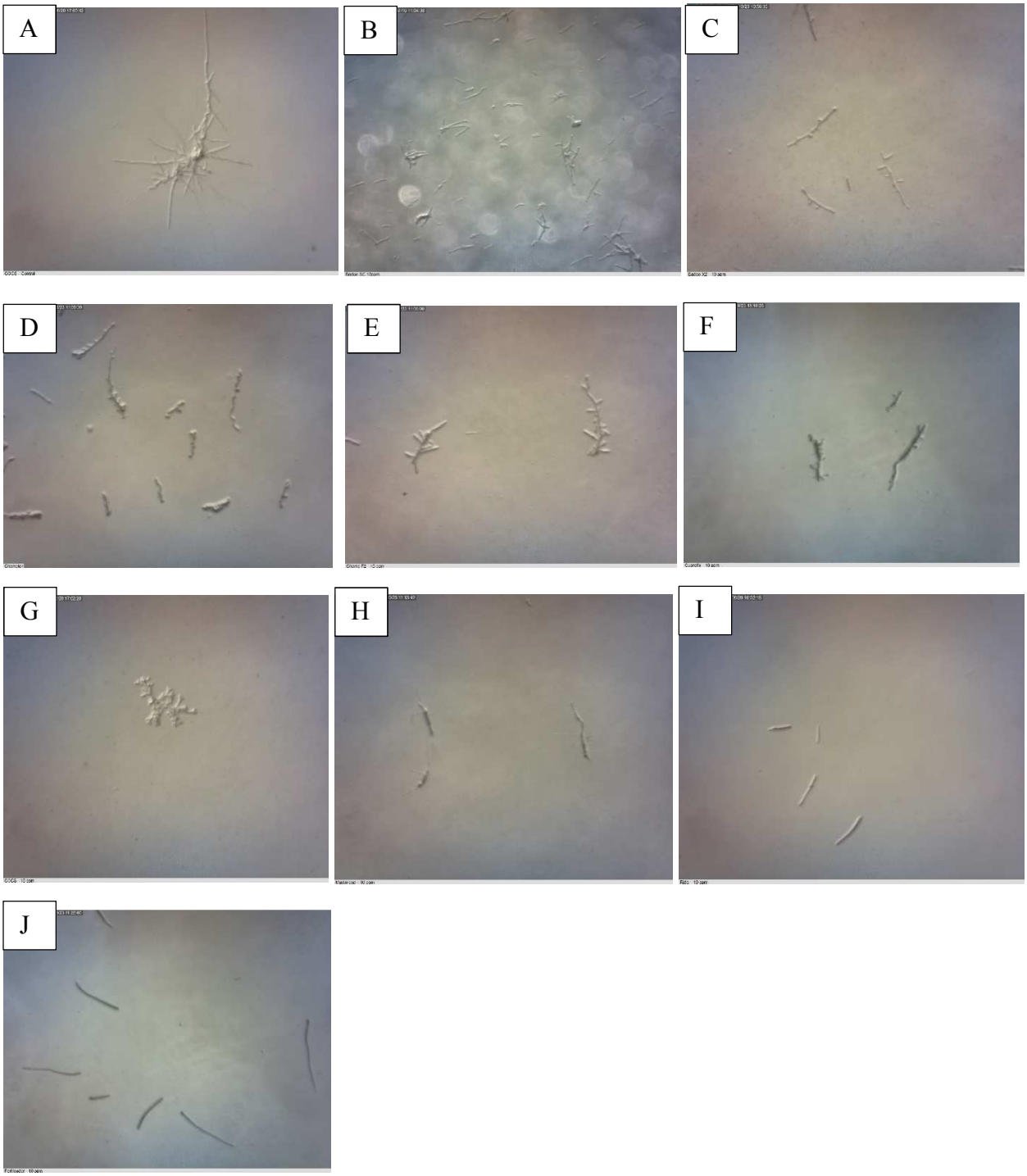

Figure 3.4: Microscopic comparison of *C. beticola* conidial germination on different water agar media after 16 hours of incubation at room temperatures without light. Microscopic photos of conidia spores were taken from water agar media with A: none of copper products; B: Badge SC at 10 µg/ml; C: Badge X2 at 10 µg/ml; D: Champion at 10 µg/ml; E: Champ F2 at 10 µg/ml; F: Cuprofix at 10 µg/ml; G: COCS at 10 µg/ml; H: Mastercop at 10 µg/ml; I: Ridomil at 10 µg/ml; J: Fertileader at 10 µg/ml.

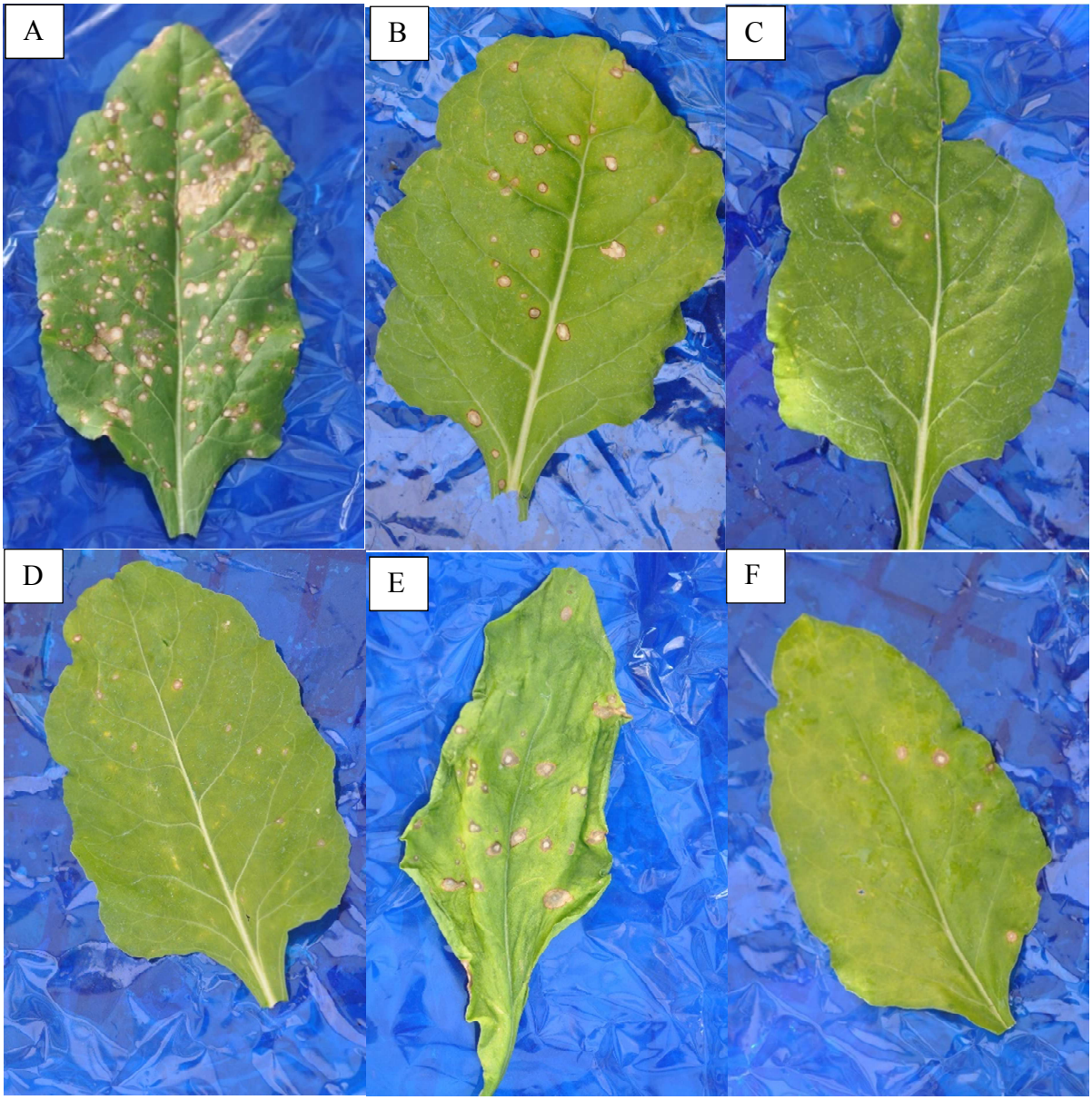

Figure 3.5: Disease symptoms caused by *Cercospora beticola* of sugar beet leaves treated with A: water (un-treated check); B: Badge SC; C: Badge X2; D: Champion; E: Champ F2; F: Cuprofix; G: COCS; H: Mastercop; I: Ridomil; K: Microthiol; L: Microthiol+Cuprofix. The phytotoxic symptoms of sugar beet leaves seen in picture "J" was treated with Fertileader.

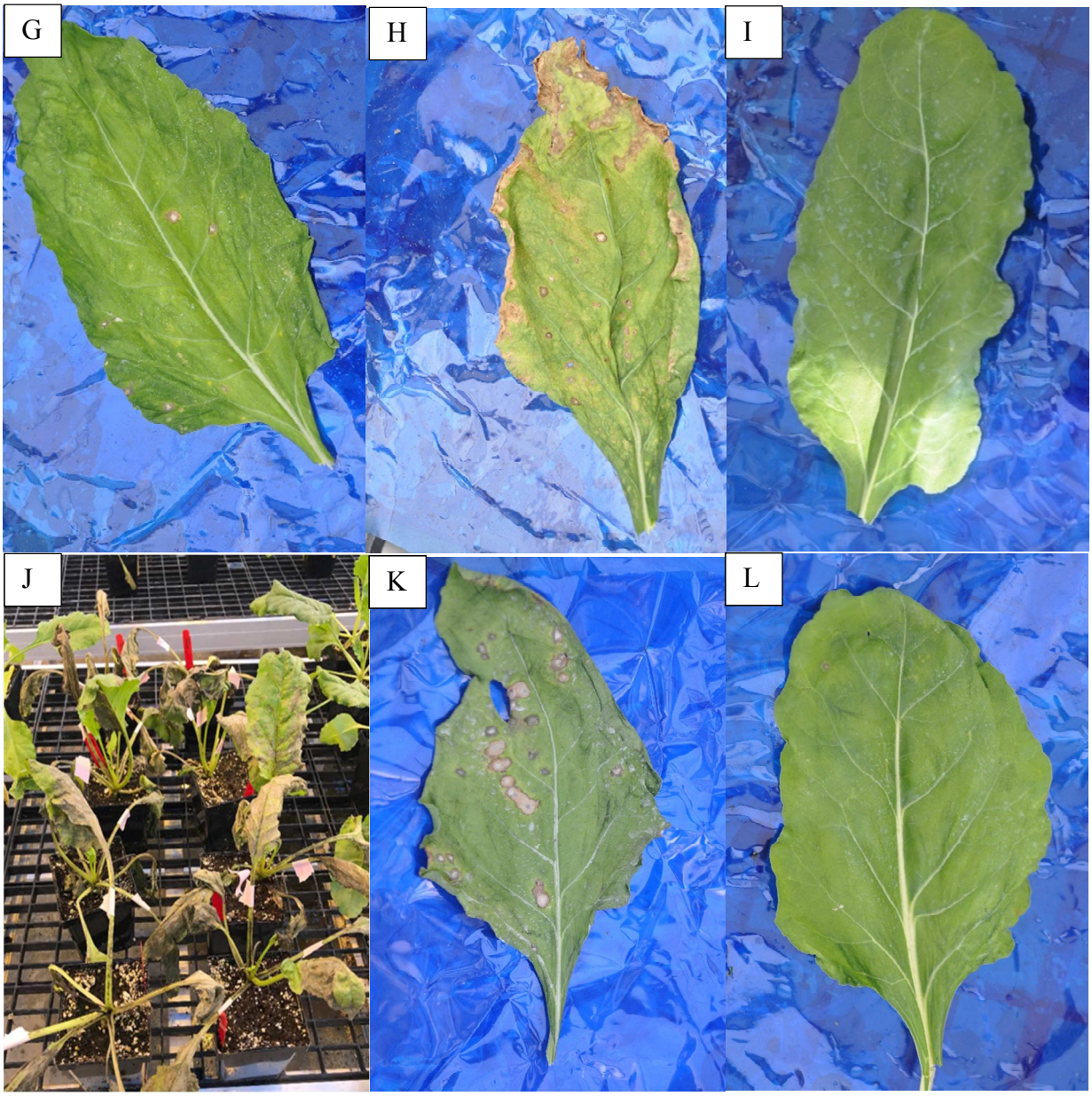

Figure 3.5: Disease symptoms caused by *Cercospora beticola* of sugar beet leaves treated with A: water (un-treated check); B: Badge SC; C: Badge X2; D: Champion; E: Champ F2; F: Cuprofix; G: COCS; H: Mastercop; I: Ridomil; K: Microthiol; L: Microthiol+Cuprofix. The phytotoxic symptoms of sugar beet leaves seen in picture "J" was treated with Fertileader (continued).

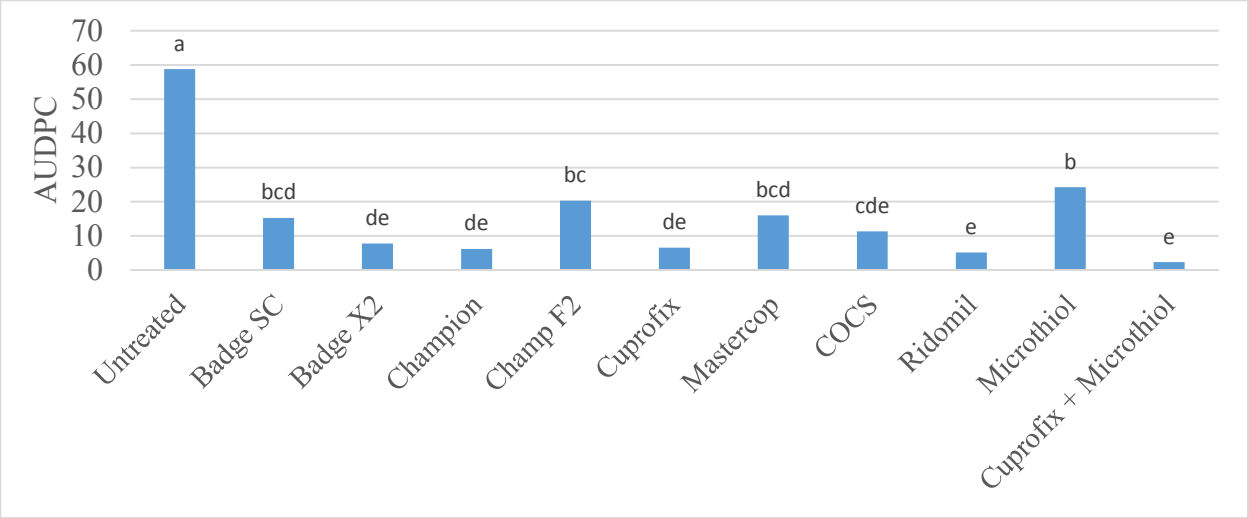

Figure 3.6: Effect of copper-based products and a check control on cercospora leaf spot (CLS) severity caused by nine *C. beticola* isolates with three of which being, respectively, resistant to QoI, DMI and both QoI and DMI on sugar beets in a greenhouse study based on the areas under the disease progress curve (AUDPC). Sugar beet plants were prayed with the copper-based products, dried for 24 h, inoculated with conidia suspension from QoI-resistant *C. beticola* isolates, and placed in humidity chamber for disease development. Disease severity was evaluated using a disease scale from 0 to 10. Means of treatments with a common letter on each bar were not significantly different using the post hoc test of the Least Significant Difference (LSD). LSD was calculated to be 9.86 at $P = 0.05$. Fertileader was excluded due to phytotoxicity in the greenhouse study.

Figure 3.7: Spray inoculation on sugar beet leaves with the conidial suspension using a Preval paint-spray gun.

3.4. Discussion

In this study, we evaluated the sensitivity to copper-based products of 47 *C. beticola* isolates resistant to multiple fungicide classes. Copper-based chemicals are multiple-site inhibitors that disrupt multiple biochemical pathways in a non-specific manner, causing damages to cell integrity and leading to pathogen death (McCallan, 1949). Therefore, both spore germination and mycelial growth assays were developed to determine the sensitivity of fungal pathogen to copper-based chemicals. Our study evaluated copper-based chemical sensitivities with the measurement of inhibited spore germination, showed that the majority of *C. beticola* isolates had EC_{50} values distributed between 1 and 10 ug/ml. On the other hand, a preliminary experiment evaluated the sensitivity of *C. beticola* isolates to both copper sulfate and copper hydroxide, showed that spore germination assay was nearly 30 times more sensitive than a mycelial assay (data not published). Other studies have suggested that spore germination was 7 to 1000-fold more sensitive than mycelial assay in testing the sensitivity of different fugnal pathogens to copper-based chemicals (Everett and Timudo-Torrevilla, 2007). Malandrakis et al. (2019) have shown that for the fungal pathogen *Botrytis cinerea* which causes gray mold on tomato leaves, spores were 50-fold more sensitive to copper hydroxide than mycelium. It indicates that copper-based chemicals inhibit *C. beticola* causing infections on leaves primarily by suppressing spore germination. Moreover, the label rate for use of copper-based chemicals is relatively high. Our preliminary study showed that a copper-based product, Cuprofix, applied at 897 g a.i./ha resulted in a rate of 12,000 µg/ml in a spray solution which is high enough to inhibit *C. beticola* mycelial growth.

In this study, copper-based chemicals showed a low mean EC_{50} value (mostly < 5 µg/ml) for *C. beticola* isolates. Malandrakis et al. (2019) tested the sensitivities of seven plant fungal

pathogens to copper hydroxide using spore germination assay, indicating that the mean EC_{50} values can vary greatly from 10.35 μg/ml for *Botrytis cinerea* to 878.24 μg/ml for *Fusarium solani*. Compared to other fungal pathogens, *C. beticola* isolates seemed to be more sensitive to copper-based chemicals although some isolates had EC_{50} concentrations reaching10 μg/ml. A discriminate dosage of 1 μg/ml of QoI and DMI was used to determine whether *C. beticola* isolates become resistant with field failure or reduced fungicide efficacy, respectively (Secor et al., 2010). However, this discriminate dosage does not apply to coppers. Copper-based chemicals with mean EC_{50} concentrations (> 1 μg/ml) are still able to control *C. beticola* isolates in the in vivo study. The label rate for coppers is nearly 3 times higher than QoIs and DMIs in field sprays (Friskop et al., 2020), mainly because coppers have a different mode of action from QoIs and DMIs. Coppers are multiple-site inhibitors while QoIs and DMIs are single-site inhibitors (Balba, 2007; Koller, 1988). It also explains that the *C. beticola* isolates possessing different resistance status to QoIs and DMIs did not significantly differ in their sensitivity to copper products.

Copper-based chemicals need to be soluble in the water to release a certain amount of copper ions at a certain range of low concentrations that is adequate for fungicidal activity, but not too high to lead to plant injury (Zitter and Rosenberger, 2013). Our study showed that with the same active ingredients, Badge X2 with high copper concentration resulted in lower mean EC_{50} values and significantly better disease control compared to Badge SC with a lower copper concentration. Similarly, Champion had better performance than Champ F2. Moreover, Badge X2, Champion, Cuprofix, COCS, Mastercop, and Ridomil either had mean EC_{50} values or provided CLS control a similar level. Other researchers suggested that copper sulfate had higher efficacy for disease control than copper hydroxide due to its relatively high solubility (Montag et

al., 2006; Shane and Sundin, 2011). No significant differences in fungicidal efficacy were observed between copper sulfate and copper hydroxide, because these copper products have various copper concentrations and are made by different chemical companies. However, excessive copper ions are toxic to plant cells. For example, an acid solution (at low pH values) would increase copper solubility producing excessive copper ions with a phytotoxic effect on the sprayed plants (Deer and Beard, 2001). Phytotoxicity, such as foliar injury, caused by copper-based chemicals was reported on cherry (McManus et al., 2007), peach and nectarine (Lalancette and McFarland, 2007).

In our study, Fertileader, a copper-based fertilizer with a high solubility and the ability to release copper ions at high concentrations in the solution, resulted in the lowest mean EC_{50} concentration among all nine copper-based products, but was also phytotoxic to sugar beet leaves under greenhouse conditions. It is possible that when sugar beet is not exposed to direct sunlight, as in the greenhouse, leaves will become more sensitive to certain chemicals. In countries where fungicide are not available for the control of fungicide resistant populations of *C. beticola*, timely application of Fertileader may be useful in controlling the pathogen's population.

Copper-based chemicals are considered as protectants and need to be applied before infection occurs (MacKenzie et al., 2009). In our in vivo study, sugar beet leaves treated with copper-based chemicals were allowed to dry for 24 hours before inoculation. Copper-based chemicals are non-systemic, and typically serve as a protectant barrier on plant surface to inhibit fungal growth or spore germination before fungal pathogens grow into the plant tissues (Gisi and Helge, 2008; MaCallan, 1949). *C. beticola* initiates infection on leaf surface where the landing conidia germinate, penetrate through stomata, and finally colonize in the mesophyll (Solel and Minz, 1971). Copper chemicals can inhibit spore germination, but their efficacy will be

significantly reduced if sprayed after the pathogen penetrates and colonizes into and inside plant tissues. All the tested copper-based chemicals showed significantly better control against CLS compared to the nontreated control check. Specifically, Badge X2, Champion, Cuprofix, COCS, and Ridomil achieved a significantly small AUDPC. Our results were consistent with Mickelson and Nielsen (2015) who reported that copper-based chemicals, such as Cuprofix, Mastercop, Badge SC, and Champion, could reduce CLS disease scores relative to a nontreated check.

Microthiol is a sulfur product that has been used to control powdery mildew on sugar beet (product label: https://gcrec.ifas.ufl.edu/static/docs/pdf/strawberry-pathology/Fung-label/2009/sulfur/microthiol-disperss.pdf). In our study, Microthiol used alone, provided poor control of *C. beticola* compared to other effective copper-based products. It is worth noting that the mixture of Microthiol with Curpofix resulted in better disease control than the individual copper-based chemicals. The mixtures of sulfur and other inorganics have been successful in different host/pathogen systems. Lime sulfur has been used to control the fungal diseases caused by *Alternaria mali* and *Venturia inaequalis* on apple, while sulfur was used with coppers at low rates to control *Botrytis cinerea* on grapevine. The synergic effects of copper and sulfur mixtures on controlling CLS in our study may be a result of sulfur, not effective by itself, playing an indirect role in increasing the availability of the copper and thus improving its efficacy (Baldwin 1950).

In North Dakota and Minnesota where QoI- and DMI-resistant *C. beticola* isolates predominate and are endemic in sugar beet growing areas (Secor et al., 2017), growers can use copper-based chemicals as a valuable fungicide resistance management tool by mixing other multi-site fungicides or with high single-site resistant risk fungicides to sustain CLS control and to help reduce the frequency of fungicide resistant populations. However, it should be noted that

copper accumulation in the soil from long-term use of copper-based chemicals could cause toxicity to sugar beet or other rotational crops such as wheat (Sayyad et al., 2009) and soybean (Kulikova et al., 2011). Therefore, copper-based products should be used judiciously especially when the population of resistant isolates decline over time or when there is a significant progress in breeding sugar beet cultivars resistant to *C. beticola*.

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4. EVALUATING THE SENSITIVITIES AND EFFICACIES OF FUNGICIDES WITH DIFFERENT MODES OF ACTION AGAINST DIFFERENT FUNGICIDE RESISTANT *CERCOSPORA BETICOLA* ISOLATES

4.1. Introduction

Sugar beet (*Beta vulgaris* L.), typically grown in cool temperate regions, is second only to sugar cane as the major source of the world's sucrose (Draycott, 2006). The United States (U.S.) ranks as the 4th largest producer of sugar beet with 450,000 ha growing areas distributed in ten states: Michigan, Minnesota, North Dakota, Colorado, Montana, Nebraska, Wyoming, California, Idaho, and Oregon (USDA-ERS, 2019). Sugar from sugar beet production supplies more than 50% of U.S. national sucrose (USDA-ERS, 2019). The sugar beet industry in North Dakota and Minnesota has been successful for decades (Shoptaugh, 1997), now contributing 51% to the total U.S. sugar beet production, generating $4.9 billion in total economic activities (Bangsund et al., 2012; USDA-ERS, 2019).

Cercospora leaf spot (CLS), caused by the fungal pathogen *Cercospora beticola*, is an economically important foliar disease of sugar beet in major producing regions around the world (Jacobsen and Franc, 2009; Weiland and Koch, 2004; Wolf and Verreet, 2002). CLS is a polycyclic fungal disease that develops and spreads rapidly to destroy all the plant leaves under favorable weather conditions. A 30% of yield loss is common when disease pressure is moderate (Lamey et al., 1996), but the damaged beet roots also have reduced sucrose and increased impurities (Shane and Teng, 1992). In North Dakota and Minnesota, CLS has always been considered as the most important threat to the local sugar beet industry because the disease outbreak typically causes losses of millions of dollars to growers (Khan, 2018). Besides crop rotation and variety resistance, the most effective CLS management is timely application of

foliar fungicides. Both quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs) have been widely applied or rotated with other protectant fungicides such as tri-phenyltin hydroxide to control CLS (Luecke and Dexter, 2003).

However, repeated applications of site-specific fungicides, such as QoIs and DMIs, lead to the development of resistant strains in *C. beticola* population. The field failure of QoI application to control CLS was first reported in Michigan and Nebraska, USA (Kirk et al., 2012). Resistances to QoIs in *C. beticola* isolates is associated with the substitution of glycine with alanine at position 143 (G143A) in the cytochrome b (*ctyb*) gene (Bolton et al., 2013). For DMIs, the reduced sensitivity of the pathogen is due to overexpression of the cytochrome P450 sterol C-14 alpha-demethylase (*cyp51*) gene (Bolton et al., 2012). In an annual program of testing fungicide sensitivities in North Dakota and Minnesota, Secor et al. (2017) demonstrated that up to 90% of *C. beticola* field isolates were found with G143A mutation conferring QoI-resistance and that the resistant factor for DMIs was even higher than previous years.

In Greece, chlorothalonil was used successfully in mixtures with QoI and DMI fungicides to manage *C. beticola* resistance in sugar beet (Karaoglanidis et al., 2002; Trkulja et al., 2015). Likewise, chlorothalonil was recommended and used in Serbia to manage *C. beticola* populations with resistance to fungicides with multiple modes of action (Trkulja et al., 2017). It will be useful to determine the usefulness of the multisite chlorothalonil for controlling fungicide resistant *C. beticola* on sugar beet in the USA so this data can be used when applying for registration or special emergency exemption. We also wanted to evaluate newer fungicides with different site specific modes of action, such as cyprodinil [anilino-pyrimidines; FRAC group D01], fluazinam [uncouplers of oxidative phosphorylation; FRAC group 29], and pydiflumetofen [succinate-dehydrogenase inhibitors (SDHIs); FRAC group C2], that are safer to people,

wildlife, and the environment. Therefore, these fungicides need to be tested for their efficacy in controlling *C. beticola* on sugar beet (FRAC, 2020). The objectives of this research were 1) to determine the sensitivity of QoI-resistant *C. beticola* isolates to cyprodinil, fluazinam, pydiflumetofen and chlorothalonil; 2) to evaluate the efficacy of cyprodinil, fluazinam, and chlorothalonil in controlling CLS on sugar beet in the field.

4.2. Materials and methods

4.2.1. Fungal isolate collection

In 2017, CLS-infected leaves were collected from sugar beet fields at Foxhome, Minnesota, USA. For each field sample, *C. beticola* spores were collected with tween 20 solution (1L distill H_2O [dH_2O], 200 µl Tween 20, and 200 mg ampicillin) from five spots per leaf from five leaves (i.e. 5x5 = 25 spots in total). The spore suspension was transferred onto 1.5% water agar media (15g Agar and 1000 ml dH_2O) amended with ampicillin (0.2 g/L). This plate was used as a source of single spore subcultures on CV-8 media (15g Agar, 100 ml V8 juice, and 900 ml dH_2O) for subsequent testing. The recovered *C. beticola* isolates were tested for their sensitivity to DMIs (difenoconazole, prothioconazole, and tetraconazole) using a radial growth procedure (Secor and Rivera, 2012); and QoIs using a PCR-based molecular procedure to test the presence of G143A conferring the QoI resistance (Bolton et al., 2013). We then used 12 QoI-resistant isolates, 12 DMI-resistant isolates, and 16 isolates resistant to both QoI and DMI to determine their sensitivity to four fungicides with different modes of action (Appendix A.).

4.2.2. In vitro fungicide sensitivity assays

Sensitivity of each *C. beticola* isolate, as measured by EC_{50} value, was calculated for technical-grade cyprodinil (a.i. 99%; Syngenta, Greensboro, NC), Omega 500F® (Fluazinam,

a.i. 40%; Syngenta, Greensboro, NC), technical-grade pydiflumetofen (a.i. 99%; Syngenta, Greensboro, NC), and Echo720® (Chlorothalonil a.i. 54%; Sipcam, Roswell, GA).

A plug of 5-mm diameter cut from a 14-day grown *C. beticola* isolate was placed on the center of petri dish (100 × 15 mm) containing amended CV-8 media (15g Agar, 100 ml V8 juice, and 900 ml dH_2O). For cyprodinil, the amended concentrations were 0, 0.01, 0.1, 1, and 10 µg/ml; for fluazinam and pydiflumetofen, the amended concentrations were 0, 0.1, 1, 10, and 100 µg/ml; for chlorothalonil, the amended concentrations were 0, 1 10, 100, and 1000 µg/ml. The inoculated plates were incubated at room temperature (21 ± 2°C) in dark. After 14 days, two perpendicular measurements of mycelial growth for each isolate were measured using a digital ruler. Each isolate was measured twice to determine mycelial growth for all fungicides and this trial was conducted twice with two replicates.

Conidia spores were induced from 14-day grown cultures following the methodology described by Secor and Rivera (2012). A sterile glass rod was used to free conidia from cultures by adding tween 20 solution (1L distill H_2O [dH_2O], 200 µl Tween 20, and 200 mg ampicillin). The spore suspension for each isolate was then evenly spread across the petri dish (100 × 15 mm) containing 1.5% water agar amended with five concentrations at 0, 0.01, 0.1, 1, and 10 µg/ml of each tested fungicide. The inoculated plates were incubated at room temperature (21 ± 2°C) in the dark. After 16 hours, 50 conidia on each plate were examined with a stereo microscope at ×50 magnification. A conidium was considered germinated if the germ tube was twice as long as the conidium. Each isolate was counted to determine spore germination for all fungicides and this trial was conducted twice with two replicates.

4.2.3. Evaluation of fungicide efficacy in field study

Field trials were conducted in 2018 and 2019 at Foxhome, Minnesota. The experimental design was a randomized complete block design (RCBD) with four replicates. Field plots comprised six 9.1-meter long rows spaced 56 cm apart. A CLS-susceptible variety, Hillshog 9528, was used in this field study (Niehaus and Moomjian, 2018). In 2018, plots were planted on 12 May and inoculated with *C. beticola* on 28 June; in 2019, plots were planted on 14 May and inoculated on 12 July. Inocula were prepared by grinding CLS-infected leaves collected from the previous year's field trial and directly placed on sugar beet leaves in each experimental plot. We recovered *C. beticola* isolates from the leaves and confirmed them as resistant against QoI or DMI fungicides. Seeds were treated with Tachigaren® (45 g/kg seed), Kabina®, Metlock Rizolex®, and Nipsit Suite® to prevent damping-off early in the season. Weeds were controlled by herbicide applications plus hand weeding throughout the summer.

Three fungicides tested in this field experiment were Inspire super® (Cyprodinil, a.i. 24.1%, Defenoconazole a.i. 8.4%; Syngenta, Greensboro, NC) at 493 g a.i. ha^{-1}, Omega 500F® (Fluazinam, a.i. 40%; Syngenta, Greensboro, NC) at 504 g a.i. ha^{-1}, and Echo 720® (Chlorothalonil, a.i. 54%; Sipcam, Roswell, GA) at 320 g a.i. ha^{-1}. Super Tin® (Triphenyltin hydroxide, a.i. 40%; United Phosphorus; King of Prussia, PA) at 113 g a.i. ha^{-1} was also applied as the industry's standard for controlling CLS. Fungicide sprays were applied with a CO_2 pressurized 4-nozzle boom sprayer with 11002 TT TwinJet nozzles calibrated to deliver 159 L ha^{-1} of solution at 413 kPa pressure to the middle four rows of plots. An untreated control inoculated with *C. beticola* was also included without any of these fungicide treatments. In 2018, four sprays were applied on 18 July, 31 July, 16 August, and 31 August. In 2019, four sprays were applied on 22 July, 1 August, 15 August and 29 August.

CLS severity was evaluated four times in both field trials during the growing season. A severity scale from 1 to 10 was used as follows: 1 = 1-5 spots/leaf; 2 = 6-12 spots/leaf; 3 = 13-25 spots/leaf; 4 = 26-50 spots/leaf; 5 = 51-75 spots/leaf; 6 = 76-99 spots/leaf; 7 = 100-124 spots/leaf; 8 = 125-149 spots/leaf; 9 = 150-200 spots/leaf; and 10 = >200 spots/leaf (Jones and Windels, 1991). The trials were harvested on 1 October in 2018 and 25 September in 2019. The middle two rows of each plot were harvested and weighed for determining root yield while 20 to 30 representative roots from each plot, excluding the roots on the ends of the plot, were sampled for quality analysis at the American Crystal Sugar Company Quality Tare Laboratory, Moorhead, MN.

4.2.4. Data analysis

In the in vitro study, the Levene's test was done to test the homogeneity of variance across the two repeats of the same experiments before combining the two data sets for analysis of variance. For each isolate with each fungicide chemical, the EC_{50} value was calculated as the effective concentration that inhibit mycelial growth or conidial germination by 50%, respectively. The mycelial or germination reduction data was linearly regressed against the $\log_{10}$ transformed concentrations of fungicides, and the EC_{50} value was determined by interpolation of the 50% intercept (Russell, 2004) using the general linear model procedure (Proc GLM) in the Statistical Analysis System (Version 9.3, SAS Institute Inc.; Cary, NC, USA).

In the field study, the CLS severity data in each treatment plot was taken four times at 14 days interval during the growing season. The disease severity data were then transformed into the areas under the disease progress curve (AUDPC) using the SAS general linear models (Proc GLM) procedure, following the formula:

$$\text{AUDPC}= \sum_{i=1}^{n-1}[(y_i + y_{i+1})/2](t_{i+1} - t_i)$$

where y_i= disease severity at the ith observation, t_i= time (days) at the ith observation, and n = total number of observations. The two-year data of AUDPC, sugar beet yield, and recovered sucrose was combined after the homogeneity was confirmed using the Levene's test. Analysis of variance (ANONA) for these data was performed, and treatment means were separated by Fisher's Least Significant Difference (LSD) at *P*= 0.05 using the SAS general linear models (Proc GLM) procedure.

4.3. Results

4.3.1. In vitro fungicide sensitivity assays

Two repeats of EC_{50} value data from mycelial growth and spore germination were combined because their homogeneity test for variance was not significantly different (*P*=0.53 and *P*=0.92, respectively). For each tested chemical, the distribution of EC_{50} values was similar between the isolates with different resistant groups.

Using mycelial growth inhibition method, the sensitivity of 40 *C. beticola* isolates resistant to QoIs and/or DMIs to cyprodinil, fluazinam, pydiflumetofen and chlorothalonil was determined and summarized in Table 4.1. The effects of different rates of each fungicide on radial growth of *C. beticola* were shown in Figure 4.1. Cyprodinil had the lowest EC_{50} value (4.95 µg/ml), followed by fluazinam (33.10 µg/ml), pydiflumetofen (99.38 µg/ml), and chlorothalonil being the highest (481.62 µg/ml). It is noted that the majority of isolates had higher EC_{50} values for pydiflumetofen where the amended media with the highest chemical concentration (100 µg/ml) did not inhibit mycelial growth by 50%.

Using spore germination inhibition method, the sensitivity of the 40 *C. beticola* isolates to cyprodinil, fluazinam, pydiflumetofen and chlorothalonil was determined and summarized in Table 4.2. The mean EC_{50} values ranged from 1 to 10 µg/ml with chlorothalonil being the lowest

(2.43 µg/ml), followed by fluazinam (4.11 µg/ml), cyprodinil (5.65µg/ml). Pydiflumetofen had the highest EC_{50} value of 9.15 µg/ml. Figure 4.2 shows that after 16 hours of incubation at room temperatures without light, the effects of each fungicide at 10 µg/ml on germination of conidial spores of *C. beticola* under microscopic views.

4.3.2. Efficacy of fungicides in controlling *C. beticola* in a two-year field study

Two-year field study data for AUDPC, sugar beet yield, and recovered sucrose yield were combined because the homogeneity test for variance was not significantly different (P=0.51, P=0.16, and P=0.93, respectively).

Effects of fungicides on AUDPC, sugar beet yield, and recovered sucrose yield are shown in Table 4.3. There were significant differences in AUDPC, beet tonnage, and recovered sucrose between fungicide treatments and the nontreated check. The nontreated check, where the plots were inoculated but without chemical sprays for CLS control, had the highest AUDPC with the lowest beet tonnage (56.6 ton/ha) and recovered sucrose (6368 kg/ha). All the fungicide treatments resulted in significantly better disease control, significantly higher beet tonnage, and recovered sucrose compared to the nontreated check. Specifically, Super Tin resulted in the best disease control and the highest beet tonnage (78 ton/ha) and sucrose (10385 kg/ha), followed by Echo 720, Inspire Super, and Omega 500F. However, none of the three fungicides tested in this study performed better than Super Tin that has been considered as the industry's standard fungicide for controlling CLS. It is likely that disease control provided by Inspire Super was probably due to the presence of difenoconazole and not cyprodinil. As such, this product may not be useful for control of CLS since difenoconazole is already present in Inspire XT (difenoconazole + propiconazole). The data suggests that chlorothalonil may provide some efficacy and a different MOA, should it be ever registered for use on beets in the U.S..

Table 4.1: Estimated chemical concentration that inhibited mycelial growth by 50% (EC_{50}) and its frequency distribution of 40 *Cercospora beticola* isolates collected from sugar beet fields at Foxhome, Minnesota, USA in 2017.

Chemical	*C. beticola* resistant isolates[a]	Isolate frequency (%) at EC_{50} range (ug/ml)				EC_{50} (ug/ml)		
		0.1-1	1-10	10-100	100-1000	Range	Mean	Total mean
Cyprodinil	QoI-resistant	0%	100%	0%	0%	1.19-8.88	4.94	4.95
	DMI-resistant	0%	100%	0%	0%	1.89-9.18	6.28	
	QoI- and DMI-resistant	19 %	81%	0%	0%	0.95-8.95	3.63	
Fluazinam	QoI-resistant	0%	17%	83%	0%	7.11-49.09	27.89	33.10
	DMI-resistant	0%	33%	64%	0%	2.55-100	44.20	
	QoI- and DMI-resistant	0%	19%	81%	0%	1.39-100	27.22	
Pydiflumetofen	QoI-resistant	0%	0%	100%	0%	87.91-100	98.64	99.38
	DMI-resistant	0%	0%	100%	0%	97.31-100	99.78	
	QoI- and DMI-resistant	0%	0%	100%	0%	95.36-100	99.71	
Chlorothalonil	QoI-resistant	0%	0%	0%	100%	306.56-746.18	468.50	481.62
	DMI-resistant	0%	0%	0%	100%	254.04-1000	516.37	
	QoI- and DMI-resistant	0%	0%	0%	100%	149.8-1000	460.00	

[a]Forty *C. beticola* isolates were used of which 12 isolates, 12 isolates, and 16 isolates were confirmed to be QoI-resistant, DMI-resistant, and both QoI- and DMI-resistant, respectively.

Table 4.2: Estimated chemical concentration that inhibited spore germination by 50% (EC_{50}) and its frequency distribution of 40 *Cercospora beticola* isolates collected from sugar beet fields at Foxhome, Minnesota, USA in 2017.

Chemical	*C. beticola* resistant isolates[a]	Isolate frequency (%) at EC_{50} range (ug/ml)				EC_{50} (ug/ml)		
		0.01-1	1-5	5-10	>10	Range	Mean	Total mean
Cyprodinil	QoI-resistant	8%	42%	50%	0%	0.88-8.14	4.62	5.65
	DMI-resistant	0%	50%	25%	25%	3.57-10	5.88	
	QoI- and DMI-resistant	0%	50%	13%	37%	1.10-10	6.47	
Fluazinam	QoI-resistant	42%	17%	33%	8%	0.10-10	3.6	4.11
	DMI-resistant	17%	41%	25%	17%	0.78-10	5.12	
	QoI- and DMI resistant	56%	13%	6%	25%	0.27-10	3.61	
Pydiflumetofen	QoI-resistant	8%	8%	0%	84%	0.34-10	8.49	9.15
	DMI-resistant	0%	0%	8%	92%	8.03-10	9.84	
	QoI- and DMI-resistant	6%	0%	25%	69%	0.93-10	9.13	
Chlorothalonil	QoI-resistant	75%	17%	0%	8%	0.18-10	1.58	2.43
	DMI-resistant	17%	66%	17%	0%	0.34-9.02	3.34	
	QoI- and DMI-resistant	44%	50%	6%	0%	0.39-8.57	2.37	

[a]Forty *C. beticola* isolates were used of which 12 isolates, 12 isolates, and 16 isolates were confirmed to be QoI-resistant, DMI-resistant, and both QoI- and DMI-resistant, respectively.

Table 4.3: Effects of four fungicides on AUDPC, sugar beet yield, and recovered sucrose yield in sugar beet fields artificially inoculated with *C. beticola* in Foxhome, Minnesota in 2018 and 2019.

Treatment[a]	AUDPC[b]	Yield (ton/ha)	Sucrose (kg/ha)
Inspire Super	200 c	67.7 b	7915 bc
Omega 500F	240 b	63.2 b	7332 c
Echo 720	193 c	66.9 b	8001 b
Super Tin	166 d	78 a	10385 a
Nontreated check	286 a	56.6 c	6368 d
LSD (*P*=0.05)[c]	26	5.4	1610

[a]Fungicide sprays were applied four times during the growing season in each year; the four fungicides were Inspire super® (cyprodinil, a.i. 24.1%, difenoconazole a.i. 8.4%; Syngenta, Greensboro, NC) at 493 g a.i. ha^{-1}, Omega 500F® (fluazinam, a.i. 40%; Syngenta, Greensboro, NC) at 504 g a.i. ha^{-1}, Echo 720® (chlorothalonil a.i. 54%; Sipcam, Roswell, GA) at 320 g a.i. ha^{-1}, and Super Tin® (Triphenyltin hydroxide, a.i. 40%; United Phosphorus; King of Prussia, PA) at 113 g a.i. ha^{-1}.

[b]Areas under disease progress curve.

[c]The means of treatments with the same letter were not significantly different according to Least Significant Difference (LSD) at $P = 0.05$.

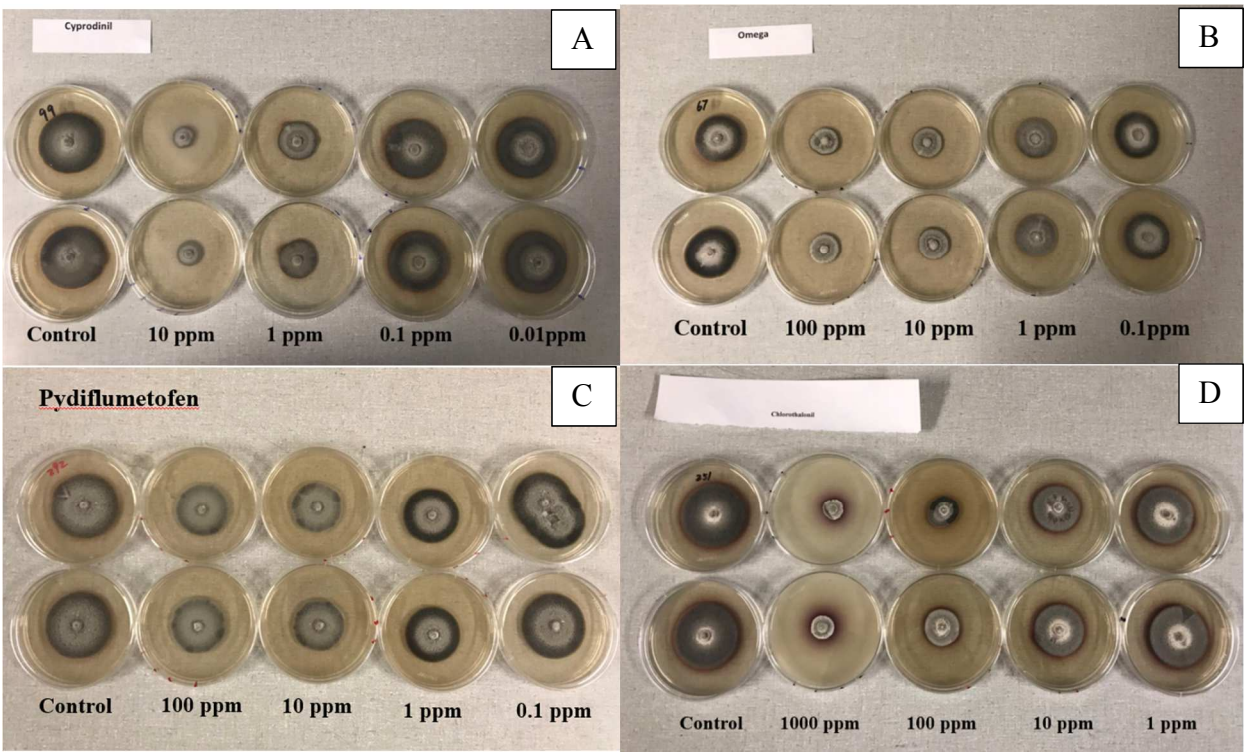

Figure 4.1: Mycelial radial growth of *Cercospora beticola* isolates at different concentrations of A: cyprodinil; B: Omega; C: pydiflumetofen; D: Chlorothalonil. Media was not amended in the controls. Other media were amended with different concentrations where the unit 'ppm' (parts per million) is equal to the unit 'ug/ml'.

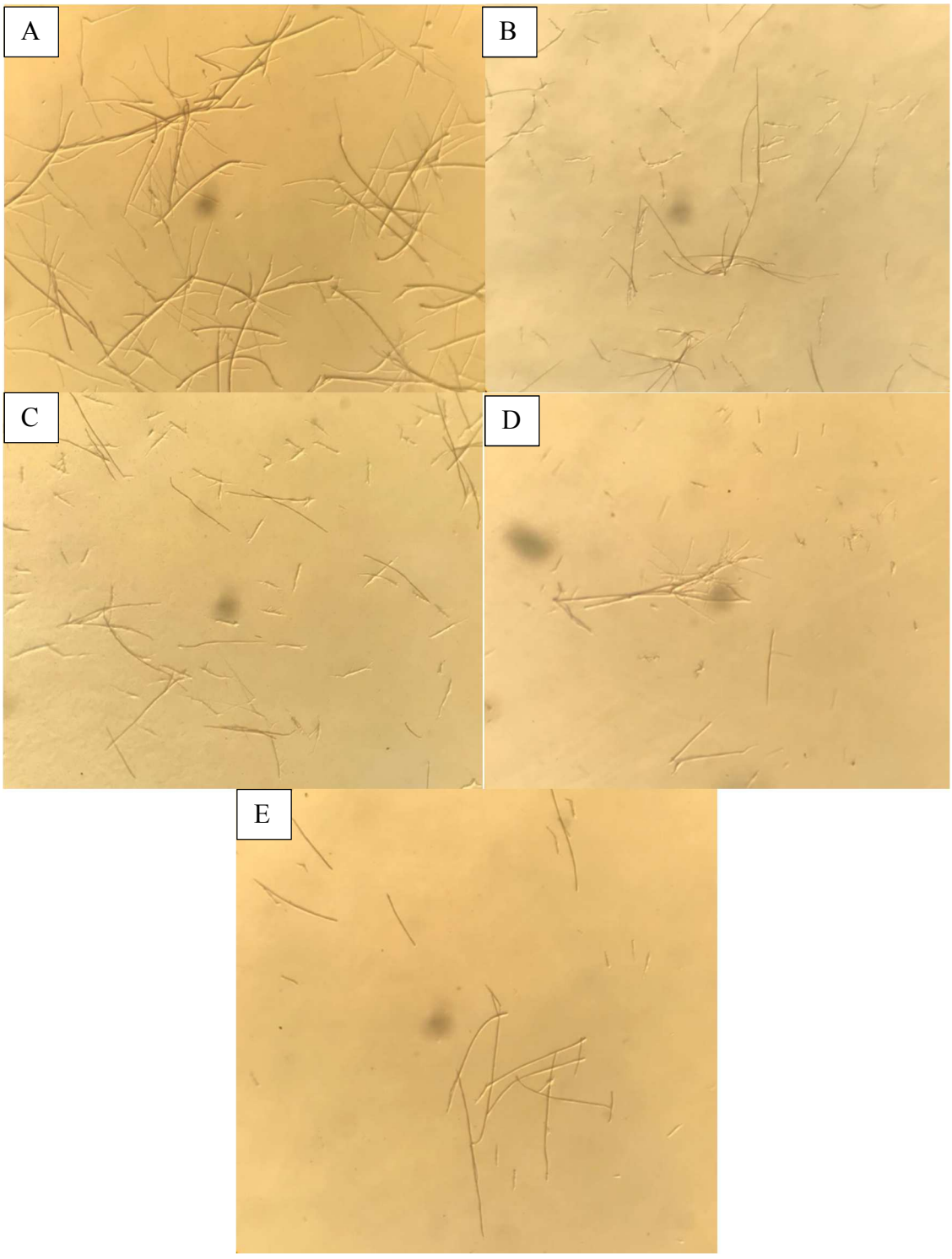

Figure 4.2: Microscopic comparison of *C. beticola* conidial germination on different water agar media after 16 hours of incubation at room temperatures without light. Microscopic photos of conidia spores were taken from water agar media with A: none of chemical products; B: cyprodinil at 10 µg/ml; C: fluazinam at 10 µg/ml; D: pydiflumetofen at 10 µg/ml; E: chlorothalonil at 10 µg/ml.

4.4. Discussion

In this research, we evaluated sensitivity of *C. beticola* isolates to different fungicides and their field efficacy to find new products for managing fungicide resistant *C. beticola* populations. Each of the tested chemicals possesses a unique mode of action (FRAC, 2020) and has never been used to control *C. beticola* or other pathogens by the sugar beet industry in North Dakota and Minnesota (Secor et al., 2010). It is practical to use resistant *C. beticola* population to test fungicide sensitivity and efficacy since the field isolates resistant to QoIs or DMIs are still prevalent according to Secor et al. (2019). The inocula used in the field study were collected from fields near Foxhome, Minnesota. These fields had known fungicide resistance issues and multiple resistances were confirmed in the laboratory (Secor et al., 2019).

In vitro study demonstrated that *C. beticola* had a different response to cyprodinil, fluazinam, pydiflumetofen and chlorothalonil using different inhibition methods. Cyprodinil inhibited both mycelial growth and spore germination at a similar concentration, while the spore germination assay was nearly 5, 10, and 300 times more sensitive than the mycelial assay for fluazinam, pydiflumetofen, and chlorothalonil, respectively. The protectant fungicides, such as copper-based chemicals, are more effective at inhibiting spore germination, while the systemic fungicides, such as DMIs, have highly inhibitive effects on mycelial growth. For example, Vega and Dewdney (2015) reported that a DMI chemical fluopyram had higher inhibitive effects on mycelial growth rather than conidial germination for a fungal pathogen *Alternaria alternata*. Another explanation is due to a fundamental difference in metabolic levels between active mycelia and dormant conidial spores (Gougouli and Koutsoumanis 2013; Liu et al., 2015). In our study, mycelial plug was cut from actively growing part of a colony, while spore suspension was

prepared from sporulation plates that requires to be incubated under light and dry conditions for several days.

Cyprodinil is a commercial pyrimidine fungicide that inhibits synthesis of amino acids in fungal pathogens of ascomycetes and basidiomycetes (Sun et al., 2011). This fungicide has been used to control *Botrytis cinerea* in different crop systems, and some studies showed that cyprodinil had little effect on spore germination but strongly inhibit the fungal growth of *B. cinerea* (Chatzidimopoulos et al., 2013; Fernández-Ortuño et al., 2013; Myresiotis et al., 2007). In our study, the mean EC_{50} values for cyprodinil from mycelial growth and spore germination assays were similar at 4.95 and 5.65 µg/ml, respectively. Moreover, both *C. beticola* and *B. cinerea* have shared the same discriminating rate at 1 µg/ml for QoI fungicides (Ishii et al., 2009; Secor et al., 2010). Myresiotis et al., (2007) suggested that the discriminating rate for *B. cinerea* being resistant against cyprodinil is 1 µg/ml using mycelial inhibition assay, indicating that cyprodinil (EC_{50} values>1 µg/ml) might not be effective at inhibiting *C. beticola*.

Fluazinam is a multi-site respiration inhibitor with an uncoupling activity of mitochondrial oxidative phosphorylation (Guo et al., 1991). This fungicide was effective for controlling pathogenic fungal pathogens including Plasmodiophora species, Phytophthora species, ascomycetes, and basidiomycetes (product label: https://www.syngenta-us.com/currentlabel/omega_500f). The sensitivity of *S. sclerotiorum* to fluazinam was determined by mycelial growth and spore germination with a discriminating dose (<0.1 µg/ml) (Lehner et al., 2017). In in vitro study, *C. beticola* was not sensitive to fluazinam with a high EC_{50} value at 33.10 and 9.15 µg/ml by mycelial growth and spore germination assay, respectively.

Pydiflumetofen, a novel succinate dehydrogenase inhibitor (Buxton et al., 2016), is labeled to control foliar diseases caused by ascomycetes including *Cercospora arachidicola* on peanut (product label: https://www.syngenta-us.com/current-label/miravis). In our study, *C. beticola* was not as sensitive to pydiflumetofen as other pathogens. The mean EC_{50} values for *C. beticola* were 99.4 and 9.2 µg/ml using mycelial and spore germination inhibition methods, respectively, and much higher than where the product is effective. Using mycelial inhibition method, *Cercospora zeae* from corn had mean EC_{50} values 0.004 µg/ml and *Fusarium asiaticum* from wheat had 0.0745 µg/ml for pydiflumetofen. Using spore germination inhibition method, the mean EC_{50} values was 0.1813 µg/ml for *F. asiaticum* (Hou et al., 2017).

Chlorothalonil is multiple-site inhibitor that has been used to control foliar diseases caused by Cercospora species, Alternaria species, and rust in different patho-systems (Bounds and Hausbeck, 2007; Holm et al., 2003) (product label: http://www.cdms.net/ldat/ld976009.pdf). Our study showed that chlorothalonil was effective at inhibiting 50% of germination of *C. beticola* conidial spores at 2.43 µg/ml. Site-specific fungicides need to have EC_{50} values less than 1 µg/ml to be considered economical and effective to use as fungicides. Most studies indicate when the EC_{50} value is more than 1 µg/ml, the chemical product become ineffective or have resistantce issues (Lehner et al., 2017; Myresiotis et al., 2017; Secor et al., 2010). However, it is different for multi-site fungicides. For example, *Alternaria solani* was still considered to be sensitive to chlorothalonil with the EC_{50} values being between 1 and 10 µg/ml (Holm et al., 2003). In our study, the low EC_{50} values (2.43 µg/ml) might indicate that chlorothalonil has the potential to effectively control *C. beticola* as was confirmed by the field study.

Cyprodinil, fluazinam, and Chlorothalonil were tested as commercial products in 2018 and 2019. In fact, pydiflumetofen was tested as Miravis Top® in 2018 field study, but the

representative from the chemical company was not interested in pursuing a registration of this product for control of *C. beticola* due to the unsatisfactory results. In the field study, cyprodinil, fluazinam, and chlorothalonil, with significantly better results than the nontreated check, failed to provide similar performances in controlling CLS, beet tonnage, and recovered sucrose compared to the industry's standard fungicide treatment, triphenyltin hydroxide. Chlorothalonil might have the potential to provide effective disease control based on its field performance and low EC_{50} values, but was not effective as the industry's standard, triphenyltin hydroxide. All the fungicides should be used judiciously, only when necessary, and according to their labels so as to preserve our wildlife, waters, and environments. It would be useful to further evaluate potential products in fungicide mixtures with different site-specific or multi-site fungicdes to achieve effective CLS control and manage *C. beticola* field population resistant to site-specific fungicides.

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APPENDIX A. THE DETAILS OF FUNGICIDE RESISTANT *CERCOSPORA BETICOLA* FIELD ISOLATES USED IN DIFFERENT RESEARCHES

No. Isolate	*C. beticola* isolates esistance to	QoIs (G143A point mutation)	DMIs			Chapter 2 study	Chapter 3 study	Chapter 4 study
			Difenoconazole	Prothioconazole	Tetraconazole			
17-67	QoI and DMI	R	1.93	>10	>10	✓	✓	
17-90	QoI and DMI	R	10	>10	>10	✓	✓	
17-99	QoI	R	0.042	0.4911	0.287	✓	✓	
17-101	QoI	R	0.025	0.6813	0.381	✓	✓	
17-123	DMI	S/r	2.837	3.902	10	✓		
17-133	QoI and DMI	R	3.572	>10	>10	✓	✓	
17-148	QoI and DMI	R	2.837	8.1548	>10	✓	✓	
17-149	QoI	R	0.023	0.2438	0.106	✓	✓	
17-150	QoI and DMI	R	2.075	1.3892	6.292	✓	✓	
17-151	QoI	R	0.049	0.3765	0.166	✓	✓	
17-157	QoI	R	0.393	0.3005	0.184	✓	✓	✓
17-164	DMI	R/S	1.408	2.3028	7.943	✓	✓	
17-168	QoI	R	0.044	0.2438	0.201	✓	✓	
17-169	DMI	S	1.664	3.1623	6.813	✓	✓	
17-179	QoI and DMI	R	10	7.8117	>10	✓	✓	✓
17-180	QoI	R	0.034	0.1497	0.1	✓	✓	
17-183	QoI	R	0.034	0.1487	0.13	✓	✓	✓
17-189	DMI	S/r	1.389	1.2925	2.88	✓		
17-191	QoI and DMI	R	5.374	2.9832	>10	✓	✓	
17-192	DMI	S	2.248	>10	>10	✓	✓	✓
17-193	QoI	R	0.031	0.0082	0.224	✓	✓	✓
17-197	DMI	S/r	4.497	6.2182	10	✓	✓	
17-200	QoI and DMI	R	1.389	1.9664	6.904	✓	✓	
17-207	DMI	S/r	2.098	1.9664	5.133	✓	✓	
17-211	QoI	R	0.041	0.0091	0.189	✓	✓	✓
17-212	QoI	R	0.032	0.3581	0.14	✓	✓	
17-217	DMI	R/S	1.296	3.8306	7.499	✓	✓	
17-219	QoI and DMI	R	3.162	>10	>10	✓	✓	
17-220	QoI and DMI	R	2.512	10	5.18	✓	✓	
17-231	QoI and DMI	R	1.958	1.2842	3.902	✓		
17-234	QoI and DMI	R	2.081	1.8962	1.389	✓		
17-240	DMI	R/S	2.081	1.2339	2.837	✓	✓	
17-244	QoI and DMI	R	1.832	2.1613	4.152	✓		✓
17-256	QoI and DMI	R	1.638	1.0938	4.988	✓	✓	✓

APPENDIX A. THE DETAILS OF FUNGICIDE RESISTANT *CERCOSPORA BETICOLA* FIELD ISOLATES USED IN DIFFERENT RESEARCHES

No. Isolate	*C. beticola* isolates esistance to	QoIs (G143A point mutation)	DMIs			Chapter 2 study	Chapter 3 study	Chapter 4 study
			Difenoconazole	Prothioconazole	Tetraconazole			
17-257	QoI and DMI	R	2.382	3.902	4.236	✓	✓	
17-258	DMI	S/r	10	>10	>10	✓	✓	✓
17-277	QoI and DMI	R	1.556	1.1058	4.497	✓	✓	
17-282	QoI and DMI	R	>10	>10	>10	✓	✓	
17-284	DMI	R/S	>10	6.2182	>10	✓		
17-286	QoI	R	0.063	0.6218	0.141	✓		
17-287	QoI and DMI	R	1.292	10	>10	✓	✓	✓
17-288	DMI	S/r	1.577	>10	>10	✓	✓	
17-290	QoI and DMI	R	>10	>10	>10	✓	✓	
17-292	DMI	S	5.133	1.2052	3.162	✓	✓	✓
17-297	QoI	R	0.023	0.2049	0.1	✓	✓	
17-300	DMI	S	>10	>10	>10	✓	✓	✓
16-33	DMI	S	>10	>10	>10	✓	✓	
16-41	None	S	3.15/33.3	4.25/33.3	5.4/33.3			✓
16-65	None	S	0.5/33.6	2.85/33.6	2.4/33.6			✓
98-25	None	S	0/36.6	0/36.6	0/36.6			✓
98-46	None	S	0/37.6	0.6/37.6	0/37.6			✓
Total isolates used						47	40	16

[a]46 C. beticola isolates were collected from Foxhome field, Minnesota in 2017. Dr. Secor from North Dakota State University, Fargo, North Dakota provided five isolates as follows: 16-33, 16-41, 16-65, 98-25, and 98-46; these isolates were collected from factory district areas of sugar beet crop in North Dakota and Minnesota.
[b]the resistant to QoIs was tested using a PCR-based molecular procedure to test the presence of G143A point mutation in isolates. The results indicated as follows: R: all spores with G143A mutation; R/s: >50% of spores with G143A mutation; S/R: equal numbers of spores with G143A mutation; S/r: <50% spores with G143A mutation; S: No spores with G143A mutation. The isolates tested to have >50% spores with the mutation were considered as QoI-resistant ones.
[c]the DMIs sensitivity (difenoconazole, prothioconazole, and tetraconazole) was evaluated using a radial growth procedure; the discriminate rate for *C. beticola* isolate resistant to DMIs was 1 ug/ml.
[d]the tick symbol indicates that the isolate was tested in research.

APPENDIX B. STATISTICAL ANALYSIS SUMMARY FOR FITNESS TRAITS OF *CERCOSPORA BETICOLA* ISOLATES IN DIFFERENT FUNGICIDE RESISTANT GROUPS

Table B.1: Combined analysis of variance for radial growth from *Cercospora beticola* isolates in fungicide resistance groups.

Source of variation	DF	Mean square	*P*
Expt	1	0.96	0.7561
Rep(Expt)	4	9.81	0.4164
Resistant group	3	7.07	0.0003
Expt x Resistant group	3	0.03	0.9937
Error	84	9.88	
Total	95		

Table B.2: Combined analysis of variance for spore production from *Cercospora beticola* isolates in fungicide resistance groups.

Source of variation	DF	Mean square	*P*
Expt	1	26.04	0.7366
Rep(Expt)	4	7.08	0.9981
Resistant group	3	9223.34	<0.001
Expt x Resistant group	3	25.91	0.9521
Error	84	228.51	
Total	95		

Table B.3: Combined analysis of variance for spore germination from *Cercospora beticola* isolates in fungicide resistance groups.

Source of variation	DF	Mean square	*P*
Expt	1	0.26	0.1715
Rep(Expt)	4	0.1	0.5537
Resistant group	3	0.21	0.2215
Expt x Resistant group	3	1.27	<0.0001
Error	84	228.51	
Total	95		