CHARACTERIZING THE DIVERSITY OF FUNGICIDE RESISTANCE IN *CERCOSPORA BETICOLA* ON SUGARBEET IN MICHIGAN AND ONTARIO

By

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ABSTRACT

CHARACTERIZING THE DIVERSITY OF FUNGICIDE RESISTANCE IN *CERCOSPORA BETICOLA* ON SUGARBEET IN MICHIGAN AND ONTARIO

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Cercospora leaf spot (CLS), caused by *Cercospora beticola* (Sacc.), is the most serious foliar disease of sugarbeet (*Beta vulgaris* L.) worldwide. Timely fungicide application is one of the most effective tools used to manage this disease. However, this strategy has been less effective because resistance has been reported in *C. beticola* to several classes of fungicides including benzimidazoles, organotin and demethylation inhibitors (DMIs). Thus knowledge of the interaction of *C. beticola* in Michigan with various fungicides is essential for making management decisions. A study has been conducted to survey the Great Lakes sugarbeet growing region for *C. beticola* isolates with sensitivity to various fungicides in use in the area. Results showed that most *C. beticola* isolates (>85%) tested were sensitive to DMI and organotin fungicides during the study (from 2012 to 2014) with EC_{50} values of less than 1 and 5 ppm respectively. Monitoring for the mutations in commercial fields indicated that resistant mutations were widespread in both Michigan and Ontario sugarbeet production areas. This data will direct the development of effective recommendations specific to production areas.

Dedicated to my beloved parents Yongzhong Jiang and Yunjian Qian;
My husband Yiqun Yang;
For their endless love, support, encouragement and sacrifices.

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Introduction to the crop - Sugarbeet

Beet (*Beta vulgaris*) has been cultivated along the seacoasts of the Mediterranean as a garden vegetable since the first century BC (Rougemont, 1989). At that time they were grown mainly for their leafy greens called “chard” (Winner, 1993). The oldest known beet-type, chard, was grown by both Ancient Greeks and Romans, but the regular cultivation of beets as leafy vegetables did not occur until the seventeenth century in Europe (Winner, 1993). The differentiation of several varieties of the root type *B. vulgaris*, distinct in form, color, and sugar content of root took place in the seventeenth and eighteenth centuries (Winner, 1993). However, the development of beet into an industrial crop grown for sugar production did not start until the second half of the eighteenth century (Winner, 1993). In 1747, Andreas Marggraf, a German chemist, demonstrated that, after a crude extraction from pulverized beetroots, the resultant crystals were indistinguishable from sugarcane crystals (Francis, 2007). This discovery was a starting point of the sugarbeet industry in Europe. Marggraf also foresaw the economic impact of that discovery, but he did not pursue it. Later, Marggraf’s student, Franz Carl Achard, invested further in the crop and developed processing methods for sugar extraction. His success and efforts established beets as an economic resource for sucrose production (Francis, 2007).

Sugarbeet (*Beta vulgaris L.*) belong to the *Amaranthaceae* family of plants. It is a relatively new crop derived from many years of breeding of domestic beets for sugar production (Bosemark, 2007). Sugarbeet is a biennial plant. The first year is a vegetative

phase, producing a circular cluster of radiating leaves and swollen roots. In the second year, the energy stored in the roots is used by plants to produce flowers and seeds after a period of cold vernalization (Elliott and Weston, 1993). Van de Sande Bakhuyzen (1949) distinguished three vegetative developmental stages of sugarbeet: a phase of leaf formation, a phase of root formation and a phase of sugar storage. The early growth of sugarbeet is dominated by the development of leaves (Reviewed by Milford, 1973).

In the United States, sugarbeet is grown in 11 states which can be grouped into three regions: the Upper Midwest (Michigan, Minnesota and North Dakota), the Great Plains (Colorado, Montana, Nebraska and Wyoming) and the Far West (California, Idaho, Oregon, and Washington). In most temperate areas, sugarbeets are planted in the spring and harvested in the late autumn. In climatic regions such as California's Imperial Valley, sugarbeets are planted as a winter crop in the fall and harvested in the spring (Bassam, 2010).

In Michigan, the long summer days and the influence of the Great Lakes are satisfactory climatic conditions for sugar beet production, more than 1,000 farmers grow sugarbeets on over 160,000 acres of land in the Saginaw Bay-Thumb area (Willie Kirk, personal communication). Beets are usually planted in mid-April and harvested from late September to November (NASS, 2010). During harvest, beet roots are lifted and delivered for processing. In Michigan, outdoor piling systems are used to store roots arriving at processing factories during concentrated harvest season (Fugate and Campbell, 2009). Fluctuating temperature, microbial activity, wind, rain, and the sun can negatively impact the quality and health of sugarbeet roots, increasing the respiration rate and building up

non-sucrose impurities, causing a reduction in the sugar yield (Martin, 2001, Fugate and Campbell, 2009).

Commercial sugarbeet fields usually yield about 50 to 100 t of sugarbeet/ha, with up to 17 to 18% sugar concentration in fresh weight, yielding 8 to 18 t sugar/ha (Milford, 2006). In 2013, Russia, France and the USA ranked 1st, 2nd and 3rd in the world for sugarbeet production respectively, followed by Germany and Turkey (Statista, 2013). In the same year, Michigan produced approximately 4,009,000 t of sugarbeet and ranked 4th in the country for production with a value of $321,239,000 (NASS, 2013).

Sugarbeet plants are vulnerable to many plant pathogens (Duffus and Ruppel, 1993; Franc et al., 2001). Some pathogens are present in the soil in various survival structures and can initiate diseases from infested soil (Windels et al., 2009; Hanson and Jacobsen 2009). Many diseases caused by soil-borne pathogens are difficult to predict, diagnose and monitor (Windels et al., 2009; Windels and Harveson 2009; Hanson and Jacobsen 2009). In addition to this, the natural soil environment is very complex, making it a challenge to understand all aspects of diseases caused by soil-borne pathogens (Franc et al., 2001). Typical symptoms of soil-borne diseases include root and crown rots, damping-off of seedlings, and vascular wilts (Leach et al., 1986; Windels and Harveson 2009). For example, *Rhizoctonia solani, Pythium* spp. and *Aphanomyces cochlioides* are most important soil-borne pathogens to cause seed rot, pre-emergence damping-off and post-emergence damping-off (Leach et al., 1986; Harveson 2009; Windels et al., 1989). They also are known to cause both seedling and root rot diseases (Campbell, 2006; Windels et al., 1989). Other root diseases caused by soil-borne pathogens include Fusarium yellows (*Fusarium oxysporum*) (Hanson et al., 2006), Fusarium root rot

(*Fusarium oxysporum* f. sp. *radices-betae*) and Rhizopus root rot (Harveson 2009). The latter two cause root rot during the later developmental stages of sugarbeet, infecting roots and crowns and leading to direct loss of yield (Harveson 2009). Management of these pathogens include disease resistant varieties, fungicide applications, avoidance of stress, and modifying the environment such as crop rotation, weed control, proper fertility management and water usage (Franc et al., 2001).

Beet leaves can be attacked by foliar pathogens (Shane and Teng, 1992; Franc, 2009). Pathogens that attack sugarbeet leaves include *Cercospora beticola* (Pool and McKay 1916b, Shane and Teng, 1992), *Alternaria* spp. (Franc, 2009), *Phoma betae* (Harveson, 2009), *Ramularia beticola* (Byford, 1975), *Erysiphe polygoni* (Hanson 2009), and *Pseudomonas syringe* (Koike, 2003). Among them, *C. beticola* is the most devastating foliar pathogen of sugarbeet worldwide (Jacobsen and Franc 2009). Because of its worldwide distribution, successful management of Cercospora leaf spot (CLS) requires a broad range of skill sets and an annual investment of resources to ensure economic crop production.

Biology of *Cercospora beticola*

Cercospora leaf spot (CLS), caused by *Cercospora beticola* (Sacc.) (Figure 1.2) is one of the most widespread and damaging foliar diseases of sugarbeet (*Beta vulgaris* L.) worldwide (Jacobsen and Franc, 2009). Under favorable environmental conditions, the pathogen can cause epidemics leading to significant yield reduction (Shane and Teng 1992). Crop losses can be as high as 40% or greater (Jacobsen and Franc 2009). Moderate and high incidences of CLS adversely affects about 33% of sugarbeet

production areas worldwide, although disease severity and incidence differ among geographical regions (Holtschulte 2000).

Lesions of CLS appear as individual, circular spots first on older leaves (Jacobsen and Franc 2009; Figure 1.1). They are usually 3 to 5 mm in diameter, light brown in color with a dark brown to purple border and are randomly distributed on the surface of leaves (Steinkamp et al. 1979, Ruppel 1986, Jacobsen and Franc 2009). As the disease develops, lesions expand, increase in number and coalesce. Elongated lesions may be found on petioles (Franc, 2010; Figure 1.1). Black fungal structures called pseudostromata are formed in the center of mature lesions (Franc, 2010; Figure 1.1). They are important diagnostic character of this disease. As CLS progresses, blighted leaves collapse and fall off the ground (Jacobsen and Franc, 2009; Figure 1.2). Phytotoxins produced by the fungus promote lesion formation and cause yellowing and rapid death of leaves (Steinkamp et al. 1981). Economic losses due to decrement in both yield and percentage of sucrose in harvested roots can approach 40% or more (Jacobsen and Franc, 2009).

The optimal temperature range for disease development is between 25 to 35ºC with relative humidity greater than 90% or free moisture on the leaf surfaces (Jacobsen and Franc 2009). The activity of *C. beticola* is greatly reduced below 15°C, with no sporulation or infection occurring at 10°C (Jacobsen and Franc 2009). For high temperatures, Pool and McKay (1916b) showed that exposure to an average of 45.5°C for three days was fatal to isolates of *C. beticola* in pure culture.

To date, no teleomorph of *C. beticola* has been found (Jacobsen and Franc, 2009; Bolton et al., 2012b; Groenewald 2006; Goodwin et al., 2001). However, results from

Europe by Groenewald (2006) have indicated an equal distribution of the mating type loci in *C. beticola* populations as well as high level of genetic variation within and between populations. This indicates that sexual recombination is likely in this fungus. Phylogenetic analysis using the internal transcribed spacer region of ribosomal DNA has identified several Cercospora species as a well-defined clade within the sexually reproducing fungus Mycosphaerella (Goodwin et al., 2001; Crous et al., 2001). Therefore if a sexual stage does exist in *C. beticola*, it is likely to be a form of *Mycosphaerella*. Additionally, high levels of genetic variation were found among isolates from the same lesion on the same sugarbeet plants in Italy (Moretti et al. 2004, 2006). The high levels of genetic variation observed in *C. beticola* suggests the existence of a means to promote genetic exchange. In the United States, equal distribution of mating type loci was reported in the Red River Valley (Bolton et al., 2012b), in agreement with what was found in Europe. However, there are areas where an uneven distribution of mating type loci have been observed indicating a less common potential sexual cycle (Bakhshi et al., 2011). Sexual recombination plays a significant role in the biology and evolution of fungal species, more studies are needed to understand the prevalence of sex in *Cercospora beticola* and allied species (Goodwin et al., 2001).

Figure 1.1. Symptoms of Cercospora leaf spot on sugarbeet leaves. A) Cercospora leaf spot lesions (arrow) caused by *Cercospora beticola* are randomly distributed, circular with dark brown to red borders. B) Sugarbeet leaf showing coalescing of Cercospora leaf spot lesions. C) Sugarbeet petiole showing elongated, rather than circular, lesions caused by *Cercospora beticola*. D) Small dark fungal structures called pseudostromata (arrow) formed in the center of a mature Cercospora leaf spot lesion.

Figure 1.2. Field view of Cercospora leaf spot caused by *Cercospora beticola* showing severe damage. Blighted leaves collapse and fall to the ground, new leaf growth can be seen emerging from the plant surrounded by collapsed leaves. (Courtesy Dr. Linda Hanson)

Disease Cycle and Epidemiology

Cercospora leaf spot follows a typical polycyclic development characterized by repeating cycles of conidial germination and infection, followed by sporulation, conidial detachment, and dissemination of conidia to host plant surfaces (Meredith, 1967; Figure 1.3). Pseudostromata serve as resting structures and primarily inoculum, conidiophores originate from pseudostromata and emerge through host stomates (Pool and McKay, 1916b). When environmental conditions are favorable for *C. beticola* to initiate disease (above), conidia are formed and released from the pseudostromata in plant debris and soil organic matter by rain, wind or irrigation water-splash (Meredith, 1967; Pool and McKay, 1916b). Conidia land on sugarbeet leaves, where they germinate and enter the

leaf through stomata (Rathaiah, 1977). A period of leaf wetness during the evening followed by a daytime drying cycle promotes infection of foliage by *C. beticola* (Franc, 2010). Foliar symptoms have been reported to appear as early as five days after infection (Rathaiah 1977，Steinkamp et al., 1979). Detectable lesions generally appear in the field after 11 to 13 days post inoculation, disease progress is slower for vareties with some degree of genetic resistance (Pool and McKay 1916b; Bosemark 2007).

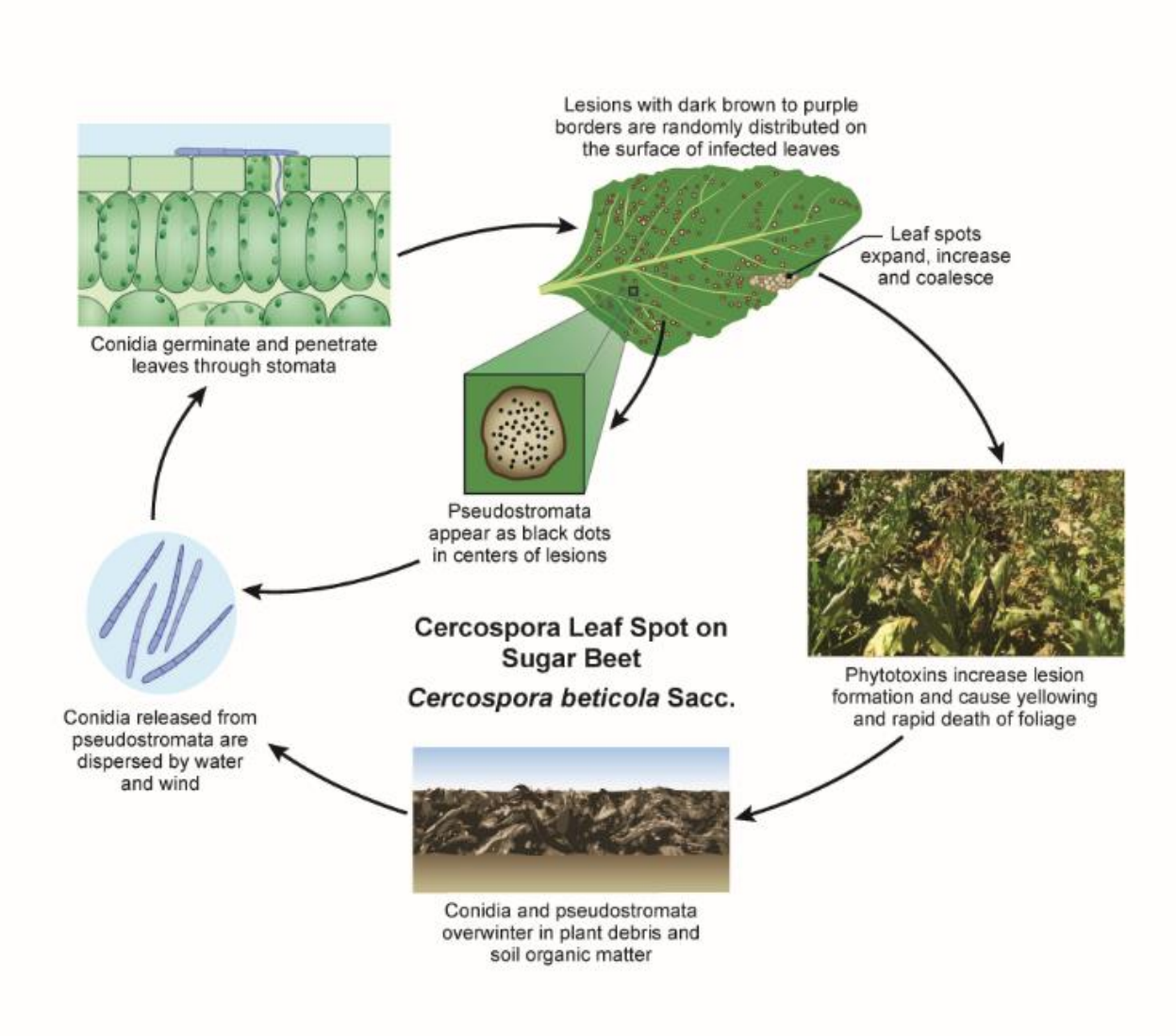

Figure 1.3. Life cycle of *Cercospora beticola*, the causal agent of Cercospora leaf spot on sugarbeet (Hanson et al., unpublished; illustration by Marlene Cameron).

Management of Cercospora leaf spot of sugarbeet

Cercospora leaf spot is a disease of concern in many sugarbeet production areas in the United States (Jacobsen and Franc, 2009). Sustainable management of CLS requires an integrated approach. Most of the strategies used are based on the foundation of reducing inoculum levels, such as removal of weed hosts, crop rotation and tillage (Smith and Gaskill 1970; Windels et al., 1998). Rotation with crops not susceptible to *C. beticola* can help reduce the level of initial inoculum in the soil (Jacobsen and Franc 2009). Crop rotations of 2-3 years or more are usually recommended (Jacobsen and Franc 2009). Incorporating leaf debris into the soil by deep plowing contribute to the reduction of initial inoculum, allowing for the decay and destruction of infected leaf residues in the soil (Windels et al., 1998; Jacobsen and Franc 2009). Additionally, the presence of resistant varieties provides a physical barrier against spore dispersal, interrupting spore movement among canopy (Bosemark, 2007)

In addition to these disease management strategies, fungicide applications continue to be one of the primary tools in CLS management in many areas of the world (Ioannidis and Karaoglanidis, 2010; Jacobsen and Franc, 2009; Wolf and Verreet, 2002). Unfortunately, the development of fungicide resistance in *C. beticola* to several commonly used fungicides is a concern for the sugarbeet industry (Weiland and Halloin, 2001; Kirk et al., 2012; Birla et al., 2012). To ensure an effective spray and delay the development of fungicide resistance, the proper fungicides need to be selected and applied at the right time of the year and frequency to protect the plant (Jacobsen 2010). To date, several models based on environmental factors and yield loss threshold parameters have been developed to help growers and producers predict the occurrence of CLS (Shane and

Teng 1984; Windels et al., 1998; Wolf and Verreet 2002). Most of these models are based on weather conditions that favor infection and sporulation of *C. beticola* (Shane and Teng 1984; Windels et al., 1998; Khan et al., 2007). Several models incorporate host resistance into CLS prediction algorithms to enhance successful prediction (Wolf and Verreet 2002; Racca and JÖrg 2007). In the Great Lakes beet production regions, the timing of fungicide applications is typically based on a weather-driven model called the BEETcast™ model (Weather Innovations, Chatham, ON, Canada). This model utilizes leaf wetness and air temperature to calculate disease severity values (DSV) on a scale of 0-4 per day. Zero DSV indicates that environmental conditions are not favorable for CLS development on that day (eg., leaves are too dry, or temperature is too cold for infection). The value of four indicates that environmental conditions are highly likely for *C. beticola* to initiate disease. In this model, DSV are accumulated until a threshold is reached, which is often used to indicate that an application of an effective fungicide is needed (Windels, 2010; Wishowski et al., 2006). Then the DSV calculation re-starts at 0 and the next application of fungicides is recommended based on the next interval of cumulative DSVs (Windels, 2010; Wishowski et al., 2006).

Fungicide resistance

Benzimidazole fungicides

Benzimidazole fungicides have been used to manage plant diseases since the 1960's (Smith, 1988; Brent and Hollomon, 2007). Since then, they have been used as foliar fungicides, seed treatments and postharvest applications (Smith, 1988). Benzimidazole fungicides were among the first commercially available fungicides for plant disease control, which provided both systemic and curative activity against many fungal

plant pathogens in agriculture (Smith, 1988). They were also the first systemic fungicides available for CLS control (Solel, 1970).

Fungicides in the benzimidazole class act by binding fungal ß-tubulin, which interferes with mitosis and disrupts the integrity of the fungal cytoskeleton (Davidse,1986). Their unique fungicidal properties such as low-use rate and systemic activity made them very attractive to growers when they were released (Davidse,1986; Smith, 1988). The use of the benzimidazole fungicides in agriculture, including benomyl (Benlate®) and thiophanate methyl (Topsin M®), was widespread during the late 1960s. The widespread and intensive use of benzimidazole fungicides led to an immense selection pressure on many fungi, leading to the failure of disease control in the field (Albertini et al., 1999; Georgopoulos and Dovas, 1973). The mutagenicity of benzimidazole derivatives has been demonstrated in several systems (Dassenoy and Meyer, 1973; Seiler 1972).

After years of intensive use, resistance was identified in a number of pathogens including *Podosphaera fusca* (Schroeder and Provvidenti, 1969), *Botrytis cinerea* (Bollen and Scholten, 1971), *Cercospora beticola* (Georgopoulos and Dovas, 1973) and *Cercospora arachidicola* (Littrell, 1974), among many others.

Resistance to benzimidazole fungicides in *C. beticola* has been documented in many geographic regions including Greece (Georgopoulos and Dovas, 1973), Italy (D'Ambra et al., 1974 , Karaoglanidis and Ioannidis, 2010), Japan (Uesugi, 1978 as reviewed by Karaoglanidis and Ioannidis, 2010), India (Pal and Mukhopadhyay, 1983), and states of the United States such as Texas (Ruppel and Scott 1974), Arizona (Ruppel, 1980), Minnesota (Weiland and Smith, 1999), North Dakota (Weiland and Smith, 1999) and Michigan (Weiland and Halloin, 2001). The finding of benzimidazole resistance in the

field on various fungi led to extensive monitoring programs to determine the resistance mechanism in plant pathogens (Ma et al., 2003b; Albertini et al., 1999).

Resistance to benzimidazole fungicides is primarily related to specific alterations in the binding sites on the ß-tubulin protein (Davidse 1986). Many mutations in the fungal ß-tubulin gene conferring resistance to benzimidazole fungicides have been identified (Cooley and Caten, 1993; Albertini et al., 1999; Ma et al., 2003b). Results from previous studies have shown that mutations at codons such as 6, 50, 167, 198, or 200 in the ß-tubulin gene of many plant pathogenic fungi can lead to different levels of benzimidazole resistance (Luck and Gillings, 1995; Koenraadt et al., 1992; Ma et al., 2003b; Albertini et al., 1999; McKay et al, 1998; Gafur et al., 1998). The most frequently reported resistance mutations occur at codon 198 (Albertini et al., 1999; Koenraadt et al., 1992). These mutations lead to change of the amino acid in the β-tubulin protein from glutamic acid (Glu) to other amino acids such as alanine (Ala), glycine (Gly), lysine (Lys) or glutamine (Gln) (Koenraadt et al., 1992; Luck and Gillings, 1995; Fujimura, et. al., 1992). All of these mutations can confer resistance, but with varying characteristics, such as different relative sensitivity toward benzimidazole fungicides (Albertini et al., 1999).

In the United States only a single mutation in the ß-tubulin gene has been found in field isolates of *C. beticola* collected in different years and geographic regions (Davidson et al., 2006). This mutation was predicted to lead to an amino acid change from glutamic acid (Glu) to alanine (Ala) at codon 198 (E198A) in the ß-tubulin gene that results in a high-level of benzimidazole resistance (Davidson et al., 2006). Recently in Serbia, a point mutation at codon 167 result in an amino acid change from phenylalanine (TTC) to tyrosine (TAC) was discovered in low-level and moderate level benzimidazole-resistant

C. beticola isolates (Trkulja et al., 2013). Other mutations have been found in other fungi that lead to varying levels of resistance, for example, a point mutation at codon 6 which alters histidine (CAT) to tyrosine (TAT) has been identified as responsible for a low-level of benzimidazole resistance in field isolates of *Monilinia fructicola* (Ma et al., 2003b). A point mutation at codon 200 from phenylalanine (TTC) to tyrosine (TAC) resulted in a moderate-level of benzimidazole resistance in field isolates of *Botrytis cinerea* (Yarden et al. 1993). A list of mutations in the β-tubulin gene shown to confer benzimidazole-resistance in various phytopathogenic fungi in the field is listed in Table 1.1.

Fungi resistant to one fungicide are resistant or tolerant to other closely related fungicides that share a similar mode of action, called cross-resistance (Brent and Hollomon, 2007). This occurs in several fungicide classes (DMIs, benzimidazole), however, the degree of cross-resistance can varied greatly (Karaoglanidis and Thanassoulopoulos, 2003). Occasionally negative cross-resistance occurs in fungi between two unrelated fungicides (Pittendrigh and Gaffney, 2001). Negative cross resistance happens when an allele that confers resistance to one fungicide makes the fungus more sensitive to another fungicide (Pittendrigh and Gaffney, 2001). In *C. beticola*, negative cross-resistance to N-phenylcarbamates (e.g. diethofencarb) is reported by the same mutation as confers a high-level of benzimidazole resistance at codon 198 (Koenraadt et al., 1992; Davidson et al., 2006). In the United States, Davidson et al. (2006) found that all benzimidazole resistant *C. beticola* isolates examined had an amino acid sequence change from GAG to GCG at codon 198. This mutation confers a strong negative correlation (R=-0.971) between *C. beticola* growth on benzimidazole and diethofencarb sensitivity. This finding agrees with the conclusion from other researchers

that this mutation confers negative-cross resistance upon the fungal isolates studied (Koenraadt and Jones, 1993; Fujimura et al., 1992). Although some benzimidazole-resistant isolates are sensitive to N-phenylcarbamates, it is not recommended to use N-phenylcarbamates or similar compounds to manage benzimidazole-resistant pathogens in the field because combined resistance to both benzimidazoles and N-phenylcarbamates has been found (Josepovits et al. 1992, Elad et al. 1992).

Benzimidazole fungicide resistance has been identified to have variable effects on fungal fitness (Smith, 1988). Ruppel (1975) reported that benomyl-resistant isolates of *C. beticola* did not differ from benomyl-sensitive isolates in growth and sporulation *in vitro*, or in virulence and sporulation *in vivo*. Similarily, Dovas (1976) demonstrated that benzimidazole-resistant and sensitive portions of natural *C. beticola* populations were equal in fitness showing equivalent survival under selection pressure imposed by various field conditions. The frequency of benomyl-resistant strains of *C. beticola* remained stable in the absence of applications of benzimidazole over a 3-yr period (Ruppel et al. 1980), further supporting that there is little difference in fitness between resistant and sensitive isolates.

Strobilurin fungicides

Strobilurin fungicides are part of the large chemical group of quinone outside inhibitor (QoI) fungicides (FRAC Group 11). The name “strobilurin” was used because strobilurins are produced naturally by the basidiomycete wood-rotting mushroom *Strobilurus tenacellus,* (Anke, 1995; Fernández-Ortuño et al., 2010). The first identified strobilurin compound (strobilurin-A) was isolated from the mycelium of *Strobilurus tenacellus* strain No.21602 by Anke et al. (1977). After the first discovery, compounds of

a similar nature were subsequently isolated and named in the order of their discovery (strobilurin B, C, D). Natural strobilurins break down rapidly in sunlight which makes them very unreliable for disease control in the field (Balba, 2007). Therefore structural modifications were made to photostabilize these compounds and maintain their fungicidal activities. In 1996 the first synthesized photo-stable strobilurin fungicides, azoxystrobin and kresoximethyl, were made commercially available, the development of similar compounds soon followed (Bartlett et al., 2002; Fernández-Ortuño et al., 2010). DuPont and Aventis have discovered famoxadone and fenamidone fungicides respectively, which are not structurally-related to the strobilurins, but they have the same mode of action. The development of these compounds with the same mode of action, collectively known as QoI fungicides.

QoI products act by binding the quinol oxidation site of cytochrome b and inhibiting mitochondrial respiration by blocking the electron transfer between cytochrome b and cytochrome c1. This interferes with the production of ATP and leads to energy deficiency in sensitive fungi and fungal-like organisms (Anke 1995; Bartlett et al. 2002). QoI fungicides have a broad-spectrum of activity against many major fungal genera because of their biochemical mode of action, (Bartlett et al. 2001, Bartlett et al. 2002). They have been used to control many plant diseases including powdery mildews, brown rusts, and downy mildews (Balba, 2007; Fernández-Ortuño et al., 2010). By 1999, only four years after their first launch, QoI fungicides became among the world's biggest selling fungicides, representing over 10% of the global fungicide market (Bartlett et al., 2002). QoI products have been registered in numerous countries for use on diverse plants such as cereals, vegetables, ornamental plants, grapevines, turf grass, and sugarbeet

(Fernández-Ortuño et al., 2010; Secor et al., 2010). Soon after their introduction, QoI fungicides became the most important additions to the fungicide spray programs for many crops because of their protective, curative and eradicative activity (Balba, 2007; Khan and Smith 2005). They are also used as in-furrow and seed treatments against some soilborne pathogens such as *Rhizoctonia solani* and *Pythium* spp. (Kirk et al., 2007; Bartlett et al., 2002; Fernández-Ortuño et al., 2010).

QoI fungicides have been used to control CLS on sugarbeet since early 2000s and showed excellent efficacy in the field (Khan and Smith 2005; Karaoglanidis and Bardas 2006, Karadimos and Karaoglanidis 2006). However, QoI fungicides were classified as "high risk" for resistance to develop by FRAC (Brent and Hollomon, 2007). The classification is based on chemical class, mode of action, experience and reported resistance in the past. High relative risk means that wide and severe decreases of fungicide effectiveness due to development of resistance occurred in most of their target pathogens, in certain regions, within a few years of their introduction (Brent and Hollomon, 2007).

Table 1.1. List of mutations in the β-tubulin gene shown to confer benzimidazole-resistance to various phytopathogenic fungi in the field (Ma, Z. and Michailides, T. J., 2005).

Codon	Substitution[3]	Pathogen	Reference	Crop	Country
6	His to Tyr	*Monilinia fructicola*	Ma et al., 2003b	Multiple stone fruits[1]	US
50	Tyr to Cys	*Cladobotryum dendroides*	McKay et al., 1998	Agaricus bisporus	Ireland
167	Phe to Tyr	*Penicillium expansum*	Baraldi et al., 2003	Pears	Italy
198	Glu to Ala	*Botrytis cinerea*	Luck and Gillings,1995	Multiple crops[2]	Australia and New Zealand
	..	*Monilinia fructicola*	Ma et al., 2003b	Multiple stone fruits[1]	US
	..	*Penicillium expansum*	Baraldi et al., 2003	Pears	Italy
	..	*Tapesias* spp.	Albertini et al., 1999	Winter wheat	France
	..	*Venturia inaequalis*	Koenraadt et al., 1992	Apple	Multiple countries
198	Glu to Gln	*Tapesias* spp.	Albertini et al., 1999	Winter wheat	France
198	Glu to Lys	*Monilinia fructicola*	Ma et al., 2003b	Multiple stone fruits[1]	US
	..	*Penicillium expansum*	Baraldi et al., 2003	Pears	Italy
	..	*Venturia inaequalis*	Koenraadt et al., 1992	Apple	Multiple countries
198	Glu to Val	*Penicillium expansum*	Baraldi et al., 2003	Pears	Italy
200	Leu to Phe	*Tapesias yallundae*	Albertini et al., 1999	Winter wheat	France
200	Phe to Tyr	*Tapesias yallundae*	Albertini et al., 1999	Winter wheat	France
200	..	*Venturia inaequalis*	Koenraadt et al., 1992	Apple	Multiple countries

1: Multiple stone fruits: nectarine, peach, plum, and prune.
2: Multiple crops: onion, grape, tomato, ornamentals, and cucumber.
3: Amino acids: Ala: Alanine, Cys: Cysteine, Gln: Glutamine, Glu: Glutamic acid, His: Histidine, Leu: Leucine, Lys: Lysine, Phe: Phenylalanine, Tyr: Tyrosine, Val: Valine.

Resistance to QoI fungicides has been reported worldwide in many pathogens of field crops including *Blumeria graminis* f. sp. *tritici* (Reviewed by Bartlett et al. 2002), *Pseudoperonospora cubensis* (Ishii et al., 2001), *Corynespora cassiicola* (Ishii et al., 2007) *Pyricularia grisea* (Vincelli et al., 2002; Kim et al., 2003), *Colletotrichum cereale* (Wong et al. 2007), *Alternaria* spp. (Rosenzweig et al., 2008; Ma et al., 2003a; Vega et al., 2014; Pasche et al., 2005), *Mycosphaerella graminicola* (Torriani et al., 2009; Siah et al., 2010), *Mycosphaerella fijiensis* (Sierotzki et al., 2000), and *Venturia inaequalis* (Köller et al., 2004). In most cases, resistance has been associated with point mutations in the mitochondrial cytochrome b gene (Cytb), resulting in one of the specific amino acid substitutions which prevents binding of fungicide at the quinol oxidation site (Pasche et al., 2005; Ma et al., 2003a, 2004; Sierotzki et al., 2000).

To date three amino acid substitutions have been reported in QoI-resistant fungi and oomycetes (Pasche et al., 2005; Gisi et al., 2002; Ishii et al., 2001; Köller et al., 2004; Sierotzki et al., 2007). The most common mechanism of resistance in these organisms is a point mutation at codon 143 in the mitochondrial cytochrome b gene which leads to an amino acid change from glycine to alanine (G143A) (Ishii, 2007; Köller et al., 2004; Ma et al., 2003a). Isolates with a G143A mutation express a high-level of resistance and this mutation is often associated with a severe loss of disease control (Fernández-Ortuño et al., 2010). Recently, resistance to QoI products was reported in field isolates of *C. beticola* in Michigan, Nebraska and the Red River Valley in the United States (Kirk et al. 2012; Bolton et al., 2013a, 2013b) and Europe (Birla et al., 2012). All the resistant isolates harbored the G143A mutation in the cytochrome b gene, which is the most common QoI-resistance mutation found in *C. beticola* to date. Other mutation sites that are less

frequently reported occur at positions 129 and 137 in the mitochondrial cytochrome b gene. They lead to substitutions of amino acids from phenylalanine (Phe) to leucine (Leu) (F129L) (Vaghefi et al., 2016; Pasche et al., 2005; Kim et al., 2003) or glycine (Gly) to arginine (Arg) (G137R) respectively (Sierotzki et al., 2007). These two mutations usually show moderate levels of QoI resistance (Pasche et al., 2005; Sierotzki et al., 2007).

It is critical to use an effective disease management program to delay the build-up of QoI resistant in *C. beticola* (Kirk et al., 2012; Birla et al., 2012; Vaghefi et al., 2016). Tank mixing QoI fungicides with other fungicides that have different modes of action and limiting the number of QoI fungicide applications in a given season can reduce the selection pressure on the fungal pathogen population. (Keinath and DuBose, 2004; Wong and Wilcox, 2001; Vincelli, 2002). Careful monitoring of QoI resistance during the growing season is very helpful for the growers to make timely and effective fungicide applications (Vincelli, 2002).

Table 1.2. Selected reports of plant pathogens with resistance to quinone outside inhibitor (QoI) fungicides

Pathogen	Resistance type	Reference
Alternaria solani	F129L	Pasche et al., 2005; Rosenzweig et al., 2008
Alternaria spp.	G143A	Ma et al., 2003a; Vega et al., 2014
Cercospora beticola	G143A	Kirk et al., 2012; Bolton et al., 2013a
Mycosphaerella fijiensis	G143A	Sierotzki et al., 2000
Mycosphaerella graminicola	G143A	Torriani et al., 2009; Siah et al., 2010
Pseudoperonospora cubensis	G143A	Ishii et al., 2001
Venturia inaequalis	G143A	Köller et al., 2004
Botrytis cinerea	G143A	Jiang et al., 2009
Corynespora cassiicola	G143A	Ishii, 2007
Pyricularia grisea	G143A, F129L	Kim et al., 2003; Vincelli et al., 2002

DMI Fungicides

Demethylation inhibitors (DMI) are the most important subgroup of the sterol biosynthesis inhibitor (SBI) fungicides and have been used for control of CLS since the 1970s (Byford 1996; Meriggi et al., 2000). The SBI fungicides are divided into four classes based on their biochemical target in disrupting the ergosterol biosynthetic pathway: DMI (Class I), amines (Class II), hydroxyanilides (Class III) and squalene epoxidase inhibitors (Class IV). Among these, the DMI fungicides target sterol 14α-demethylation that disrupts sterol synthesis at a single biochemical site (Köller, 1988). DMI fungicides belong to a group of site-specific fungicides and thus they are prone to the development of resistance by fungi (Georgopoulos and Skylakakis 1986; Köller, 1987). However, the actual development of resistance to DMI fungicides has been slow and quantitative (Karaoglanidis and Ioannidis, 2010) and the loss of fungicidal activity is usually not

complete in *C. beticola* (Karaoglanidis and Ioannidis, 2010). Unlike benzimidazole or QoI resistance, which are caused by single genetic mutations with effects on certain fungal biochemical pathways, resistance to DMI fungicides appears to incorporate many genetic steps (Hollomon et. al., 1984; van Tuyl, 1977; Kalamarakis et al., 1989). These steps influence the activity of DMI fungicides in the field. Therefore it is tough to relate monitoring data to product performance in the field.

To date, reduced sensitivity to DMI fungicides has been reported in many pathogens, including *Venturia inaequalis* (Stanis and Jones, 1985), *Rhynchosporium secalis* (Kendall et al., 1993), *Penicillium digitatum* (Eckert et al., 1994), *Mycosphaerella fijiensis* (Romero and Sutton, 1997), *Erysiphe graminis* (Delye et al., 1998) and *Sphaerotheca fuliginea* (Schepers, 1985b*)*. In *Cercospora beticola* reduced sensitivity to DMI fungicides has been reported in Greece (Karaoglanidis et al., 2000), Italy (D'Ambra et al., 1974, Karaoglanidis et al., 2010), and states of United States including North Dakota and Minnesota (Bolton et al., 2012a).

Three mechanisms responsible for DMI resistance have been described in members of the ascomycota, the first of these is target-site modification in the C-14α-demethylase (CYP51) (Delye et al., 1997, 1998). This has been examined in the grape powdery mildew fungus *Uncinula necator*. For example, Delye et al. (1997) showed that a single mutation occurred at codon 136, leading to a substitution of phenylalanine for tyrosine. This mutation was associated with a high-level of resistance to a DMI fungicide, triadimenol. A second resistance mechanism is overexpression of the 14α-demethylase gene (CYP51) during ergosterol formation (Hamamoto et al., 2000; Schnabel and Jones, 2001). This has been demonstrated in *Penicillium digitatum* (Hamamoto et al., 2000) and

Venturia inaequalis (Schnabel and Jones, 2001). The third resistance mechanism is the increased activity of ATP-binding cassette (ABC) transporter genes. For example, Leroux and Walker (2013) showed that BcatrD and BcmfsM2 are the most important transporters for DMI efflux, with BcatrB and Bcmfs1 playing a secondary role in field isolates of *Botrytis cinerea.*

Reduced sensitivity to DMI fungicides has been observed in *C. beticola* in the field (Karaoglanidis et al., 2000; Bolton et al., 2012a). However the mechanism of resistance is not well studied in this genus. Recently the 14α-demethylase gene (CYP51) was isolated from *C. beticola* (Bolton et al., 2012a) and no mutations associated with resistance were identified in the gene or the promoter region. Bolton et al. (2012a) found that the CbCyp51 gene was overexpressed in *C. beticola* isolates with high effective fungicide concentration which inhibit 50% of mycelial growth (EC_{50} values) compared with isolates with low EC_{50} values. Similarly, a study of transcriptional levels of the CYP51 gene of *C. beticola* showed that overexpression of the target gene was strongly associated with highly resistant isolates (Nikou et al., 2009).

The fitness cost associated with DMI resistance varies among the fungi studied (Henry and Trivellas, 1989; Kalamarakis et al., 1989; Peever and Milgroom, 1994). In *C. beticola*, Henry and Trivellas (1989) showed that *C. beticola* strains resistant to DMI-fungicides had lower virulence compared to sensitive strains after fungicide-induced mutagenesis in the laboratory. Results in this study indicated that resistance to DMI fungicides could be associated with reduced fitness. Another study carried out by Karaoglanidis et al. (2001) compared six fitness parameters of fungi with the DMI fungicide flutriafol. They tested nine flutriafol-resistant and twelve flutriafol-sensitive isolates of *C. beticola* obtained from the field. Fitness parameters measured in the study

include mycelial growth, spore germination, germ tube length, incubation period, virulence, and spore production. Results showed that resistant isolates had significantly lower virulence and spore production compared to the sensitive isolates, supporting a fitness cost to resistance in the field. Although resistance was reported in *C. beticola*, there are no reports of major failures in disease management of CLS at this point (Karaoglanidis et al., 2001). This may be due to the low-level of resistant isolates in the population or fitness penalty associated with resistant isolates.

Organotin resistance

Organotin compounds have constituted an effective fungicide class for management of CLS on sugarbeet since the 1960s (Stallknecht and Calpouzos 1968). Triphenyltin hydroxide (TPTH) is the only fungicide in this class currently registered for use on sugarbeet (Mueller et al., 2013). Although organotin was used extensively in the 1960s, until the introduction of target-specific fungicides, benzimidazoles, no resistance had been reported (Giannopolitis, 1978). Organotin resistance of *C. beticola* was first reported in Greece in the 1970s (Giannopolitis, 1978). In Greece, use of tins was stopped, but after detection of benzimidazole resistance, organotin fungicides were re-introduced into fungicide programs to manage CLS in 1973 (Karaoglanidis and Ioannidis, 2010). After three years of renewed use, decreased effectiveness of the fungicide was documented in 1976 (Giannopolitis, 1978). After the first report of resistance to tin fungicides in Greece, strains of *C. beticola* resistance to organotin compounds was reported in other countries such as Italy (Cerato and Grassi, 1983), Yugoslavia (Reviewed by Karaoglanidis and Ioannidis, 2010), and the United States (Bugbee 1995).

C. beticola strains resistant to organotin fungicides have been reported to have reduced fitness compared to the sensitive strains (Giannopolitis and Chrysayi-Tokousbalides, 1980). Resistant strains do not compete well with sensitive *C. beticola* in the absence of the fungicide (Giannopolitis and Chrysayi-Tokousbalides 1980). This phenomenon can be used in resistance management because resistant isolates will decrease in the population over time when the organotin fungicides are not used, due to the lower competitive ability of resistant strains, which may allow the efficient use of this class of fungicide (Secor et al., 2010).

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Chapter 2: An examination of fungicide sensitivity in *Cercospora beticola* from Michigan and Ontario sugarbeet (*Beta vulgaris*) fields over a three year period

Abstract

Cercospora leaf spot (CLS), caused by *Cercospora beticola* (Sacc.) is an annual concern for sugarbeet production worldwide. In the Midwestern USA management of CLS mostly relies on timed fungicide applications and CLS-resistant sugarbeet varieties. The objective of this study was to determine the sensitivity of *C. beticola* to commonly used fungicides and the fungicide variability in sensitivity among fungal strains. Isolates were collected from throughout the east-central growing region of Michigan and the southern region of Ontario from 2012 to 2014. The spiral gradient dilution method (SGD) was used to estimate the fungicide concentration that caused a 50% inhibition (EC_{50}) in mycelial growth or spore germination. Results indicate that resistance to quinone outside inhibitor (QoI) and benzimidazole fungicides are widespread in the Great Lakes sugarbeet growing region. However, most isolates (>85%) tested were sensitive to demethylation inhibitor and organotin fungicides with EC_{50} values of less than 5 ppm. This indicates the need for the development of additional fungicides with alternative modes of action or use of other methods for long term viability of CLS management.

Introduction

Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola* (Sacc.), is one of the most serious foliar diseases of sugarbeet (*Beta vulgaris L*.) worldwide especially under warm (25-35˚C) and humid (>90% relative humidity) growing conditions (Jacobsen and Franc, 2009). During severe epidemics losses caused by CLS can be as high as 40% or greater (Shane and Teng 1992). Losses are manifested by reduction in root weight, sugar purity, and sugar yield as a result of a decrease in photosynthetic capacity (Smith et al. 1978, Shane and Teng 1992, Jacobsen and Franc 2009). Moreover storability is reduced in sugarbeet roots with lower sugar content due to foliar infection (Smith et al. 1978). Moderate and high incidence of CLS adversely affects about 33% of sugarbeet production areas worldwide although disease severity and incidence differ among geographical regions (Holtschulte 2000).

In the Great Lakes sugarbeet production region evidence of CLS is often found on beet leaves from mid-July to early August and continues through the fall (Poindexter 2006). Nature inoculum of *C. beticola* in sugarbeet field begin as pseudostromata (Pool and McKay, 1916b). When environmental conditions are favorable conidiophores and clusters of conidia develop on the pseudostromata (Franc 2010). Conidia land on leaves of susceptible sugarbeet and alternate host to initiate the disease. Early symptoms include randomly distributed small, circular, grey-brown lesions with defined reddish-brown margins on older leaves (Steinkamp et al. 1979, Ruppel 1986, Jacobsen and Franc 2009). Black pseudostromata can be found in the center of mature lesions (Franc, 2010). As the disease develops, lesions expand, increase in number and coalesce, resulting in blighted areas. Phytotoxins (e.g. beticolin and cercosporin) produced by the fungus

promote lesion formation and cause yellowing and rapid death of leaves (Steinkamp et al. 1981). Secondary spread of *C. beticola* begins when conidia are produced on foliar lesions and carried to neighboring leaves and plants by water-splash or the wind (Jacobsen and Franc 2009).

Fungicide applications (Windels et al., 1998) and host resistance (Panella and McGrath, 2010) are the major management strategies used for CLS. Timed fungicide applications are typically based on weather-driven disease forecasting models (Windel et al. 1998) such as the BEETcast™ model (Weather Innovation Inc., Chatham, ON, Canada). Growers use these models to generate the recommended fungicide application dates for CLS management. In addition to host resistance and chemical control, cultural practices to reduce the inoculum level have been incorporated into CLS management programs as well, including removal of weed hosts, crop rotation, and tillage (Jacobsen and Franc 2009, Khan 2013). Incorporating leaf debris into the soil by deep plowing contribute to the reduction of initial inoculum, allowing for the decay and destruction of infected leaf residues in the soil (Ruppel, 1986; Khan et al., 2008). A 2-3 year rotation using crops not susceptible to *C. beticola* may reduce problems caused by CLS (Jacobsen and Franc 2009). Among these options, chemical fungicide application is the most commonly used practice in the Great Lakes beet production region, particularly under high disease pressure conditions (Michigan Sugarbeet Research and Education Advisory Council 2015, Kirk and Schafer 2013, 2014).

Fungicides are classified in a number of ways, including fungicide biochemical mode of action (MOA) [fungicide resistance action comittee (FRAC Code List, 2015)], role in protection, breadth of activity, chemical type, and mobility (Mueller et al., 2013;

McGrath, 2004). In this chapter, fungicides labeled for CLS management on sugarbeet will be classified into two basic groups based on their form of mobility: i.e. contact residual fungicides or penetrant fungicides. Contact residual fungicides have limited mobility, do not move into new tissue (Mueller et al., 2013), and the availability of active ingredient decreases quicker compare to penetrant fungicides after application due to weathering (Wise et al., 2013). Fungicides in this group should, therefore, be applied more frequently to re-establish protection on previously treated plants (Wise et al., 2013). Fungicides used on beet that fall into this category include organo-tin fungicides (TPTH, Supertin®, AgriTin®), ethylene-bis-dithiocarbamates (EBDC, Dithane®, Manzate®, Penncozeb®) and copper compound products (Kocide, Basicop, Champ). In contrast, penetrant fungicides have more mobility and can penetrate into the plant tissue and redistribute inside the plant, they are less likely to be washed off compared to contact residual fungicides (van Tuyl, 1977). Therefore availability of the active ingredient may last longer after application (van Tuyl, 1977). Penetrant fungicides are further divided into four categories based on the direction and degree of movement once they have been absorbed and translocated within the plant (McGrath, 2004): xylem-mobile fungicides (move upward from the point of entry through the plant's xylem, also called acropetal fungicides), translaminar fungicides (able to move through the leaf to the opposite side of the leaf)，locally systemic fungicides (move short distance within the leaf from the application site), and true systemic fungicides (move throughout the plant through its xylem and phloem systems). Fungicides evaluated in this study are summarized in Table 2.1.

Table 2.1. Fungicides evaluated for effect on *Cercospora beticola* in this study including product name [(fungicide resistance action committee (FRAC) code], the active ingredient (fungicide class), formulation (and percentage of active ingredient), years that fungicides were used in the study, and the current manufacturer.

Product name (FRAC[a] Group)	Active ingredient (fungicide class)	Formulation[b] and (percent active ingredient)	Years used in study	Current Manufacturer
Super Tin (30)	Triphenyltin hydroxide (Organotin)	4L (40.0)	2012-14	United Phosphorus Inc. King of Prussia, PA. USA, 19406
Eminent (3)	Tetraconazole (DMI)	125SL (11.6)	2012-14	Sipcam Agro USA, Inc., Roswell, GA, USA, 30076
Enable (3)	Fenbuconazole (DMI)	2SC (23.5)	2013-14	Dow AgroSciences LLC., Indianapolis, IN, USA, 46268
Inspire (3)	Difenoconazole (DMI)	2.08SC (23.2)	2012-14	Syngenta Crop Protection Inc., Greensboro, NC, USA, 27419
Topguard (3)	Flutriafol (DMI)	1.04SC (11.8)	2013-14	Cheminova Inc., Research Triangle Park, NC, USA, 27709
Proline (3)	Prothioconazole (DMI)	480SC (41.0)	2012-14	Bayer Cropscience LP, Research Triangle Park, NC, USA, 27709
Headline (11)	Pyraclostrobin (QoI)	2.09SC (23.6)	2012	BASF Corporation LP, Research Triangle Park, NC, USA, 27709
Topsin-M (1)	Thiophanate-methyl (Benzimidazole)	70WDG (70)	2012	United Phosphorus Inc. King of Prussia, PA. USA, 19406

[a]FRAC: Fungicide Resistance Action Committee. (2015). FRAC Code List: Fungicides sorted by mode of action (including FRAC Code numbering). Online document available at http://www. frac. info. CropLife International, Brussels, Belgium. Product names are listed for reader's information, analytical standard grades of each fungicide was used in the experiment.

[b] Formulation: L = flowable concentrate; SC = suspension concentrate; SL = soluble liquid concentrate; WDG = water dispensable granule; WP = wettable powder.

DMI fungicides

DMI fungicides

The DMI fungicides are now the largest class of agricultural fungicides used in the world (Leadbeater 2014). They have been used on many crops (e.g. vegetables, fruit trees, turf grass, and field crops) against many diseases including powdery mildew, downy mildew, rusts, anthracnose and CLS (Köller, 1988; Russell 2005; Wise et al., 2013). Their unique fungicidal properties such as translaminar and xylem systemic movement (Klittich and Ray 2013; Dahmen and Staub 1992), low-use rate, protective and curative properties (Tsuda et al., 2004) made them very attractive to growers when they were released. Currently DMI fungicides labeled for CLS management on sugarbeet in the USA include tetraconazole (Eminent), difenoconazole (Inspire), fenbuconazole (Enable), prothioconazole (Proline), and flutriafol (Topguard) (Table 2.1).

Resistance to DMI fungicides has been reported in many pathogens (Stanis and Jones, 1985, Kendall et al., 1993, Eckert et al., 1994, Romero and Sutton, 1997, Delye et al., 1998, Schepers, 1985). In *C. beticola*, resistance to DMI fungicides has been reported in Greece (Karaoglanidis et al., 2000), Italy (D'Ambra et al., 1974, Karaoglanidis et al., 2010), North Dakota and Minnesota in the USA (Bolton et al., 2012a). To date, three mechanisms for DMI resistance have been described, including target-site modification in the C-14α-demethylase (CYP51) gene (Delye et al., 1997, 1998), overexpression of the CYP51 gene (Hamamoto et al., 2000; Schnabel and Jones, 2001) and increased activity of ATP-binding cassette (ABC) transporter genes (Hayashi et al., 2002; Leroux and Walker 2013). Mechanisms of resistance is not well studied in *C. beticola*. Although resistance to DMI fungicides has been observed in the field, no consistent or known resistance mutations in the Cyp51 were found in this fungus (Nikou

et al., 2009; Bolton et al., 2012a). Bolton et al. (2012a) found that there was a stronger positive correlation between CYP51 gene expression and tetraconazole exposure in isolates with relatively high EC_{50} values (>1.0 ppm) than for isolates with a low EC_{50} values (<0.006 ppm). Similarly, a study of transcriptional levels of the CYP51 gene showed that overexpression of CYP51 was strongly associated with highly resistant [resistant factor (the ratio of EC_{50} of mutant: EC_{50} of the wild type.) between 65 to 115] isolates (Nikou et al., 2009). These results indicate that the mechanism of overexpression is likely responsible for highly-resistant *C. beticola* isolates to tetraconazole.

QoI fungicides

QoI products act by binding the outer quinol-oxidation (Qo) site of cytochrome b and inhibiting mitochondrial respiration by blocking the electron transfer between cytochrome b and cytochrome c1. This interferes with the production of ATP and leads to energy deficiency in sensitive fungi and fungal-like organisms (Anke 1995; Bartlett et al. 2002). QoI fungicides have a broad-spectrum of activity against many major fungal genera because of their biochemical mode of action, (Bartlett et al. 2001, Bartlett et al. 2002). They have been used to control many plant diseases (Balba, 2007; Fernández-Ortuño et al., 2010). By 1999, only four years after their first launch, QoI fungicides became among the world's biggest selling fungicides, representing over 10% of the global fungicide market (Bartlett et al., 2002).

QoI products have been registered in numerous countries for use on diverse crops such as cereals, vegetables, ornamental plants, grapevines, turf grass, and sugarbeet (Fernández-Ortuño et al., 2010; Secor et al., 2010). They are also used as in-furrow and

seed treatments against some soilborne pathogens such as *Rhizoctonia solani* and *Pythium* spp. (Kirk et al., 2007; Bartlett et al., 2002; Fernández-Ortuño et al., 2010).

QoI fungicides have been used to control CLS on sugarbeet since early 2000s and showed excellent efficacy in the field (Khan and Smith 2005; Karaoglanidis and Bardas 2006, Karadimos and Karaoglanidis 2006). However, QoI fungicides were classified as "high risk" for resistance to develop by FRAC (Brent and Hollomon, 2007). The classification is based on chemical class, mode of action, experience and reported resistance in the past. High relative risk means that wide and severe decreases of fungicide effectiveness due to development of resistance occurred in most of their target pathogens, in certain regions, within a few years of their introduction (Brent and Hollomon, 2007).

Resistance to QoI has been reported in many fungi and oomycetes (Kirk et al., 2012; Pasche et al., 2005; Gisi et al., 2002; Ishii et al., 2001; Köller et al., 2004; Sierotzki et al., 2007). The major mechanism of resistance in studied organisms are point mutations in the mitochondrial cytochrome be gene (Kirk et al., 2012; Bolton et al., 2013a; Rosenzweig et al., 2008; Sierotzki et al., 2000). The most prevalent of these mutations is a single point mutation at codon 143 which leads to an amino acid change from glycine to alanine (G143A) (Ishii, 2007; Köller et al., 2004; Ma et al., 2003a). In some cases, isolates with a G143A mutation express a high-level (EC_{50}> 100 ppm) of resistance, and the mutation is often associated with a partial loss of disease control (Fernández-Ortuño et al., 2010). Recently resistance to QoI fungicides was reported in *C. beticola* of sugarbeet in Michigan, Nebraska (Kirk. et al. 2012), and the Red River Valley in the USA (Bolton 2013b) as well as in Europe (Birla et al., 2012) and Canada (Trueman et al., 2013).

All the resistant isolates recovered had the G143A mutation. In other fungi other mutation sites that are less frequently reported occur at codons 129 and 137 in the cytochrome b gene. They lead to substitutions of amino acids from phenylalanine (Phe) to leucine (Leu) (F129L) (Pasche et al., 2005; Kim et al., 2003) or glycine (Gly) to arginine (Arg) (G137R), respectively (Sierotzki et al., 2007). These two mutations usually show moderate levels ($0.1<EC_{50}<10$ ppm) of QoI resistance.

Benzimidazole fungicides

Benzimidazole fungicides were the first systemic fungicides available for CLS control (Solel, 1970). They were very popular when they first released due to their systemic and curative activity against *C. beticola* and many other pathogens (Davidse, 1986; Smith, 1988). The use of the benzimidazole fungicides, including benomyl (Benlate®) and thiophanate methyl (Topsin M®), was widespread during the late 1960s (Solel, 1970). After years of intensive use, resistance was reported in many fungi including *C. beticola* (Georgopoulos and Dovas, 1973) and related fungi such as *Mycosphaerella fijiensis* (Cañas-Gutiérrez et al., 2006), *Cercospora kikuchii* (Imazaki et al., 2006) and *Cercospora arachidicola* (Littrell, 1974). Resistance to benzimidazole fungicides in isolates of *Cercospora beticola* has been documented in many geographic regions including Greece (Georgopoulos and Dovas, 1973), Italy (D'Ambra et al., 1974, Karaoglanidis and Ioannidis, 2010), Japan (Uesugi, 1978 as reviewed by Karaoglanidis and Ioannidis, 2010), India (Pal and Mukhopadhyay, 1983), Texas (Ruppel and Scott 1974), Arizona (Ruppel, 1980), Minnesota (Weiland and Smith, 1999), North Dakota (Weiland and Smith, 1999) and Michigan in the USA (Weiland and Halloin, 2001).

Benzimidazoles act by binding and interrupting fungal ß-tubulin, thus interfering with mitosis and disrupting the integrity of the fungal cytoskeleton (Davidse,1986). Resistance to benzimidazole fungicides is usually caused by single nucleobase mutation in the fungal ß-tubulin gene that result in an altered amino acid sequence at the benzimidazole binding site (Cooley and Caten, 1993). Results from previous studies have shown that mutations at different codon sites (e.g. 6, 50, 167, 198, 200) in the ß-tubulin gene of plant pathogenic fungi resulted in different levels of benzimidazole resistance (Luck and Gillings, 1995; Koenraadt et al., 1992; Ma et al., 2003b; Albertini et al., 1999; McKay et al, 1998; Gafur et al., 1998). The most frequently reported mutations causing benzimidazole resistance in the field occur at codon 198 (Koenraadt et al., 1992; Davidson et al., 2006). These mutations can lead to various changes, including changing the amino acid at this site in the β-tubulin protein from glutamic acid to alanine (Ala), glycine (Gly), lysine (Lys) or glutamine (Gln) (Koenraadt et al., 1992; Luck and Gillings 1995; Fujimura et al., 1992).

In the United States, only a single mutation in the ß-tubulin gene has been found in field isolates of *C. beticola* collected in different years and geographic regions (Davidson et al., 2006). This mutation is predicted to lead to an amino acid change from glutamic acid (GAG) to alanine (GCG) at codon 198 (E198A). Such a mutation results in a high-level (EC_{50}>100 ppm) of benzimidazole resistance. Davidson et al. (2006) also confirmed previous reports that the E198A mutation confers a negative cross-resistance to another fungicide class, the N-phenylcarbamates (e.g. diethofencarb). This finding agrees with the conclusion from other researchers that this mutation confers negative-cross resistance upon *Neurospora crassa* isolates studied (Koenraadt and Jones, 1993;

Fujimura et al., 1992). Recently study in Serbia, a point mutation at codon 167, resulting in an amino acid change from phenylalanine (TTC) to tyrosine (TAC), was discovered in benzimidazole-resistant *C. beticola* isolates (Trkulja et al., 2013). This or other mutations may be present in *C. beticola* in other regions as isolates with benzimdazole resistance that do not show negative cross-resistance to N-phenylcarbamates have been found in some areas (Koenraadt and Jones, 1993; Fujimura et al., 1994). The presence of multiple different resistance mutations is known to occur in other fungi (Ma et al., 2003b; Baraldi et al., 2003; Albertini et al., 1999).

Organotin fungicides

Organotin compounds are another effective fungicide group for *C. beticola* management on sugarbeet and have been used since the 1960s (Stallknecht and Calpouzos, 1968). They are single site mode of action products that inhibit oxidative phosphorylation and ATP synthases (von Ballmoos et al., 2004). They have very limited therapeutic effect during disease development and must be applied in anticipation of fungal infection (Bock, 1981).

Resistance to organotin compounds was first found in Greece (Giannopolitis, 1978). The use of organotins was stopped due to the presence of resistance and benzimidazole fungicides were used for replacement (Giannopolitis, 1978). After the development of resistance to benzimidazole fungicides in the field in Greece (Giannopolitis, 1978), organotin fungicides were re-introduced into the fungicide program to control CLS in 1973 (Karaoglanidis and Ioannidis, 2010). Three years after re-introduction a decrease in the effectiveness of organotin fungicide was observed in the field, and nearly 36% of tested isolates were tolerant to tin fungicides at that time

(Giannopolitis, 1978). After the first report of resistance to tin fungicides, strains of *C. beticola* resistant to organotin compounds were reported in other countries such as Italy (Cerato and Grassi, 1983), Yugoslavia (Karaoglanidis and Ioannidis, 2010), and the USA (Bugbee 1995). Although the resistance to organotin fungicides has been observed in many geographic regions, the mechanism of resistance is not known. As well as the fungicides listed above, some additional fungicides are used for CLS control. These will not be discussed in the current work as the above include the major classes in which resistance has been reported and are the focus for ongoing survey work (Kirk and Schafer, 2013, 2014; Secor et al., 2010).

This is part of an ongoing project in the Kirk lab (Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI) to monitor fungicide sensitivity of *C. beticola* field isolates to various fungicides used in the Great Lakes production region. Results were distributed to the Michigan sugarbeet industry to help maintain effective fungicide recommendations and provide information for resistance management recommendations for CLS specific to the MI/ON production region.

Material and Methods

Sampling

Sugarbeet leaves with symptoms of CLS were sampled from fields in east-central Michigan (Figure 2.1) and southern Ontario during late August to early October of 2012, 2013 and 2014. Samples of up to 10 leaves were taken from symptomatic sugarbeet every year with the generous help of Michigan sugar agriculturalists. Global positioning system (GPS) coordinates of the fields selected for sampling were recorded using a Mobile GPS Unit (Trimble Navigation Ltd., Sunnyvale, CA). Sugarbeet growers were

asked to provide information on the types of fungicides used and the number of fungicide applications as part of the annual CLS survey. Leaf samples were placed in ice-filled coolers and shipped overnight to Michigan State University (East Lansing, MI). Samples from Ontario were delivered by the end of the second business day after being shipped. Leaf samples were stored at 4˚C until processed.

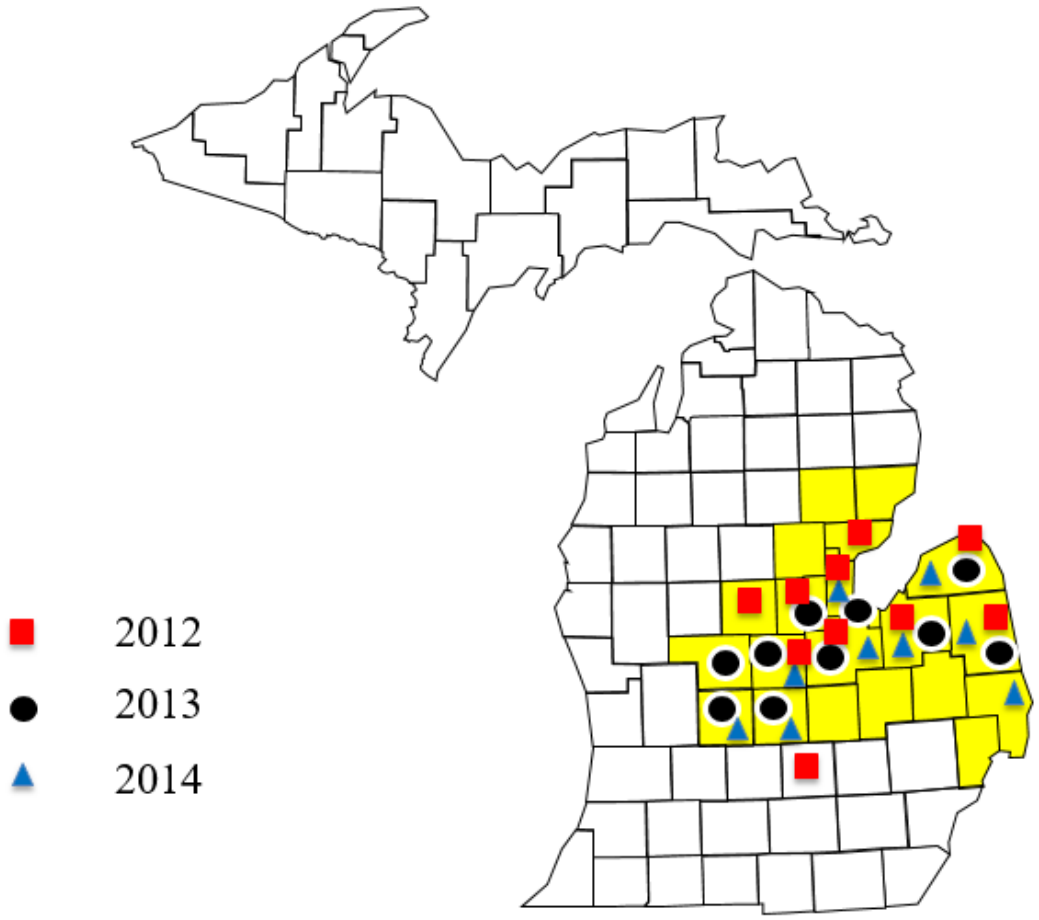

Figure 2.1. Distribution of sampling locations in Michigan from which sugarbeet leaves with symptoms of Cercospora leaf spot were collected over a three year period. Yellow area represents the commercial sugarbeet production region. ■ represent sampling locations for 2012; ● represent sampling locations for 2013; ▲ represent sampling locations for 2014.

Cercospora beticola Isolates

To collect isolates for the survey sugarbeet leaves from a given field were placed in sealable 27cm x 27 cm plastic bags (Ziploc® SC Johnson Inc., Racine, WI) with a 26 cm x 23 cm paper towel moistened with sterile distilled water and incubated at room temperature with ambient light for 48 hrs to promote spore production. Lesions were observed under a dissecting microscope (Olympus Inc., Tokyo, Japan) and identification of *C. beticola* was done based on morphological characteristics (Crous and Braun 2003). Pure cultures were obtained by single spore transfer (Choi et al., 1999) and C*. beticola* cultures were maintained on half-strength V8 agar (Miller 1955) plates at room temperature (23-24˚C) and transferred every three weeks.

Preparation of media and fungicide stock solutions

Clarified V8-juice was prepared as described by Miller (1955) with the following modifications: three grams of calcium carbonate ($CaCO_3$) was added to 1 L of V8 juice (Campbells Soup Company, Camden, NJ, USA), the mixture was stirred by a glass rod thoroughly and clarified by centrifuging (Sorvall RC 6 Plus, Thermo Scientific, Waltham, MA, USA) the mixture at 6750 x g for 10 min in nalgene bottles (Nalge Nunc International Corporation, Rochester, NY, USA). The supernatant was collected and used for making clarified half strength V8 juice agar (Miller, 1955, used half as much V8 compared to the full strength).

Analytical standard grades of the eight fungicides, tetraconazole, fenbuconazole, prothioconazole, flutriafol, difenoconazole, pyraclostrobin, thiophanate methyl, and TPTH were used (Sigma-Aldrich, St Louis, MO, USA). Fungicide stock solutions were prepared by dissolving 10 mg of technical-grade fungicide in 1 mL of 100% ethanol and

mixing the mixture thoroughly for 10 seconds on a vortex mixer (Fisher Scientific™ Analog Vortex Mixer, Thermo Fisher Scientific, Waltham, MA) to make 10,000 ppm fungicide stock solutions for each compound.

Each isolate was classified as sensitive or resistant based on the following. QoI resistance was defined as an EC_{50} value of >10 ppm based on the work by Kirk et al. (2012). Benzimidazole resistance was defined as EC_{50} > 5 ppm (Davidson et al., 2006). Organotin resistance was defined as EC_{50} >1 ppm (Bugbee, 1995). DMI resistance was defined as EC_{50} > 5 ppm (Kirk et al., unpublished). The levels that classified as resistant in this study are still well below the labelled field application rates (Kirk and Schafer, 2013, 2014).

Spore suspension

To induce spore production, *C. beticola* were grown on clarified V8 agar medium and incubated under 10: 14 h light: dark photoperiod at 28˚C for 7 days (Khan et al., 2008). Spore suspensions were prepared by adding 0.1% sterile tween 20 in sterile water to plates and scraping with an L-shaped plastic spreader (USA-Scientific, Ocala, FL). Each suspension was strained through double layers of sterile cheesecloth and the spore concentration was estimated with a hemocytometer and adjusted to 1×10^4 conidia/ml with sterile water. Suspensions were kept on ice to retard spore germination during the experiment.

Mycelial suspension

Cultures were incubated at room temperature in ambient light conditions for 3 weeks until the colony covered about 60% of most 100 x 15 mm plates (VWR International, Radnor, PA). Mycelial suspensions were made by pouring 2 ml of sterile distilled water

over plates, scraping with a sterile plastic spreader and transfer suspension into a sterile 1.5 ml tube.

In vitro fungicide sensitivity assay using the spiral gradient dilution method

The spiral gradient dilution method (SGD) was used to estimate the fungicide concentration which caused 50% inhibition of mycelial growth or spore germination (EC_{50}) as described by Förster et al. (2004). Gachango (2012) showed that there is no significant difference in fungicide sensitivity classifications between SGD and a serial dilution plate (SDP) method so results from this study could be compared to previous work using SDP (Kirk et al. unpublished). Molten V8 juice agar (50 ml) was poured into 15-cm diameter petri dishes using an electronic pipette (Pipet-Aid® XP2, Broomall, PA) and allowed to solidify at least 12 h before applying the fungicide solution. Using a spiral plater (Eddyjet II SGE, Spiral Botech, Inc. Norwood, MA, USA), 54.3-ul of each fungicide stock solution was spirally applied in a gradient on separate plates. Control plates were plated with 100% ethanol in a gradient. Plates were incubated overnight to allow the fungicide to diffuse into the agar and form a gradient of concentration along the radius. For the QoI fungicide tested, plates (including control) were supplemented with salicylhydroxamic acid (SHAM) at 100 ug/ml to block the alternate oxidation pathway (Olaya 1998).

The fungicide-amended V8 agar plates were placed on a template provided by the spiral plater manufacturer and 15 ul droplets of the mycelial or spore suspension (above) were evenly applied along a radial line across the fungicide concentration gradient. Eight isolates were applied on each plate with a sterile glass stirring rod (Figure 2.2). Three replicates per isolate were used for each fungicide. Plates were sealed with paraffin film (Parafilm, Bemis Company, Inc., Neenah, WI, USA) and incubated for 7 d in the dark at

room temperature (24±1˚C) before examination for mycelial growth. Following incubation for 48 hrs, spore germination was recorded from three replicates. Germination was recorded as positive when germ tube was at least half the length of the conidium. Using the manufacturer's software the 1-d incubation option was used for calculation of the local concentrations of fungicides where 50% growth inhibition was observed compared to the control plate. The EC_{50} values were determined by the manufacturer's software for each isolate by entering the end radius (ER) and tail end radius (TER) values. The EC_{50} was calculated using R studio software (Package developed by Gabriel Andres Torres-Londono, unpublished). Plate screening was done in 2012 for all fungicides. In 2013 and 2014 a different testing method was used for the QoI and benzimidazole fungicide classes which will be discussed in chapter 3.

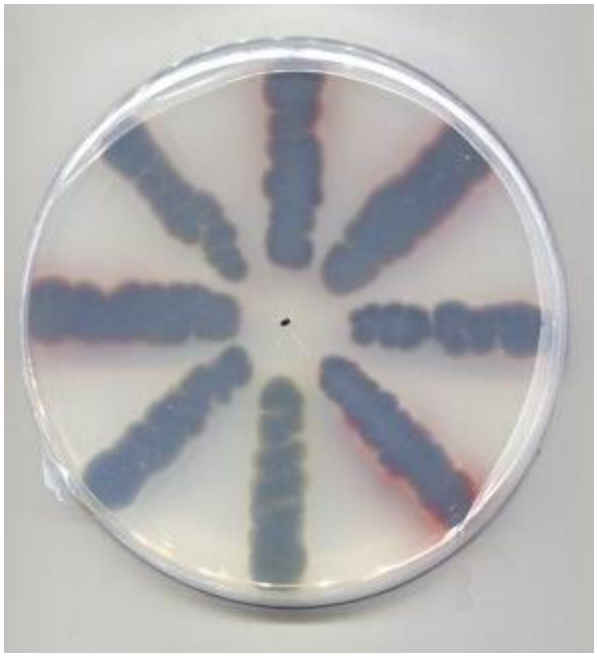

Figure 2.2. Image of a spiral plate gradient dilution assay plate. Shown is a control plate (ethanol applied to V8 agar) with the growth of *eight Cercospora beticola* isolates. The plate is shown after seven days incubation at 24 ± 1˚C in the dark after mycelial suspensions were applied.

Results

Fungicide use

Survey results indicated that DMI fungicides were widely used, with three most common difenoconazole (50%), prothioconazole (36.54%), tetracoazole (34.62%), also used were QoI fungicides (44%), TPTH (19.23%) and other products such as EBDC, copper compounds and biological control products (Figure 2.3).

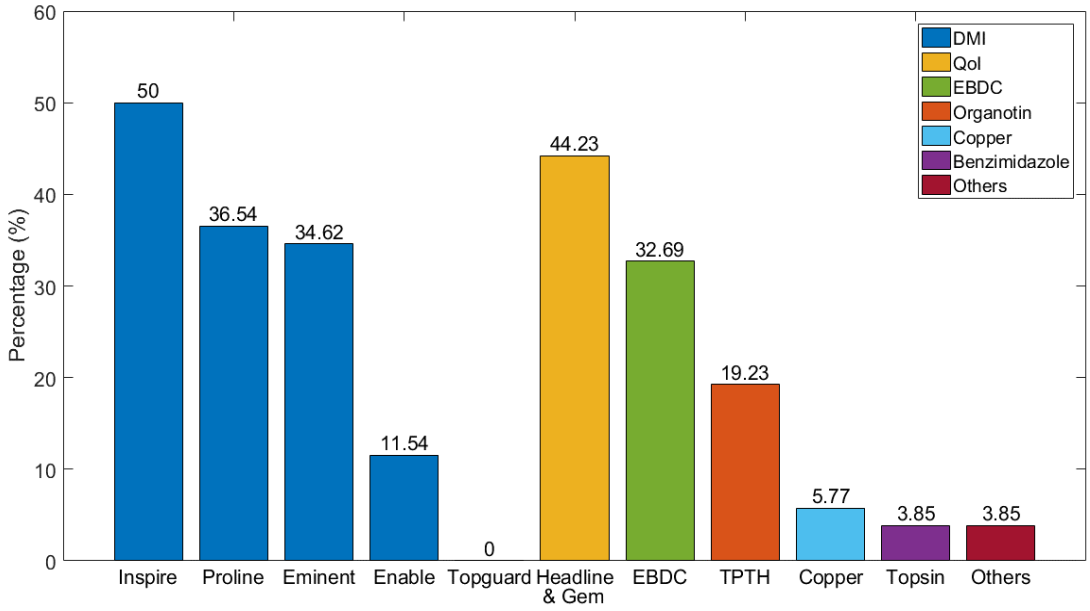

Figure 2.3. Distribution of type and proportion of fungicides used by Michigan and Ontario sugarbeet growers to control Cercospora leaf spot from 2012 to 2014. Different colors represents the different fungicide classes. Results add up to more than 100% because more than one spray may be used.

Fungicide sensitivity

A summary of the fungicide sensitivity test results from 2012 to 2014 are shown in figures 2.4-2.7.

DMI fungicides

The majority (>85%) of *C. beticola* isolates tested were sensitive to DMI fungicides from 2012 to 2014 with EC_{50} values less than 5 ppm. The average EC_{50} of difenoconazole and fenbuconazole were consistently lower than those of flutriafol, tetraconazole, and prothioconazole (Figure 2.4). The percentage of isolates with EC_{50} values greater than 1 ppm varied between different compounds within the DMI class. Less than 10% of isolates

tested had EC_{50} values greater than 10 ppm and the actual proportion varied for the various compounds tested (Figure 2.4).

QoI fungicides

Resistance to the QoI fungicide class was widespread in the current study. More than 80% of isolates tested had EC_{50} values greater than 100 ppm to the QoI fungicide pyraclostrobin in 2012. Sensitive isolates accounted for less than 20% of the material collected (Figure 2.7).

TPTH fungicides

The majority of *C. beticola* isolates (~80%) examined from all three years were sensitive to TPTH with EC_{50} values of less than 1 ppm (Figure 2.5). In 2012 and 2013, no isolates tested had EC_{50} values >5 ppm. In 2014 four isolates out of 251 had EC_{50} values between 5 and 10 ppm, and 14 isolates had EC_{50} values greater than 10 ppm.

Benzimidazole (Thiophanate methyl)

In 2012, 50 *C. beticola* isolates were tested for sensitivity to the benzimidazole fungicide thiophanate methyl. Approximately 90% of the isolates had EC_{50} values greater than 100 ppm. Only 10% of the isolates were sensitive with EC_{50} values less than 1 ppm. A subset of isolates (31) were screened for sensitivity to the N-phenylcarbamate diethofencarb. 86% of the isolates were sensitive with EC_{50} values less than 1.0 ppm (Figure 2.6).

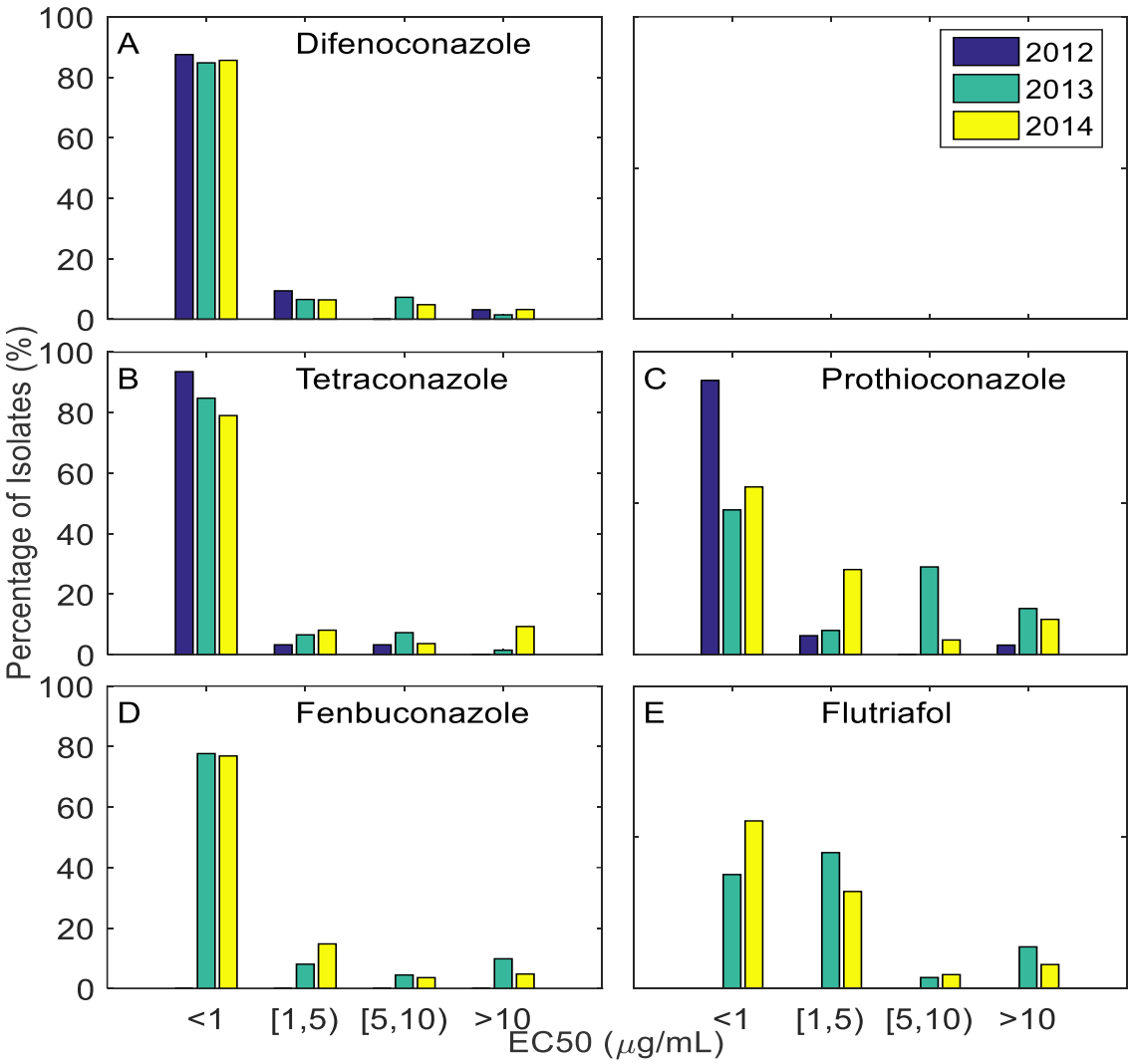

Figure 2.4. The percentage of *Cercospora beticola* isolates from 2012 (blue), 2013 (green) and 2014 (yellow) identified in each of four fungicide concentration ranges that caused a 50% inhibition of mycelial growth (EC_{50}) based on a spiral gradient plate test. Results are shown for five demethylation inhibitor (DMI) fungicides, A) difenoconazole, B) tetraconazole, C) prothioconazole, D) fenbuconazole, and E) flutriafol. The categories are EC_{50} <1 µg/ml, (1, 5) =1 to 4.99 µg/ml, (5, 10) =5 to 9.99 µg/ml and >10 µg/ml. For the number of fungicides tested in 2012, 2013, and 2014 were 32,138 and 250 for difenoconazole, 31, 111, and 248 for tetraconazole, 32, 138, and 249 for prothioconazole, 0, 112, and 250 for fenbuconazole, and 0, 138, and 240 for flutriafol, respectively.

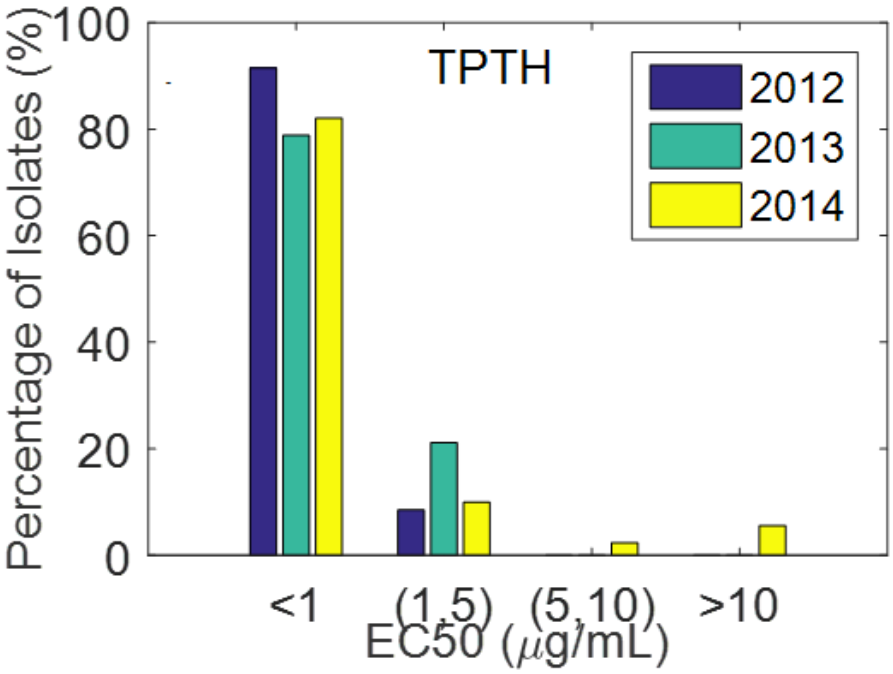

Figure 2.5. The sensitivity of *Cercospora beticola* isolates from Michigan and Ontario sugar beet fields to triphenyltin hydroxide (TPTH) over a three year period. The EC_{50} categories are EC_{50} <1 µg/ml, (1, 5) = 1 to 4.99 µg/ml, (5, 10) = 5 to 9.99 µg/ml and >10 µg/ml. The numbers of isolates tested in 2012 (blue), 2013 (green), and 2014 (yellow) were 47, 137 and 251 respectively.

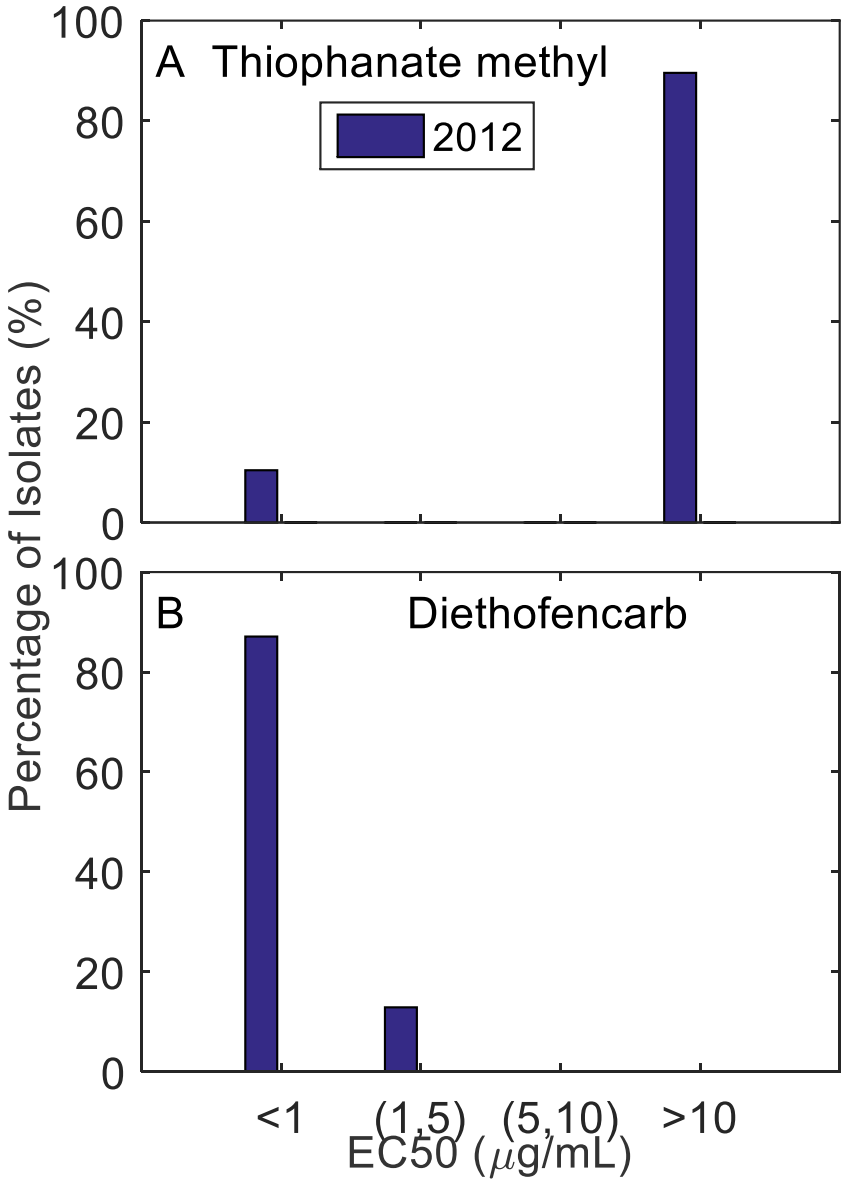

Figure 2.6. The sensitivity of *Cercospora beticola* isolates from Michigan and Ontario sugar beet fields to A) benzimidazole fungicide thiophanate methyl and B) the N-phenylcarbamate fungicide diethofencarb in 2012. The number of isolates tested for thiophanate methyl and diethofencarb was 50 and 31 respectively. The categories are EC_{50} <1 ug/ml, (1, 5) = 1 to 4.99, (5, 10) = 5 to 9.99 and >10 µg/ml.

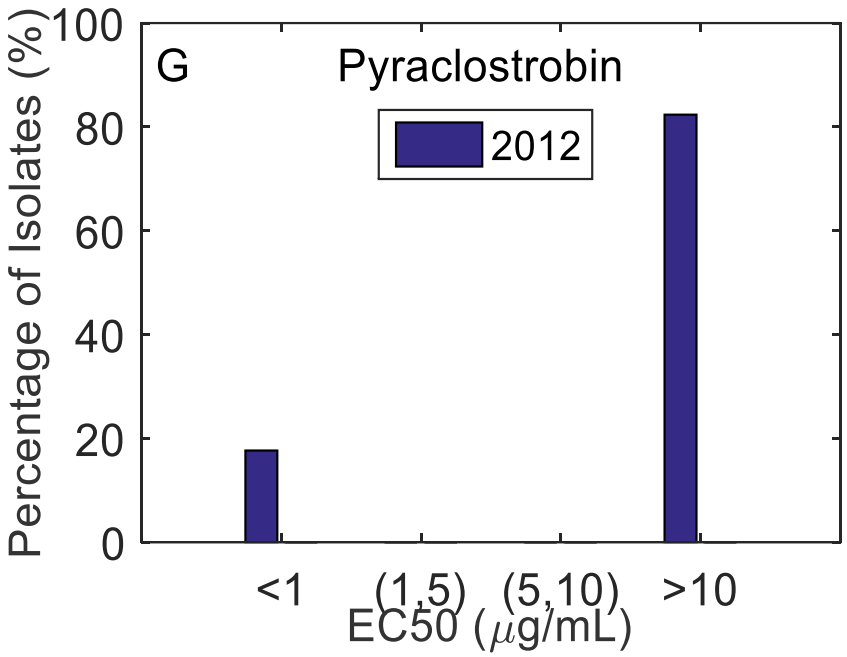

Figure 2.7. The sensitivity of 53 *Cercospora beticola* isolates from Michigan and Ontario sugar beet fields to pyraclostrobin in 2012. The categories are EC_{50} <1 µg/ml, (1, 5) =1-4.99 µg/ml, (5, 10) =5-9.99 µg/ml, and >10 µg/ml.

Discussion

Monitoring fungicide sensitivity of *C. beticola* to common fungicides can be an excellent tool to direct the development of effective recommendations specific to the Great Lakes sugarbeet production area (Kirk et al., 2012; Rosenzweig et al., 2015; Kirk and Schafer, 2013, 2014). As the agrochemical industry develops fewer fungicides it is vital that this cooperative effort maintains those fungicides that are still available. The reduced efficacy of a particular fungicide could raise concerns about the presence of resistance (Karaoglanidis and Ioannidis, 2010; Kirk et al., 2012; Bolton et al., 2013b; Trueman et al., 2013). Sampling Michigan and Ontario sugarbeet growing region for *C.*

beticola isolates and determining their sensitivity levels for fungicides registered for CLS management can help detect changes that might occur in the population.

The finding of a large portion of isolates resistance to thiophanate methyl (a benzimidazole) and pyraclostrobin (a QoI) fungicides in 2012 indicates that resistance to these fungicide classes is widespread in the Michigan sugarbeet production region. The level (~90%) of benzimidazole resistance found in this study is an increase from the range of 40-50% resistant isolates that had been observed in the area by Dr. Kirk's program between 2002 and 2011 (Kirk et al., unpublished). This indicates that resistance was maintained in the population even with little use of benzimidazole (Kirk et al., unpublished).

Benzimidazole resistance had already been known in *C. beticola* (Davidson et al., 2006), but had only recently QoI resistance been identified in the region (Kirk et al., 2012). Results from this study revealed that, only shortly after QoI was first identified in the area (Kirk et al. 2012), more than 80% of isolates tested were resistant to this class of fungicides. This indicated that QoI resistance had quickly become widespread in the Michigan and Ontario growing region.

The presence of resistance to both fungicide classes limit the number of QoI or benzimidazole fungicides applications in a given season. It is recommended that QoI or benzimidazole fungicides should never be applied alone (Kirk et al., 2012; Rosenzweig et al., 2015; Michigan Sugarbeet Research and Education Advisory Council 2015; Kirk and Schafer, 2013, 2014). They should only be applied once for CLS control as a tank-mix partner with other fungicides (e.g., DMI fungicides, TPTH, EBDC, copper) during a season.

In this study the majority of isolates tested were sensitive to DMI fungicides with EC_{50} less than 5 ppm which is consistent with results from Dr. Kirk's program in previous

years (unpublished). However, percent of isolates with EC_{50} values greater than 1 ppm have gradually increased over the past 3 years, indicating a shift toward reduced sensitivity (Figure 2.4). In the Red River Valley, a slow overall increase in the average EC_{50} values for *C. beticola* field isolates to DMI fungicides was observed between 1997 and 2005 (Secor et al., 2010), a similar phenomenon may be occurring in Michigan and Ontario growing region.

Different fungicides in the DMI class varied in their efficacy in vitro according to EC_{50} values observed in the study (Figure 2.4). Difenoconazole and fenbuconazole constantly had the lowest EC_{50} values, followed by tetraconazole, prothioconazole and flutriafol. For example, in the year of 2013, the percentage of isolates with EC_{50} values greater than 1 ppm for these five DMI fungicides was 10.8% for difenoconazole, 18.5% for fenbuconazole, 18.6% for prothioconazole, 24.4 % for tetraconazole, and 30.7% for flutriafol. While the EC_{50} values of flutriafol are higher than those of either difenoconazole or fenbuconazole, this may be more of a reflection of intrinsic activity of fungicides rather than higher resistant levels in the field, since there is no loss of disease management in the fungicide efficacy field trails (Kirk and Schafer, 2013, 2014) and data from multiple years need to be compared in order to make such conclusion.

From 2012 to 2014, a small portion of isolates (<10%) with EC_{50} values greater than 10 ppm was observed in DMI fungicide class, especially for some of the weaker fungicides such as flutriafol and prothioconazole (Figure 2.4). This raises concern for the future disease management as it could develop field level resistance. In the sugarbeet industry, loss of DMI fungicides will have a significant impact on resistance management of CLS, since several of the major alternate fungicides, such as QoI and benzimidazole

fungicides, has already developed field resistance in the Great Lake growing region (Weiland and Halloin, 2001; Davidson et al., 2006; Kirk et al., 2012; Trueman et al., 2013). Therefore, DMI fungicides have become the most important fungicide class for CLS management because of their high fungicidal efficacy (Meriggi et al. 2000). Growers could not afford to lose this class of fungicide.

Although the majority of isolates tested in this study were sensitive to most of the DMI fungicides, frequent use of this chemical class could result in continued high selection pressure for *C. beticola* with reduced sensitivity to DMI fungicides in the production area. There is evidence from other growing areas as reduced sensitivity to DMI fungicides has been reported in Greece (Karaoglanidis et al., 2000), Italy (D'Ambra et al., 1974, as reviewed by Karaoglanidis et al., 2010), and other parts of the United States including North Dakota and Minnesota (Bolton et al., 2012a).

From 2012 to 2014, the majority (>80%) of *C. beticola* isolates had EC_{50} values less than 1 ppm to TPTH (Figure 2.5). In 2012 and 2013, no isolates tested had EC_{50} value in the categories of 5 to10 ppm or greater than 10 ppm. However, in 2014, four isolates had EC_{50} values greater than 10 ppm. This is the first time that we started to identify isolates with higher EC_{50} values. TPTH is currently not used heavily in the Michigan growing region (Figure 2.3). It is an excellent contact fungicide which bring a different mode of action for disease management (Mueller et al., 2013). As part of the current CLS resistance management recommendations from Michigan Sugar Cooperative are to mix or rotate with different chemistries (Michigan Sugarbeet Research and Education Advisory Council, 2015). One of the recommendations is to use more organotin fungicides like TPTH. Since resistance to TPTH has been reported

in other areas (Bugbee 1995, Cerato and Grassi, 1983), it is important to monitor fungicide sensitivity of *C. beticola* to organotin to assess efficacy of resistant management programs in Michigan.

C. beticola strains resistant to organotin fungicides have been reported to have reduced fitness (Giannopolitis and Chrysayi-Tokousbalides, 1980) compared to tin-sensitive strains. Resistant strains usually don't compete well with sensitive *C. beticola* in the absence of the fungicide (Giannopolitis and Chrysayi-Tokousbalides 1980). This can be important in resistance management because the resistant isolates may decrease in the population over time when the organotin fungicides are not used (Giannopolitis, 1978; Secor et al., 2010).

The Great Lakes sugarbeet industry is facing a significant challenge with respect to CLS control due to the loss of effective fungicides such as benzimidazoles (Weiland and Halloin, 2001; Davidson et al., 2006), QoI fungicides (Kirk et al., 2012; Trueman et al., 2013; Rosenzweig et al., 2015) and a lack of products from other fungicide groups to supplement the small number of fungicides currently available. The DMI fungicides have an enormous economic impact on sugarbeet production industry and are now under considerable pressure because they are the most effective fungicide class left for CLS management (Kirk et al., 2012, Weiland and Halloin, 2001; Rosenzweig et al., 2015). This indicates the need for the development of additional fungicides with alternative modes of action or use of other methods for disease management for long term viability of CLS management.

Twenty *C. beticola* isolates resistant to both to DMI fungicides and TPTH were recovered over the course of this study. It is recommended to continue to monitor

sensitivity of *C. beticola* to commonly used fungicides in this region, especially fungicides in the DMI class as the widespread application of DMI fungicides on sugarbeet and the potential for failure of under field condition if isolates continue to shift toward decreased sensitivity to this class of fungicides.

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Chapter 3: Using PCR-RFLP to monitor *Cercospora beticola* from Michigan and Ontario, Canada sugarbeet (*Beta vulgaris*) fields for differing fungicide sensitivity

Abstract

Cercospora leaf spot (CLS) is a disease of concern to many sugarbeet production areas in the USA. With the finding of high-level resistance to several classes of fungicides in the field, grower's spray options are limited, and thus CLS management may become an increasing challenge in the future. Molecular techniques for detection of fungicide resistance have the potential to provide reliable and rapid identification data. A PCR-RFLP assay was assessed to screen *Cercospora beticola* for point mutations known to cause high levels resistance to two classes of fungicides. The presence of the two mutations (G143A and E198A) were monitored within *C. beticola* from Michigan and Ontario sugarbeet production fields in 2012, 2013 and 2014. In 2012, the mutations were detected in over 90% of the field samples originating from the Michigan and Ontario sugarbeet production area. Monitoring for the mutations in fields located in 14 counties and 58 townships indicated that the resistant mutations were widespread in both Michigan and Ontario. Overall, PCR-RFLP is a high-speed, reliable and sensitive tool for detecting QoI and benzimidazole resistance in *C. beticola*.

Note: Data from 2012 has already been published. Rosenzweig, N., Hanson, L. E., Clark, G., Franc, D., Stump, W. L., Jiang, Q. W., Stewart, J., and Kirk, W. W. (2015). Use of PCR-RFLP analysis to monitor fungicide resistance in *Cercospora beticola* populations from sugarbeet (*Beta vulgaris*) in Michigan, United States. Plant Disease 99:355-362.

Introduction

Cercospora leaf spot (CLS), caused by *Cercospora beticola* (Sacc.) is an annual concern for sugarbeet (*Beta vulgaris* L.) production in many growing regions (Jacobsen et al. 2009). CLS is the major foliar disease on sugarbeet especially during growing seasons with high humidity (>90%) and warm temperatures (25-35˚C) (Franc, 2010). CLS can cause significant yield losses, with potential for losses of up to 40% (Shane and Teng 1992). Economic losses may occur due to reductions in harvested yield and percent of extractable sucrose as a result of a decrease in photosynthetic capacity (Shane and Teng 1992, Jacobsen et al. 2009). Moreover root storability can be reduced due to foliar infection (Jacobsen et al. 2009). In most areas where *C. beticola* is a problem, management of CLS primarily depends on timed fungicide applications, disease forecasting models and the use of CLS-tolerant sugarbeet varieties (Kaiser et al. 2010, Khan et al. 2007, Windel et al. 1998). Several fungicides are used to manage CLS, including two, the quinone outside inhibitor (QoI) and benzimidazole fungicide classes, which will be discussed in this chapter.

QoI fungicides are widely used to manage plant diseases because of their wide range of efficacy against many agriculturally important fungal pathogens (Bartlett et al. 2002). First launched in 1996, QoI fungicides had become one of the world's biggest selling fungicides four years later, representing over 10% of the global fungicide market (Bartlett et al., 2002). QoIs have been registered in numerous countries for use on diverse plants such as cereals, vegetables, ornamental plants, grapevines, turf grass and sugarbeet. (Fernández-Ortuño et al., 2010; Secor et al., 2010). They are used to control many plant diseases, including powdery mildews, brown rusts and downy mildews (Balba,

2007; Fernández-Ortuño et al., 2010).

QoI fungicides have a broad-spectrum of activity a large variety of pathogenic fungi primarily due to their specific mode of action (Bartlett et al. 2002). QoI fungicides act by binding the quinol oxidation (Qo) site of cytochrome b and inhibiting mitochondrial respiration by blocking the electron transfer between cytochrome b and cytochrome c1, which interferes with the production of ATP and leads to an energy deficiency in organisms (Anke 1995; Bartlett et al. 2002). This target site is an integral membrane protein complex that it is an essential part of fungal respiration (Anke 1995; Bartlett et al. 2002).

While QoI fungicides have been very popular and successful for disease control, they have some downsides as well. In particular, they are at high risk for resistance development (FRAC, 2015). Resistance to QoI fungicides was first discovered in Europe in isolates of *Blumeria graminis* f. sp. *tritici,* the causal organism of powdery mildew on wheat (Sierotzki et al. 2000b). Product efficacy had declined compared to earlier disease control levels in many commercial and experimental fields in Europe (Chin et al. 2001, Sierotzki et al. 2000). After the first report resistance has been reported in many fungi, including *Pseudoperonospora cubensis* (Ishii et al., 2001), *Corynespora cassiicola* (Ishii et al., 2007) *Pyricularia grisea* (Vincelli et al., 2002; Kim et al., 2003), *Cercospora beticola* (Kirk et al., 2012; Bolton et al., 2013a, 2013b) , *Colletotrichum cereale* (Wong et al. 2007), *Alternaria* spp*.*, (Ma et al., 2003a; Vega et al., 2014; Pasche et al., 2005), *Mycosphaerella graminicola* (Torriani et al., 2009; Siah et al., 2010), *Mycosphaerella fijiensis* (Sierotzki et al., 2000), and *Venturia inaequalis* (Köller et al., 2004).

Resistance to QoI fungicides in the field most commonly is caused by a point

mutation in the fungal cytochrome b gene (CYTB) which results in an altered amino acid sequence at the quinol oxidation binding site (Pasche et al., 2005; Gisi et al., 2002; Ishii et al., 2001; Sierotzki et al., 2007). This type of resistance is commonly referred to as "qualitative resistance" because the resistance results from the modification of a single major gene and is characterized by a sudden loss of fungicide effectiveness (Ma et al., 2003a, 2004; Sierotzki et al., 2000). To date three amino acid substitutions have been reported in QoI-resistant fungi and oomycetes from the field (Pasche et al., 2005; Gisi et al., 2002; Ishii et al., 2001; Köller et al., 2004; Sierotzki et al., 2007). The most common resistance mutation is at codon 143 in the mitochondrial cytochrome b gene which leads to an amino acid change from glycine to alanine (G143A) (Ishii, 2007; Köller et al., 2004; Ma et al., 2003a). Isolates with a G143A mutation express a high-level of resistance, and the mutation is often associated with a severe loss of disease control (Fernández-Ortuño et al., 2010). Recently resistance to QoI products was reported in field isolates of *C. beticola* in Michigan, Nebraska and the Red River Valley in the United States (Kirk. et al. 2012; Bolton et al., 2013a, 2013b) and other regions [e.g. in Canada (Trueman et al., 2013) and European country such as Italy (Birla et al., 2012)]. All the highly resistant *C. beticola* isolates identified had the G143A mutation. Other mutation sites that are less frequently reported occur at positions 129 and 137 in the mitochondrial cytochrome b gene. They lead to substitutions of amino acids from phenylalanine (Phe) to leucine (Leu) (F129L) (Pasche et al., 2005; Kim et al., 2003) or glycine (Gly) to arginine (Arg) (G137R) respectively (Sierotzki et al., 2007). These two mutations usually show moderate levels of QoI resistance (Sierotzki et al., 2007).

The benzimidazole class of fungicides acts primarily by binding to fungal β-tubulin,

thus interfering with mitosis and disrupting the integrity of the fungal cytoskeleton (Davidse et al. 1986, Sisler et al. 1988). Resistance was found among *C. beticola* in Michigan in the 1990s (Weiland et al. 2001). Studies on several benzimidazole-resistant phytopathogenic fungi have shown that the most common source of resistance is mutations in the target β-tubulin (Davidson et al., 2006; Koenraadt et al., 1992; Ma et al., 2003b). One of the more commonly mutated codons in field isolates is codon 198, with resulting amino acid substitutions such as E198A, E198K, E198Q or E198V (Koenraadt et al., 1992; Luck and Gillings, 1995; Fujimura, et. al., 1992; Baraldi et al., 2003). Mutations at other codons, including 6, 50, 167, 198, 200 or 240, also are reported from field isolates of pathogenic fungi and can cause different levels of benzimidazole resistance (Luck and Gillings, 1995; Koenraadt et al., 1992; Ma et al., 2003b; Albertini et al., 1999; McKay et al, 1998; Gafur et al., 1998). The E198A mutation has been shown to be responsible for high-level of benzimidazole resistance in field isolates of many fungal species compare to mutations at other codons (Yarden et al. 1993, Ma et al., 2003b, Baraldi et al., 2003).

In addition to conferring resistance to benzimidazoles, the E198A mutation confers a negative cross-resistance to N-phenylcarbamate fungicides (Koenraadt et al., 1992; Davidson et al., 2006). This might raise the possibility of using N-phenylcarbamate fungicides for management of benzimidazole resistance when this mutations is present. However previous researchers determined that the E198A mutation did not remain the primary mechanism of resistance very long when N-phenylcarbamates were used (Josepovits et al. 1992, Elad et al. 1992). Thus it is not recommended to use N-phenylcarbamates to manage benzimdazole-resistant fungi. Hwever the negative cross-

resistance can be used as a method to help screen for the type of resistance mutation present in a fungus (Davidson et al., 2006). There are other amino acid substitutions that confer negative cross resistance at codon 198 of the β-tubulin gene (Koenraadt et al., 1992; Davidson et al., 2006) but mutations at other codons, and some at codon 198 do not confer this resistance (Koenraadt et al., 1992; Fujimura et al., 1992).

Because of the slow speed and high cost of the traditional method, rapid cost-effective detection methods are needed for diagnosis and detection of resistant *C. beticola* in sugarbeet fields, monitoring *C. beticola* field population(s) and evaluating fungicide resistant programs (Rosenzweig et al., 2015). The development of this method will allow diagnostic labs to provide rapid, cost-effective results to sugarbeet industries. The objectives of this study were to (i) field test two PCR-based detection assays developed by the Hanson lab for benzimidazole and QoI resistance in *C. beticola* in eastern Michigan, (ii) determine the distribution of benzimidazole- and QoI- resistant *C. beticola* from CLS in sugarbeet production fields in Michigan over time.

Materials and Methods

Sampling and Isolation

Leaves with symptoms of CLS were sampled from sugarbeet fields in east-central Michigan and southern Ontario during late August to early October of 2012, 2013 and 2014 with the generous assistance of agriculturalists from Michigan Sugar Cooperative. Samples of up to 10 leaves (depending on the severity of symptoms and number of leaf spots) were taken at random from symptomatic sugarbeet from a selection of 14 counties and 58 townships in Michigan (Figure 3.1). Global positioning system (GPS) coordinates of the fields were recorded using a mobile GPS unit (Trimble Navigation Ltd., Sunnyvale,

CA). Leaf samples were sent overnight to Michigan State University (East Lansing, MI) for evaluation. Identification of *C. beticola* was done in the Hanson lab based on morphological characteristics (Crous and Braun 2003). Leaf discs (~5mm) were cut from leaves using a hole-punch. Both leaves and leaf discs were stored at 4˚C until processed.

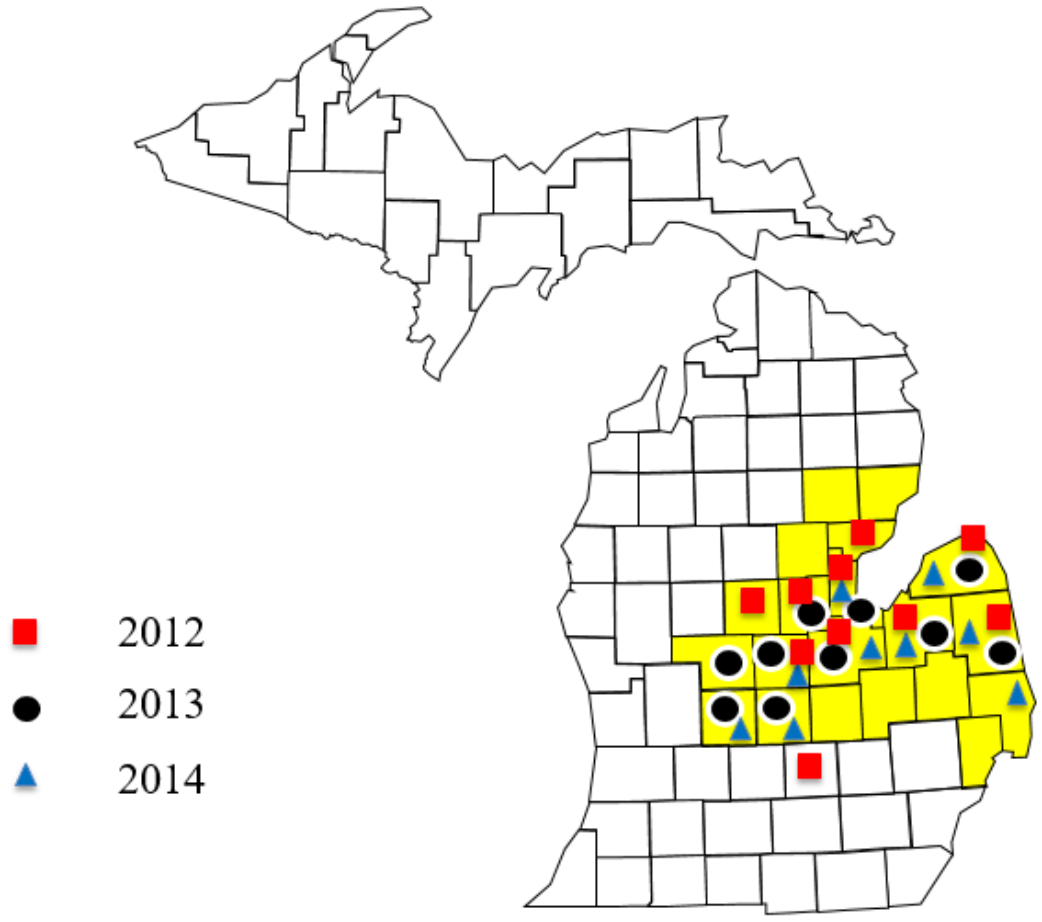

Figure 3.1. Distribution of sampling locations in Michigan from which sugarbeet leaves with symptoms of Cercospora leaf spot were collected from 2012 to 2014. Yellow areas represent the commercial sugarbeet production region. ■ represent sampling counties for 2012; ● represent sampling locations for 2013; ▲ represent sampling locations for 2014.

Medium Preparation-*Clarified V8® medium*

Three grams of calcium carbonate ($CaCO_3$) was added to 1L of V8 juice to prevent the acidity of V8 from hydrolyzing the agar (Miller 1955), the mixture was stirred by a glass rod thoroughly and clarified by centrifuging (Sorvall™ RC 6 Plus, Thermo Scientific, Waltham, MA, USA) the mixture at 6750 × g for 10 min in nalgene bottles (Nalge Nunc International Corporation, Rochester, NY,USA). The supernatant was collected and used for making clarified half strength V8 juice agar (Miller, 1955, used half as much V8 compared to the full strength).

Isolation

Leaves from the field samples were incubated in plastic bags (SC Johnson Inc., Racine, WI) with two layers of 26 x 23 cm paper towels moistened with 50 ml of sterile distilled water and incubated at room temperature with ambient light for 48 hrs to promote spore production (Hanson, unpublished). Lesions were observed with a dissecting microscope (Olympus Inc., Tokyo, Japan) and identification of *C. beticola* was done based on morphological characteristics (Crous and Braun 2003). To collect spores a wire loop was flame sterilized, dipped in sterile distilled water, and scrapped across the lesion surface. Spores were spread onto 1.5% (w/v) water agar. Pure cultures were obtained by transferring an individual germinating conidium with water agar onto clarified V8 agar plates under a dissecting microscope.

Reference isolates of *C. beticola* known to be sensitive to benzimidazole and QoI fungicides that had been originally collected from infected sugarbeet leaves were maintained on V8 agar during the experiment (Davidson et al., 2006; Kirk et al., 2012) and used for comparison to the materials collected during the study.

Fungicide sensitivity test

A subset number (32) of isolates were tested for their fungicide sensitivity using a spiral gradient dilution plate growth bioassay (Forster et al. 2004) on half strength V8 agar amended with a technical grade benzimidazole (thiophanate methyl, Sigma-Aldrich, St Louis, MO, USA) or with a QoI fungicide (pyraclostrobin, Sigma-Aldrich, St Louis, MO, USA). For the QoI fungicide salicylhydroxamic acid (SHAM) was added to block the alternate oxidation pathway (Olaya et al. 1999). Stock solutions were prepared by dissolving 10 mg of technical-grade fungicide in 1 mL of 100% ethanol and mixing thoroughly with a vortex mixer to make 10,000 ppm fungicide stock solutions. 54.3 µL of each fungicide was spirally deposited on 150 × 15 mm petri dishes (Fisher Scientific International Inc, Hampton, New Hampshire, USA) using the exponential model in the spiral plater (SGE^{TM}; Spiral Biotech, Inc. Norwood, MA). The effective concentration for 50% reduction (EC_{50}) in mycelial growth (for benzimidazoles) or spore germination (for pyraclostrobin) values for each isolate was calculated using R studio software (Package developed by Gabriel Andres Torres-Londono, unpublished). Isolates were classified as sensitive if the EC_{50} values were < 0.1 µg/ml and <0.01 ug/ml for benzimidazole and QoI fungicides respectively (Davidson et al. 2006; Kirk et al., 2012). Isolates were classified as resistant if the EC_{50} values were > 10 ug/ml for benzimidazole and QoI fungicides respectively. Isolates with EC_{50} values >100 ug/ml are considered resistance at field-level.

DNA extraction from fresh sugarbeet leaf tissue

Seven 5-mm diameter leaf disks with actively growing *C. beticola* were punched out of either a single leaf or up to 10 different leaves from the same field using a hole-punch and transferred to sterile 2-ml plastic tubes. A 1/4″ceramic sphere bead was added

to each tube (FastPrep, MP Biomedicals Inc., Solon, OH). A bead beater (FastPrep, MP Biomedicals Inc., Solon, OH) was used to disrupt plant tissue according to the manufacturer's instructions. Total DNA was extracted using a DNA extraction kit (DNeasy Plant Mini Kit, Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. DNA was quantified at 260/280 nm wavelength using a spectrophotometer (NanoDrop, Wilmington, DE).

DNA extraction from C. beticola pure culture

V8 broth cultures were prepared by transferring a plug of agar from a two week-old actively growing culture of each isolate into 250-ml sterile flasks containing 50 ml of half strength clarified V8 broth. Flasks were shaken at 130 rpm and 28°C for one week in a shaking incubator (New Brunswick Scientific, NJ, USA) in the dark. After a week the fungal materials were washed twice with sterile distilled water and transferred to sterile 15-ml plastic tubes **(**USA Scientific, Inc., Ocala, FL) using a stainless steel lab spatula. All the tubes were kept in a -20˚C freezer overnight, transferred to a -80˚C freezer at least overnight and lyophilized (Genesis Pilot Lyophilizer, SP Scientific, Gardiner, NY). Lyophilised mycelium was ground into a fine powder using a tissue grinder (GenoGrinder 2000, OPS Diagnostics LLC, Lebanon, NJ, USA). A DNA extraction kit (DNeasy Plant Mini Kit) was used for DNA extraction according to the manufacturer's instruction. DNA was quantified as described above.

PCR-RFLP

The primer pair CbBtrF 5′-ATTCTCCGTCATGCCATCTC-3′ and CbBtrR 5′-GAAACGCAGACAGGTTGTCA-3′ (Obuya et al., 2008; Rosenzwig et al., 2015) was used to amplify a predicted 500 bp amplicon from the β-tubulin gene that includes condon 198, the site of previously reported resistance mutations (Davidson et al., 2006). Another primer set, cytbFu 5′-ACAAAGCACCTAGAACATTGG-3′ and cytbRu 5′-GAAACTCCTAAAGGATTACCTGAACC-3′ (Malandrakis et al. 2011), were used to amplify a predicted amplicon from the cytochrome *b* gene that includes codon 143, the site of previously reported resistance mutations. PCR reactions consisted of ~ 30 ng of genomic DNA template, 10 μL of 5X reaction buffer with 7.5 mM $MgCl_2$ (GoTaq, Promega Co., Madison, WI), 1 μL of 10 mM dNTP's (Promega Co., Madison, WI), 1.25 μL of 10 μM of each primer (Synthesized by Integrated DNA Technologies, Inc., Coralville, IA) and 5U of Taq (GoTaq, Promega Co., Madison, WI), in a 25 μL reaction volume. For the cytochrome b PCR reaction, amplifications were performed in a thermocycler (MyCycler, Bio-Rad, Hercules, CA) with a denaturation of 95 °C for 5 min denaturation followed by 28 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 35 s with a final 10 min extension at 72 °C. For the β-tubulin PCR reaction, amplifications were performed with the following parameters: initial denaturation of 94°C for 5 min, 35 cycles of 94°C for 1 min, 52°C for 30 sec, and 72°C for 1 min final extension at 72°C for 5 min. Five microliters of the PCR products were separated by gel electrophoresis in a 1% (w/v) agarose gel in 0.5×Tris-borate-EDTA (5.4g Tris-base, 2.75g boric acid, 2 ml 0.5M EDTA), stained with nucleic acid stain (GelRed, Phenix Research Products, Chandler, NC) and visualized by UV exposure using a transilluminator (Gel Doc 2000 Bio-Rad, Hercules, CA).

In 2013 and 2014 GoTaq® Green Master Mix (Promega, Madison, USA) premixed ready-to-use solution was used for PCR. It is a solution which already contains TaqDNA polymerase, $MgCl_2$, dNTPs and reaction buffers. PCR reactions were conducted and performed as described above.

After amplification with PCR, a 10 µL volume of the PCR reaction was digested overnight with 1.5 U of restriction endonuclease Fnu4HI for cytochrome b gene or BstUI for the β-tubulin gene (New England Biolabs, Ipswich, MA) at 37˚C or 60°C respectively. Restriction fragments were separated on 2.5% agarose gels in 0.5×TBE by electrophoresis and stained with ethidium bromide (10 mg/ml). Gels were visualized using a UV transilluminator and documented using Image J (National Institutes of Health, Bethesda, Maryland, USA).

Products from 32 PCR reactions were sequenced at the Michigan State University Research Technology Support Facility using a sequencing kit (BigDye3.1 Terminator, Applied Biosystems Inc. Foster City, CA) and analyzed on a sequencer (ABI3730XL capillary, Applied Biosystems). The sequence of the QoI-resistant *C. beticola* (Kirk et al., 2012) had 100% identity to QoI-resistant isolates from Genbank (GenBank accession JQ619933 and JQ360628). QoI-sensitive isolates had 100% identity with a sensitive isolate (GenBank accession EF176921.1). Sequence results revealed that each resistant isolate contained a change at codon 143 in the cytochrome b gene that was predicted to lead to an amino acid change from glycine to alanine (G143A) (Kirk et al., 2012) which confers QoI resistance in several other fungi (Ishii, 2007; Köller et al., 2004).

Results

PCR-based detection of the G143A mutation in C. beticola

The molecular-based PCR-RFLP method differentiated isolates which contained the resistance mutation for QoI fungicides from those with the wild-type, sensitive sequence. Strong amplification and digestion results were obtained from DNA extracted from pure cultures and similar results were found for DNA extracted from symptomatic leaf tissues (Figure 3.2). After digestion with *Fnu*4HI, three DNA fragments were obtained from the single PCR amplification product in QoI-resistant isolates whereas the PCR product from the sensitive isolates was cleaved only once yielding two products (Figure 3.2).

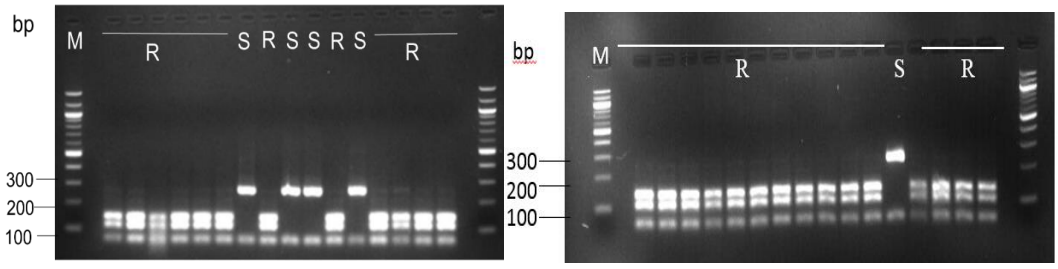

Figure 3.2. Fnu4HI restriction patterns of a 325-bp-long fragment of the cytochrome b gene of *Cercospora beticola* including the site of the quinone outside inhibitor-sensitive (S) and -resistant (R) sequences. The first lane (M) on the left contains the 100-bp DNA ladder. On the left is an example of a gel from amplification from symptomatic leaf tissues. On the right is an example of a gel from *C. beticola* isolates.

PCR-based detection of the E198A mutation in *C. beticola* β-tubulin

After digestion, two DNA fragments were obtained from benzimidazole-resistant isolates whereas the wild-type isolates were not cut (Figure 3.3). (Note for both caption that the sensitive sequence is generally considered the wild-type and not a mutant).

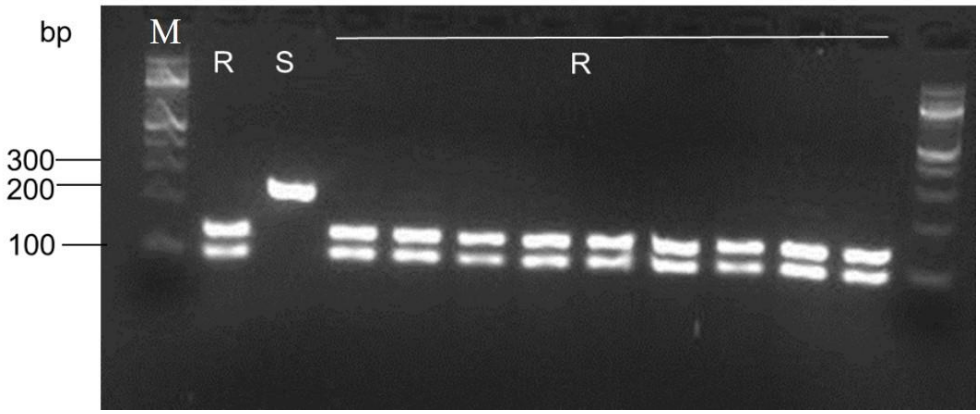

Figure 3.3. BtsUI restriction pattern of a fragment of the β-tubulin gene from *Cercospora beticola* that includes the site of a mutation associated with varied benzimidazole sensitivity. Shown are results of benzimidazole-sensitive (S) and resistant (R) leaf disc samples. Each sample included 7 leaf discs from sugar beet leaves with Cercospora leaf spot symptoms. M indicates the standard 100-bp DNA ladder.

The PCR-RFLP assay was tested in 2012 (Rosenzweig et al., 2015), 2013 and 2014. Results showed that resistance to benzimidazole and QoI fungicides is widespread in the Michigan sugarbeet growing region. In 2012, a total of 123 samples, for a total of 861 leaf discs, each representing a single lesion were processed using the PCR-RFLP detection method for both the QoI and benzimidazole fungicide resistance mutations (Table 3.1). For QoI fungicides, 82 samples (67%) tested had the QoI-resistant digestion pattern, 34 samples (27.7%) had both the resistant and sensitive digestion pattern, and seven samples showed only a sensitive digestion pattern. For benzimidazoles, 98 samples (80%) of those tested had the benzimidazole-resistance pattern, 22 samples

(17.9%) had both resistance and sensitive digestion patterns, and three samples had only the sensitive digestion pattern. Wild-type (sensitive) mutations were detected in samples from two counties (Gratiot and Huron) and three counties (Gratiot, Huron, and Saginaw) using the G143A and the E198A detection methods respectively (Figures 3.4 and 3.5). In 2013, only pure cultures were used, about 89.5% of the *C. beticola* isolates (114) tested contained the G143A mutation and 95.8% of the isolates (118) contained the E198A. In 2014 nearly 99% of samples tested had the resistance mutation for QoI fungicides, 69 samples (75.8%) from fields contained the E198A mutation, 22 samples (24.2%) showed a sensitive digestion pattern, 53 samples (58.2%) had only resistant digestion pattern, and 16 leaf samples (17.6%) had both a sensitive and resistant digestion pattern.

Table 3.1. Summary of *Cercospora beticola* monitoring efforts from Michigan commercial sugarbeet fields from 2012 and 2014. Results are based on PCR-based detection methods from leaf discs containing Cercospora leaf spot lesions. "Resistant" indicates that only the resistant mutation was identified in the composite sample, "Sensitive" indicates that only the sensitive wild-type sequence was identified and "Mixed" indicates that both mutant and wild-type material was detected.

	QoI assay[a]	Benzimidazole assay[b]
2012		
Resistant	82 (67%)	98 (80%)
Sensitive	7 (5.7%)	3 (2.4%)
Mixed	34 (27.7%)	22 (17.9%)
Number of samples[c]	123	123
Number of lesions[d]	861	861
2014		
Resistant	166 (98.8%)	53 (58.2%)
Sensitive	0 (0%)	22 (24.2%)
Mixed	2 (1.2%)	16 (17.6%)
Number of samples[c]	168	91
Number of lesions[d]	1176	637

a: Assay based on PCR-RFLP using the Fnu4HI restriction enzyme to differentiate the G143A mutation in the *Cercospora beticola* cytochrome b gene that confers resistance to Quinone outside inhibitor (QoI) fungicides from the wild-type, sensitive sequence.

b: Assay based on PCR-RFLP using the BstUI restriction enzyme to differentiate the E198A mutation in the *Cercospora beticola* β-tubulin gene that confers resistance to benzimidazole fungicides from the wild-type, sensitive sequence.

Table 3.1 (cont'd)
c: Total number of samples processed in the study, each sample consists of seven 5-mm diameter leaf disks with actively growing *C. beticola*.
d: Total number of leaf disks (with actively growing *C. beticola*) tested in the study.

Table 3.2. Summary of *Cercospora beticola* monitoring efforts from Michigan commercial sugarbeet fields in 2013. Shown are the number of isolates tested. Results are based on PCR-RFLP detection methods. "Resistant" indicates that the resistant digestion pattern was identified in the pure culture, "Sensitive" indicates that the sensitive (wild-type) digestion pattern was found.

	Benzimidazole assay[a]	QoI assay[b]
2013 Number of Isolates	118	114
Resistant	113 (95.8%)	102 (89.5%)
Sensitive	5 (4.2%)	12 (10.5%)

a: Assay based on PCR-RFLP using the BstUI restriction enzyme to differentiate the E198A mutation in the *Cercospora beticola* β-tubulin gene that confers resistance to benzimidazole fungicides from the wild-type, sensitive sequence.
b: Assay based on PCR-RFLP using the Fnu4HI restriction enzyme to differentiate the G143A mutation in the *Cercospora beticola* cytochrome b gene that confers resistance to Quinone outside inhibitor (QoI) fungicides from the wild-type, sensitive sequence.

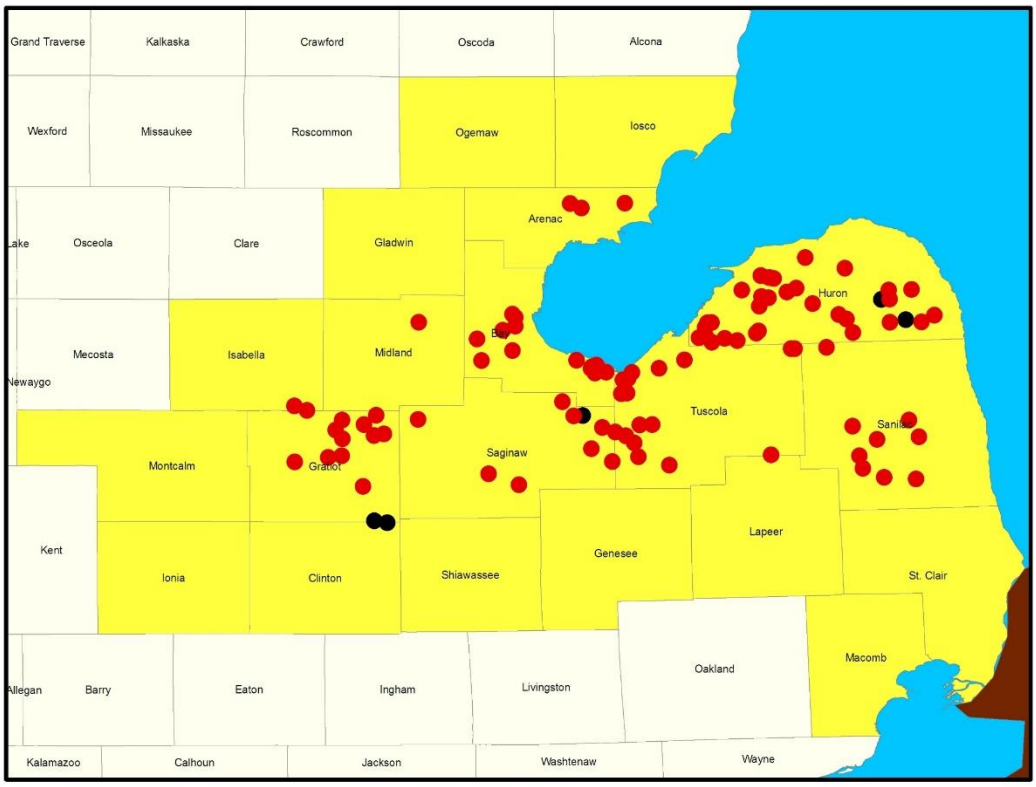

Figure 3.4. Distribution of QoI-sensitive and -resistant samples detected by a PCR-RFLP method in Michigan *Cercospora beticola* sampled in 2012. The county names are shown within the border. The yellow area represents the commercial sugarbeet production region. ●, represents detection of QoI resistant samples; ●， represents detection of QoI sensitive samples.

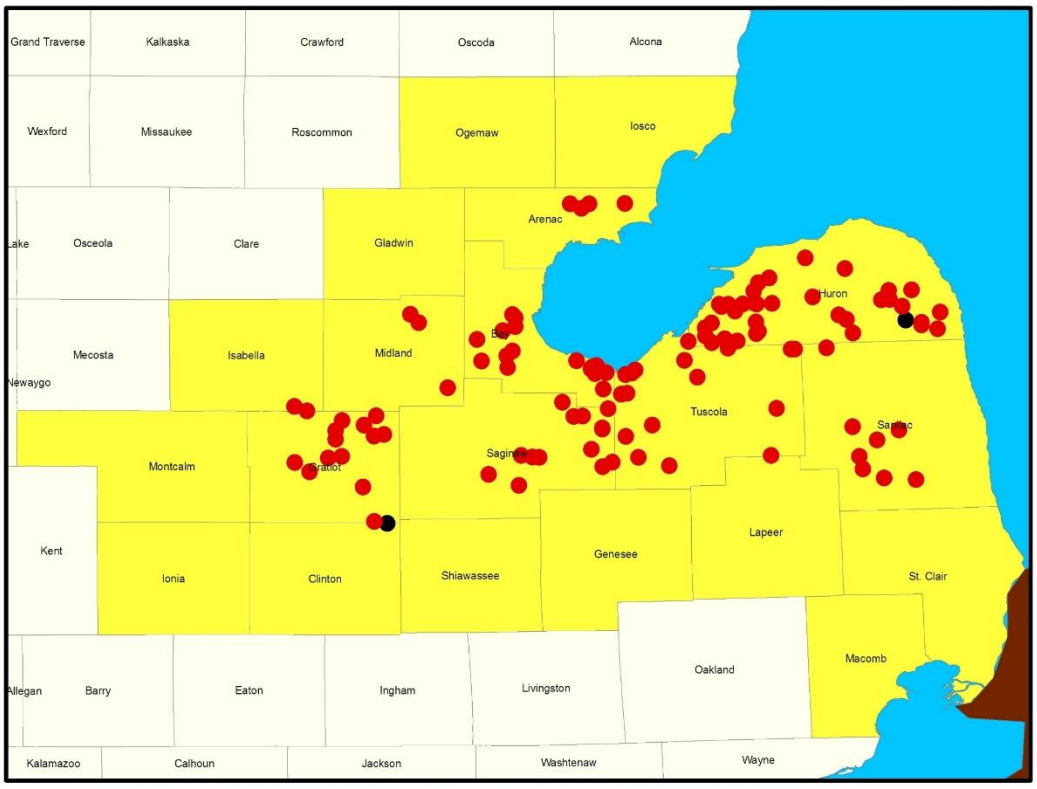

Figure 3.5. Distribution of the benzimidazole-resistant and -sensitive samples detected by a PCR-RFLP method in Michigan *Cercospora beticola* sampled in 2012. The county names are shown within the border. The yellow area represents the commercial sugarbeet production region. ●, represents detection of benzimidazole-resistant sample; ●, represents detection of benzimidazole sensitive samples

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Discussion

This study confirmed the widespread presence of two known fungicide resistance mutations, the G143A mutation (Kirk et al., 2012; Bolton et al., 2013a; Ma et al., 2003a) and the E198A mutation (Davidson et al., 2006; Ma et al., 2003b) in *C. beticola* from the Great Lakes sugarbeet fields. For QoI fungicides the resistance was first identified in *C. beticola* in Michigan in 2011 (Kirk et al., 2012). Only one year after the initial discovery of QoI fungicide resistance in Michigan the resistance was already widespread (Rosenzweig et al., 2015). For benzimidazoles, the resistance in *C. beticola* had been reported in the

area in the late 1990s (Weiland and Halloin, 2001). Benzimidazole use had decreased in the area (Dr. William Kirk, personal communication), however the current study still found widespread resistance to benzimdazoles. This level indicated a potential increase during the time of the study compared to monitoring done between 2002 and 2011 (Kirk et al., unpublished).

DNA-based approaches to detect single nucleotide polymorphisms (SNP) responsible for QoI resistance have been tested in various fungi including *Mycosphaerella fijiensis* (Sierotzki et al., 2000), *Alternaria. Solani* (Pasche et al. 2005; Rosenzweig et al. 2008a), *Cercospora beticola* (Kirk et al., 2012; Bolton et al. 2013b), *Venturia inaequalis* (Fontaine et al. 2009). The results of the current work agree with previous studies that the presence of a single point mutation causing fungicide resistance enables the use of rapid DNA-based detection methods for monitoring for resistance in a plant pathogen population (Malandrakis et al. 2011, Rosenzweig et al., 2015). Resistance management is an effort for long-term conservation of fungicide effectiveness (Brent 2012). For effective resistance management knowledge of the types of resistance present and a means to track resistance in the population are necessary. Thus such a rapid detection method would allow a rapid and simple detection of resistance mutations at field level.

Both sensitive and resistant digestion patterns were observed in some samples tested (Table 3.1). This is probably because these DNA samples were directly extracted from CLS leaf discs with more than one disc per sample. There is a possibility that one sample contained both resistant and sensitive *C. beticola.* Previous studies have shown that there can be genetically variable *C. beticola* on a single lesion (Moretti et al., 2004) or single leaf (Bolton et al., 2012b) and these results agree with such studies.

Rosenzweig et al. (2015) reported that both resistant and sensitive mutations could be detected with varying proportions of DNA from resistant and sensitive strains, with up to a 20%:80% ratio being detectable. This would be consistent with being able to detect at least one out of seven of the leaf discs in each combined sample with a different mutation pattern.

One advantage of this PCR-RFLP method compared to the traditional method using pure culture is that results can be produced within 48 hrs after leaf samples are received (Rosenzweig et al., 2015; Malandrakis et al., 2012). With the traditional methods it takes more than 21 days to get results (Kirk, unpublished). Additionally, a larger number of samples, with multiple lesions per sample, can be processed with the PCR-RFLP method that provides a better picture of the prevalence of resistance mutations in *C. beticola* under field conditions in the testing area (Rosenzweig et al., 2015) than can be obtained with pure cultures during the same time period. On the other hand, this PCR-RFLP method has some drawbacks as well. Because DNA was extracted directly from CLS lesions, no isolates were obtained. Therefore there is no material to study the biology of the causal organism. Another disadvantage of this method is that it could not detect other resistance mutations due to specificity of the primer and restriction enzyme combination (Rasmussen, 2012). Therefore this method could miss some types of resistance.

Overall, the reduced availability of effective chemical fungicides for plant disease management, the evolution of fungicide resistant strains (Kirk. et al. 2012; Bolton et al., 2013a, 2013b), as well as the United States Environmental Protection Agency restrictions on continued use of select chemistries necessitates the development evaluation and

deployment of effective resistance management programs. This PCR-RFLP diagnostic method provided an efficient tool for high-throughput processing of fungi that can be used to evaluate fungicide resistance management strategies for CLS (Rosenzweig et al., 2015) and can be helpful in rapidly monitoring for changes in sensitivity mutations in *C. beticola*.

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