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FUNGICIDE SENSITIVITY OF
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FUNGICIDE SENSITIVITY OF CHERRY PATHOGENS

By

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ABSTRACT

FUNGICIDE SENSITIVITY OF CHERRY PATHOGENS

By

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Michigan populations of *Blumeriella jaapii* (Rehm) Arx, the causal agent of cherry leaf spot (CLS), have documented resistance to sterol demethylation inhibitor fungicides (DMIs), which were frequently used to control the pathogen. A bioassay was completed to determine the current sensitivity status of *B. jaapii* isolates to dodine. Dodine has been used sporadically to control CLS for over 50 years, but the sensitivity of *B. jaapii* to dodine was unknown. When *B. jaapii* isolates from Michigan were screened *in vitro*; a wide range of minimum inhibitory concentrations (MIC) were found. MIC values ranged from 0.05 ppm to greater than 400 ppm dodine, and demonstrated a reduction in the sensitivity of *B. jaapii* isolates collected from dodine-exposed sites.

American brown rot (ABR) is caused by *Monilinia fructicola* (Wint.) Honey, and significantly impacts cherry production. DMIs are currently the most effective and frequently used fungicides for ABR control, but resistance to DMI fungicides is well documented in various fungal pathogens, including *M. fructicola* from Georgia and New York. Due to the single-site mode of action, and the history of resistance development in DMIs, the risk of resistance developing in Michigan *M. fructicola* populations is substantial. An *in vitro* assay was performed to determine the status of *M. fructicola* sensitivity to fenbuconazole (a DMI) in Michigan. Effective dose values, by orchard, ranged from 0.0038-0.0379 ppm fenbuconazole, a significant amount of variability that may be an indicator of the fungal populations ability to shift toward reduced sensitivity.

To my parents John and Joyce Taylor,
who never had a doubt.

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LITERATURE REVIEW

FUNGICIDE RESISTANCE IN PATHOGENS OF STONE FRUIT

INTRODUCTION

Michigan's cherry industry began in the late 1800's with the planting of the first tart cherry orchard on Old Mission Peninsula in northwest Michigan. The cultivation of tart cherry trees (*Prunus cerasus* L.), and sweet cherry trees (*Prunus avium* L.) has prospered in the region thanks to sloping terrain, well-drained soils and mild weather mediated by the proximity of the area to Lake Michigan. According to the USDA National Agriculture Statistics Service, the 2006 tart cherry crop was worth an estimated $53.4 million nationally (78% of which was produced in Michigan), and the sweet cherry crop was estimated at $487.4 million nationally (7% of which was produced in Michigan); (74). Fungal diseases, including cherry leaf spot, American brown rot, Armillaria root rot, European brown rot and powdery mildew cause significant losses annually. *P. cerasus* (Montmorency) is most desirable and prevalent tart cherry cultivar in the U.S. Montmorency cherry trees possess no innate resistance to the more damaging fungal diseases, cherry leaf spot and American brown rot, the two diseases that are the focus of this review. This lack of inherent disease resistance drives the cherry industry's heavy reliance on fungicides. Genetic mutations are the most prevalent cause of fungicide resistance and exist at very low frequencies within the fungal population (8). In conventionally managed cherry orchards, sterol demethylation inhibitor (DMI) fungicides are often applied more than five times in a single season, this is heavy fungicide pressure and selects for resistant isolates. Under heavy fungicide pressure

resistant isolates may increase disproportionately to normal population structure. Resistant mutants typically exist at a frequency of roughly 1:100 million, but the proportion of resistant mutants in the population increases under fungicide pressure because they tolerate the fungicide treatment and survive to propagate (8). The increase in this less sensitive population may ultimately lead to a lack of disease control in the field, or practical field resistance (40). An estimated 1:10 to 1:100 ratio of resistant isolates is required in the population before the effect of resistance is readily detectable in the field (8). Resistance has developed to most major chemical classes of fungicides in various plant pathogens. The fungicides affected include; dodine (35), dicarboximides (17, 63), benzimidazoles (35, 49) organic fungicides (24), strobilurins (29), and DMI's (20, 30). Resistance is a major concern for Michigan cherry growers because of heavy fungicide pressure and the history of fungicide resistance development in many fungicide classes.

IMPORTANT DISEASES OF CHERRY

Cherry Leaf Spot

Cherry leaf spot (CLS), caused by the ascomycete *Blumeriella jaapii* (Rehm) Arx, is arguably the most damaging fungal pathogen of tart cherry, and has the potential to significantly reduce profits for growers in the Great Lakes region of the U.S. (54). There has been relatively little information published on the biology and resistance issues associated with *B. jaapii*. CLS primarily affects foliage and as a result reduces the photosynthetic ability of the tree. Infected tart cherry leaves will turn yellow and defoliate prematurely if disease is not effectively controlled. Sweet cherry tree leaves

turn yellow but are retained. Less than 50% defoliation by early September is considered acceptable CLS control in Michigan. When significant defoliation occurs before harvest, fruit is soft and immature, has low soluble solids and ripens unevenly (37). Significant defoliation can be quantified based on the standard that at least two leaves are needed to effectively ripen each cherry on tart cherry trees (57). Following two or more years with significant defoliation early in the season, trees are susceptible to winter injury. This is due to the loss of photosynthates and therefore stored carbohydrates in roots. Blossom production is also reduced for at least two subsequent years (37).

B. jaapii overwinters on fallen leaves on the orchard floor and produces apothecia (sexual spore-bearing structures) in the spring (37). The primary infection period may last 2-6 weeks depending on conditions. Optimal apothecial development occurs between 6-16°C, with ascospore discharge increasing with temperature between 8-30°C (23). Ascospore release occurs as tissue dries following the thorough wetting of mature asci (39). Germination occurs on the surface of the leaf and infection occurs through the leaf stomata. Leaves remain susceptible throughout the growing season (37), contrary to the *in vitro* evidence of ontogenic, or age-related resistance in leaves (18). Following infection, acervuli develop on the underside of the leaf and produce a visible mass of white conidia. Conidia are dispersed from leaf to leaf by wind or rain and the infection cycle can be repeated several times during a single season, depending on conditions. The conidial stage was the basis for the development of an effective infection model by Eisensmith, an adaptation of which is still in use today (18).

All commercially cultivated cherry cultivars are susceptible to cherry leaf spot (37), although less susceptible cultivars have been found (77). The primary method of

CLS management is through fungicide application, with five to seven applications recommended per season, depending on disease pressure (62). The most common fungicides used to control CLS include: chlorothalonil, captan, strobilurins, and several sterol demethylation inhibitors (fenbuconazole, tebuconazole, myclobutanil, and fenarimol); (54). Salts containing the Cu^{2+} ion (copper hydroxide or copper sulfate), and dodine are also used. *B. jaapii* has a history of cross resistance to DMI fungicides in Michigan (62).

American Brown Rot

American brown rot (ABR) is caused by the ascomycete *Monilinia fructicola* (Wint.) Honey, and is an important pathogen on apricot, peach, nectarine, plum and cherry (particularly sweet cherry varieties); (37). The fungus attacks fruit, blossoms, spurs, and shoots with ideal infection conditions initiating epidemic inoculum levels in as little as 24 hours. ABR causes fruit rot before and after harvest, greatly reducing the quality and quantity of the yield (37). Fungicide applications help control the disease until harvest, but the development of ABR in harvested fruit remains a problem in fresh fruit markets. Processors suffer losses from ABR during the storage of fruit and ripening process (31, 34). The most distinctive sign of infection is gray/tan conidia that appear on the surface of discolored and decaying fruit. Infected fruit may rot and abscise, or persist through the winter in a mummified form. In cherry, mummified fruit provide the vast majority of the primary inoculum for infection the following spring (37) in the form of asexual conidia. Conidial production is greatest between 15 and 23°C (76). The optimal temperature for conidial germination is between 20 and 25°C (11, 59). Apothecia (sexual fruiting structures) form on fruit on the orchard floor producing ascospores in the spring,

but only in areas where the soil is moist (31); generally apothecia formation on cherry is rare in Michigan. Although injury to the fruit may lead to increased infection, the fungus readily infects when no wound or fruit-to-fruit contact is present (55). Direct infection occurs through the fruit cuticle (37), or the trichomes (31) and conidial production can occur within as little as three days (37).

ABR infection is dependent on inoculum levels and occurs more readily on mature fruit (31, 48, 56); therefore the secondary infections that occur immediately prior to harvest should be emphasized in disease management strategies (47, 48, 56). Losses during shipment and at market can be great, as the fungus spreads rapidly when fruit is removed from cold storage (1). Additionally, avoiding damage to the fruit is an important factor in minimizing disease (37), and there is evidence that hot water treatments can decrease postharvest fruit infection (34). Removal of inoculum sources such as mummies is recommended but not practical for cherry orchards. Fungicides (commonly sterol demethylation inhibitors) are heavily relied upon for control (78). Resistance of *M. fructicola* to DMI fungicides has been reported in stone fruit production outside of Michigan (12, 20, 67, 81).

Armillaria Root Rot

Armillaria root rot (ARR) is caused by fungi in the genus *Armillaria,* including; *A. gallica* (Marxmüller and Romagnesi), *A. mellea* (Vahl: Fr.) Kummer, *A. ostoyae* (Romagnesi) Herink, and *A. bulbosa* (Barla, Kile and Watling) (37). These fungi are also pathogenic to many forest and ornamental species (79). When land is cleared for cherry production, *Armillaria* spp. may already be present in the soil, colonizing debris left over from previously infected forest trees. The situation is exacerbated by the fact that the

Armillaria species appear able to persist saprophytically on woody debris for many years and populations are not eliminated by fumigation. Trees on sandy, well drained soils are more susceptible to ARR, often showing reduced growth of the terminal shoots. Leaves on infected trees may change color earlier in the season and fail to abscise normally in the fall (37). Tree mortality typically spreads in a circular pattern, emanating from the initial infection site. Armillaria root rot is diagnosed by removing soil from the base of a suspected tree and looking for a thick, white, mycelial fan between under the bark and woody xylem. Black rhizomorphs (root-like structures) and honey-colored mushrooms, which form in autumn around the base of recently killed trees, are also signs of *Armillaria* spp. Infrequently, the disease may also be introduced through contaminated soil or equipment (37).

On Mazzard rootstock, sweet cherry cultivars are more resistant than tart cherry. Currently there is no known control for *Armillaria* spp., except planting on sites where infection has not occurred (37). The acreage of useable land for cherry production will continue to diminish until an effective treatment for *Armillaria* spp. is found. Apple rootstocks are considered moderately susceptible to armillaria root rot.

European Brown Rot

European brown rot is caused by the ascomycete *Monilinia laxa* (Aderhold and Ruhland), and infects tart cherry, specifically the cultivars Meteor, English Morello and Balaton. The most common sweet cherry cultivars and Montmorency tart cherry are reasonably resistant to *M. laxa*, rarely requiring fungicide use, except during years with cool, wet spring weather. *M. laxa* damages blossoms and spurs, with a wetting period of only one day required for severe blossom infection. Newly infected blossoms turn brown

and the fungus sporulates on withered tissue. Leaves at the base of infected blossoms may also be killed; systemic infection of the spurs quickly follows. The systemic infection causes the formation of cankers (2.5-7.6 cm long) at the base of the spur. The cankers, as well as blossom debris and dead spurs, produce conidia during subsequent seasons. Fruit infections are rare and require direct contact with infected blossoms. Decreasing leaf drying time, and the application of DMI fungicides can help reduce disease development in susceptible varieties (37).

Powdery Mildew

Powdery mildew of cherry is caused by the ascomycete *Podosphaera clandestina* (Wall. Fr) Lev, an obligate parasitic. The infection first appears as circular, felt-like patches of mycelium on the leaf surface and can spread rapidly. Severely infected leaves become brittle and bunch up. Fruit infections manifest as red shiny blotches, often with mycelium and spores in the center. Conidia are spread via wind and reinfect new leaves during the repeating, secondary infection cycle. As the season progresses, cleistothecia (dark, spherical fruiting bodies) are produced. The fungus overwinters as cleistothecia on fallen leaves which then initiate the primary infection the following spring when ascospores are released. The disease commonly reduces the growth of tart cherry trees in nurseries and young orchards. Fungicide application is the primary method used to control powdery mildew. Additional cultural practices such as pruning and the removal of hedges may aid in creating a less favorable environment for disease development (37).

THE HISTORY OF FUNGICIDES

Since the first European settlers reached North America, tree fruit have been an important commodity. Losses due to disease were an issue from the beginning and only increased in severity with the development of high density agriculture (10). By the early 1900's, relief arrived from Europe after grape growers in France began using Bordeaux mixture, lime, and sulfur to control fungal diseases. These chemistries were phytotoxic and marginally effective at controlling tree fruit diseases (70); however, these mixtures were the only known control for fungal pathogens. In Michigan, these fungicides became important in controlling fungal pathogens in newly established tart cherry orchards. A 1937 University of Wisconsin research bulletin recommended using Bordeaux mixture and lime sulfur (a mixture of calcium polysulfides) to control CLS (39).

When discussing the history of fungicide development, it is important to note the evolution of fungicide modes of action, from multi-site to single-site; as well as the associated resistance issues. An overview of the history of important fruit fungicides, their modes of action and resistance histories is shown in Table 1. Copper was the first effective fungicide and is still considered valuable today (70). Broad spectrum protectants, such as dithiocarbamates (mancozeb), were patented as early as 1934 (64). During the 1950's, captan and dodine were introduced and had multi-site modes of action that affected the critical processes of the fungi.

In the late 1960s, new site-specific fungicides were introduced and widely adopted by growers. The mechanism of action in single-site fungicides involves the disruption of a single metabolic pathway or structural site of an enzyme. These

Table 1. List of fungicide, or chemistry introductions relevant to tree fruit production, the associated specificity, and resistant pathogens.

Date introduced	Chemistry Group or Fungicide	Target Site Specificity	Risk of Resistance*	Resistant Pathogens (date published)
1900	Copper	Multi-site	Low	N/A*
1952	Captan	Multi-site	Low	*Botrytis cinerea*
1959	Dodine	Multi-site	Low	*V. inaequalis* (1968)
1966	Chlorothalonil	Multi-site	Low	N/A*
1972	Benzimidazole	Single-site	High	*V. inaequalis, M. fructicola* and *B. jaapii* (1975-80)
1974	Dicarboximide	Multi-site	Low	*M. fructicola (1979)*
1986	Sterol demethylation inhibitors	Single-site	High	*V. inaequalis* (1989) and *B. jaapii* (2006)
1996	Strobilurins	Single-site	High	*P. viticola* (2002)

* Risk of resistance as determined by the Fungicide Resistance Action Committee. Table modified from Sutton, 1996 and Russell, 1995. *N/A means no known resistant fungal pathogens.

fungicides also often possessed post-infection activity, meaning that the fungicide could be absorbed by plant tissue and kill the fungi that had already invaded. The expanded use of single-site fungicides in the 1970's resulted in the increased appearance of resistant pathogen populations (62).

Contemporary public opinion is forcing many regulatory agencies to eliminate effective fungicides from the market and pushing the agrochemical industry toward discovering new and safer modes of action. The demand for new fungicides with low toxicity to non-target organisms is increasing as heightened concern over agricultural inputs increase and more stringent regulatory rules are enforced. As of the mid 1990's, an estimated $570 million was spent annually on research and development by leading agrochemical companies (52). Novel modes of action are also important in combating pathogen resistance and reduced sensitivity in existing chemistries. Specialty crops such as cherry are often overlooked when new fungicides are introduced to the market. Many specialty crops are only added to the list of approved crops long after introduction of the fungicide.

FUNGICIDE RESISTANCE IN FRUIT PATHOGENS

Due to the lack of innate resistance to pathogenic fungi in tart cherry cultivars and sweet cherry cultivars; cultural practices and sanitation are frequently helpful in controlling fungal diseases. Fungicides are ultimately are required to maximize yield and quality. The intensive use of site-specific fungicides is particularly risky in terms of resistance development. Intensive use provides a potent selective pressure that increases the frequency of fungicide-resistant isolates in a pathogen population (62). As the

frequency of resistant isolates increases, a resistant subpopulation may develop. This subpopulation can increase over time and lead to a reduced level of disease control in that population (40). This is commonly referred to as practical field resistance, and is defined as the point at which the frequency and levels of resistance are great enough to limit the effectiveness of disease control in the field (62). In the late 1960's, the first reports of sporadic fungicide resistance were documented in Europe (24). In 1969, reduced sensitivity of *Venturia inaequalis* (Cke.) Wint., the causal agent of apple scab, to dodine was reported in New York (71). This report was the first documented case of field resistance to any fungicide used in fruit production in the United States. By the late 1980's, over 60 resistant fungal genera in hundreds of crop systems had been documented (69). Currently, all of the systemic fungicide groups (sterol inhibitors, benzimidazoles, strobilurins, phenylamides, and dicarboximides) have been affected by resistance (69).

Dodine

Dodine was introduced to the U.S. market in 1959. Dodine, a broad spectrum fungicide, was used intensively to control important tree fruit diseases such CLS and apple scab, caused by *Venturia inaequalis.* Dodine is a guanidine-based, protectant fungicide currently classified as having multi-site activity (21). Ten years after the introduction of dodine, resistance was reported in *V. inaequalis* from New York (71), Michigan (36) and Europe (53, 80). Resistance appears to be stable (there is no fitness penalty for resistance) and is present in orchards where Dodine has not been used for 20 years (41). The development of resistance in *V. inaequalis* populations to dodine suggest resistance problems may develop in other pathogens. Dodine use for the control of apple scab and CLS was greatly reduced when DMI fungicides were introduced in 1986. With

the recent confirmation of DMI cross resistance in Michigan *B. jaapii* populations (62) dodine is needs to be reevaluated to determine the feasibility of widespread use to help control CLS in Michigan.

Benzimidazole

Benzimidazole fungicides (e.g. benomyl and thiophanate) are locally systemic, single-site fungicides with a high potential for resistance development (73). After the introduction of benzimidazole in 1972, the scientific community thought that fungicide resistance would not affect the fruit industry, and were optimistic about the use of benomyl to control *M. fructicola* (46). In the early 1970's, many pathogens, on a diverse range of crops, rapidly developed resistance to benzimidazoles. The first case of benzimidazole resistance developed in the peanut leaf spot fungus, *Cercospora arachidicola* (Mulder and Holliday), in the southeastern U.S. (13). Within three years of the 1972 registration for use on stone fruit, and 1973 registration for use on pome fruit, benomyl-tolerant isolates of *M. fructicola* and *V. inaequalis* were found in Michigan (35, 36). Benzimidazole-resistant phenotypes of *V. inaequalis* are fit and have continued to persist in the field; consequently benzimidazoles are rarely used in Michigan (35). New technological advances have been made in testing for fungicide resistance, and PCR based assays are now available for detecting benzimidazole resistance in *M. fructicola* from California stone fruit (49).

Dicarboximides

Dicarboximides (iprodione or vinclozolin) were introduced to the market in 1974 (43) and were used globally. Dicarboximides are locally systemic and have a multi-site mode of action (73). Dicarboximide resistance first appeared in *Botrytis cinerea* (Pers:

Fr) on the European grape crop (33). Reduced sensitivity also developed in *M. fructicola* isolates (58, 63, 72), and *M. laxa* (38). Lab and field studies were conducted to assess the general risk of resistance to dicarboximides. The results indicated that resistant isolates were easily selected *in vitro*, but development was slower in the field (17, 45). In the absence of dicarboximide, resistant strains were generally less fit than their wild-type counterparts and returned at a very low level in the *in vitro* population (51).

Sterol Demethylation-Inhibiting Fungicides

Sterol demethylation inhibiting fungicides (e.g fenbuconazole, tebuconazole, or propiconazole) are single-site, locally systemic, and are considered at high risk for resistance development (73). DMI fungicides inhibit the sterol C-14 alpha-demethylation of 24-methylenehydrolanosterol, a precursor of ergosterol, a critical fungal cell membrane component (7). When first introduced to the market, DMI fungicides were effective and contributed greatly to the relatively new management strategies of integrated pest management programs (46). DMI fungicides have become essential in the management of fungal pathogens in fruit production (42) and have been used by commercial fruit growers since the mid to late 1980's. DMI fungicides can work as a protectant, but are also effective after infection occurs (41), allowing for some flexibility in spray timing if an application is missed. *In vitro* testing proved early on that resistance and cross resistance could be readily induced in *Aspergillus nidulans* (Eidam) G. Wint, (14). Resistance of *V. inequalis* to DMI fungicides has been reported in experimental apple orchards (6, 30, 68). As of 2004, statewide cross-resistance of *B. jaapii* isolates to four DMI fungicides was reported in all the commercial cherry producing regions in Michigan (62). Cherry pathogens such as *M. fructicola* should be evaluated for DMI

sensitivity to aid in preventing fungicide resistance development. Monitoring pathogen sensitivity now may help develop spray recommendations in subsequent seasons and detect a significant change in DMI sensitivity that would lead to practical field resistance.

Resistance to DMI fungicides appears to be due to the combined effects of many incremental genetic changes (32, 65, 75). These small genetic differences cumulatively cause a reduction in fungicide sensitivity, and decrease the efficacy of DMI fungicides in the field (8). The slow development of DMI resistance makes it difficult to monitor for practical field resistance. The loss of efficacy is generally not complete, and can sometimes be overcome by shortening the spray interval or increasing the spray rate. (69); not always feasible options due to application guidelines and cost.

Strobilurins

Strobilurin fungicides were introduced on 1996 and are important to the management of many pant pathogens, and account for $620 million in fungicide sales during 1999, or over 10% of the global fungicide market (60). Strobilurin fungicides act as both a contact and translaminar fungicide, with a single-site mode of action (73). Strobilurin fungicides are based on a substance produced by wood-rotting fungi from the subphylum Basidiomycotina. Strobilurins inhibit mitochondrial respiration by binding to the Q_o site, blocking electron transfer and subsequently disrupting the production of ATP (5).

Azoxystrobin (synthetic strobilurin) is a broad-spectrum fungicide and oomycete pesticide that controls organisms from four major taxonomic groups of plany pathogens; Ascomycota, Basidiomycota, Zygomycota and Oomycota (25, 28). Strobilurin fungicides strongly inhibit spore germination and have both preventative and curative

properties. Strobilurins are particularly effective against *P. clandestina* and *V. inaequalis* in tree fruit (2, 9).

Possible modes of resistance were recognized before the release of the product, but the speed at which resistance developed was not expected. In 2002 Heaney reported that *Plasmopara viticola* (Berk. and M.A. Curtis) Berl & De Toni in Sacc., on grape was resistant to strobilurins (29). The group is now considered high risk, and when used strict resistance management strategies are recommended (8).

FUNICIDE RESISTANCE MECHANISMS

There are numerous known fungicide resistance mechanisms including: (i) alteration of the biochemical target site so that it is no longer sensitive; (ii) increased production of the target protein; (iii) development of an alternative metabolic pathway that bypasses the target site; (iv) metabolic breakdown of the fungicide; and (v) exclusion or expulsion of the fungicide through ATP-dependent transporter proteins (8).

The most common resistance mechanism appears to be an alteration of the biochemical target site of the fungicide (8). This could explain why many of the earlier, multi-site products have not encountered resistance problems. Once they have penetrated the fungal cell, the earlier fungicides act as general enzyme inhibitors, affecting many target sites. All of these target sites in the fungus would have to change concurrently in order to stop the fungicide from working. The chances of the numerous, needed genetic changes happening at the same time are slight, and would likely cause the organism to become avirulent if not completely nonviable (8).

In contrast to the earlier, multi-site fungicides, modern fungicides act on single target sites and are referred to as "single-site" fungicides. DMI fungicides have a single-site mode of action. A single gene mutation can cause an alteration of the target site, resulting in decreased sensitivity to the fungicide, making modern fungicides much more susceptible to resistance development. The rapid advances over the past ten years of PCR-based diagnostic methods for detection of point mutations has aided in the identification of resistance mechanisms, especially those with changes in the target site (8).

In the case of DMI fungicides, three major mechanisms for resistance have been described, including: mutations in the target enzyme, 14 alpha-demethylase (*CYP51*), leading to a decreased affinity of DMI fungicides to the target protein (3, 15, 16); overexpression of the *CYP51* gene leading to increased production of the target enzyme (26, 66); and overexpression of the ATP-binding cassette (27, 82). Recently published data has shown that the overexpression of the *CYP51* gene in *B. jaapii* mediates DMI resistance (50). A PCR detection test that screens for the presence of a promoter associated with the overexpression of the *CYP51* gene was also developed (50).

MANAGMENT TO DELAY FUNGICIDE RESISTANCE

To increase the time a fungicide can effectively control a given pathogen management strategies should minimize the risk of resistance development. New chemistries need to be carefully analyzed before widespread use on a particular pathogen in a given agricultural system (69). Thoroughly scrutinizing the interaction of a pathogen and fungicide before widespread use can make withdrawal from the market less likely by

anticipating possible issues (69). Predicting the risk of fungicide is difficult (69) and predictions are often unreliable. In the case of dicarboximides resistance developed more slowly than expected (44) and DMI fungicides developed resistance despite predictions that it was at low risk for pathogen resistance (22). Monitoring the response of a pathogen population to repeated fungicide exposures is the only way aspects of pathogen fitness, such as the disproportionate survival of resistant isolates under fungicides pressure, are sufficiently taken into account (69).

Preserving earlier fungicides for use on plant pathogens is very important to mitigating the development of fungicide resistance to new chemistries. Preserving earlier fungicides provides a greater number of rotation partners for use within the season. With bans on certain residual partners, the selection of alternative fungicides is becoming insufficient for many pathogens (4). Abandoning conventional, protectant fungicides would therefore be in poor judgment and new fungicide groups are needed (69). Management strategies such as tank mixing fungicides and rotating the mode of action have successfully delayed or contained resistance in the past (13, 69), and is a current resistance management strategy used today. Additionally, resistance management could be potentiated by collecting baseline fungicide sensitivity data in populations of plant pathogens of particular commodities. If pathogen populations were monitored for small changes in sensitivity levels, preemptive management strategies could be adopted to avoid the loss of efficacy (69).

SUMMARY

Resistance to DMI fungicides has recently been confirmed in *B. jaapii* populations in Michigan (62). With the loss in efficacy of DMI fungicides, it has become important to determine the current sensitivity status of *B. jaapii* isolates to dodine, which is a probable and available alternative. Establishing baseline sensitivity levels may aid in predicting practical field resistance, and assist in evaluating the value of the fungicide resistance management strategies being employed.

DMI fungicides are currently the most frequently utilized fungicides for controlling *M. fructicola* in Michigan. Resistance to DMI fungicides is well documented in various fungal pathogens of fruit, including *M. fructicola* populations in stone fruit orchards from New York and Georgia (20, 72, 81). Fenbuconazole (a DMI fungicide) is being applied extensively in Michigan cherry orchards, and because of the single-site mode of action, is at risk for resistance development. Determining the status of *M. fructicola* sensitivity to fenbuconazole in Michigan is an important facet of managing fungicide resistance. Tracking changes in fungicide sensitivity over time can aid in predicting practical field resistance, and assist in evaluating the value of current fungicide resistance management strategies.

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CHAPTER 1

SENSITIVITY OF *BLUMERIELLA JAAPII* TO DODINE

INTRODUCTION

Cherry leaf spot (CLS), caused by the ascomycete *Blumeriella jaapii* (Rehm) Arx, is the most damaging fungal pathogen of tart cherry trees (*Prunus cerasus* L.). After a severe outbreak in 1993, thousands of tart cherries died during the following winter (11). Cherry leaf spot also affects sweet cherry (*P. avium* L.) production. The impact on the Michigan cherry industry is exacerbated by the fact that the Montmorency cultivar, which constitutes more than 90% of tart cherry production in Michigan, is highly susceptible to CLS. With Michigan producing upwards of 80% of the national tart cherry crop (19), losses due to CLS not only affect Michigan, but the national tart cherry market as well.

Cherry leaf spot causes the formation of small, purple lesions on the leaf that reduces the photosynthetic ability of the tree. As leaves accumulate CLS lesions, they turn yellow and on tart cherry trees leaves prematurely defoliate. When tart cherry trees become severely defoliated before harvest, fruit is soft and immature, has low soluble solids, and ripens unevenly (11). During seasons with high disease pressure CLS can cause defoliation as early as midsummer. In the years following a severe outbreak with early defoliation, bud formation and fruit set is significantly reduced (8). Trees defoliated extremely early (July-August) are also less able to cope with cold winters and may be killed by freezing temperatures.

All commercially cultivated cherry cultivars are susceptible to cherry leaf spot, (11) most notably Montmorency. Currently, management of CLS relies heavily on

fungicides and requires five to seven applications per year, depending on disease pressure (18). The extensive use of sterol demethylation inhibitor (DMI) fungicides has led to newly documented resistance in *B. jaapii* populations (20), and has increased the need for alternative fungicides to control CLS, such as dodine.

Although the mode of action of dodine is unknown, it was an effective and widely utilized asset for controlling CLS before the introduction of DMI fungicides. After the introduction of DMI fungicides, the use of dodine was greatly reduced in Michigan. With DMI resistance in Michigan *B. jaapii* populations (20) new options for CLS control are needed. Dodine should be reevaluated for potential widespread application to control CLS in Michigan. Baseline sensitivity levels of *B. jaapii* need to be established to aid in monitoring *B. jaapii* for the development of dodine resistance under more widespread use.

Dodine was introduced to the U.S. fungicide market in 1959 and was used intensively in northeastern North America to control economically important pathogens of cherry and apple, particularly *Venturia inequalis*, the causal agent of apple scab (5). Dodine is a guanidine-based, protectant fungicide classified as having multi-site activity (4). Dodine also has post-infection properties. Generally, multi-site fungicides are thought to have a lower probability of developing resistance issues than single-site fungicides (1). Multi-site fungicides have broad fungicidal activity and target metabolic pathways or processes; versus a single enzyme as is the case in single-site fungicides. Despite expectations of low risk, resistance to dodine was found in Michigan *V. inequalis* isolates in 1981 (9), and then internationally shortly thereafter (17). Resistance in *V. inaequalis* appears to be genetically stable and has been documented in orchard

populations from sites where dodine has not been used for 20 years (14). The potential use of dodine for the control of CLS and the risk of resistance development in *B. jaapii* populations, needed to be carefully evaluated before it is recommended.

DMI fungicides have been a major part of most management strategies in commercial cherry orchards in Michigan. Dodine may become a more popular control method for CLS because of the development of resistance in *B. jaapii* populations to DMI fungicides (20). Monitoring pathogen populations for changes in fungicide sensitivity can aid in detecting the early stages of resistance, before it becomes severe enough to cause practical field resistance (2). Determining the sensitivity of *B. jaapii* populations in Michigan now will contribute to monitoring dodine for resistance development in subsequent seasons by allowing for comparison of current sensitivity levels to future measurements. The objectives of this study were to determine the efficacy of dodine in controlling CLS in Michigan orchards, and determine the *in vitro* sensitivity of *B. jaapii* populations to dodine.

MATERIALS & METHODS

Dodine field trials

During the 2005 and 2006 growing seasons experiments to determine the field efficacy of dodine for the control of CLS were conducted at the Michigan State University Northwest Horticultural Research Station (NWMHRS) located in Leelanau County, Michigan. Fungicide sprays were applied to a block of 12-yr-old Montmorency and 11-yr-old Balaton tart cherry trees (0.99 hectares) or a 26-year-old Montmorency block (0.56) hectares. Sprays were applied at 2,809 liters of water per hectare, using a custom

built research plot sprayer with a JBS model 705 hand gun (John Bean Sprayer Co., AR); (Figure 1.A). The experimental design was a randomized complete block with single-tree plots, replicated four times per treatment. Randomization occurred each year. Twenty terminal branches from each tree were rated and disease incidence was recorded as the percentage of leaves infected or defoliated. Fisher's Protected Least Significant Difference test was used to determine the significance of variability in disease incidence between treatments.

In addition to dodine (Syllit 65W; Platte Chemical Company, Geeley, CO), other fungicides commonly used to control CLS were tested for comparison, including a triflumizole (Procure 4SC; Uniroyal Chemical Company, Middlebury, CT), boscalid (Endura 70 WDG; BASF, Germany), trifloxystrobin (Flint; Syngenta Crop Protection, Switzerland), chlorothanlonil (Bravo Ultrex 82.5 WDG; Syngenta Crop Protection, Switzerland), and two elemental copper materials (Cuprofix Disperss 40DF; Cerexagri Incorporated, 402 Wilmington, DE; and Kocide 2000, DuPont, DuPont, WA).

2005 trial

The focus of the 2005 field trial was to compare the efficacy of dodine and copper fungicides in controlling CLS. These fungicides were integrated into spray programs based on the resistance management concept of rotating the mode of action within the season (Table 1.3). Spray dates, respective cherry growth stages and CLS infection periods were recorded (Table 1.1). From June 1 to 16, rainfall totaled 2.6 centimeters (Appendix A, Table A.1). Cherry leaf spot disease data was not collected until September 12, after sufficient infection had occurred. Five terminals from each tree quadrant (20 terminals per tree) were evaluated.

Figure 1.A. Custom built research plot sprayer with a JBS model 705 hand gun, used to apply fungicides for efficacy trials at the Northwest Horticultural Research Station.

2006 trial

Fungicide rotations were evaluated in a 0.56 hectare block, 27-yr-old Montmorency cherry trees at the NWMHRS. The block contained a total of 88 trees. This experiment examined chemicals commonly recommended for the management of CLS. Individual fungicides were applied for all applications during the growing season (5 cover sprays); (Table 1.4). Fungicides were applied with a handheld applicator to run-off (equivalent to 2,809 liters/hectare). Treatments were arranged in a randomized complete block using four replications of single-tree plots. Spray dates, respective growth stages and cherry leaf spot infections were recorded (Table 1.2). There were 10 days (May 9-18) with measurable rainfall totaling 5.77 centimeters. Mid-summer was dry; June had 7 days with rainfall that totaled 4.22 cm, and July had 8 days of rainfall totaling 4.09 centimeters (Appendix A, Table A.2). CLS disease assessment was conducted on July 28; a second assessment was performed on September 18. Leaf spot foliar incidence and defoliation were calculated from observations on 20 shoots per tree.

Table 1.1. The 2005 spray dates (in bold), respective growth stages, and cherry leaf spot infections periods* as recorded at the Northwest Horticultural Research Station.

Date	Event
9-May	Low disease severity
10-May	**Bloom**
19-May	Low disease severity
24-May	Low disease severity, **Shuck Split**
3-Jun	**First cover**
8-Jun	Low disease severity
13-Jun	**Second cover**
23-Jun	Moderate disease severity, **Third cover**
4-Jul	Low disease severity
5-Jul	**Fourtha and final cover**
21-Jul	Harvest
24-Jul	Low disease severity
25-Jul	Moderate disease severity
28-Jul	Low disease severity
4-Aug	Low disease severity
10-Aug	Low disease severity
12-Aug	Low disease severity
18-Aug	High disease severity
12-Sep	Disease rating

*Cherry leaf spot infection periods as defined by www.enviroweather.msu.edu.

Table 1.2. The 2006 spray dates (in bold), respective growth stages, and cherry leaf spot infections periods* as recorded at the Northwest Horticultural Research Station.

Date	Event
2-May	Low disease severity
10-May	**Petal fall**
11-May	Low disease severity
12-May	Moderate disease severity
19-May	**Shuck split**
25-May	High disease severity
28-May	Low disease severity
30-May	**First cover**
31-May	High disease severity
6-Jun	Moderate disease severity
9-Jun	**Second cover**
18-Jun	High disease severity
19-Jun	**Third cover**
26-Jun	Moderate disease severity
28-Jun	Moderate disease severity
1-Jul	Moderate disease severity
3-Jul	**Fourth and final cover**
9-Jul	Moderate disease severity
17-Jul	Moderate disease severity
21-Jul	Harvest
26-Jul	Low disease severity
28-Jul	Disease rating
1-Aug	High disease severity
9-Aug	Low disease severity
14-Aug	Low disease severity
23-Aug	Moderate disease severity
24-Aug	Low disease severity
26-Aug	Moderate disease severity
18-Sep	Disease rating

*Cherry leaf spot infection periods as defined by www.enviroweather.msu.edu.

B. jaapii isolate collection

The eight hundred and sixty *B. jaapii* isolates used in the *in vitro* dodine assay were from the DMI survey collection of *B. jaapii*, established by T. Proffer (20). In 2003-2004, Proffer collected *B. jaapii* isolates from commercial orchards in all four of the major tart cherry producing areas of Michigan: Traverse City, Benzie, Hart/Shelby, and the Southwest region. Proffer also collected isolates from a commercial orchard in eastern Michigan; research orchards from Michigan State University; dooryard trees near Salem, OH; and an abandoned sweet cherry orchard in Berlin Center, OH. Additional *B. jaapii* isolates were from black cherry (*P. serotina*), choke cherry (*P. virginiana*), pin cherry (*P. pensylvanica*), mahaleb (*P. mahaleb*), and dwarf cherry (*P. fruticosa*) trees in Michigan and Ohio. Cherry leaves were selected at random to be used as the source of monoconidial isolates. Individual germinated conidia were transferred from 2% water agar to modified malt extract agar (MMEA) plates and maintained on MMEA (recipe in Appendix A) slants at 5°C (20).

Determination of *in vitro* sensitivity of *B. jaapii* isolates to dodine

Previous *in vitro* fungicide sensitivity studies have used reduced mycelial growth to measure differences in sensitivity (6, 15, 16, 22). There are two methods to measure mycelial response to fungicides; quantitative and qualitative. The quantitative method is based on the fungicide concentration at which mycelial growth is reduced 50%, relative to growth in unamended medium; this is referred to as the Effective Concentration 50 (EC_{50}). The alternative, qualitative method involves determining the minimum concentration at which colony growth is completely inhibited, commonly referred to as the Minimum Inhibitory Concentration (MIC) (6, 15, 16, 22). It is important to note that

B. jaapii isolates are very slow growing and monoconidial isolates often take two weeks or more to produce colonies of only a few millimeters in diameter. Due to slow growth, the qualitative MIC method was used to measure dodine sensitivity.

The sensitivity of eight hundred and sixty *B. jaapii* isolates to dodine was tested during 2006. Dodine was dissolved in 95% ethanol to prepare stock solutions. Quantities of these stock solutions were added to modified malt extract agar (MMEA) after it had been autoclaved for sterilization and cooled to 50°C. The concentration of active ingredient (a.i.) was adjusted to 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 200, and 400 µg/ml. MMEA amended with 125 µl of ethanol (equal to the highest concentration of ethanol in the treatments) was used as a control. Mycelial plugs, 1.0 mm in diameter, were transferred to MMEA plates amended with the previously mentioned 11 levels of dodine concentrations and the control; this was replicated three times for each isolate. After 21 days of incubation at 23 to 25°C, the plates were examined for colony expansion and the MIC of fungicide for each isolate was recorded (Figure 1.B). The MIC reported is the concentration at which the growth of at least 2 of 3 isolate replicates was inhibited. The MIC was defined as no mycelial expansion of *B. jaapii* from the inoculation plug onto the amended MMEA agar plate.

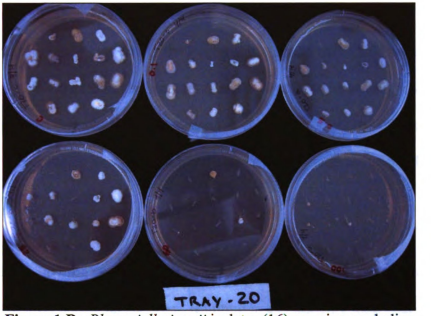

Figure 1.B. *Blumeriella jaapii* isolates (16) growing on dodine amended modified malt extract agar to determine the minimum inhibitory concentration.

RESULTS

Dodine efficacy field trials

2005

Treatments utilizing dodine were as effective as those utilizing copper hydroxide but were also comparable to infection levels in trees not treated with fungicides (Table 1.3). Treatments with copper sulfate were more effective at 2 of the 3 spray rates screened than any other treatment and disease levels were significantly lower than in the unsprayed control, most likely due to the long residual action associated with copper sulfate. No significant defoliation occured. Trees sprayed with four sprays of copper hydroxide exhibited some darkening between the veins (phytotoxicity) on the underside of the leaf, but defoliation was not a problem despite of temperatures up to 34 °C in early August (Appendix A, Table A.1).

2006

The 2006 growing season was dry (Appendix A, Table A.2) and slowed the development of CLS during midsummer, resulting in an unusually long interval between the last spray on July 3 and the final leaf spot disease rating on September 18. Under these dry conditions, control of CLS early in the season was outstanding with all products except triflumizole. The boscalid maintained the best late control of CLS with dodine and chlorothalonil showing similarly effective potency for disease suppression and acceptable levels of defoliation (Table 1.4). On the September 18 more than 95% of the remaining leaves on unsprayed control trees were infected with CLS and more than 90% had been defoliated.

Table 1.3. Incidence of cherry leaf spot infection during the 2005 growing season based on use of copper and dodine in a resistance management spray regime.

Fungicide and rate per hectare	Spray Timing	Incidence (% leaves)
Chlorothalonil (3.4 kg) Trifloxystrobin (183.4 ml) Dodine (2.3 kg) Tebuconazole (440.6 ml)	Bloom (5/10); Shuck split (5/24) First cover (6/3) Second (6/13); Third cover (6/23) Fourth cover (7/5)	55.1 bcd
Chlorothalonil (3.4 kg) Trifloxystrobin (183.4 ml) Copper sulfate (4.0 kg) Tebuconazole (440.6 ml)	Bloom (5/10); Shuck split (5/24) First cover (6/3) Second (6/13); Third cover (6/23) Fourth cover (7/5)	33.5 ef
Chlorothalonil (3.4 kg) Trifloxystrobin (183.4 ml) Copper sulfate (2.0 kg) Tebuconazole (440.6 ml)	Bloom (5/10); Shuck split (5/24) First cover (6/3) Second (6/13); Third cover (6/23) Fourth cover (7/5)	55.9 bc
Chlorothalonil (3.4 kg) Trifloxystrobin (183.4 ml) Copper sulfate (1 kg) Tebuconazole (440.6 ml)	Bloom (5/10); Shuck split (5/24) First cover (6/3) Second (6/13); Third cover (6/23) Fourth cover (7/5)	34.1 ef
Chlorothalonil (3.4 kg) Trifloxystrobin (183.4 ml) Copper hydroxide (2.3 kg) Tebuconazole (440.6 ml)	Bloom (5/10); Shuck split (5/24) First cover (6/3) Second (6/13); Third cover (6/23) Fourth cover (7/5)	50.9 bcd
Chlorothalonil (3.4 kg) Trifloxystrobin (183.4 ml) Copper hydroxide (4.5 kg) Tebuconazole (440.6 ml)	Bloom (5/10); Shuck split (5/24) First cover (6/3) Second (6/13); Third cover (6/23) Fourth cover (7/5)	53.6 bcd
Untreated control		64.5 ab

* Means followed by the same letter are not significantly different according to Fisher's Protected LSD ($\alpha = 0.05$).

Table 1.4. Cherry leaf spot incidence during the 2006 growing season based on season long applications of fungicides commonly used for control.

Treatment and product per hectare	Infected leaves (%)		Defoliation (%)
	28-Jul	18-Sep	18-Sep
Trifloxystrobin (219.6 ml)	2.9 b	97.7 a	49.1 c
Triflumizole (585.6 ml)	54.1 a	100.0 a	90.5 a
Triflumizole (1171.2 ml)	30.7 c	100.0 a	78.5 b
Boscalid (585.6 ml)	3.9 b	16.0 b	3.4 d
Chlorothalonil (3.2 kg)	0.7 b	79.7 c	11.3 d
Dodine (1976.4 ml)	0.7 b	75.9 c	32.6 e
Untreated control	61.5 a	95.1 a	90.9 a

***In vitro* sensitivity of *B. jaapii* isolates to dodine**

The *in vitro* MIC of the 860 *B. jaapii* isolates ranged from 0-400 ppm dodine (Figure 1.C). The overall population profile formed a bell shaped curve, with most of the isolates having a moderate MIC value (Figure 1.C). The difference in MIC values between isolates from dodine-exposed, and unexposed sources was significant according to a Chi-square test ($P = 0.1$). *B. jaapii* isolates from sites that had likely been exposed to dodine required a higher dosage of dodine to inhibit mycelial growth than isolates from sites with limited or no exposure to dodine (Figure 1.D).

Additionally, one set of isolates (AH), were collected from an orchard that was abandoned in the early 1980's. The source of the AH isolates was probably only exposed to dodine for 30 years, versus the more than 50 years of possible exposure in conventional orchards. The isolates from the abandoned orchard had intermediate MIC values when compared with those from exposed and unexposed sites (Figure 1.D).

Greater than 69% of the isolates from sources not exposed to dodine were controlled at a dodine concentration of less than or equal to 10 ppm. Conversely, almost 20% of *B. jaapii* isolates from sources exposed to dodine required greater than 100 ppm of dodine to inhibit mycelial expansion. Figure 1.D illustrates a clear the shift in the bell-shaped, population profiles. Respective curves move from lower MIC values in *B. jaapii* isolates from unexposed sources, to the higher MIC values in isolates from sources exposed to dodine.

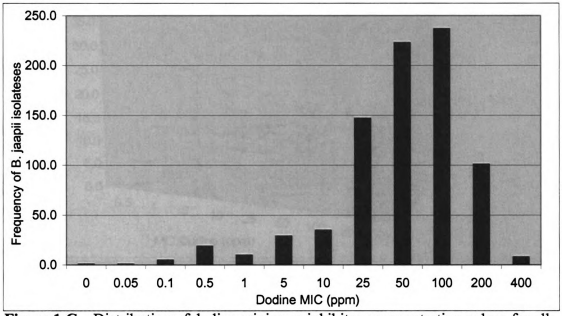

Figure 1.C. Distribution of dodine minimum inhibitory concentration values for all *Blumeriella jaapii* isolates.

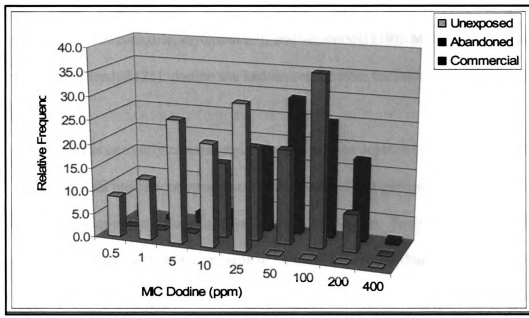

Figure 1.D. Relationship of dodine exposure of the isolate source to *in vitro* sensitivity of *Blumeriella jaapii* isolates, as measured by the minimum inhibitory concentration. Unexposed isolate sources included dooryard trees, wild cherry, and black cherry. Exposed isolates were collected from commercial cherry orchards. The abandoned orchard isolates source has not been exposed to dodine since the 1980s. A Chi-square test was performed and indicated a significant difference between the exposed and unexposed sources ($P = 0.1$).

DISCUSSION

The management of CLS relies heavily on fungicides and requires five to seven applications per year, beginning at petal fall and continuing into late summer (18). The most recent fungicide recommendations include an additional post harvest spray to reduce initial inoculum the following year. The fungicides used to control *B. jaapii* include chlorothalonil, captan, strobilurins and several sterol demethylation inhibitors (fenbuconazole, tebuconazole, myclobutanil, and fenarimol) (19). More recently dodine has been recommended (21); dodine use had decreased after the introduction of DMI fungicides.

Each of the currently recommended fungicides has potential problems, limiting use. Chlorothalonil has serious residue concerns because it is carcinogenic (3), as a result applications are limited to before shuck-split and after harvest. Fenbuconazole has been used extensively because of low cost, season long availability, and action against multiple pathogenic fungi such as brown rots and powdery mildew (10,12,19). This extensive use of the DMI fungicides has led to newly documented resistance in *B. jaapii* populations (20). Strobilurin fungicides were registered for use on tart cherry in 2002 and have proven effective in controlling CLS (12, 19). Strobilurins are prone to fungicide resistance because they have a single-site mode of action, which may be overcome by a single pathogen mutation (1, 7). Due to the previously listed issues with common fungicides used to control CLS, it is critical to evaluate all available and effective treatments. It is also imperative that an integrated program is developed to minimize the development of fungicide resistance.

Spray trial results confirmed that dodine is as effective as many recommended fungicides currently used to control CLS (Table 1.4). The 2005 growing season was the driest since weather data recording began at the NWHRS in 1982 (Appendix A, Table A.1). Dry conditions slowed CLS development and disease pressure was low, pushing the efficacy evaluation date back to September. At harvest (July 21), few leaves were infected and CLS lesions were hard to find, even on the unsprayed control trees. The length of time between final spray (July 5) and evaluation (September 12) was unusually long. The 2005 spray trial suggests that dodine could control CLS as well as most copper products. This is impressive as copper had been highly effective in the control of CLS in past seasons (13) and has also been proven effective under current conditions (18). Low disease pressure during the 2005 growing seasons (Table 1.3) did not allow conclusive data on the overall efficacy of dodine in the field. The 2006 growing season did produce significant disease pressure (Table 1.4), due to substantial rainfall in the northwest region of the state (Appendix A, Table A.2). Dodine was shown to suppress CLS as effectively as representatives from many popular fungicides including, strobilurins, boscalids and chlorothalonil (Table 1.4). Dodine was also effective at reducing defoliation (Table 1.4). Dodine has proven a viable fungicide for the control of CLS in Michigan cherry orchards, but additional efficacy trials are needed.

The overall baseline dodine sensitivity data was variable, with *in vitro* sensitivities ranging from 0.05 to more than 400 ppm of dodine. Wide variability in baseline data (8,000-fold) may be an indicator of a higher risk for resistance development. It is thought that the broader the base-line range is, the greater the proclivity of the population for subsequent population shifts towards lower levels of

sensitivity under fungicide selection pressure (1). The *in vitro* assay also showed a population shift towards reduced dodine sensitivity in *B. jaapii* isolates when evaluated based on their previous exposure to dodine. The population shift (Figure 1.D) is a common precursor of reduced fungicide sensitivity (2). To slow the reduction in *B. jaapii* sensitivity to dodine, fungicide resistance management strategies should be utilized. Rotating fungicide classes within the growing season, and tank mixing with an appropriate broad spectrum fungicide, are critical facets of resistance management (1). Further tracking of the sensitivity to dodine in Michigan *B. jaapii* populations will be critical in determining the long term use of dodine, and in amending future recommendations based on subsequent findings.

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CHAPTER 2

SENSITIVITY OF *MONILINA FRUCTICOLA* TO FENBUCONAZOLE

INTRODUCTION

Monilinia species cause a wide range of stone fruit diseases worldwide, including fruit rots and blossom blight. Only *M. laxa* and *M. fructicola* are currently found in the United States (11). *Monilinia fructicola*, is the causal agent of American brown rot (ABR), and is an important pathogen on cherry, particularly varieties of sweet cherry. ABR also affects apricot, peach, nectarine and plum fruit. ABR reduces cherry yield by causing fruit rot before and after harvest. Direct infection through the fruit cuticle occurs during a 2-3 day period of warm, wet and humid weather. The entire secondary infection cycle takes only three days to complete, allowing for epidemic outbreaks in a matter of days. The fungus attacks fruit, blossoms, spurs, and shoots, with infection occurring more readily on mature fruit as the season progresses. The most distinctive sign of the disease is the gray/tan conidia that appear on the surface of the fruit. Infected fruit may rot and abscise or persist through the winter in a mummified form. Mummified cherry fruit provide the primary source of conidia that act as the primary inoculum in the spring. In rare cases *M. fructicola* form apothecia (sexual fruiting structures) on the orchard floor, producing ascospores in the spring. (13).

Sterol demethylation inhibitor fungicides (DMI fungicides) are currently the most common chemistries used to control ABR. Cultural practices such as the removal of mummies and blighted twigs are recommended to reduce inoculums sources, but are not feasible in commercial orchards. Avoiding damage to the fruit is critical to minimizing

disease. Cultivars currently in production have exhibited no resistance to the pathogen and fungicides are the primary strategy for disease control (13).

Prior to 1970, most fungicides had multi-site modes of action, and interfered with multiple metabolic pathways, inhibited spore germination, and acted as protectants. The development of resistance to these fungicides was rare, but problems with toxicity to non-target organism, such as insects and humans, were considerable. Conversely, in the late 1960's, DMI fungicides were introduced and disrupted only a single metabolic process or structural site in the fungus (21). The use of DMI fungicides has typically involved multiple applications during a growing season, and provides conditions for intense selection of fungicide resistant isolates. Genetic mutations are the most prevalent cause of fungicide resistance in fungi and resistant isolates exist at very low frequencies within most pathogen populations (3). When fungicide pressure is present, as is the case in conventionally managed cherry orchards, isolates with fungicide resistance are selected. The population size of resistant isolates may then increase disproportionately to the normal population structure, when compared to population structure in the absence of fungicides. The increase in less sensitive individuals may ultimately lead to a lack of disease control in the field, or practical field resistance (15).

DMI fungicides specifically inhibit the sterol C-14 alpha-demethylation of the 24-methylenehydrolanosterol, a precursor of ergosterol; a critical fungal cell membrane component (3). Resistance in *M. fructicola* populations to DMI fungicides was first documented in a laboratory study (18). Currently, DMI resistance has been confirmed in field populations of *M. fructicola* in peach orchards (27), specifically in Georgia (23) and New York (19). Resistance of *V. inequalis* isolates to DMI fungicides in experimental

apple orchards has also been reported (2, 10, 24). In 2004, cross-resistance of *B. jaapii* isolates to four DMI fungicide groups was reported in Michigan's commercial cherry orchards (21). Three major mechanisms for resistance to DMI fungicides have been previously described, including: mutations in the target enzyme, 14 alpha-demethylase (*CYP51*), that leads to a decreased affinity of DMI fungicides to the target protein (1, 5, 6); overexpression of the *CYP51* gene leading to increased production of the target enzyme (8, 17, 22); and overexpression of the ATP-binding cassette (9, 28).

The objectives of this study were to: (i) determine the current sensitivity levels of Michigan *M. fructicola* populations to fenbuconazole (a DMI commonly used in Michigan cherry orchards); (ii) evaluate the significance of these sensitivity levels with regard to disease control; and (iii) establish whether the most and least sensitive isolates showed significant genetic variations in the *CYP51* gene.

MATERIALS AND METHODS

Monilinia fructicola isolate collection

Stone fruit infected with *M. fructicola* were randomly collected from 24 commercial cherry or peach orchards from around Michigan, including Grand Traverse, Leelanau, Benzie, Berrien, Montmorency, and Oceana County. Infected cherries were pressed gently on the surface of a water agar plate, transferring conidia from the fruit's surface to the agar plate. These conidia were then spread with a sterile glass rod. Water agar plates were used to reduce bacterial growth. Conidia were incubated for 24 hours at temperatures between 23 and 25°C, and single conidial isolates were transferred to potato

dextrose agar (Appendix B, Table B.1). Isolates were maintained on potato dextrose agar (PDA), between 23 and 25°C.

PCR-based species confirmation

Monilinia laxa and *M. fructicola* are both found in Michigan cherry orchards and have similar morphology so PCR was used to confirm that the isolates tested for DMI sensitivity were *M. fructicola*. Tissue for PCR analysis was prepared using a bead-beater centrifuge to disrupt the fungal cell membrane. The protocol section of Appendix B describes the bead-beater procedure. Actively growing mycelium (0.1 g) was collected from two randomly selected representative isolates from each sampling location (Table 2.1). The ITS1Mfcl and ITS4Mfcl primers were used (12). A Qiagen, DNeasy Plant Mini Kit was used for DNA extraction; the mini protocol was followed. Electropheresis was then used to confirm the amplification of the ITS region. *M. laxa* primers (ITSMlx and ITS4Mlx) were also used to exclude cross-amplification as a possibility (12). The thermocycler program and template preparation are described in the protocol section of Appendix B. The PCR products were run on a 1% agarose gel in 1x sodium borate (SB) buffer, at 94 volts for 45 minutes. KBPlus (LI-COR) was used to determine product size and one well was loaded with master mix and sterile water to serve as a negative control. Additionally, one *M. fructicola* isolate (DRAD-29) was randomly selected and sequenced; identification was then confirmed through comparison with GenBank sequences of *M. fructicola*.

Table 2.1. List of suspected *Monilinia fructicola* isolates screened to determine species, including the source, locus and associated PCR code.

Isolate Code	Gel Code	Source	Locus*
500-4	5	Tart Cherry	NWHRS
500-5	8	Tart Cherry	NWHRS
BRC-15	10	Tart Cherry	NW MI
BRC-7	4	Tart Cherry	NW MI
BRR-16	40	Tart Cherry	NW MI
BRR-19	39	Tart Cherry	NW MI
DRA-6	14	Sweet Cherry	NW MI
DRA-9	13	Sweet Cherry	NW MI
DRAD-14	38	Sweet Cherry	NW MI
DRAD-29	37	Sweet Cherry	NW MI
EMP-16	16	Sweet Cherry	NW MI
EMP-21	15	Sweet Cherry	NW MI
ENT1000-15	11	Tart Cherry	NWHRS
ENT1000-18	6	Tart Cherry	NWHRS
ENT450-10	1	Sweet Cherry	NWHRS
ENT450-7	12	Sweet Cherry	NWHRS
HH-26	18	Sweet Cherry	NW MI
HH-27	17	Sweet Cherry	NW MI
HRG-18	43	Tart Cherry	Hart, MI
HRG-4	44	Tart Cherry	Hart, MI
KHG-16	41	Sweet Cherry	Gillspier, MI
KHG-18	42	Sweet Cherry	Gillspier, MI
LAB-14	2	Tart Cherry	Benzie Co.
LAB-4	7	Tart Cherry	Benzie Co.
LAR-1	3	Tart Cherry	Montmorency Co.
LAR-2	9	Tart Cherry	Montmorency Co.
MRC-2	27	Sweet Cherry	Coopersville, MI
MRC-4	28	Sweet Cherry	Coopersville, MI
PFR-6	26	Peach	Benton Harbor, MI
PFR-8	25	Peach	Benton Harbor, MI
RBT-24	30	Tart Cherry	NW MI
RBT-26	29	Tart Cherry	NW MI
RFS-20	33	Sweet Cherry	NW MI
RFS-21	34	Sweet Cherry	NW MI
VNL-12	20	Tart Cherry	Hart, MI
VNL-6	19	Tart Cherry	Hart, MI
VPD-3	22	Sweet Cherry	NW MI
VPD-5	21	Sweet Cherry	NW MI
VPDS-2	32	Sweet Cherry	NW MI
VPDS-8	31	Sweet Cherry	NW MI
WIL-13	23	Peach	Benton Harbor, MI
WIL-2	24	Peach	Benton Harbor, MI
YBR-20	36	Tart Cherry	NW MI
YBR-21	35	Tart Cherry	NW MI

* NWHRS is the Northwest Horticultural Research Station and NW MI stands for Northwest Michigan.

***Monilinia fructicola in vitro*, fenbuconazole assay**

Fungicide resistance studies often use reduced mycelial growth to measure sensitivity (7, 15, 16, 24). There are two acceptable methods of measuring mycelial response to a fungicide; quantitative and qualitative. The quantitative method is based on the fungicide concentration at which mycelial growth is reduced 50%, relative to normal growth, this is referred to as the effective concentration 50 (EC_{50}). The alternative, qualitative method involves determining the minimum concentration at which colony growth is completely inhibited, commonly referred to as the minimum inhibitory concentration (MIC) (7, 15, 16, 24). *M. fructicola* is fast growing, only taking seven days to reach colony diameters of 8 cm, for this reason the quantitative EC_{50} value was used for this study.

In 2007, the sensitivity of *M. fructicola* isolates to fenbuconazole was measured. Fenbuconazole (Indar WSP; Dow AgroSciences, Indianapolis, IN) was dissolved in sterile water to prepare stock solutions. After autoclaving the PDA for sterilization and allow it to cool to 50°C, quantities of the stock solutions were added. The fenbuconazole concentrations were selected based on a prior subsampling of the collection. The preliminary assay screened five *M. fructicola* isolates at fenbuconazole concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 μg/ml. The preliminary isolates were all controlled by less than 0.1 μg/ml of fenbuconazole so the concentrations for the assay were adjusted to below 0.1 μg/ml. Fenbuconazole concentrations used for the assay were 0, 0.001, 0.005, 0.01, 0.05, and 0.1 μg/ml fenbuconazole. A total of one hundred and forty-eight *M. fructicola* isolates were tested. Mycelial plugs one millimeter in diameter from each isolate were transferred to PDA plates amended with fenbuconazole or an unamended

control (Figure 2.A). Each treatment was replicated three times. After five days of incubation at 23 and 25°C the plates were examined for colony expansion, two diameter measurements were recorded for each isolate and the diameters for the three replicates of each treatment were averaged. The EC_{50} values were calculated using a regression analysis function included in SigmaPlot (Systat Software Incorporated) statistical software. The EC_{50} reported is the fungicide concentration at which the growth of the colony would be reduced by 50%.

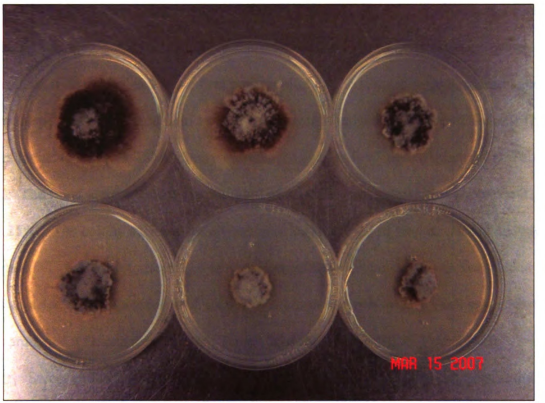

Figure 2.A. *Monlinia fructicola* isolates growing on PDA plates amended with six concentrations of fenbuconazole (0, 0.001, 0.005, 0.01, 0.05, and 0.1 μg/ml).

Inoculation trial

A fruit assay was performed to establish if there is a difference in the ability of *M. fructicola* isolates to cause disease in the presence of fenbuconazole based on the previously determined *in vitro* EC_{50} values. The assay was designed to test the ability of the four isolates with the lowest EC_{50} values (LAB-3, MRC-20, WIL-3 and LAR-3) and highest EC_{50} values (RFS-10, VNL-18, BRC-16, and KHG-1) to produce infections on fruit that had been dipped in a range of fenbuconazole concentrations. Peaches were inoculated with the isolates to confirm pathogenicity. The isolates were then recovered from the peaches and plated onto three types of agar to induce conidial production. The agars included acidified PDA, cherry concentrate agar, and V8 agar (Appendix B, Table B.1). The fungi were grown for a minimum of seven days at temperatures between 23 and 25°C. The colony surfaces were washed twice with 3 ml of sterile water to dislodge propagules. A hemacytometer was used to determine propagule concentration, which was adjusted to a concentration of 10^5/ml by dilution in sterile water. Each suspension was amended with one drop of 10%, Tween 20 Solution (Sigma, St. Louis, MO).

Sweet and tart cherry fruit were randomly collected from untreated trees at the NWHRS approximately 2 weeks prior to their respective harvest dates. The cherries were prepared by briefly dipping them in one of three concentrations of fenbuconazole. Fenbuconazole concentrations were based on the recommended field rate of 146 ml of fenbuconazole/ 2,810 liters water/ ha, and represented rates equivalent to 25, 50, and 100% of the labeled spray rate. A control set of cherries were also dipped in sterile water. Thirteen sweet cherries and twenty tart cherries were tested for each isolate, at each fungicide concentration. One 20 µl droplet of *M. fructicola* suspension was placed

on each sweet cherry. In the case of the smaller tart cherries, fungal inoculum was sprayed until runoff using an atomizer. One set of cherries was not inoculated to determine if latent *M. fructicola* infections were present at the time of collection. Cherries were suspended on hardware cloth over water in aluminum trays and covered with plastic wrap. These trays were placed in a 23°C incubator for 14 days. The number of cherries with ABR infections was then recorded.

Propiconazole calibration

M. fructicola populations in Georgia have established EC_{50} propiconazole (DMI) levels from baseline *M. fructicola* populations and EC_{50} values associated with practical field resistance (Figure 2G). Michigan *M. fructicola* isolates with known fenbuconazole sensitivites (LAB-3, MRC-20, WIL-3, LAR-3, RFS-10, VNL-18, BRC-16, and KHG-1) were tested for propiconazole sensitivity. Comparison of the sensitivity of Michigan *M. fructicola* populations to fenbuconazole and propiconazole were measured in an attempt to develop a means of calibrating the differences in reported EC_{50} values.

Propiconazole (Orbit; Syngenta Crop Protection, Switzerland) was dissolved in sterile water to prepare stock solutions. Quantities of these stock solutions were added to PDA after it had been autoclaved for sterilization and cooled to 50°C. The propiconazole concentrations were selected based on discriminatory dose information from Georgia (Schnabel, in publication). Five isolated were screened at propiconazole concentrations of 0.005, 0.01, 0.05, 0.1, 0.15, 0.2 and 0.25 μg/ml.

Mycelial plugs 1.0 mm in diameter from each isolate were transferred to PDA plates amended with propiconazole. Each treatment was replicated three times for each isolate. After five days of incubation at temperatures between 23 and 25°C, the plates

were examined for colony expansion and the diameters of the replicates were measured twice to account for colonies not forming perfect circles, these values were then averaged. The EC_{50} values were calculated using a regression analysis function included in SigmaPlot (Systat Software Inc) statistical software. The EC_{50} reported is the fungicide concentration at which the growth of the colony was reduced by 50%.

CYP51 sequencing

Tissue for PCR analysis was prepared by using a bead-beater centrifuge to disrupt the fungal cell wall, as described in the protocol section of Appendix B. Actively growing mycelium (0.1g) was collected from isolates with the highest and lowest EC_{50} values determined in the fenbuconazole sensitivity screening (LAB-3, MRC-20, WIL-3, LAR-3, RFS-10, VNL-18, BRC-16, and KHG-1). The MfCYP51-F and MfCYP51-R primers (23) were used. A Qiagen, DNeasy Plant Mini Kit was used; the mini protocol was followed. The thermocycler program and template preparation are described in the protocol section of Appendix B. The PCR products were run on a 1% agarose gel in 1x SB buffer at 94 volts for 45 minutes. KBPlus (LI-COR) was used to determine product size. The PCR product was then sequenced at the Michigan State University Research and Technology Support Facility.

RESULTS

PCR-based species confirmation

Of the forty-four isolates tested, PCR testing confirmed that all, except 500-4, were *M. fructicola*. Isolate 500-4 was also not amplified by the *M. laxa* primers. No cross amplification with the *M. laxa* primer was observed (Figures 2.B-2.D).

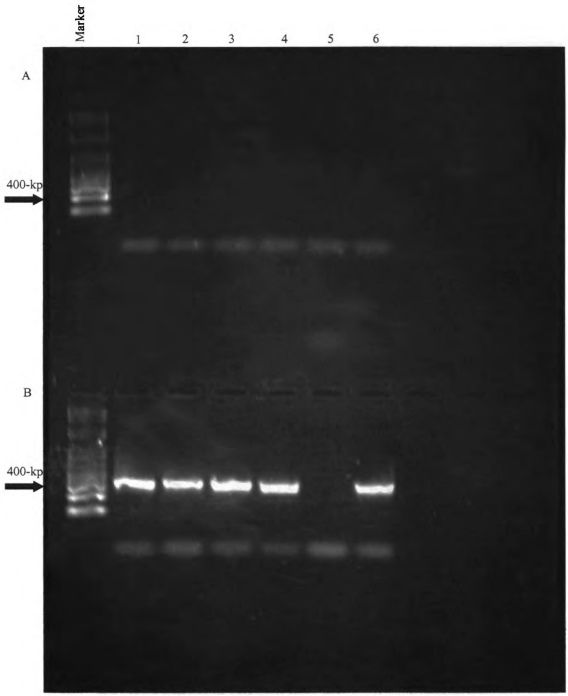

Figure 2.B. PCR amplification of the ITS regions using (A) *Monilinia laxa* ITS primers 1 and 4, or (B) *Monilinia fructicola* ITS primers 1 and 4 for suspected *Monilinia fructicola* isolates 1-6.

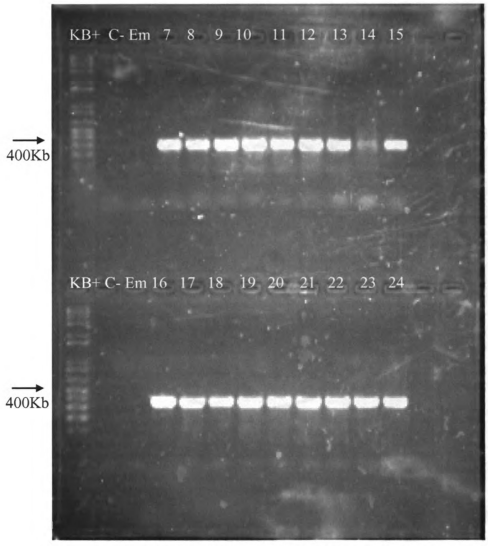

Figure 2.C. PCR amplifications of the ITS1 and ITS4 regions using *Monilinia fructicola* ITS primers 1 and 4, for suspected *Monilinia fructicola* isolates 7-24

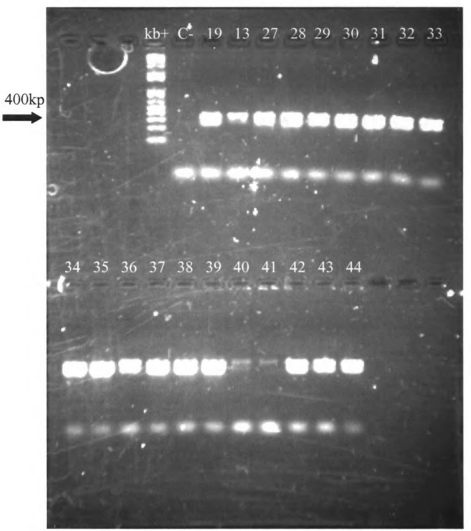

Figure 2.D. PCR amplifications of the ITS1 and ITS4 regions using *Monilinia fructicola* ITS primers 1 and 4, for suspected *Monilinia fructicola* isolates 13, 19 and 27-44.

Monilinia fructicola in vitro, fenbuconazole assay

A greater than 10-fold difference in fenbuconazole sensitivity was found among *M. fructicola* populations when comparing isolates from different orchards (Table 2.2). Overall EC_{50} values were extremely low when compared to the average fenbuconazole field rate of 37.45 ppm; indicating the probability of highly sensitive isolates.

Table 2.2. *In vitro*, mean fenbuconazole EC_{50} value and standard error based on location, and isolate source of *Monilinia fructicola* isolate.

Farm Code	Source	Mean	Std Error
PFR	Peach	0.0061	0.0029
PRL	Peach	0.0045	0.0018
WIL	Peach	0.0045	0.0008
DRA	Sweet cherry	0.0038	0.0010
DRAD	Sweet cherry	0.0049	0.0010
EMP	Sweet cherry	0.0228	0.0047
ENT450	Sweet cherry	0.0156	0.0026
HH	Sweet cherry	0.0243	0.0049
KHG	Sweet cherry	0.0344	0.0085
MRC	Sweet cherry	0.0057	0.0009
RFS	Sweet cherry	0.0379	0.0117
VPD	Sweet cherry	0.0113	0.0019
VPDS	Sweet cherry	0.0286	0.0070
500	Tart cherry	0.0123	0.0007
BRC	Tart cherry	0.0318	0.0087
BRR	Tart cherry	0.0165	0.0026
ENT1000	Tart cherry	0.0182	0.0035
HRG	Tart cherry	0.0351	0.0092
LAB	Tart cherry	0.0049	0.0020
LAR	Tart cherry	0.0050	0.0004
RBT	Tart cherry	0.0101	0.0035
VNL	Tart cherry	0.0245	0.0073
YBR	Tart cherry	0.0049	0.0008

Monilinia fructicola fruit assay

The overall trend for *M. fructicola* fruit infections in inoculated tart and sweet cherries showed a decrease in infection as fenbuconazole concentration increased, regardless of isolate sensitivity (Tables 2.3 and 2.4). The infection rates of all of the isolates were relatively low in the inoculated cherries dipped in water. In both fruit assays, there were no latent *M. fructicola* infections on uninoculated fruit, establishing that the infections on inoculated fruit likely occurred as a direct result of inoculation. The results of the tart cherry assay (Figure 2.E) showed that as a group, the isolates least sensitive to fenbuconazole in the *in vitro* study were more able to produce infections on the inoculated cherries at all fenbuconazole concentrations including the water-dipped cherries. The sweet cherry assay (Figure 2.F) produced a pattern similar to the tart cherry assay. Infection of the sweet cherries with *M. fructicola* decreased as fenbuconazole concentrations increased and the least fenbuconazole-sensitive isolates caused significantly more infection than the most sensitive isolates. Sweet cherries had a lower overall infection rate than tart cherries, and the percentage of ABR infections on the sweet cherries dropped more drastically as the concentration of fenbuconazole increased.

Propiconazole calibration

No direct association could be determined between the fenbuconazole and propiconazole sensitivity of *M. fructicola* isolates from Michigan (Table 2.5). The data did show Michigan isolates are as sensitive to propiconazole as baseline, unsprayed *M. fructicola* populations from Georgia (Figure 2.G).

Table 2.3. Percent tart cherries infected, fifteen days after inoculation with a 10^5 /ml *Monilinia fructicola propagules*. Cherries were dipped in three concentration of fenbuconazole*.

	Most Sensitive Isolates					
Fenbuconazole rate	LAB-3	MRC-20	WIL-3	LAR-3	Mean	Std Error
Control (0 µl/ml)	95.0	57.0	30.0	50.0	58.0	0.0304
Quarter (9.36 µl/ml)	20.0	25.0	5.0	85.0	34.0	0.03936
Half (18.45 µl/ml)	5.0	0.0	15.0	5.0	6.0	0.00703
Full (37.45 µl/ml)	0.0	0.0	5.0	0.0	1.0	0.0028
	Least sensitive isolates					
Fenbuconazole rate	RFS-10	VNL-18	BRC-16	KHG-1	Mean	Std Error
Control (0 µl/ml)	50.0	86.0	80.0	80.0	74.0	0.01809
Quarter (9.36 µl/ml)	29.0	70.0	55.0	55.0	52.0	0.01927
Half (18.45 µl/ml)	0.0	50.0	50.0	25.0	31.0	0.02676
Full (37.45 µl/ml)	0.0	75.0	48.0	0.0	31.0	0.0415

	Uninoculated Control
Fenbuconazole rate	Percent infection
Control (0 µl/ml)	0.0
Quarter (9.36 µl/ml)	0.0
Half (18.45 µl/ml)	0.0
Full (37.45 µl/ml)	0.0

*Fenbuconazole rates of quarter, half, and full concentration are based on the reccommended field rate of 146 ml fenbuconazole/2,810 liters water/hectare.

Table 2.4. Percent sweet cherries infected, fifteen days after inoculation with a 10^5 /ml Monilinia fructicola propgaules. Cherries were dipped in three concentration of fenbuconazole*.

	Most Sensitive Isolates					
Fenbuconazole rate	LAB-3	MRC-20	WIL-3	LAR-3	Mean	Std Error
Control (0 µl/ml)	36.0	43.0	7.0	50.0	33.9	0.021
Quarter (9.36 µl/ml)	0.0	14.0	0.0	0.0	3.6	0.00799
Half (18.45 µl/ml)	0.0	0.0	0.0	7.0	1.8	0.00399
Full (37.45 µl/ml)	0.0	0.0	7.0	7.0	3.6	0.00461

	Least sensitive isolates					
Fenbuconazole rate	RFS-10	VNL-18	BRC-16	KHG-1	Mean	Std Error
Control (0 µl/ml)	93.0	71.0	93.0	29.0	71.4	0.03388
Quarter (9.36 µl/ml)	36.0	14.0	93.0	0.0	35.7	0.04564
Half (18.45 µl/ml)	50.0	21.0	64.0	7.0	35.7	0.02916
Full (37.45 µl/ml)	38.0	21.0	14.0	0.0	18.3	0.01743

	Uninoculate Control
Fenbuconazole rate	Percent infection
Control (0 µl/ml)	0.0
Quarter (9.36 µl/ml)	0.0
Half (18.45 µl/ml)	0.0
Full (37.45 µl/ml)	0.0

*Fenbuconazole rates of quarter, half, and full concentration are based on the reccommended field rate of 146ml fenbuconazole/2,810 liters water/hectare.

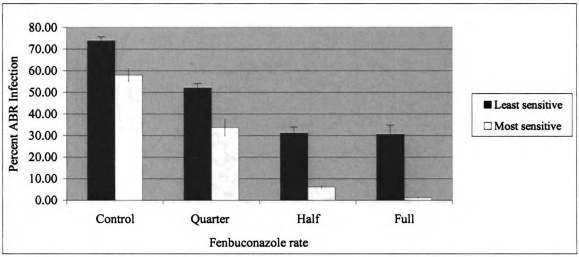

Figure 2.E. Average incidence of tart cherries infected with *Monilinia fructicola*, based on isolate sensitivity, and fenbuconazole rate (Full rate is 146 ml/2,810 liter water/ hectare).

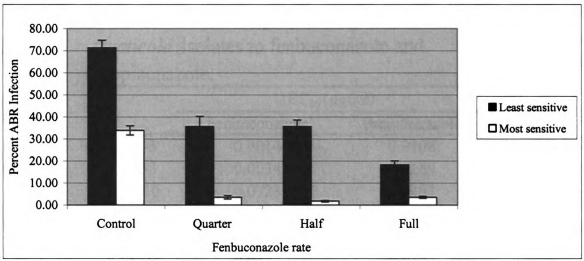

Figure 2.F. Average incidence of sweet cherries infected with *Monilinia fructicola,* based on isolate sensitivity, and fenbuconazole rate (Full rate is 146 ml/2,810 liter water/ hectare).

Table 2.5. Sensitivity of Michigan *Monilinia fructicola* isolates to fenbuconazole and propiconazole.

	EC_{50} (µg/ml)	
	Fenbuconazole	Propiconazole
LAB-3	0.0014	0.0108
LAR-3	0.0034	0.0169
RFS-10	0.0723	0.0191
WIL-3	0.0018	0.0113
KHG-1	0.1024	0.0098
VNL-18	0.0447	0.0133
MRC-20	0.0033	0.0069
BRC-16	0.0562	0.0079

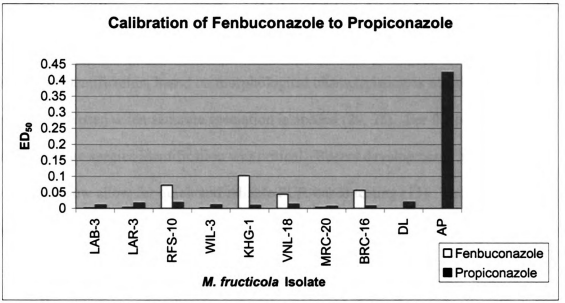

Figure 2.G. Comparison of fenbuconazole and propiconazole sensitivity of Michigan *Monilinia fructicola* isolates (LAB-3, LAR-3, RFS-10, WIL-3, KHG-1, VNL-18, MRC-20, BRC-16) with baseline propiconazole levels from Georgia (DL), and an isolate resistant to propiconazole (AP).

CYP51 sequencing

Variability was found in the *CYP51* gene sequences of the *M. fructicola* isolates tested (LAB-3, MRC-20, WIL-3, LAR-3, RFS-10, VNL-18, BRC-16, and KHG-1). However, the base pair differences in sequence did not differentiate isolates based on fenbuconazole sensitivity. No consistent differences in sequence were found between the groups of the most and least sensitive *B. jaapii* isolates.

DISCUSSION

Historically two brown rot fungi (*M. laxa*, and *M. fructicola*) have been identified based on colony morphology and germ tube growth when grown *in vitro* (4, 26). This method of identification, based on morphological characteristics, is not always reliable enough to be used when accurate speciation is needed (20, 25). For the purposes of this paper accurate species identification was critical. Recent developments in molecular techniques now allow for quick and reliable PCR-based testing (12). This molecular technique confirmed that all but one of the isolates screened were *M. fructicola* (Figure 2.B-2.D). Isolate 500-4 was neither *M. laxa* nor *M fructicola*.

Relating the *in vitro* fungicide sensitivity levels of a pathogen, to efficacy in the field can be difficult. Practical field resistance to DMI fungicides in *M. fructicola* populations has not occurred in Michigan to date, so the *in vitro* sensitivity of field resistant isolates remains unknown. *M. fructicola* populations in Georgia have established *in vitro* EC_{50} propiconazole levels associated with both baseline sensitivities practical field resistance (Figure 2.G). If a direct correlation between the *in vitro* efficacy of propiconazole and fenbuconazole on Michigan isolates could have been determined,

then an EC_{50} value of fenbuconazole at which practical field resistance would occur could be estimated. The sensitivity of Michigan *M. fructicola* populations to fenbuconazole were compared with propiconazole sensitivity in an attempt to develop a means of calibrating the differences in EC_{50} values. No direct relationship could be determined (Table 2.5). In some cases more propiconazole than fenbuconazole was required for the MIC, in others more fenbuconazole than propiconazole was required (Figure 2.G). The baseline propiconazole data did show Michigan isolates are as sensitive to propiconazole as baseline *M. fructicola* populations from Georgia. This development is surprising as both fungicides are triazoles (sterol demethylation inhibitors) and thought to have a similar mode of action, making them susceptible to the development of cross resistance. In this case it appears propiconazole could be used in Michigan to control ABR, but larger samplings are needed to be sure.

Molecular techniques were used to look for a genetic difference in *M. fructicola* isolates, based on their *in vitro* fenbuconazole sensitivity. Three major mechanisms for resistance to DMI fungicides have been previously described, including: mutations in the target enzyme, 14 alpha-demethylase (*CYP51*), leading to a decreased affinity of DMI fungicides to the target protein (1, 5, 6); overexpression of the *CYP51* gene leading to increased production of the target enzyme (8, 18, 23); and overexpression of the ATP-binding cassette (9, 28). In Michigan, *M. fructicola* populations are sensitive to fenbuconazole in the field. Moreover, differences in the *CYP51* gene sequence between isolates, failed to correlate to specific isolate sensitivities. Base pair changes were seen in both the most and least fenbuconazole-sensitive isolates, none of the base pair differences were exclusively found in sensitive or less sensitive *M. fructicola* isolates. The

differences found in the base pair sequence of the *CYP51* gene do not affect fenbuconazole sensitivity in a significant way.

Characterizing the *CYP51* genes of inherently sensitive isolates will be useful in future endeavors. If a significant change in DMI sensitivity occurs in *M. fructicola* populations in Michigan and a genetic cause is suspected, this screening will provide baseline sequence data for comparison. Sequencing the *CYP51* gene may also aid in identifying the mechanism of resistance being utilized.

The overall *in vitro* fenbuconazole sensitivity data was variable, with *M. fructicola* sensitivity varying up to 10-fold between orchards with EC_{50} values ranging from 0.0038-0.0379 μg/ml (Table 2.2). Variability in baseline data may be an indicator of a population's proclivity for shifting towards reduced sensitivity, however a 10-fold difference is considered only moderately variable (3).

The fruit assay demonstrated a significant difference in the ability of the most and least fenbuconazole-sensitive isolates to cause infection in both sweet and tart cherry fruit (Figure 2.E and 2.F). Sweet cherries had a lower overall infection rate than tart cherries and the percentage of ABR infections on the sweet cherries dropped more drastically as the concentration of fenbuconazole increased. This is likely due to the differences in inoculation techniques. Sweet cherries received one droplet of inoculum, while the entire surface of the tart cherries was misted with inoculum using an atomizer that covered more of the surface with inoculum and increasing the chance of infection. Overall, the fruit assay illustrated that the *M. fructicola* sensitivity levels, determined by the *in vitro* fenbuconazole screening, do affect the ability of the pathogen to infect fruit in the presence of fenbuconazole.

Rotating fungicide classes within the growing season, and tank mixing with an appropriate broad spectrum fungicide, are vital facets of managing for resistance (3). Establishing baseline fenbuconazole sensitivity levels was critical to monitoring the sensitivity of Michigan's *M. fructicola* populations in subsequent years. Monitoring fungicide sensitivity will be vital to the development of subsequent recommendations that may help prolong the useful lifespan of DMI fungicides against CLS.

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APPENDIX A

MMEA Recipe

Combine 20.0g malt extract, 1.0g yeast extract, 20.0g agar, and 1.0 liter DI water. The mixture needs to be autoclaved (20 minutes) for sterilization.

Table A.1. Weather conditions during the 2005 growing season in Suttons Bay, Michigan, including; temperature, wetting period duration and rainfall.

Start of Wetting Period	End of Wetting Period	Duration (Hrs.)	Avg. Temp (C)	Rainfall (cm)	Cherry Leaf Spot
		Wet: 13			
5/9 4-5PM	5/10 8-9AM	Span: 16	16.9	0.23	Moderate
		Wet: 14			
5/10 9-10PM	5/11 Noon-1PM	Span: 15	6.9	0.03	None
		Wet: 51			
5/13 7-8AM	5/16 8-9AM	Span: 73	6.1	1.04	None
		Wet: 24			
5/19 8-9AM	5/20 8-9AM	Span: 24	9.1	1.37	None
		Wet: 21			
5/22 7-8AM	5/23 Noon-1PM	Span: 29	11.2	0.76	Low
		Wet: 10			
5/23 11-12PM	5/24 9-10AM	Span: 10	9.8	0.05	None
		Wet: 4			
5/26 9-10AM	5/26 1-2PM	Span: 4	11.9	0.05	None
		Wet: 3			
5/27 5-6AM	5/27 8-9AM	Span: 3	9.5	0.08	None
		Wet: 2			
6/5 8-9AM	6/5 10-11AM	Span: 2	20.7	0.05	None
		Wet: 2			
6/5 11-12PM	6/6 1-2AM	Span: 2	20.6	0.05	None
		Wet: 7			
6/8 1-2AM	6/8 8-9AM	Span: 7	16.7	0.30	Low
		Wet: 23			
6/14 2-3PM	6/15 9-10PM	Span: 31	15.8	0.74	High
		Wet: 1			
6/23 1-2PM	6/23 2-3PM	Span: 1	17.6	0.08	None
		Wet: 5			
6/26 3-4AM	6/26 9-10AM	Span: 6	17.6	0.03	None
		Wet: 1			
6/30 7-8AM	6/30 8-9AM	Span: 1	20.8	0.03	None
		Wet: 5			
7/1 8-9AM	7/1 2-3PM	Span: 6	14.7	0.05	None
		Wet: 6			
7/4 6-7AM	7/4 Noon-1PM	Span: 6	19.0	1.22	Low
		Wet: 3			
7/13 6-7AM	7/13 9-10AM	Span: 3	21.4	0.03	None
		Wet: 1			
7/17 3-4PM	7/17 4-5PM	Span: 1	28.2	0.84	None
		Wet: 2			
7/18 6-7AM	7/18 8-9AM	Span: 2	24.4	0.05	None
		Wet: 9			
7/24 3-4AM	7/24 Noon-1PM	Span: 9	21.0	0.64	Low
		Wet: 15			
7/25 11-12PM	7/26 2-3PM	Span: 15	20.3	2.21	Moderate
		Wet: 12			
7/28 9-10PM	7/29 9-10AM	Span: 12	16.1	0.53	Low
		Wet: 9			
8/4 3-4AM	8/4 2-3PM	Span: 11	23.1	0.89	Low
		Wet: 11			
8/10 1-2AM	8/10 Noon-1PM	Span: 11	21.0	0.05	Low
		Wet: 12			
8/12 Midnight-1AM	8/12 Noon-1PM	Span: 12	18.6	0.76	Moderate
		Wet: 40			
8/18 Noon-1PM	8/20 Noon-1PM	Span: 48	19.0	3.81	High
		Wet: 7			
8/21 10-11PM	8/22 9-10AM	Span: 11	14.0	0.13	None
		Wet: 7			
8/27 5-6AM	8/27 Noon-1PM	Span: 7	18.5	0.84	Low

Table A.2. Weather conditions during the 2006 growing season in Suttons Bay, Michigan, including; temperature, wetting period duration and rainfall.

Start of Wetting Period	End of Wetting Period	Duration (Hrs.)	Avg. Temp (°C)	Rainfall (cm)	Cherry Leaf Spot
5/9 4-5PM	5/10 8-9AM	Wet: 13 Span: 16	16.9	0.23	Moderate
5/10 9-10PM	5/11 Noon-1PM	Wet: 14 Span: 15	6.9	0.03	None
5/13 7-8AM	5/16 8-9AM	Wet: 51 Span: 73	6.1	1.04	None
5/19 8-9AM	5/20 8-9AM	Wet: 24 Span: 24	9.1	1.37	None
5/22 7-8AM	5/23 Noon-1PM	Wet: 21 Span: 29	11.2	0.76	Low
5/23 11-12PM	5/24 9-10AM	Wet: 10 Span: 10	9.8	0.05	None
5/26 9-10AM	5/26 1-2PM	Wet: 4 Span: 4	11.9	0.05	None
5/27 5-6AM	5/27 8-9AM	Wet: 3 Span: 3	9.5	0.08	None
6/5 8-9AM	6/5 10-11AM	Wet: 2 Span: 2	20.7	0.05	None
6/5 11-12PM	6/6 1-2AM	Wet: 2 Span: 2	20.6	0.05	None
6/8 1-2AM	6/8 8-9AM	Wet: 7 Span: 7	16.7	0.30	Low
6/14 2-3PM	6/15 9-10PM	Wet: 23 Span: 31	15.8	0.74	High
6/23 1-2PM	6/23 2-3PM	Wet: 1 Span: 1	17.6	0.08	None
6/26 3-4AM	6/26 9-10AM	Wet: 5 Span: 6	17.6	0.03	None
6/30 7-8AM	6/30 8-9AM	Wet: 1 Span: 1	20.8	0.03	None
7/1 8-9AM	7/1 2-3PM	Wet: 5 Span: 6	14.7	0.05	None
7/4 6-7AM	7/4 Noon-1PM	Wet: 6 Span: 6	19.0	1.22	Low
7/13 6-7AM	7/13 9-10AM	Wet: 3 Span: 3	21.4	0.03	None
7/17 3-4PM	7/17 4-5PM	Wet: 1 Span: 1	28.2	0.84	None
7/18 6-7AM	7/18 8-9AM	Wet: 2 Span: 2	24.4	0.05	None
7/24 3-4AM	7/24 Noon-1PM	Wet: 9 Span: 9	21.0	0.64	Low
7/25 11-12PM	7/26 2-3PM	Wet: 15 Span: 15	20.3	2.21	Moderate
7/28 9-10PM	7/29 9-10AM	Wet: 12 Span: 12	16.1	0.53	Low
8/4 3-4AM	8/4 2-3PM	Wet: 9 Span: 11	23.1	0.89	Low
8/10 1-2AM	8/10 Noon-1PM	Wet: 11 Span: 11	21.0	0.05	Low
8/12 Midnight-1AM	8/12 Noon-1PM	Wet: 12 Span: 12	18.6	0.76	Moderate
8/18 Noon-1PM	8/20 Noon-1PM	Wet: 40 Span: 48	19.0	3.81	High
8/21 10-11PM	8/22 9-10AM	Wet: 7 Span: 11	14.0	0.13	None
8/27 5-6AM	8/27 Noon-1PM	Wet: 7 Span: 7	18.5	0.84	Low

APPENDIX B

PROTOCOLS

Bead-beater protocol

One hundred milligrams of fresh mycelium was aseptically placed in shatter-resistant, 2ml centrifuge tubes with two glass beads. Centrifuge tubes are then submerged in liquid nitrogen for 30 seconds and placed in the centrifuge. Short intervals (30 seconds) at moderate RPMs are used until the mycelia become a chalk-like powder.

PCR reaction formula for all reactions

PCR reaction volumes were 50µl and included: 5µl of Invitrogen PCR Buffer, 1.5µl Invitrogen MgCl Buffer, .4µl dNTPs, 1.25µl Taq DNA polymerase, 41.1µl sterile DI water, 1µl of each primer and 5µl template from each isolate.

Thermocycler program for PCR confirmation of *M. fructicola* species

Amplifications were performed with the following thermocycler program: 94°C for 3 minutes; 36-cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1.5 minutes; 10 minutes at 72°C; and finally held at 4°C.

Thermocycler program for PCR amplification of the *CYP51* gene

Amplifications were performed with the following thermocycler program: 94°C for 3 minutes; 30-cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; followed by a final extension step of 5 minutes at 72°C.

Table B.1. Culture media for inducing conidia formation in *Monilinia fructicola*

Acidified PDA agar: 39g of potato dextrose agar with 1 liter deionized water and autoclave for 20 minutes. Allow agar to cool to 50°F and add 1.3ml of 25% lactic acid, pour plates.
Cherry concentrate agar: Mix 12g agar, 400ml deionized water, and 60ml commercially available cherry concentrate diluted per the labeled instructions. Autoclave (20 minutes) and then adjust the pH to 5 (should take around 40ml of 10% KOH).
V8 agar: Mix 125ml V8, 375ml deionized water, and 15g of agar, then autoclave (20 minutes).