EVALUATION OF RESISTANCE OF *BLUMERIELLA JAAPII* TO SUCCINATE DEHYDROGENASE INHIBITOR FUNGICIDES

By

Jacqueline Costa Gleason

A THESIS

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

Plant Pathology – Master of Science

2020

ABSTRACT

EVALUATION OF RESISTANCE OF *BLUMERIELLA JAAPII* TO SUCCINATE DEHYDROGENASE INHIBITOR FUNGICIDES

By

Jacqueline Costa Gleason

Cherry leaf spot (CLS), caused by the fungus *Blumeriella jaapii* (Rehm) v. Arx. is a devastating disease of tart cherry (*Prunus cerasus* 'Montmorency') in the Great Lakes region of the U.S. Michigan is the largest producer of tart cherries in the United States, and control of CLS is critical for the maintenance of high-yielding, healthy trees. Succinate dehydrogenase inhibitor (SDHI) fungicides are widely used for CLS management, but fungicide resistance has been reported for the SDHI fungicide boscalid, and other alternative SDHI fungicides have been in use commercially since 2013. In 2016, 36 tart cherry orchards in Michigan were sampled, and 883 single conidium isolates were established. In 2017, 42 orchards were sampled, and 898 isolates were established. These isolates were tested *in vitro* for sensitivity to the SDHI fungicides fluopyram, fluxapyroxad, and boscalid, and minimal inhibitory concentrations (MICs) were determined. Experiments using fungicide-treated potted trees inoculated with *B. jaapii* isolates with known MICs were done to establish the relevance of MIC to fungicide efficacy in the field. Results suggest that populations of *B. jaapii* have shifted towards resistance to fluopyram and fluxapyroxad. Cross-resistance between boscalid, fluopyram and fluxapyroxad is confirmed by the number of tested isolates that are resistant to the three of them (n=418), which represents 26% of the isolates tested. Management of disease must be consistent, and it is the most important form of preventing against the development of resistance to the SDHIs.

To my son Levi William Gleason

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my supervisor Dr. George Sundin for the opportunity to be part of his great team of graduate students, for his guidance, encouragement, and for always trusting me. My deep appreciation to Dr. Tyre Proffer, who with all expertise, patiently taught me to work with fungi. His guidance helped me to believe I could do it. Thank you to Dr. Martin Chilvers for giving me the feedbacks needed and for always being available to talk.

I would like to thank Cory Outwater for the friendship, for helping me in the field, in the office, or in the laboratory anytime I needed, for sharing his knowledge and experience with me; Calla Sundin for being the best undergraduate someone could have, Linda Colon for her thoughtful and kind endless help, and Mikaela Breunig for being my best friend on this journey.

Thank you to Nikki Rothwell and Dave Jones for the effort in making sure I would get the right and most precise places to collect my samples from, and to my lab mates and friends that graduate school gave me: Luisa Castiblanco, Jie Wang, Emma Sweeney, Jingyu Peng, Suzanne Slack, Jeff Schachterle, Roshni Kharadi, Salta Mambetova and Kerry Vermeulen. Thank you for always being there for me.

I am grateful for the support of my family, who has always been there for me, especially my mom, who is my great inspiration to follow my dreams with no fear. Thank you to my father for the great example, to my siblings for the emotional support and encouragement, and to my in laws Patty and Michael for the never-ending support and love.

I would like to recognize my husband Matt for the unconditional love and daily stimulus to achieve my long-dreamed degree. I would not have accomplished this without you, and so this achievement is yours as it is mine.

Most sincere thanks to my little boy Levi, to whom I dedicate this thesis with the intention of one day inspiring him to make his professional decisions based on what makes the best of him.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

µg ml-1 Microgram per Milliliter

ABR American Brown Rot

ALB Alternaria Late Blight

cfu/ml Colony-forming units per milliliter

CLS Cherry Leaf Spot

CWA Coffee Agar Media

DMIs Demethylation Inhibitor Fungicidess

FRAC Fungicide Resistance Action Committee

g/ha grams per hectare

GLYE Glycerol Yeast Media

IPM Integrated Pest Management

MIC Minimal Inhibitory Concentrations

ml/ha Milliliter per hectare

MMEA Malt Extract Agar

MSU Michigan State University

NC North Carolina

NWMHRC Northwest Michigan Horticultural Research Center

QoIs Quinone outside Inhibitors (Strobilurin)

sdhB Succinate Dehydrogenase Complex Iron Sulfur Subunit B

SDHI Succinate dehydrogenase inhibitor

SREC Southwest Research and Extension Center

µL Microliter

CHAPTER 1

INTRODUCCTION

Cherries (*Prunus* sp.) are one of the most important crops in the temperate fruit growing regions of the world, with Turkey being the largest producer of cherries in the world and the United States the second largest. Michigan is the primary tart cherry (*Prunus cerasus* L.) producer in the United States, accounting for almost 75% of the total quantity produced nationwide (Proffer, Lizotte, Rothwell, & Sundin, 2013). Annual production of tart cherries in Michigan has averaged 91.4 million kilograms (201.5 million pounds) per year (NASS, 2018). In 2016, Michigan produced 101.5 million kilograms (224 million pounds) of tart cherries with a value of $54 million. The states of Utah and Washington follow as the second and third largest producers, respectively. Michigan is also the fourth largest producer of sweet cherries in the U.S., accounting for 20% of the production nationwide (NASS, 2015).

Tart cherry trees originated from the Carpathian Basin in Hungary, where *P. avium* can be found as a wild or cultivated tree. Cultivated cherries were introduced to the Americas in the 1600s and cultivated by the French settlers as they colonized Michigan. The first cultivated traditional variety of tart cherry, named Pandy, was identified, vegetatively propagated and distributed across the countryside in approximately 1848 ("History of Tart Cherries - Department of Horticulture," n.d.). In 1857, Peter Dougherty, a Presbyterian missionary living in northern Michigan, began the production of modern-day cherries near Traverse City. After more than 40 years, the first commercial orchard was planted near Dougherty's farm on the Old Mission Peninsula. In the early 1900s, the tart cherry industry was already established from Benton Harbor to Elk Rapids, and with the establishment of the first cherry processing facility, fruit started being shipped to Detroit, Chicago and Milwaukee ("History of Cherries," n.d.).

Cherries are grown commercially along the west side of Michigan, and Leelanau is the county with the greatest production of tart cherries in Michigan, producing 26% of the tart cherries, 48% of the sweet cherries, and 30% of all cherries produced in the state. This county is also the one with the largest number of acres in production nationwide (Dunckel, 2011). The counties of Oceana, Antrim, and Grand Traverse are also major producers of tart cherries. The tart cherry growing areas in northwest Michigan are benefited by the short distance to Lake Michigan, which is responsible for balancing temperature extremes and helping to moderate the temperatures in the spring. Lake effect snowfall is also responsible for insulating the ground, protecting trees from severe winter temperatures, and offering an onshore breeze that moderates warm temperatures during the summer. The soil in this area is favorable to the production of cherries because it is characterized as sandy loam to loamy sand that offers good drainage (Crop Profile for Tart Cherries in Michigan, 2003).

Tart cherries are mostly sold frozen, dried, and canned, or as juice or wine (Yılmaz et al., 2019). A minimal proportion is sold fresh, and a new way to add value to the fresh market are the “you-pick” operations, where consumers go to the orchard, harvest their own cherries and pay per pound picked (Boriss et al., 2006). Tart cherries are not only appreciated for their taste but also are rich in polyphenolic contents, which are compounds that act as antioxidant, antimutagenic and anticarcinogenic. These compounds help in the treatment of chronic pain, oxidative stress, inflammation, muscle damage, osteoarthritis, fibromyalgia, the recovery of soft tissue injuries, and in the treatment of diseases that lead to more serious problems like cardiovascular disease, stroke, diabetes, cancer, Alzheimer’s and other age-related diseases (Tiernan, Imrhan, Prasad, Vijayagopal, & Juma, 2016). The intake of anthocyanins and other flavonoids, antioxidants found naturally in cherries, are proved to reduce lipids, glucose, insulin and fatty liver and consequently prevent against hyperlipidemia, obesity, and atherosclerosis (Seymour et al., 2008). Cherries are also rich in melatonin, a critical molecule that acts in regulating the sleep cycle in humans, being beneficial to sleep duration and quality (Howatson et al., 2012). They also contain a significant amount of vitamin C, beta-carotene, potassium, magnesium, fiber and iron, and for this it is called "America's superfruit" ("Michigan Agriculture Facts & Figures," n.d.)

From a disease perspective, there is a major downside to tart cherry production in Michigan, namely, that the industry relies almost exclusively on the 'Montmorency' cultivar, which is highly susceptible to cherry leaf spot (CLS), the most important disease affecting tart cherry production in the state (McManus et al., 2007). Cherry leaf spot is caused by the fungus *Blumeriella jaapii* (Rehm) v. Arx. (formely *Coccomyces hiemalis* Higgins), which is an ascomycete that belongs to the order *Helotiales*. This fungus was first described in Europe in 1884 by Karsten. In 1907, Rehm found the fungus on *Prunus padus* in Europe and named it as *Pseudopeziza jaapii* (Farr, D.F., & Rossman, A.Y., 2018). In 1913, Bascombre Higgins published a description of the fungus, in the journal *Science New York* (Murril, 1915). He discovered and proved that the fungus actually had an asexual but also sexual stage, naming it *Coccomyces hiemalis*. The fungus was then renamed in 1961 by J. A. von Arx to *Blumeriella jaapii* (Rehm) v. Arx (Arx, J.A. Von,1961), which is the currently accepted name by the International Code of Nomenclature for algae, fungi and plants (Farr, D.F., & Rossman, A.Y., 2018).

Blumeriella jaapii has been reported in species of the genus *Prunus* such as *P. avium* (sweet cherry), *P. cerasus* (tart chery), *P. domestica* (European plum), *P. emarginata* (bitter cherry), *P. melanocarpa* (black chokecherry), *P. padus* (European bird cherry), *P. pennsylvanica* (fire cherry), *P. serotina* (black cherry), and *P. virginiana* (common chokecherry) as hosts, but it is more prevalent and causes more serious problems in tart cherries (*P. cerasus*) causing chlorosis and premature defoliation of the trees (Jones & Sutton, 1996). CLS is primarily a disease of the foliage, but in severe infection situations, symptoms can also be observed on fruit stems, petioles and fruits. This disease has spread to all areas in Europe and the U.S. where cherries are grown and is present in 100% of cherry orchards of Michigan (Pedersen H., Jensen B., Munk L., Bengtsson M., Trapman M., 2012). All commercial cherry varieties are susceptible to CLS, and so this disease is responsible for the large numbers of fungicide applications in tart cherry production throughout the season (Proffer et al., 2006).

Cherry Leaf Spot Disease Cycle

Blumeriella jaapii overwinters in fallen infected dead leaves on the orchard floor. In the spring, cup shaped fruiting bodies called apothecia develop on the surface of old leaves, and ascospores are then produced inside asci, which will later be ejected to the tree canopy for a time period of 6 to 8 weeks when the temperatures are optimum for the discharge. These ascospores are dispersed through water or through the wind to new open leaves (Jones & Sutton, 1996). Apothecia develop at temperatures between 4 and 24°C with optimum temperature at 16.6° C, and it is also influenced by the relative wetness. Ascospores are discharged at temperatures between 8 and 30° C, but discharge is greatest at temperatures above 16°C, associated with heavy rain (Garcia & Jones, 1993). These temperatures and humidity conditions usually happen when trees are blooming, and petals are dropping. The knowledge of ascospore maturation timing may be very important in the development of a spray recommendations calendar that could help growers know what dates fungicide application would be more effective.

Once on the leaf surface, ascospores or conidia will germinate, and the growing germ tube will penetrate the leaf through open stomata, then hyphae start growing throughout intercellular spaces of the mesophyll (primary location of photosynthesis in the plant) and haustoria (the hyphal tip) penetrate cell walls (Gruber, Davies, & McManus, 2010). Heavy infections are usually caused by a long rainy season in summer and fall. The two main factors for germination and infection of plants are temperature and humidity. Time of incubation will also depend on these two factors; only a few hours of wetness during the ideal temperature is enough for the germination of spores. Infection on the primary cycle can be overseen because leaves are usually small, and infection is low. Usually by early summer the supply of ascospores is exhausted, and this is when secondary infection begins. Secondary cycle is possible because conidia is produced from late spring to late fall. Secondary infections occur from conidia produced on the overlooked lesions from primary cycle. (Jones, Alan L.,Ehret, G. R. , Garcia, S.M., Kesner, C.D., Klein, 1993).

The second phase of the life cycle is more efficient in the dissemination of the disease because the number of spores produced in the lesions is larger than number of spores produced in apothecia; also, for the distance between leaves in the plants. This distance is insignificant when compared to the distance from the orchard ground to the leaves on trees canopy, which is the way made in the first phase of life cycle (Sundin & Rothwell, 2013). Lesions form 10 to 15 days after infection starts depending on temperature and humidity conditions. When lesions have developed, white masses of spores are formed in acervuli, open fruiting bodies that expose spores on the underside of leaves. These spores are spread by water and cause new infections in other leaves. This process will repeat, and each new lesion will again produce thousands of spores and infect t other leaves (Díaz, Zas, & Fernández-López, 2007). In the fall, when leaves have dropped and become litter, *B. jaapii* shifts from a parasitic lifestyle to a saprophytic lifestyle (Holb, 2013).

Symptoms of the Disease

The symptoms of CLS are mainly small round purple to brownish spots, about 3 mm or less in diameter, on the upper surface of the leaves becoming black when older, and white to pink masses on the underside of the leaves made by thousands of conidiospores that distinguish CLS from other diseases that cause spots on the leaves of tart cherry trees such as bacterial canker. These acervuli with thousands of conidiospores will rupture the epidermis of the leaves and liberate cirrhii, the masses of conidia. Leaves will become yellow and defoliation of the trees will occur, affecting tree ability to overwinter (Wilcox, 1993) and to receive the photoperiodic stimulus that generate the origin of acclimation, and so they lose their ability to measure daylength through their phytochrome system, delaying acclimation of wood and flower buds in the fall, and accelerating deacclimation in the Spring. Leaves should stay in the trees as long as possible, with the first leaves to be lost naturally in the first frost of the season (Howell & Stackhouse, 1973). After six to eight weeks, the lesions have become necrotic and separate from the health part of the leaves, but this only happens on sweet cherry trees. On tart cherries, with the appearance of lesions in about 14 days after infection, the leaves are dropped, and this is the reason CLS is more severe on tart cherries (Pedersen H., Jensen B., Munk L., Bengtsson M., Trapman M., 2012).

Blumeriella jaapii also infects sweet cherry trees causing CLS. Its symptoms are not as devastating as in tart cherry trees. The lesions are smaller and usually appear 2-4 days later than in tart cherries. Sporulation is reduced, and the number of conidia is much lower than in tart cherries, so consequently defoliation in sweet cherry trees caused by CLS happen in a much slower rate (Sjulin et al., 1989).

If not controlled, CLS can reduce yields by 100%. Cherry trees are susceptible to CLS from May through September, and severe defoliation can be observed as early as July. Trees defoliated by August will have decrease on their productivity due to poor flower bud formation, and plants defoliated during the spring or early summer may die over the winter for lack of nutrients that are obtained during summer through photosynthesis. Reduction of blossom production, soft and immature fruits, fruits ripened unevenly, reduction of fruit set for at least two seasons, susceptibility to winter injury due to the loss of photosynthesis and carbohydrates stored in roots are some of the symptoms that terrify tart cherry growers. All varieties of tart cherries are susceptible to CLS, but the main commercial variety 'Montmorency' is the most susceptible to the disease (McManus et al., 2007).

Control of Cherry Leaf Spot

Management of CLS is difficult because once infection is established it becomes almost impossible to eradicate the disease. The use of resistant varieties would be the ideal method for the control of CLS, if growers actually had that option. Previous studies have detected species that are considered tolerant to CLS such as sweet cherry (*P. avium*) that are also *B. jaapii* hosts, but are much less affected than tart cherries for producing smaller and fewer lesions, and therefore fewer conidia; tart cherry (*P. cerasus* cv. North Star) that even though gets infected as the other tart cherry species, produces much less conidia causing consequently less defoliation; and the wild species *P. canescens* and *P. maackii* that are believed to trigger host response before the biotrophic proliferation is well established, preventing sporulation to occur (Sjulin, T.M., Jones, A. L., Andersen, 1989), (Wharton, Pathology, Iezzoni, & Jones, 2003). Research is active hoping to find more loci responsible for resistance and tolerance. Until date only one locus responsible for the control of CLS has been identified and the efforts to breed tart cherries to obtain resistant or tolerable varieties with multiple resistance alleles are still ongoing (Andersen, Sebolt, Sundin, & Iezzoni, 2017).

The combination of not being able to rotate cultures in orchards and the ability of surviving in extreme temperatures like very cold winters make control of CLS even harder to do. One option small orchards and organic producers have is to collect as much leaf litter and mulching under the trees. In some cases, these practices may be useful to help with reduction of inoculum for the following spring. In order to achieve good results though, these practices should be combined with other disease control methods such as the use of resistant cultivars, plant inducers and resistant cultivars. This option though is impractical for big growers because of cost and great amount of work (Holb, 2013).

The attempts of breeding for a resistant cultivar with a superior fruit quality than Montmorency cherries, and that could maintain yields through the years have been a continuous effort in Europe and also in the USA. After World War II, Dr. Maliga Pal started a project with the objective of identifying the high-quality varieties among the native tart cherries in the villages of Hungary. He found Újfehértói Fürtös to be the most promising variety. In 1970, this variety was released, and it represents at least 30% of Hungarian production in the present. In 1981, Dr. Amy Iezzoni initiated a breeding program at Michigan State University with same goals of Dr. Maliga Pal, but focusing in resistance to disease and quality of fruits. She travelled to Europe to get varieties from a larger germplasm because since tart cherries are not native to the USA, there is not diversity available. After years collecting and testing, researchers from MSU and the Institute in Hungary that helped funding this research released Újfehértói Fürtös in the United States under the name of Balaton, in 1998 ("History of Tart Cherries - Department of Horticulture," n.d.).Balaton cherries have been cultivated in a much smaller scale than the traditional Montmorency because trees don't produce as much as Montmorency and the fruits have different characteristics, but the possibility of being bred to be resistant to CLS is a necessity.

The most efficient method of control of CLS is done with fungicide applications. Fungicides are much more efficient when applied in early season, before the inoculum load is high, and should start before blooming, if considered that *B. jaapii* can infect bract leaves and start an early infection, which could lead to a possible epidemic (Sundin & Rothwell, 2013). These applications need to be repeated every 7-10 days during the growing season until harvesting. Applying fungicide at the right time will delay the progress of the disease, and the trees will be able to accumulate the carbohydrates necessary for fruit growth and their survival. Only one delayed or missed spray can result in major consequences. Fungicide applications need to offer complete coverage of the canopy, but even with this aggressive fungicide application program, it does not offer security of full control of the fungus. Fungicide treatment rarely eradicates the fungus (Proffer, Lizotte, Rothwell, & Sundin, 2013).

Fungicide applications are also the source of major expenses in production of cherries, but it is the most effective way to control CLS. Before 1970, fungicides were used as protectants; they inhibited spore germination, interfering with metabolic pathways. There was no evidence of resistance to them and the possibility is still low at present.

Control Alternatives

The implementation of IPM has helped growers to minimize costs reducing fungicide application frequency. They predict the weather and invest in innovative spray application technology. With dense canopy, spraying cherry trees uniformly becomes a difficult task. Most of the time it results in inadequate coverage of the tree, concentrating spraying in the lower external tree canopy to get enough coverage in the upper centers of the tree and so inadequate coverage of tree is done to get a satisfactory control on the top of the trees. For this, engineers at Michigan State University created an "air curtain" spray technology that makes fungicide application uniform and avoids contamination in the middle of the tree. This tool ensures that fungicide coverage is optimal, reduces amounts of fungicide waste and allows faster application speeds. As a result of this technology, the number of preventative fungicide applications have been reduced (Edson, Laubach, Nugent, & Thornton, 1998)

Organic orchards can use sulfur and copper–based fungicides, however they are often less effective and more phytotoxic than synthetic fungicides used in the conventional commercial orchards (Holb, 2013). The use of copper-based fungicides can also affect the lives of beneficial organisms like earthworms (when copper build up in the soil), and of some fishes and invertebrate aquatic animals, and so precaution has to be used when these fungicides are applied (McManus et al., 2007).

Programs with at least 3 applications of copper-based fungicides are more effective in the control of CLS, even though phytotoxicity and reduction on the size of fruits can be observed (McManus et al., 2007). Little effects caused by copper compound fungicides are proved to be minimal when compared to the benefits of fungicide resistance and disease control achieved with a copper spray program. Copper-associated leaf bronzing does not affect fruit fresh weight compared to fresh fruit weight of synthetic programs or nonsprayed control, and so copper compounds can be used to control CLS with minor phytotoxicity problems (Gruber et al., 2010). Lime is often used in the attempt to mitigate the phytotoxicicty associated with these fungicides. Copper-based fungicides are also cheaper than QoIs, DMIs, SDHIs or Chlorothalonil. Fungi don't become copper resistant because of its mode of action, which is altering the characteristic properties of the proteins (McManus et al., 2007).

The application of urea to leaf litter on the fall can reduce *Blumeriella jaapii* biomass because of the combination of ammonia toxicity that results in fungicide effect, increase in leaf pH, and antagonism (competition with other microorganisms that are actually favored by urea application), and increases the potential of leaf litter decomposition, but these applications are expensive, require much work and inoculum can come from neighboring orchards which discourage growers to try this process (Green et al., 2006).

The equilibrium between genetic resistant cultivars, and the use of bio controls, chemical products, and biochemical sources are equally important for the control of diseases. The integration of these technologies with sanitation and cultural methods are very important for grower's success.

Population Resistance to Fungicides

Some of the most important multi-site fungicides like captan, dodine and chlorothalonil were introduced in the 1950s and 1960s. They offered exceptional control of CLS when used as protectant fungicides, preventing against high infection, but some restrictions are applied to the use of them because of residues left on the fruits. These fungicides are still utilized; however, it is required that spray happens in the beginning of the season and as a component of a tank mix compound (C. A. Outwater, 2014). Next generation of fungicides was the benzimidazoles, including thiabendazole, benomyl, and thiophanate methyl. They are single-site inhibitors of fungal microtubule assembly during mitosis and are active against many pathogens. They were introduced in 1968, and allowed farmers to reduce the number of fungicide applications and to lower the rates applied, but only one year later the first case of resistance to them occurred in powdery mildew (Morton & Staub, 2008).

Demethylation inhibitors (DMIs) were then introduced and widely used in the 1980's in the control of CLS and brown rot. Some farmers even used them as exclusively form of prevention because of their efficacy and for offering slight curative action. This exhaustive utilization resulted in DMI-resistant population of *B. jaapii* in Michigan. A research done for 17 years, from 1989 to 2005 was conducted in the Northwest Michigan Horticultural Experiment Station, in Leelanau County. In the first years, Fenbuconazole (Indar 75W) and Tebuconazole (Elite 45 DF), some of the most effective and important fungicides of this class, were highly effective in the control of CLS, but with the continuous use of this only class of fungicides, populations of *B. jaapii* became resistant to them. The analyses of the data reported scaled percentage of infection, relating the number of infections to the control number of infections. It is possible to see in the data reported that in 1995 there was a high increase of almost 70% of infection. In 1999, numbers of infection are the same as the control numbers, and resistance problems continued to be reported by growers (Proffer et al., 2006). In 2006 DMIs presented total failure in the control of CLS in Michigan, and with the loss of this important class of fungicides growers started using copper-based fungicides again, which they had stopped because of their phytotoxicity to tart cherry leaves, and to the assessment of other potential modes of action (Proffer et al., 2006).

Succinate Dehydrogenase Inhibitors (SDHIs) are a more recently introduced class of single-site fungicides that was introduced in 2004 against fungal pathogens for many crops and are increasingly important in the management of pathogens that affect the production of fruits and vegetables. This class of fungicides inhibits the enzyme succinate dehydrogenase, which is a functional part of the tricarboxylic cycle and of the mitochondrial electron transport chain, interfering with mitochondrial respiration. They belong to the Fungicide Resistance Action Committee (FRAC) code 7, which are categorized to offer medium to high risk of fungal populations to develop resistance to them, and as examples there are boscalid, fluopyram and fluxapyroxad used in the control of CLS (McGrath, 2004).

Boscalid is a fungicide that belongs to the first generation of the carboxamines (SDHIs). Growers started applying it when it became available in the market in the year of 2004. This fungicide is mainly effective in the suppression of spore germination, and germ tube elongation, but can also inhibit mycelial growth and appressoria formation. This fungicide is rapidly absorbed by the leaf, being translaminarily transported to the other side of the leaves, or acropetally transported in the xylem making untreated parts of the plant to be protected against certain pathogens (Stammler, Brix, Nave, Gold, & Schoefl, 2008). Pristine (BASF Corporation, Research Triangle Park, NC) is a premix of the SDHI boscalid and of pyraclostrobin, a QoI (Quinone outside inhibitor) that inhibits fungal respiration at complex III of the respiratory chain. This compound was highly used in the control of CLS, but due to the site-specific mode of action, populations of *B. jaapii* developed resistance to it, and growers stopped using it in 2014 when the use of it was not preventing against the disease anymore. The highly specific mode of action of the SDHIs make them prone to resistance development (C. A. Outwater, 2014).

Altered sensitivity to boscalid that have occurred in the commercial orchards of Michigan is due to a non-synonymous mutation in the *sdhB* gene, a single mutation in the succinate dehydrogenase gene, when an amino acid converts from histidine to arginine. Mutation of gene H260R in *B. jaapii* populations correlates to the sensitivity to boscalid in these orchards (C. Outwater, Proffer, Rothwell, Peng, & Sundin, 2019).

Fluopyram and fluxapyroxad are the most used fungicides in the actuality to treat CLS. They belong to the second generation of SDHIs and are very important in the control of many diseases since 2012 when they were first introduced on the market. Luna Sensation (BAYER Crop Science, Research Triangle Park, NC) is a premix of the SDHI fluopyram and the strobilurin trifloxystrobin, and Merivon (BASF Corporation, Research Triangle Park, NC) is a premix of the SDHI fluxapyroxad and of the strobilurin pyraclostrobin. They are broad-spectrum fungicides that inhibit spore germination, germ tube elongation, mycelium growth and sporulation, and are effective against many other diseases such as powdery mildew and Botrytis gray mold ("Fluxapyroxad | New Active Ingredient Review," 2012); (Kim & Xiao, 2011).

Cross-Resistance Between SDHI Fungicides

Because of the excessive and not always correct application of the SDHIs, fungi populations are adapting to fungicide treatments by mutations and becoming resistant to them. In some cases, they are acquiring cross-resistance, which happens when an isolate is resistant to more than one fungicide of the same chemical group.

Botrytis cinerea was one of the first organisms for which cross-resistance was described. Isolates of *B. cinerea* collected between 2005-2013 from strawberry fields in Florida were tested for resistance and cross-resistance between fluopyram, fluxapyroxad, penthiopyrad and boscalid. Cross-resistance between boscalid and penthiopyrad and boscalid and fluxapyroxad were confirmed (Achour Amiri, Heath, & Peres, 2014). In the years 2014, 2015 and 2016 resistance to fluopyram, fluxapyroxad and penthiopyrad was investigated in strawberry fields in Spain for *B. cinerea*. The risks of cross-resistance between fungicide treatments and with boscalid was also investigated, and results confirmed cross-resistance between boscalid and penthiopyrad, fluxapyroxad and penthiopyrad, and fluxapyroxad and fluopyram (Fernández-Ortuño et al., 2017). Resistance to fluopyram and fluxapyroxad was later reported in 2017 for *B. cinerea* from commercial apple orchards in Washington. This fungus causes gray mold disease in more than 240 plants, causing excessive economic damages. Fungicide applications are needed to control this disease and the repeated applications have resulted in the selection for resistance (Amiri, Mulvaney, Pandit, & Angelis, 2017).

Alternaria late blight (ALB), caused by the fungus *Alternaria alternata*, is a very important disease affecting the production of pistachios by causing tree defoliation and shell staining. In 2015, 35 orchards in California were surveyed and cross-resistance to the SDHIs was frequently found between fluxapyroxad and penthiopyrad, but there was only a moderate correlation between fluopyram and penthiopyrad and fluxapyroxad and also between boscalid and fluxapyroxad and penthiopyrad (Lichtemberg et al., 2018).

Gummy stem blight is a disease that affects leaves, stems and fruits of cucurbits. This disease is caused by the *Didymella bryoniae,* which has a history of developing resistance to single-site fungicides. Researchers have investigated the cross-resistance between boscalid and penthiopyrad and their sensitivity to fluopyram and it was concluded that there is evidence of cross-resistance between boscalid and penthiopyrad, but not between them and fluopyram (Avenot et al. 2012).

Cross-resistance to SDHIs is also found in one of the most important organisms in Michigan, *Venturia inaequalis*, which causes apple scab in apples. Fluopyram, penthiopyrad and benzovindiflupyr were tested for cross-resistance and correlation was found between fluopyram and penthiopyrad and between fluopyram and benzovindiflupyr (Villani et al. 2016).

After resistance to DMIs and to the first generation of SDHI fungicide (boscalid) have been confirmed in *B. jaapii* populations, it is important to determine the status of *B. jaapii* populations sensitivity to the SDHIs in use for the control of CLS, fluopyram and fluxapyroxad.

Other Fungal Diseases Affecting Cherry Trees

Powdery mildew is also a very important disease affecting tart cherries in Michigan. This disease is caused by an obligate biotrophic fungus called *Podosphaera clandestina* (Wallr.:Fr.) Lev. that causes yellow mottling, brittle leaves and distortion and also makes them susceptible to defoliation during harvest. Fruiting bodies called chasmothecia release ascospores in response to rain or irrigation and then infect cherry leaves or shoots in the spring (Grove, n.d.). The first symptoms of powdery mildew can be seen seven to ten days after the first irrigation on new and expanding leaves, where germination and fungal growth are favored by high humidity levels and warm temperatures. With leaves covered by hyphae, severe infection can also affect the vigor of the trees because of their inability to photosynthesize. Cultural control to prevent powdery mildew can be done by pruning trees to avoid dense canopies, by keeping the grass short to reduce the humidity in the orchard, and by removing fallen leaves from the orchard ground. Control can also be accomplished by using fungicides, especially preventative ones in order to avoid infection of the young leaves and to ensure continued coverage until petal fall and the application of post-harvest fungicides that prevent buildup of overwinter inoculum. Clearly, control is a season-long challenge in preventing powdery mildew, even though cherry leaves increase their levels of resistance to powdery mildew as they mature (Grove, 1991).

American brown rot is the most important disease of stone fruits, and it is also a disease that greatly affects the production of tart cherries in Michigan. It is caused by the ascomycete fungus *Monilinia fructicola* (G.Winter) Honey, and causes blossom blight, twig blight, branch canker, and fruit rot (Cox, Quello, Deford, & Beckerman, 2009). American brown rot is more prevalent in rainy and moderate temperatures, and fruits wounded by environmental conditions, insects, or cultural orchard management are more susceptible to this disease. Epidemic inoculum levels can be reached in only 24 hours, destroying the entire crop overnight (Lizotte & Sundin, n.d.). The first sign of the disease is a small tan-brown circular spot that in just a few days will be bigger and covered by an ash-gray-brown "powder" of spores, infecting the fruits that are in direct contact with them. Rotted fruits usually hold onto the branches, dehydrate, maintain their forms and become black fungal mummies that will not disintegrate. and are the first source of inoculum the next spring. American brown rot can be managed by removing mummies and blighted twigs from the trees and ground in an orchard, thereby reducing the inoculum for the next season. ABR can also be managed by applying the fungicide at an appropriate time and by the post-harvest control, which can be done using fungicides, avoiding injuries to the fruits while picking and moving the fruits, cooling them right after harvest and using clean containers to hold the fruits (Ritchie, 2000).

After understanding how impactful fungal diseases can be to growers, especially CLS, it is important to evaluate if populations of *B. jaapii* are acquiring resistance to the newest SDHI fungicides available, and if there is cross-resistance between these single-site mode of action fungicides.

BIBLIOGRAPHY

BIBLIOGRAPHY

Amiri, A., Heath, S. M., & Peres, N. A. (2014). Resistance to Fluopyram, Fluxapyroxad, and Penthiopyrad in Botrytis cinerea from Strawberry. *Plant Disease*, *98*(4), 532–539. https://doi.org/10.1094/PDIS-07-13-0753-RE.

Amiri, A., Mulvaney, K. A., Pandit, L. K., & Angelis, D. R. (2017). First Report of Resistance to Fluxapyroxad and Fluopyram in *Botrytis cinerea* From Commercial Apple Orchards in Washington State. *Plant Disease*, *101*(3), 508–508. https://doi.org/10.1094/PDIS-09-16-1384-PDN.

Andersen, K. L., Sebolt, A., Sundin, G. W., & Iezzoni, A. (2017). Assessment of the Inheritance of Resistance and Tolerance in Cherry (Prunus sp. *Plant Pathology*, *67*(3). https://doi.org/10.1111/ppa.12765.

Arx, J.A.Von. 1961.Uber Cylindrosporium padi. Phytopathologische Zeitschrift. 42 (2): 161-166.

Avenot, H. F., Thomas, A., Gitaitis, R. D., Langston, D. B., & Stevenson, K. L. (2012). Molecular characterization of boscalid- and penthiopyrad-resistant isolates of Didymella bryoniae and assessment of their sensitivity to fluopyram. *Pest Management Science*. https://doi.org/10.1002/ps.2311.

Boriss, H., Brunke, H., & Kreith, M. (2006). *Commodity Profile: Cherries, Sweet and Tart*.http://www.ers.usda.gov/data/foodconsumption/FoodAvailQueriable.aspx

Cherry Festival impact is $26.7 million | Business | record-eagle.com. (n.d.). Retrieved April 28, 2020, from https://www.record-eagle.com/news/business/cherry-festival-impact-is-26-7-million/article_8727cc4a-88b4-5a17-9e0d-7688f4ec2ceb.html

Cox, K. D., Quello, K., Deford, R. J., & Beckerman, J. L. (2009). A Rapid Method to Quantify Fungicide Sensitivity in the Brown Rot Pathogen *Monilinia fructicola*. *Plant Disease*, *93*(4), 328–331. https://doi.org/10.1094/PDIS-93-4-0328.

Dept of Agriculture, M. (2012). Fluxapyroxad | New Active Ingredient Review. Retrieved from http://www.mda.state.mn.us/chemicals/pesticides/regs/~/media/Files/chemicals/reviews/nair-fluxapyroxad.ashx.

Díaz, R., Zas, R., & Fernández-López, J. (2007). Genetic variation of Prunus avium in susceptibility to cherry leaf spot (Blumeriella jaapii) in spatially heterogeneous infected seed orchards. *Annals of Forest Science*. https://doi.org/10.1051/forest.

Dolores Fernández-Ortuño, Alejandro Pérez-García, Chamorro, M., Peña, E. de la, Vicente, A. de, & Torés, J. A. (2017). Resistance to the SDHI Fungicides Boscalid, Fluopyram, Fluxapyroxad, and Penthiopyrad in Botrytis cinerea from Commercial Strawberry Fields in

Spain. *Plant Disease*, *Volume 101*(Number 7), 1306–1313.

Dunckel, M. (2011). Michigan leads the nation in the production of blueberries and tart cherries | MSU Extension. Retrieved February 16, 2018, from http://msue.anr.msu.edu/news/michigan_leads_the_nation_in_the_production_of_blueberries_and_tart_cherrie.

Edson, C. E., Laubach, J. E., Nugent, J. E., & Thornton, G. E. (1998). Integrated sour cherry (Prunus cerasus) production in Northwest lower Michigan. In *Acta Horticulturae* (Vol. 468, pp. 505–513).

Farr, D.F., & Rossman, A.Y. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved February 22, 2018, from /fungaldatabases/

Garcia, S. M., & Jones, A. L. (1993). Influence of Temperature on Apothecial Development and Ascospore Discharge by Blumeriella jaapii. *The American Phytopathological Society*. Retrieved from http://www.apsnet.org/publications/PlantDisease/BackIssues/Documents/1993Articles/PlantDisease77n08_776.PDF.

Green, H., Bengtsson, M., Duval, X., Pedersen, H. L., Hockenhull, J., & Larsen, J. (2006). Influence of urea on the cherry leaf spot pathogen, Blumeriella jaapii, and on microorganisms in decomposing cherry leaves. *Soil Biology and Biochemistry*, *38*(9), 2731–2742. https://doi.org/10.1016/j.soilbio.2006.04.027.

Grove, G. (n.d.). Washington State University Extension.Cherry Powdery Mildew.

Grove, G. (1991). Powdery Mildew of Sweet Cherry: Influence of Temperature and Wetness Duration on Release and Germination of Ascospores of Podosphaera clandestina. *Phytopathology*, *81*(10), 1271–1275.

Gruber, B. R., Davies, L. R. R., & McManus, P. S. (2010). A copper-based fungicide has minimal effects on tart cherry fruit quantity and quality. *HortScience*, *45*(1), 48–53.

History of Cherries. (n.d.). Retrieved February 16, 2018, from https://www.cherryfestival.org/p/about/history/337

History of Tart Cherries - Department of Horticulture. (n.d.). Retrieved February 16, 2018, from https://www.canr.msu.edu/profiles/dr_amy_iezzoni/balaton_cherries/history_of_tart_cherries

Holb, I. J. (2013). Effect of sanitation treatments on leaf litter density and leaf spot incidence in integrated and organic sour cherry orchards. *Plant Disease*, *97*(7), 891–896. https://doi.org/10.1094/PDIS-09-12-0811-RE.

Howatson, G., Bell, P. G., Tallent, J., Middleton, B., McHugh, M. P., & Ellis, J. (2012). Effect of tart cherry juice (Prunus cerasus) on melatonin levels and enhanced sleep quality. *European Journal of Nutrition*, *51*(8), 909–916. https://doi.org/10.1007/s00394-011-0263-7.

Howell, G. S., & Stackhouse, S. S. (1973). The effect of defoliation time on acclimation and dehardening in tart cherry (Prunus cerasus l.). *Amer Soc Hort Sci J*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201303245830.

Jones, Alan L.,Ehret, G. R. , Garcia, S.M., Kesner, C.D., Klein, W. M. (1993). Control of Cherry Leaf Spot and Powdery Mildew on Sour Cherry with Alternate-Side Apllications of Fenarimol, Myclobutanil, and Tebuconazole. *The American Phytopathological Society*, *77*, 703–706.

Jones, A. L., & Sutton, T. B. (1996). Cherry Leaf Spot. In *Diseases of Tree Fruits in the East* (pp. 57–59). East Lansing.

Kim, Y. K., & Xiao, C. L. (2011). *Stability and Fitness of Pyraclostrobin-and Boscalid-Resistant Phenotypes in Field Isolates of Botrytis cinerea from Apple*. *101*(11), 1385. https://doi.org/10.1094/PHYTO-04-11-0123

Lichtemberg, P., Puckett, R., Felts, D., Luo, Y., Doster, L., Rodriguez, D., & Michailides, T. (2018). Survey of the pathogen of Alternaria late blight reveals different levels of carboxamide fungicide resistance on the main pistachio producing regions of California. *California Agriculture*. https://doi.org/0.3733/ca.2018a0022.

Lizotte, E., & Sundin, G. (n.d.). American brown rot management in cherries for 2011 - MSU Extension for thesis.

McGrath, M. T. (2004). What are Fungicides? *The Plant Health Instructor*. https://doi.org/10.1094/phi-i-2004-0825-01. Updated 2016.

McManus, P. S., Proffer, T. J., Berardi, R., Gruber, B. R., Nugent, J. E., Ehret, G. R., … Ma, Z. (2007). Integration of Copper-Based and Reduced-Risk Fungicides for Control of Blumeriella jaapii on Sour Ch. *APS Journal*, *91*(3), 294–300.

Michigan Agriculture Facts & Figures. (n.d.). Retrieved from http://www.michigan.gov/documents/mdard/MI_Ag_Facts__Figures_474011_7.pdf.

Morton, V., & Staub, T. (2008). A Short History of Fungicides. *APSnet Feature Articles*. https://doi.org/10.1094/APSnetFeature-2008-0308.

Outwater, C. A. (2014). DETECTION OF BOSCALID RESISTANCE AND THE H272R MUTATION IN THE SDHB GENE OF BLUMERIELLA JAAPII By Submitted to Michigan State University for the degree of Plant Pathology – Master of Science.

Outwater, C., Proffer, T. J., Rothwell, N., Peng, J., & Sundin, G. W. (2019). Boscalid resistance in Blumeriella jaapii: distribution, effect on field efficacy, and molecular characterization. *Plant Disease*.

Pedersen H., Jensen B., Munk L., Bengtsson M., Trapman M. (2012). Reduction in the use of fungicides in apple and sour cherry production by preventative methods and warning systems. Pesticides Research, 139. Proffer, T. J., Berardi, R., Ma, Z., Nugent, J. E., Ehret, G. R., McManus, P. S., … Sundin, G. W. (2006). Occurrence, Distribution, and Polymerase Chain Reaction-Based Detection of Resistance to Sterol Demethylation Inhibitor Fungicides in Populations of Blumeriella jaapii in Michigan. *Phytopathology*, *96*(7), 709–17. https://doi.org/10.1094/PHYTO-96-0709.

Proffer, T. J., Lizotte, E., Rothwell, N. L., & Sundin, G. W. (2013). Evaluation of dodine, fluopyram and penthiopyrad for the management of leaf spot and powdery mildew of tart cherry, and fungicide sensitivity screening of Michigan populations of Blumeriella jaapii. *Pest Management Science*, *69*(6), 747–754. https://doi.org/10.1002/ps.3434.

Ritchie, D. F. (2000). Brown rot of stone fruits. *The Plant Health Instructor*. https://doi.org/DOI: 10.1094/PHI-I-2000-1025-01.

Seymour, E. M., Singer, A. A. M., Kirakosyan, A., Urcuyo-Llanes, D. E., Kaufman, P. B., & Bolling, S. F. (2008). Altered Hyperlipidemia, Hepatic Steatosis, and Hepatic Peroxisome Proliferator-Activated Receptors in Rats with Intake of Tart Cherry. *Journal of Medicinal Food*, *11*(2), 252–259. https://doi.org/10.1089/jmf.2007.658.

Sjulin, T.M., Jones, A. L., Andersen, R. L. (1989). Expression of partial resistance to cherry leaf spot in cultivars of sweet, sour, duke, and European ground cherry. *Plant Disease*, *73*:56–61.

Stammler, G., Brix, H. D., Nave, B., Gold, R., & Schoefl, U. (2008). Studies on the biological performance of boscalid and its mode of action. *Modern Fungicides and Antifungal Compounds V: 15th International Reinhardsbrunn Symposium, Friedrichroda, Germany, May 6-10, 2007*, 45–51. Retrieved from https://www.cabdirect.org/cabdirect/abstract/20153030375.

Sundin, G. W., & Rothwell, N. (2013). Cherry leaf spot: get an early start on protection before the fungus gets started on infection | MSU. Retrieved March 12, 2016, from http://msue.anr.msu.edu/news/cherry_leaf_spot_get_an_early_start_on_protection_before_the_fungus_gets_st.

Tiernan, C., Imrhan, V., Prasad, C., Vijayagopal, P., & Juma, S. (2016). Tart cherry in amelioration of pain in the elderly. *Nutrition and Aging*, *3*(2–4), 203–217. https://doi.org/10.3233/NUA-150060.

Villani, S. M., Katrin Ayer, & Kerik D. Cox. (2016). Molecular Characterization of the sdhB Gene and Baseline Sensitivity to Penthiopyrad, Fluopyram, and Benzovindiflupyr in

Venturia inaequalis, (August), 1709–1716. https://doi.org/10.1094/PDIS-12-15-1512-RE.

Wharton, P. S., Pathology, P., Iezzoni, A., & Jones, A. L. (2003). Screening Cherry Germ Plasm for Resistance to Leaf Spot. *Plant Disease*, *87*(Table 1), 471–477.

Wilcox, W. F. (1993). Cherry Leaf Spot, 4–5.

W. A. Murrill (1915) Notes, News and Reviews, Mycologia, 7:1, 45-50, DOI: 10.1080/00275514.1915.12021689.

Yılmaz, F. M., Görgüç, A., Karaaslan, M., Vardin, H., Ersus Bilek, S., Uygun, Ö., & Bircan, C. (2019). Sour Cherry By-products: Compositions, Functional Properties and Recovery Potentials – A Review.*Critical Reviews in Food Science and Nutrition*, *59*(22), 3549–3563.https://doi.org/10.1080/10408398.2018.1496901

CHAPTER 2

Assessment of SDHI fungicides sensitivity in Michigan populations of *Blumeriella jaapii*

Introduction

The United States produced over 159 million kilos of tart cherries in 2018, and tart cherry production in Michigan totaled 119 million kilos. Michigan produces almost 75 % of the tart cherries produced in the United States, followed by Utah with 12%, Washington with almost 7%, and New York and Wisconsin with 3% each. Michigan also produced about 20% of sweet cherries, making it the 4th in nation for sweet cherry production (NASS, 2018).

Almost 99% of the tart cherries grown in the U.S. are canned, frozen, dried or juiced. (Quero-Garcia, Iezzoni, Pulawska, & Lang, 2017). This processing industry creates jobs and improves the economy of the state of Michigan. There is also the cherry festival that was created in 1924, in Traverse City, and for almost a century has attracted people from all over the country at the end of June or beginning of July when cherries traditionally ripen in northwest Michigan. With contests, sale of cherries, parades, and tourism, the regional impact of the cherry festival is approximately $26.7 million dollars annually *(Cherry Festival Impact Is $26.7 Million | Business | Record-Eagle.Com*, n.d.).

Among disease that affect cherries, cherry leaf spot (CLS), caused by *Blumeriella jaapii* is the most important disease affecting tart cherry trees in the Great Lakes region of the United States. Tart cherry (*Prunus cerasus* 'Montmorency') is a highly susceptible cultivar that accounts for >95% of tart cherry production in Michigan. The fungus overwinters in infected fallen leaves on the orchard floor, and in the Spring when temperature and humidity are ideal, primary ascospores are ejected to the tree canopy and infect the leaves, causing round purple to brownish spots, about 3 mm or less in diameter, on the upper surface of the leaves, and white to pink masses on the underside of the leaves containing thousands of spores. Infected leaves become yellow and drop prematurely. If defoliation occurs before harvest, it affects fruit quality and yields, making them unmarketable. If defoliation is severe after harvest, it will reduce fruit bud survival, the fruit setting in the next year and it can impact the ability of trees to survive over the winter (C. A. Outwater, 2014).

Control of cherry leaf spot

Cultural control methods for CLS such as pruning for good light penetration and air circulation can help in the disease management but are ineffective in controlling the disease. Growers depend on season long fungicide programs to produce tart cherries and protect the trees. Because mycelium penetrates the leaves through the stomata, fungicide applications must start at petal fall or shortly after leaves are unfolded and continue every seven to 10 days until harvest. Post-harvest fungicide applications are required only if the trees have heavy infection to reduce overwinter inoculum and to keep leaves attached to the trees as long as possible to produce more carbohydrates.

It is necessary to prolong the effectiveness of the fungicides that are successful in the control of CLS. Shifts in the populations towards resistance can result in reduced efficacy and in field resistance. If resistance is monitored, the practical resistance in the fields can be avoided (Proffer et al., 2013). The many fungicide applications required to control CLS lead to selection of fungicide resistant isolates. Increasing the number of resistant individuals in the population may lead to the development of a resistant subpopulation (Proffer et al., 2006).

The most common method of testing for fungicide resistance is through the sampling of isolates in orchards to determine sensitivity profiles for the population of fungi in that orchard and then compare to population patterns in research plots with control data. With this information is possible to better inform the growers of potential failures for compound in use.

<u>History of fungicides on the control of cherry leaf spot</u>

Sulfur and copper were the first fungicides used in the control of CLS; they were multi-site inhibitors and were used as protectants. These broad-spectrum fungicides provided good control of this disease, but they also caused phytotoxicity, especially when applied in high amounts or in hot dry weather (McManus et al., 2007).

In the late1960s site-specific fungicides were introduced to the market and became widely used because of their non-targeting effects and for being less toxic to other organisms. These site-specific fungicides disrupt a single metabolic site or structural site of an enzyme affecting the survival of the fungus (Proffer et al., 2006). The compound long persists in the trees and have systemic activity, which improve the control of disease. However, while single-site chemistries are more efficient in the control of a specific pathogen, they also offer a higher risk for fungicide resistance to occur. Only one mutation in the fungus can make the population resistant to a fungicide that is until then effective (McGraph, 2004).

The demethylation inhibitor fungicides (DMIs) were introduced in 1990 for the control of CLS and were highly effective as protectants and also as curatives. They could be used season long and were also active against powdery mildew and brown rot. Grower reliance on these fungicides, using them intensively and sometimes exclusively, led populations of *B. jaapii* that were resistant to demethylation inhibitors (DMIs) and led to the loss of DMIs for CLS. Resistance to DMIs was reported by 2001 (Proffer et al., 2006) and practical resistance was documented in 2005.

With resistance to DMIs, Pristine (BASF Corporation, Research Triangle Park, NC) was the first carboxamide fungicide used in the control of CLS, a premix of boscalid (SDHI) and pyraclostrobin (strobilurin). Boscalid was launched in 2003 and registered to the control of CLS in 2004 as Pristine. This fungicide interferes with cell respiration and with the production of energy. Boscalid was great in controlling the disease and growers relied on it for about eight years. It was widely used, and its efficacy was remarkable until 2012 when complaints from growers were coming from all over the cherry growing areas in Michigan. Resistance was definitely proven in 2012 when boscalid was tested in the experimental orchard at the Northwest Michigan Horticultural Research Center (NWMHRC) (C. A. Outwater, 2014). Growers stopped using it when practical resistance to boscalid was confirmed by the failure of it in commercial orchards.

After detection of resistance, isolates tested *in vitro* were analyzed, and a mutation that confers resistance to boscalid was identified at the *sdhB* gene. The mutation from a C to a G at nucleotide 816 of *sdhB* gene was identified and correlated to boscalid resistance because this exchange from a histidine to an arginine, at the amino acid position H260R, was detected only in the resistant isolates of *B. jaapii* (C. Outwater, Proffer, Rothwell, Peng, & Sundin, 2019).

A newer class of SDHIs was subsequently launched and fluopyram and fluxapyroxad replaced boscalid on the control of CLS. These fungicides were registered for CLS in 2012 (Herrick, 2012), ("Advanced Xemium Powered Fungicides Receive EPA Registration | BASF Crop Protection Specialty Product," 2012), and are still in use.

Studies indicate that the binding that causes resistance to fluopyram or to fluxapyroxad are different than the binding that causes resistance to other fungicides like the first SDHI generation boscalid. For example, cross-resistance between fluxapyroxad and boscalid has been reported in *Botrytis cinerea* in commercial strawberry fields in Spain. They are associated with the H272Y mutation or with N230I allele in the SdhB subunit (Fernández-Ortuño et al., 2017). Strong cross-resistance patterns between boscalid and fluxapyroxad were also detected in *B. cinerea* in Florida (Achour Amiri, Heath, & Peres, 2014).

Growers rely on SDHIs for the management of CLS, but loss in the efficacy on the research trials was noticed, and the present study aims to evaluate the sensitivity of *B.jaapii* populations to fluopyram and fluxapyroxad and to determine their efficacy for the control of CLS. This study also aims to understand if there is a correlation of resistance between boscalid and fluopyram, boscalid and fluxapyroxad, and between fluopyram and fluxapyroxad, and consequently help growers to maintain production without major losses due to cherry leaf spot.

Materials and methods

Orchard sampling

During the growing seasons of 2016 and 2017, leaves displaying symptoms of CLS were collected from 36 and 42 orchards in Michigan, respectively, to generate mono-conidial isolates. A total of 35 sampled sites from 2016 were commercial tart cherry orchards; an additional sampling site was a research orchard at the Northwest Michigan Horticultural Research Center (NWMHRC), where fungicide efficacy experiments are conducted. In the 2017 survey, leaves were collected from 39 commercial orchards, two research plots at the NWMHRC, and one forest stand in Ohio containing black cherry (*Prunus serotina*). The sampled commercial orchards were located in several Michigan counties including Antrim, Benzie, Kent, Leelanau, Manistee, Mason, Oceana, and Traverse City. Baseline samples were collected from a forest in Columbiana county, Ohio which was never sprayed with fungicides and was located far from commercial cherry orchards.

Isolation Method

Approximately 30 leaves were collected at random from 30 different cherry trees (1 leaf per tree) from each orchard, placed in paper bags, and brought on ice to the laboratory. To obtain mono-conidial isolates, one lesion per leaf was randomly selected, and conidia emerging from the lesion were streaked onto coffee agar medium (CWA; 20% brewed coffee, 2% agar). After 24 hrs, single germinated conidia were transferred and maintained on malt extract agar (MMEA; 2% malt extract, 0.1% yeast extract, 2% agar). Voucher specimens of each isolate were transferred to a MMEA slant, which was maintained at 5°C for long-term storage. A total of 876 and 897 isolates were collected in 2016 and 2017, respectively. In 2016, the collection consisted of 838 isolates from commercial orchards and 38 from the NWMHRC. In 2017, 843 isolates were obtained from commercial orchards, 34 isolates from the NWMHRC, and 20 isolates from the forest in Columbiana, Ohio.

In vitro sensitivity determination of *Blumeriella jaapii* to SDHI fungicides

A minimum inhibitory concentration (MIC) method was used to evaluate the sensitivity of *B. jaapii* to the SDHI fungicides boscalid, fluopyram, and fluxapyroxad. The MIC method is the most appropriate method of evaluation for *B. jaapii* because of the very slow growth rate of the fungus of only a few millimeters of colony expansion over a 15-day time period. The MIC method identifies the lowest concentration of fungicide that completely inhibits fungal growth. Fungicide stock solutions were prepared using the commercial formulation of Endura® (BASF), which contains 70% a.i. boscalid, the commercial formulation of Luna Privilege® (BAYER) that contains 41.5% of fluopyram, and the technical grade of Fluxapyroxad (BASF). They were prepared by dissolving the fungicides in acetone, which were later added to autoclaved, cooled glycerol yeast extract agar media (GLYE), which contains 10 g of glycerol, 10 g of yeast extract, 6 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, and 15 g of agar in 1 liter of water. For fungicide screening, a phenotypic sensitivity rating was assigned based on colony growth at the tested concentrations (0.01, 0.1, 0.5, 0, 2.5, 5, 10, 25, 35 and 40 $\mu g\ ml^{-1}$ of active ingredient). Concentrations greater than 40 $\mu g\ ml^{-1}$ a.i. were not assessed because of issues with precipitation of the studied fungicides in solution. Control medium, not amended with any fungicide, contained an equivalent amount of acetone as fungicide-amended medium. Mycelial plugs (1 mm) from actively-growing cultures on MMEA medium were transferred to the GLYE fungicide-amended media and incubated for 15 days at 23°C after which the plates were examined for colony expansion, and the MIC for each isolate was determined depending on the concentration at which the colonies failed to grow.

Sensitivity of *Blumeriella jaapii* to SDHIs on fungicide-treated trees

Nine *B. jaapii* isolates differing in SDHI fungicide MIC, and from different orchards and different years (Table 2.1), were assessed for their ability to cause CLS symptoms on trees treated with SDHI fungicides used at label rates. Since boscalid, fluopyram, and fluxapyroxad are all components of fungicide premixes, we only used the SDHI component for these tests, at the rate that it would be present in the commercial fungicide premix used at a label rate. Experiments were conducted on 10-year-old 'Montmorency' tart cherry trees maintained in the field at the Michigan State University Plant Pathology farm or on 1 year old potted 'Montmorency' tart cherry trees maintained outside the research greenhouses on the Michigan State University campus. When leaves were fully expanded, trees were sprayed with boscalid, fluopyram, or fluxapyroxad using the commercial formulations of Endura® (BASF) which contains 70% a.i. boscalid, Luna Privilege® (BAYER) that contains 41.5% of fluopyram, and

Sercadis® (BASF) composed of 26.5% of fluxapyroxad, at the same field rates used in commercial orchards, 147.418 g/ha, 182.694 ml/ha, and 343.466 ml/ha respectively.

Table 2.1 MIC values found on the *in vitro* assay for each of the isolates tested.

	Fungicides and isolate MICs		
Isolates	Boscalid	Fluopyram	Fluxapyroxad
15PETB-10	>40	>40	>40
16DMVB-30	40	>40	40
16EBTB-26	40	>40	25
16LAUB-1	5	>40	10
16SWOB-13	2.5	35	2.5
17VSOB-25	5	25	5
17DRSB-3	>40	>40	>40
17HKTB-9	>40	2.5	10
17DRTB-11	40	40	25

All isolates were cultured on MMEA medium at 23°C under ambient light conditions for at least 42 days, to induce conidial production. Suspensions of *B. jaapii* conidia containing 2.5x106 spores ml-1 of water were prepared in the lab immediately prior to inoculation. Masses of spores were placed in sterile 50 ml plastic tubes with plastic beads and dispersed in suspension by vortexing for 30 seconds. The conidial suspensions were then sifted through gauze and maintained on ice until inoculation. Inoculations were done using a hand-held mist sprayer; conidial suspensions were applied to runoff on trees 24 hours after fungicide application. To keep humidity high, inoculated branches were covered with white plastic bags for 12 hours after inoculation. The interior of the bags was sprayed with sterile water before covering. Experiments were conducted from the end of May to October, when temperature and humidity are considered to be optimum for CLS infection.

Statistical analyzes and comparisons between treatments

Statistical analysis of cross-resistance between treatments on the control of CLS was accomplished by the use of Spearman's rank correlation coefficient, using base R package “stats” (R Core Team, 2015) to determine the correlation between fungicides. This evaluation was done using the MICs obtained from the *in vitro* assay and analyzed the relationship between resistance in boscalid and fluopyram, boscalid and fluxapyroxad, and between fluopyram and fluxapyroxad. Isolates with MICs greater than 40 were not included in the analysis because the exact MIC was not determined for those isolates. A total of 739 isolates were tested comparing MICs between fluopyram and boscalid, 1,006 isolates comparing MICs between fluxapyroxad and boscalid and 958 isolates comparing MICs between fluopyram to fluxapyroxad.

The interaction between counties, years and fungicides was analyzed through ANOVA type II test in conjunction with the Tukey’s HSD test comparison within treatments to find means that are significantly different from each other, using $\alpha=0.05$, on R package “agricolae”.

Results

Orchard Sampling

A total of 874 *B. jaapii* isolates from 36 orchards, and 898 isolates from 42 orchards were collected in 2016 and 2017, respectively. In 2016, 836 isolates were collected from commercial orchards and 38 isolates were obtained from the Northwest Michigan Horticultural Research Center (NWHRC). Commercial isolates were compared to those obtained from NWHRC, as isolates from this specific location have been demonstrated to exhibit practical resistance. From the isolates collected on 2017, a total of 844 isolates were collected from commercial orchards, 34 isolates were obtained from the NWHRC. An additional 20 isolates that had never been in contact with fungicides were obtained from orchards in Columbiana County, Ohio.

Commercial orchard isolates were collected from five different Michigan counties over two regions; the West Central region included 25 orchards in Manistee, Mason, and Oceana counties, and the Northwest region included Antrim, Benzie, Grand Traverse, and Leelanau counties, comprising 30 orchards. Some of these orchards were sampled in both years for comparison, and in larger orchards, samples were obtained from multiple locations that reflected different fungicide spraying histories, and hence they were analyzed as separated locations. In total, the West Central region collection consists of 800 isolates (353 from 2016 and 447 from 2017), and the Northwest region collection consists of 952 isolates (521 from 2016 and 431 from 2017).

Sensitivity of *B. jaapii* to SDHIs on fungicide-treated trees

A total of nine isolates with different MIC profiles, determined from *in vitro* assays for boscalid, fluopyram and fluxapyroxad, were chosen from the *B. jaapii* collections of 2015, 2016 and 2017 to be inoculated on cherry trees, and to assess if the differences in MIC corresponded to different levels of fungicide control in *in vivo* inoculations.

Inoculations with isolate 15PETB-10, resulted in a high number of lesions in all treatments, which correlates to the results obtained on the *in vitro* test, where it presented a MIC greater than 40 $\mu g\ ml^{-1}$, for each one of the treatments. Statistical comparison of variance (ANOVA) showed that, for isolate 15PETB-10, there are not significant differences between the treatments and the untreated control (Figure 2.1).

Interestingly, results showed that isolate 16LAUB-1 is significantly less sensitive in the field than *in vitro*: while lesions produced by this isolate in the *in vivo* experiments are not statistically different between fungicides and the untreated control, this isolate exhibited statistically different MICs for each one of the treatments (5 μg ml^{-1} for boscalid, 10 μg ml^{-1} for Fluxapyroxad and greater than 40 μg ml^{-1} for fluopyram (Figure 2.1).

Isolate 16SWOB-13 showed similar results in the in *vivo* and *in vitro* experiments. The lesion number evidenced in the untreated control is significantly higher than the lesions observed on cherry trees treated with fluxapyroxad. Likewise, statistical differences were found between the number of lesions produced of fluxayroxad-treated cherry trees and those treated with fluopyram or boscalid, where no lesions were found. Moreover, these *in vivo* results showed that all treatments are still controlling the disease in the field. *In vitro* experiments demonstrated that the MIC of this isolate for boscalid and fluxapyroxad was 2.5 μg ml^{-1} and 35 μg ml^{-1} for fluopyram (Figure 2.1).

For isolate 16DMVB no significant statistical differences were observed in neither the *in vivo* nor the *in vitro* experiments, between the untreated control and treatments. A high number of lesions produced after inoculations on cherry trees with this isolate (figure 2.1) correlates with the results found *in vitro* and suggest that this isolate as a resistant to all of the treatments. The MIC for boscalid is 40 μg ml^{-1}, for fluopyram is greater than 40 μg ml^{-1} and for fluxapyroxad is 25 μg ml^{-1}. If the whole population of *B. jaapii* in an orchard were at this level, no treatment would be able to control CLS.

Similarly, *in vitro* analyses showed that, for isolate 16EBTB-26, the MIC for boscalid is 40 μg ml^{-1}, for fluopyram is greater than 40 μg ml^{-1} and for fluxapyroxad is 25 μg ml^{-1}, in agreement with the results *in vivo,* which demonstrate that that treatments are not able to control disease in the field, as no statistical significant differences were found between the untreated control and the treatments (figure 2.1).

Lesions caused by isolate 17VSOB-25 on inoculated cherry trees demonstrated a statistical difference between the untreated control and the treatments, but no significant difference in between treatments. According to the *in vitro* tests, the MIC for boscalid and fluxapyroxad was 5 $\mu g\ ml^{-1}$, and for fluopyram was 25 $\mu g\ ml^{-1}$, for this isolate, suggesting that boscalid and fluxapyroxad should be able to control disease in the field, but not fluopyram. Even though the *in vivo* test shows that all treatments were able to decrease the number of lesions when compared to the untreated control, it does not mean that disease was controlled by these treatments because number of lesions was still high.

For isolate 17DRSB-3, *in vitro* and *in vivo* tests show that the three fungicides evaluated cannot control disease, since a high number of lesions and MIC values were observed for this isolate. There are no statistically significant differences between the untreated control and the treatments in this case (figure 2.1).

When tested *in vivo*, the number of lesions caused by isolate 17DRTB-11 did not show significant differences between boscalid and fluopyram and the untreated control, in agreement with very high average number of lesions per leaf observed for these treatments, but they were significantly higher than fluxapyroxad (figure 2.1). When tested *in vitro*, MIC values for this isolate are greater than 40 $\mu g\ ml^{-1}$ for boscalid and fluopyram and 25 $\mu g\ ml^{-1}$ for fluxapyroxad, supporting the results observed in the *in vivo* assays.

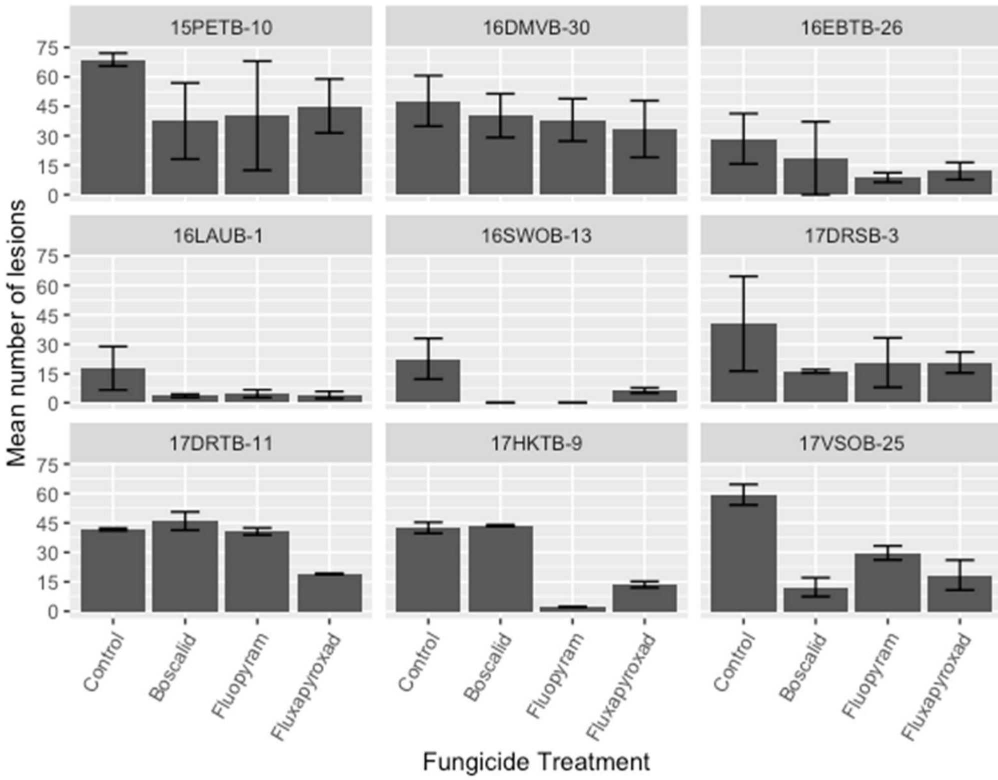

Figure 2.1 Results of *in vivo* experiment testing isolates from different orchards.

Results of *in vivo* assay showing which treatments are still controlling the disease in the field. The number of lesions per leaf were compared between control and fungicide sprayed trees and compared to the MIC found on the *in vitro* test.

In vivo inoculations with isolate 17HKTB-9 showed that the number of lesions produced in the untreated control and boscalid treatments are not significantly different from each other, but they are significantly different from fluxapyroxad, which is also significantly higher than fluopyram. The high average number of lesions per leaf is in concordance with MICs, which are greater than 40 µg ml^{-1} for boscalid, suggesting that this isolate is resistant to this fungicide, 10 µg ml^{-1} for fluxapyroxad, which would classify this isolate as shifting towards resistance and 2.5 µg ml^{-1} for fluopyram, which according to the suggested classification, is still sensitive to this fungicide.

Sensitivity of *B. jaapii* to SDHIs (*In vitro* test)

In order to investigate the distribution of *B. jaapii* isolates with reduced sensitivity to fluopyram and fluxapyroxad, isolates were categorized into three different groups based on the *in vitro* assays. For fluopyram and fluxapyroxad, isolates with MICs smaller or equal than 2.5 μg ml^{-1} are considered sensitive to these fungicides; populations with MICs greater than 2.5 and less than 25 μg ml^{-1} are considered shifting towards reduced sensitivity, and isolates with MICs equal or greater than 25 μg ml^{-1} are classified as resistant to these fungicides. The range of boscalid resistance was determined based on previous studies where isolates with MICs smaller or equal to 5 μg ml^{-1} were considered to be sensitive, 10μg ml^{-1} were considered to have reduced sensitivity, and 25 μg ml^{-1} or greater were considered resistant to boscalid. (Outwater, 2012). Each isolate was then classified into different categorization as their *B. jaapii* populations are sensitive, shifting towards reduced sensitivity (moderate), or reduced in sensitivity to the fungicides boscalid, fluopyram or fluxapyroxad. This resulted in 27 unique categories and the classification for both years is shown in table 2.2.

Isolates collected in 2016 and 2017 that came from commercial orchards presented MICs ranging from 2.5 μg ml^{-1} to greater than 40 μg ml^{-1}. Out of the 876 isolates analyzed for boscalid resistance in 2016, 262 (30%) of them were considered sensitive. A total of 176 isolates, 20% of the total were classified as shifting towards resistance, and the other 438 individuals (50%) were considered resistant to boscalid (figure 2.2).

Isolates from 2017 were compared to baseline isolates from Ohio, which presented MICs ranging from 0.001 to 0.5 μg ml^{-1}. Out of 20 baseline isolates, 16 of them were inhibited by a concentration of 0.01 μg ml^{-1} and the other four were inhibited by concentrations of 0.1 μg ml^{-1}. Out of 869 isolates collected from commercial orchards, 173 isolates, which represent 20% of commercial isolates, were considered as sensitive. A total of 141 isolates, representing 15% of the total number, were classified as shifting towards resistance, and 565 isolates, 65% of the total, were considered resistant to boscalid (Figure 2.2).

Table 2.2 Population distribution of isolate sensitivity groups

S: sensitive, M: moderately resistant, R: resistant. Number and percentage of isolates classified to each of these 27 categories making possible the analyses of the number of isolates that are resistant to each of the fungicides and the number of these isolates that are resistant to more than one of these SDHIs.

Sensitivity/Resistance to Boscalid, Floupyram and Fluxapyroxad				
Boscalid	Fluopyram	Fluxapyroxad	Number of isolates	Percentage of isolates
S	S	S	112	7.0
S	S	M	2	0.1
S	S	R	0	0.0
S	M	S	144	8.9
S	R	S	85	5.3
S	M	R	5	0.3
S	R	R	15	0.9
S	M	M	6	0.4
S	R	M	15	0.9
M	M	M	15	0.9
M	S	S	9	0.6
M	S	M	0	0.0
M	M	S	23	1.4
M	R	R	48	3.0
M	S	R	0	0.0
M	M	R	5	0.3
M	R	S	114	7.1
M	R	M	63	3.9
R	R	R	418	26.0
R	S	S	22	1.4
R	S	M	14	0.9
R	M	M	42	2.6
R	M	S	83	5.2
R	R	S	169	10.5
R	S	R	5	0.3
R	M	R	16	1.0
R	R	M	180	11.2

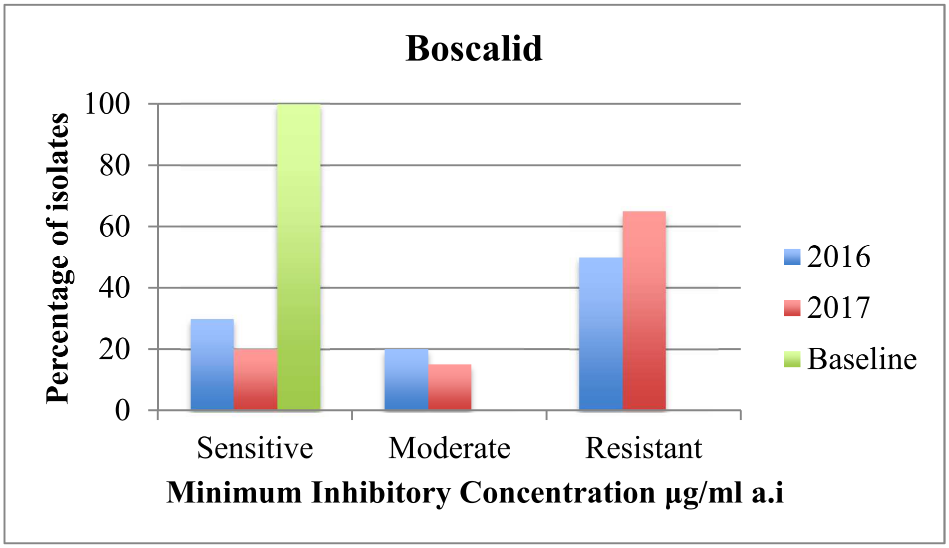

Figure 2.2 Distribution of MIC values determined by *in vitro* assays for boscalid resistance in 2016 and 2017 (n=1,765).

Isolates collected in 2016 and 2017 from commercial orchards and compared to baseline isolates. In green, baseline isolates inhibited at concentrations below 1µg ml^{-1}. In blue the profile of isolates in 2016, and in red the population profile of the 2017 isolates.

A total of 758 and 868 isolates were assayed for fluopyram sensitivity in 2016 and 2017, respectively. Out of the 758 isolates collected in 2016, only 4 isolates were considered sensitive; 82 isolates (10.8%) of isolates were suggested as shifting towards resistance, and 672 (88.7%) of isolates were classified as resistant. None of the isolates from 2017 were inhibited by a concentration of 2.5 µg ml^{-1}. 61 isolates (7% of the total) were considered shifting towards resistance and the other 807 (93%) were considered resistant to fluopyram (Figure 2.3).

A total of 872 and 877 isolates were tested for fluxapyroxad resistance in 2016 and 2017, respectively. Based on *in vitro* MIC doses for Fluxapyroxad in 2016, 23.3% of the isolates tested (203 individuals) was considered to be sensitive, 38.2% (333 isolates) were classified as shifting towards resistance, and 38.5% of the isolates (336 isolates) were considered to be resistant to fluxapyroxad. In 2017, 64 isolates (7.3% of the population) were considered sensitive to fluxapyroxad. The number of isolates shifted towards resistance increased to 29.5% in that year,

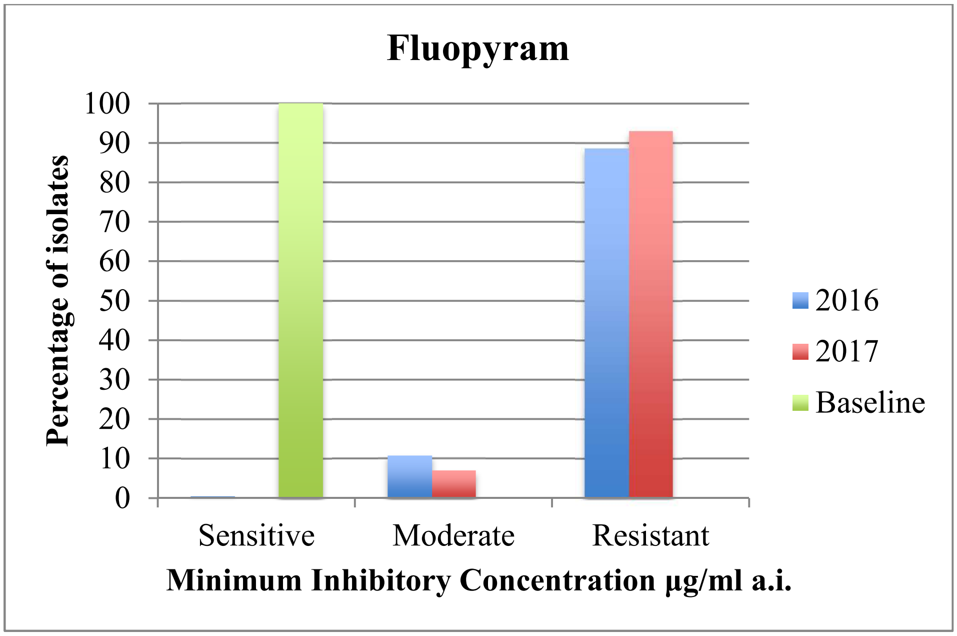

Figure 2.3 Distribution of MIC values determined by *in vitro* assays for fluopyram resistance in 2016 and 2017.

Isolates collected in 2016 and 2017 from commercial orchards and compared to baseline isolates. In green, baseline isolates inhibited at concentrations below 1µg ml^{-1} of fluopyram. In blue the profile of isolates in 2016, and in red the population profile of the 2017 isolates (n=1,626).

and the number of isolates found to be resistant to fluxapyroxad increased to 63.2% of the collection (figure 2.4).

Sensitivity profiles were also analyzed at the orchard level. To consider an orchard as resistant, at least 30% of the isolates analyzed to boscalid, fluopyram and fluxapyroxad resistance need to present MICs of 25 µg ml^{-1} or greater, which was established based on the results from the NWHRC *in vivo* experiments and from field experiments where individual isolates were sprayed on trees treated with these fungicides.

In 2016, a total of 80% of the 36 orchards analyzed were considered resistant to boscalid and the other 20% of total were considered sensitive. In 2017, 90.5% of the 42 orchards analyzed were considered resistant and only 9.5% had populations considered to be sensitive to boscalid.

Out of the 36 orchards screened in 2016 for boscalid resistance analyzes, 21 were located in the Northwest region and 15 orchards were located in the West Central region of the State of Michigan. A total of 4 orchards of the Northwest region were considered sensitive to boscalid and the other 17 orchards were considered resistant. In the West Central region, out of the 15 orchards analyzed, 3 orchards were considered sensitive to boscalid and the other 12 were resistant. In 2017, out of 42 orchards analyzed, 17 belong to the Northwest region and 25 orchards were located in the West Central region. Out of the 17 orchards in the Northwest region, 3 of them were sensitive to boscalid, and 14 were resistant. In the West Central region only one orchard was classified as sensitive to boscalid and the other 24 of the orchards were considered resistant to this fungicide (Figure 2.5).

In 2016, all of the 34 orchards analyzed to fluopyram were considered to be resistant to this fungicide on the *in vitro* assay. In 2017, the profile of 40 commercial orchards was analyzed and 97.5% of the orchards were considered to be resistant to fluopyram and only 2.5% of the orchards were sensitive.

Out of the 34 orchards analyzed in 2016 for fluopyram, 22 belonged to the Northwest region and the remaining 12 orchards belonged to the West Central region. All of these orchards in both regions were resistant to this fungicide. In 2017, 17 orchards were located in the Northwest region, and the other 23 orchards out of the 40 screened belonged to the West Central region. There was only one orchard considered sensitive, which was located in the West Central region. The other 39 orchards were found to be resistant to fluopyram (Figure 2.6).

In 2016, 35 commercial orchards were analyzed for fluxapyroxad sensitivity and a total of 68% of the orchards presented populations of *B. Jaapii* resistant to this fungicide. The remaining orchards had less than 30% of their isolates with MICs equal or greater than 25 µg ml^{-}

[1]. In 2017, of 40 orchards, (15%) were considered sensitive and 85% of them had more than 30% of their isolates with MICs equal or greater than 25µg ml^{-1}.

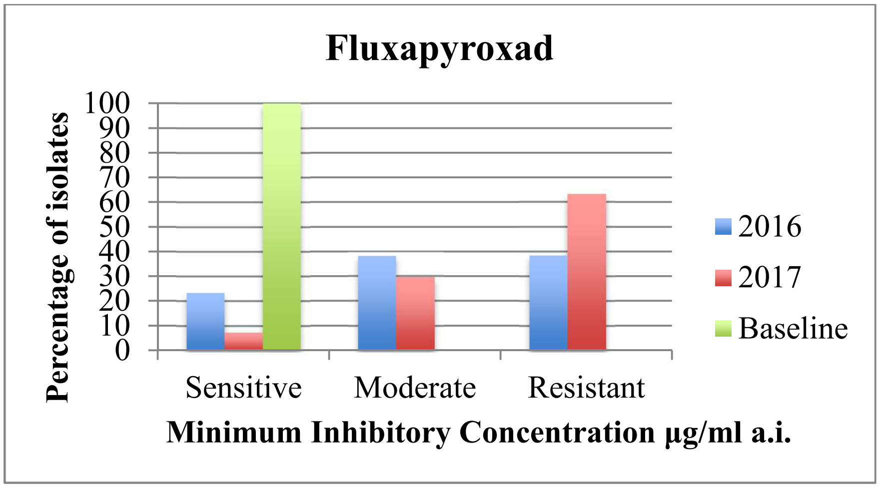

Figure 2.4 Distribution of MIC values determined by *in vitro* assays for fluxapyroxad resistance in 2016 and 2017.

Isolates collected in 2016 and 2017 from commercial orchards and compared to baseline isolates. In green, baseline isolates inhibited at concentrations below 1µg ml^{-1} of fluxapyroxad. In blue the profile of isolates in 2016, and in red the population profile of the 2017 isolates (n=1,749).

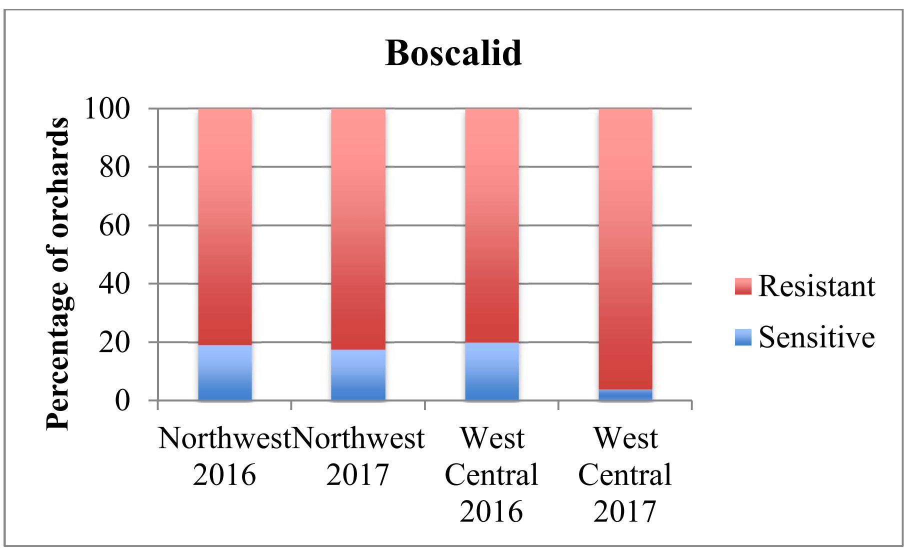

Figure 2.5 Boscalid sensitivity profile of commercial orchards by region and year

Orchards classified as resistant had average Minimum Inhibitory Concentrations of 25 µg ml^{-1} or greater. The percentage of orchards considered to have a *B. jaapii* population resistant to boscalid is indicated in red and the percentage of orchards considered to have a *B. jaapii* population sensitive to boscalid is indicated in blue.

In 2016, 14 orchards located in the Northwest region were found to be resistant to fluxapyroxad and 7 were considered sensitive to this fungicide. In the West Central region, 10 orchards were classified as resistant while 4 remained sensitive. In 2017, out of 40 orchards were screened, 17 orchards were located in the Northwest region and 23 orchards in the West Central region. In the Northwest region, 14 orchards were found to resistant to Fluxapyroxad and the remaining 3 were considered sensitive. In the West Central region, 20 orchards were considered resistant, and 3 orchards were found to be sensitive to fluxapyroxad (Figure 2.7).

Fluopyram is the only fungicide that maintained MIC average in the years of this study, with the average of 28.9 µg ml-1 in 2016 and 27.4 µg ml-1 in 2017. The MICs average for fluxapyroxad isolates increased from 12 µg ml-1 in 2016 to 19 µg ml-1 in 2017. Boscalid also showed a significant increase in the MIC average, which was 11.6 µg ml-1 in 2016 and 16.5 µg ml-1 in 2017 (Figure 9). Overall results showing the percentage of orchards resistant to boscalid, fluopyram and fluxapyroxad in the years of 2016 and 2017 by county are displayed on figure 8.

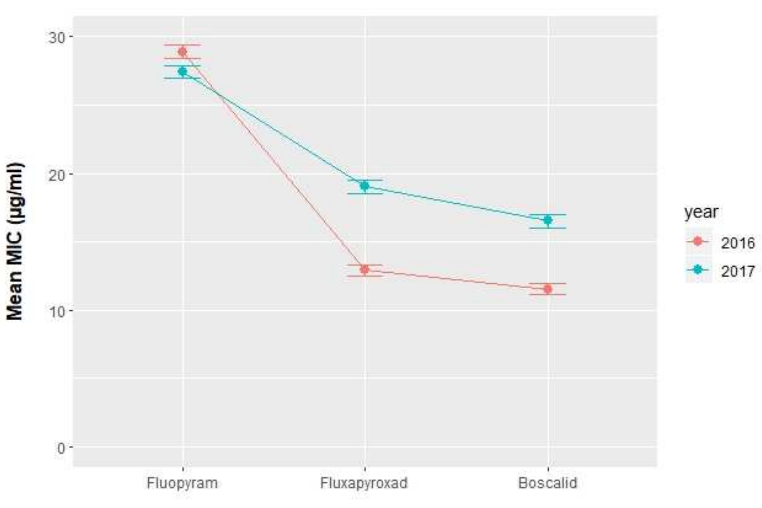

Figure 2.9 Average of MICs in 2016 and 2017 for boscalid, fluopyram and fluxapyroxad

Fluopyram is the only fungicide that maintained MIC average in the years of this study. The average MIC in the year of 2017 was significantly higher than the average MIC in 2016 for boscalid and for fluxapyroxad.

Sensistivity of *B. jaapii* to SDHIs in Research Plots

Results from research plots conducted at the NWMHRC where boscalid, fluopyram and fluxapyroxad were tested in the years of 2012, 2016 and 2017 show that by August 1, 2012, 47.8% of leaves presented CLS lesions and 30.3% of trees canopies were defoliated when treated with Pristine. This fungicide was tested again in 2018, and the results of the data showed even higher percentage of infection (87%) and of defoliation (46%) when compared to the 2012 data.

By August 1, 2012, trees treated with fluopyram and fluxapyroxad had a defoliation percentage of 9.4% and 3.9%, respectively. The number of infections recorded was 22.1% for fluopyram and 22.4% for fluxapyroxad. By August 2, 2017, a total of 85.1% of leaves presented CLS lesions and 40.9% of trees were defoliated when treated with Sercadis. When treated with

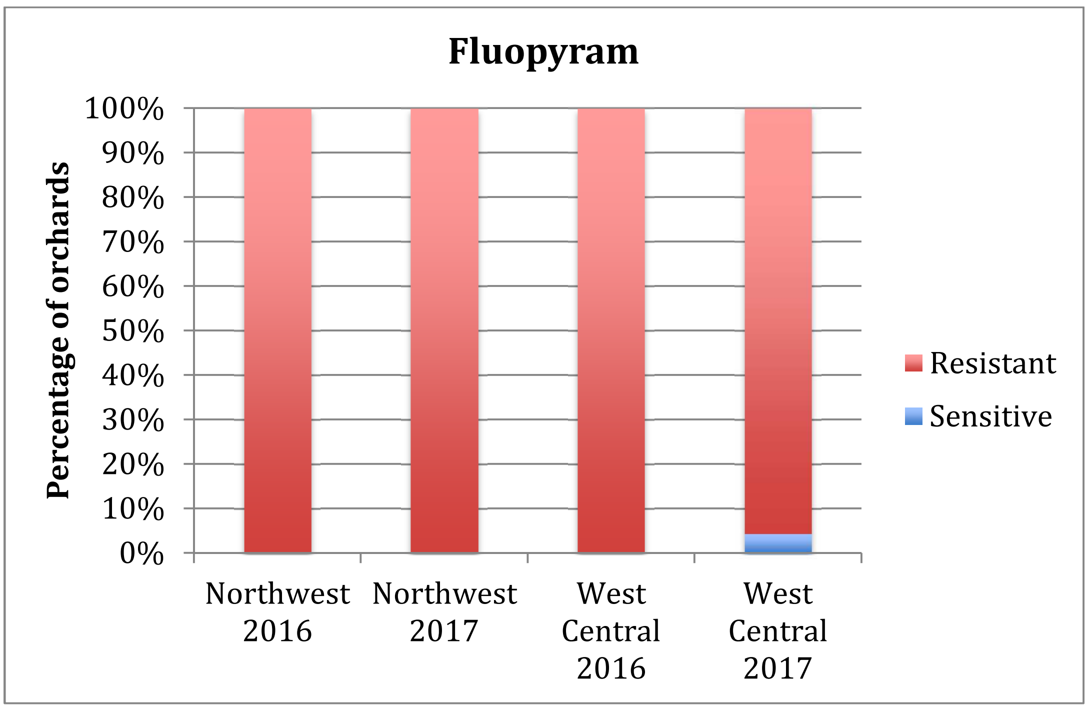

Figure 2.6 Fluopyram sensitivity profile of commercial orchards by region and year

Orchards classified as resistant had average Minimum Inhibitory Concentrations of 25 $\mu g\ ml^{-1}$ or greater. The percentage of orchards considered to have a *B. jaapii* population resistant to

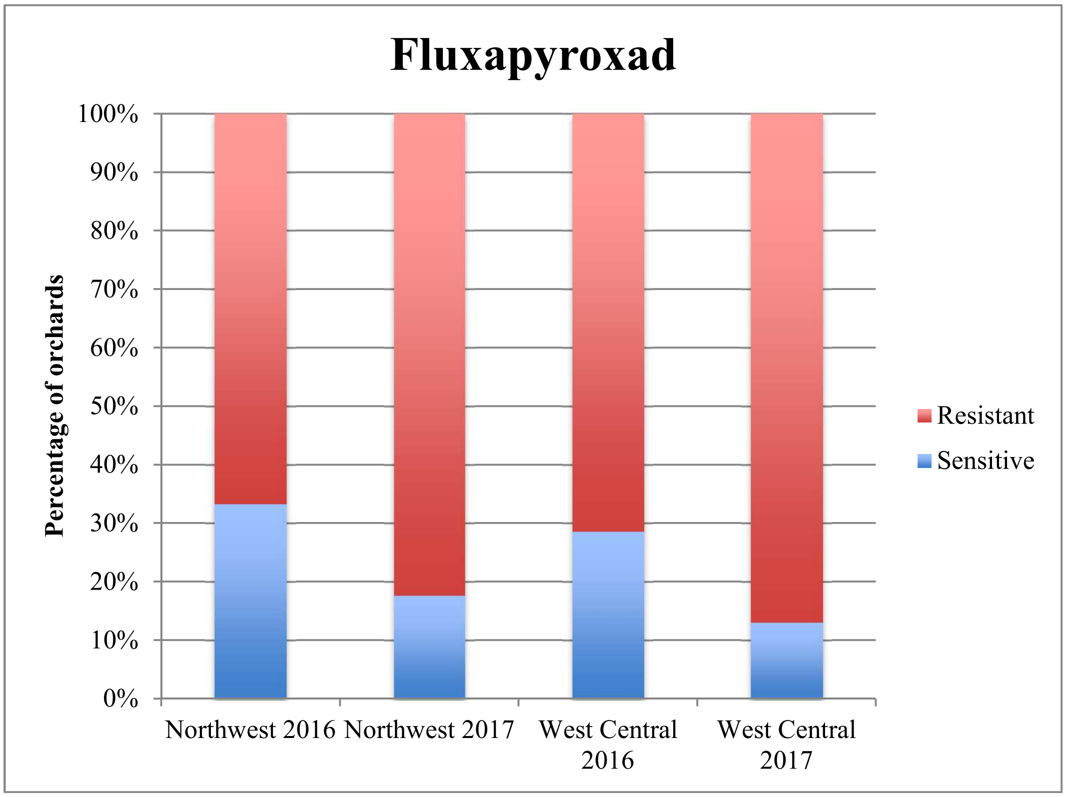

Figure 2.7 Fluxapyroxad sensitivity profile of commercial orchards by region and year
fluopyram is indicated in red and the percentage of orchards considered to have a *B. jaapii* population sensitive to fluopyram is indicated in blue.

Merivon, 41.3% of leaves were infected, but the percentage of defoliation was of only 5% by the same date. Sercadis and Merivon were tested again in 2018, and the results of the data showed even higher numbers of lesions by August 30. When trees were treated with Sercadis, the percentage of infection was compared to the previous year, but the percentage of defoliation increased to 56%. When treated with Merivon, both numbers increased. The percentage of infection was 56.1% and the percentage of defoliation was 26.7%.

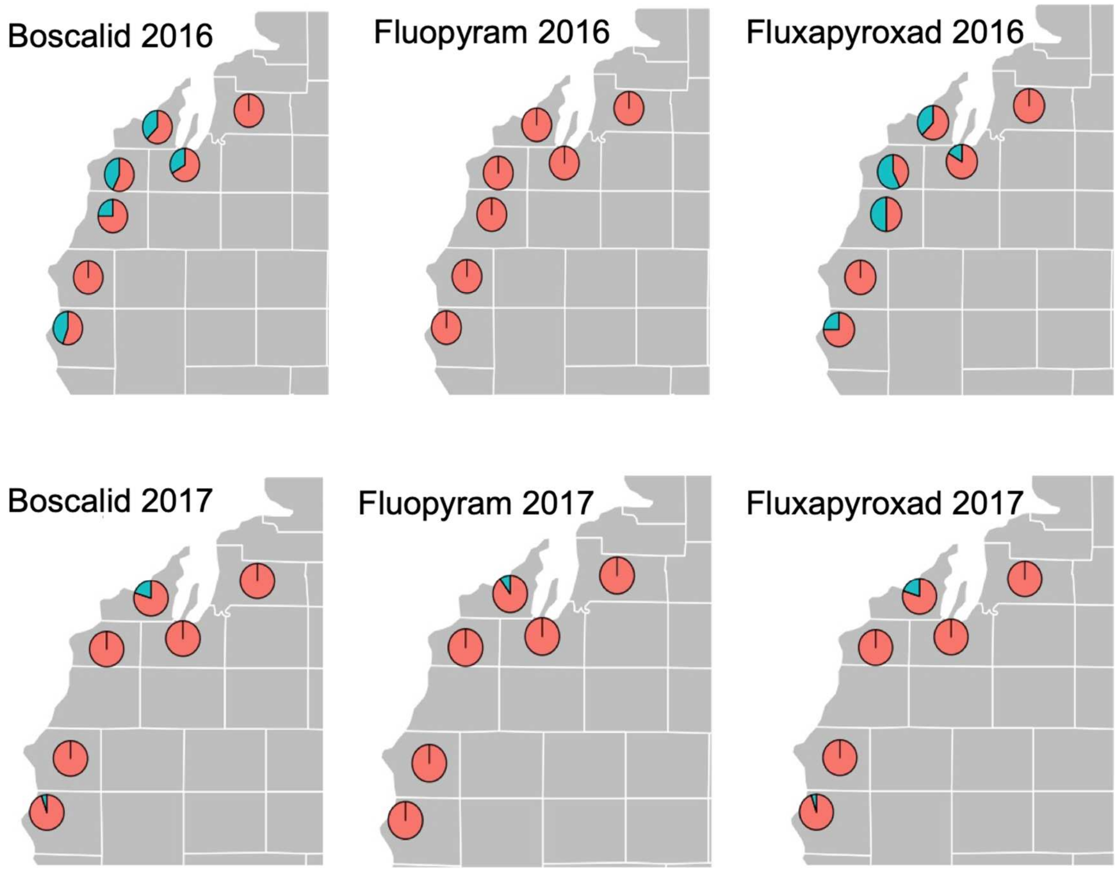

Figure 2.8 Profile of commercial orchards by county

Red shows the number of orchards by county that have at least 30% of their isolates with MICs at or greater than 40 µg ml^{-1}, and blue shows the orchards that are considered to have populations of *B. jaapii* still sensitive to the respective fungicides.

In 2017, three different plots were treated with three different programs containing Luna Sensation at 5 fl oz. Trees that were sprayed with the minimum rate required of 5 fl oz/A, Luna Sensation (fluopyram and tryflosxytrobin), presented defoliation percentages of 5.8%, 18% and 12.2%, and the percentage of infected leaves were of 39.4%, 62.9% and 63.3% by August 2, 2017. In 2018, the percentage of infection was of 84.4% by that time in the season and the percentage of defoliation was of 45.6%. At rates of 6 fl oz/A, Luna Sensation showed 20.9% and 25.4%percentage of infection and 5.4% and 19.8% of defoliation by August 30, 2018.

Statistical analyzes and comparisons between treatments

SDHI cross-resistance was tested for the studied population with the exception of isolates with MICs greater than 40. Correlation between fluopyram and boscalid showed a positive relationship (r=0.503, P= 2.20E-16). Resistance to fluxapyroxad showed a positive relationship to boscalid resistance (r=0.612, P= 2.20E-16) and resistance to fluopyram was positively correlated to fluxapyroxad resistance (r=0.597, P= 2.20E-16).

The year of 2017 showed significantly higher MIC than 2016, with means of 20.7 and 16.9 respectively (Figure 2.9). When analyzed, significant differences were found between counties and years, counties and fungicides and between fungicides and years (table 2.3).

Table 2.3 Analysis of significance difference between variables.

Analyses of data done using Anova type II test. Significant differences were found between counties and years, counties and fungicides and between fungicides and years.

Anova Type II Test			
Response: MIC	**Df**	**F value**	**Pr(>F)**
County	8	44.8477	<2.2e-16
Year	1	33.7567	6.79E-09
Fungicide	2	535.3665	<2.2e-16
Year:fungicide	2	25.3889	1.12E-11
County:year	4	6.3421	4.41E-05
County:fungicide	16	3.3794	5.65E-06

In the pool of isolates that were considered resistant to boscalid, only 19% of them were considered sensitive to fluopyram, and 54% to fluxapyroxad. When isolates were considered resistant to fluopyram, 31% of them were considered sensitive to boscalid and 57% to fluxapyroxad. When isolates were considered resistant to fluxapyroxad, 14% and 7% were considered sensitive to boscalid and fluopyram, respectively.

Discussion

Sensitivity of *Blumeriella jaapii* to SDHIs on fungicide-treated trees

Overall, the results of this study show that resistance to fluopyram and fluxapyroxad are present in populations of *B. jaapii* throughout the major tart cherry production regions in Michigan. These results are especially significant because both of these fungicides have been registered since only 2012. Although these results suggest that fluopyram and fluxapyroxad are not able to fully control CLS in most of the commercial orchards in Michigan, they are at present the only available options for single-site fungicides, by themselves or in mixes, for the control of this disease. As of the publication of this thesis, there is no indication that any new chemicals are being tested as substitutes for either fluopyram or fluxapyroxad. This differs from previous years when populations of *B. jaapii* have shown resistance to other classes of fungicides. For example, in the late 1990s when resistance to DMIs evolved, boscalid was launched and replaced the use of DMIs. Then later, around 2012, when *B. jaapii* populations evolved resistance to boscalid, fluopyram and fluxapyroxad were ready to be introduced into the market.

Then, even though fluopyram and fluxapyroxad are the best options for the control of CLS, caution should be used when applying either of these fungicides because coverage and the manner in which they are applied to the orchards really matters. Some methods of spraying interfere in shifting of populations towards resistance by the end of the season, such as the alternate row-middle spraying. Using this method, the outside rows and the row ends are fully covered, and the middle sections of alternative rows are sprayed with the fungicides, still providing acceptable coverage of the whole canopy. This process is frequently used because it saves money and because growers believe it works. However, this practice can increase the chances of poor coverage and the reduction on the dosages of the fungicides. This can play a role in the potential resistance to the fungicide and consequently cause fungicide failure. Wind, high temperatures, low humidity, fast speeding and poor pruning practices are some of the factors that can affect the coverage of the whole canopy when this method of spraying is used. In order to avoid affecting the efficacy of the fungicide when this method of spraying is used, the fungicide coverage should be confirmed and the environmental conditions should be verified as appropriate before the fungicide is applied.

Another problem with the resistance of *B. jaapii* populations is that SDHIs are not only used for the treatment of CLS but also for the treatment of other fungal diseases that occur in the field at the same time. When application is done to control another disease, additional selective pressure is imposed upon populations of *B. jaapii*.

The results obtained from trees inoculated with *B. jaapii* isolates show that there is a strong relationship with the MICs found *in vitro*. It is possible to say that isolates like the 15PETB-10, for which MICs for boscalid, fluopyram and fluxapyroxad are very high, perform poorly when tested *in vivo*. When 15PETB-10 was tested, the number of lesions on fungicide-treated trees was very similar to the number of lesions on the control trees, which proves that there is a relationship between the high MICs and the infection.

The isolate 17VSOB-25, which is considered sensitive to boscalid and fluxapyroxad and resistant to fluopyram, showed significant differences between treatments and control. The average number of lesions on boscalid-treated trees was reported as at least 80% less than the number of lesions on the control trees. As for fluxapyroxad, for which MIC was 5 $\mu g\ ml^{-1}$, the number of lesions reported was higher than the numbers reported for boscalid, but still 69% less than the number of lesions reported on the control trees. The average number of lesions on fluopyram-treated trees, for which MIC was 25 $\mu g\ ml^{-1}$, showed a reduction of 50%. Even though the number of lesions was still very high, and fungicide would not be able to provide control of the disease, fluopyram treatment offered a reduction of 50% *in vivo*, probably because of all the environmental variations offered in the orchards.

Isolate 17HKTB-9, for which MIC for boscalid was >40 μg ml^{-1}, demonstrated no reduction in the number of lesions when inoculated on trees and compared to the control trees. When inoculated on fluopyram-treated trees, for which the MIC was 2.5 μg ml^{-1}, the number of lesions was drastically reduced, demonstrating that fungicide is able to control disease in the field. When compared to control trees, the fluxapyroxad-treated trees showed a significant reduction in the number of lesions. However, fluxapyroxad is still considered to have reduced sensitivity, just like on the *in vitro* results when the MIC obtained was 10 μg ml^{-1}.

The MIC numbers obtained for the isolates of *B. jaapii* from 2016 and 2017 and how *B. jaapii* populations are distributed towards resistance are able to tell how fungicides are performing in the orchards and can help growers more effectively manage CLS. According to the *in vitro* results, fluopyram is the fungicide with the greatest MIC average number, probably because it is the fungicide that was used as a substitute for boscalid and because it has been used by growers longer than fluxapyroxad. The MIC average number for boscalid also increased, and because this fungicide is not being used anymore, it is possible that this increase is because of cross-resistance with fluopyram and/or fluxapyroxad.

After MICs were determined on an *in vitro* assay for each of the isolates, they were classified as sensitive, reduced sensitive or resistant to either of the fungicides. Frequency distributions of MICs were then established, and the MIC populations could determine if *B. jaapii* population from a determined location was sensitive, shifting towards resistance, or resistant to a specific fungicide. Table 2.2, where numbers from 2016 and 2017 are combined, shows that only 7% of the isolates were considered sensitive to all the chemistries, while 26% were considered resistant to all of them. These numbers show that resistance is progressively taking place in the tart cherry orchards of Michigan and that CLF is not fully controlled by SDHIs anymore. The isolates collected were harvested in the middle of the season from commercial orchards, which use whatever they can to avoid CLS, and SDHI is certainly one of the treatments in their program. If there were infected leaves, either those isolates survived the application of fungicides (resistant individuals) or the fungicide coverage was so poor that those leaves were not targeted.

As Table 2.2 shows, most of the isolates that were sensitive to all three fungicides were the isolates obtained from Columbus County, Ohio, that were never exposed to SDHIs. The isolates were tested, and the MICs show that this population is sensitive to these fungicides, which means that *B. jaapii* populations are not reduced in sensitivity or resistant to SDHIs before they are introduced to these fungicides. Because selection for resistance has not been done through the usage of fungicides, it is very rare that a reduced sensitivity or resistant isolate can be found.

The next higher numbers on Table 2.2 are of isolates considered resistant to boscalid and fluopyram and either reduced in sensitivity (11.2%) or sensitive (10.5%) to fluxapyroxad. These results make sense because resistance to boscalid was found years ago, and practical resistance forced growers to stop using Pristine, the combination of boscalid and pyraclostrobin. Also, growers have started to complain about the efficacy of fluopyram in the orchards. In 2012 fluopyram started being used as a substitute fungicide for boscalid to control CLS. Just as growers had relied on boscalid to effectively control this disease, they now relied on fluopyram. However, the extensive usage of fluopyram for so many years has caused populations to become resistant to it. Fluxapyroxad is the newest fungicide in use, and even though it was registered in 2012, the same year as fluopyram, growers did not really start using it until the following year. Consequently, of the three fungicides compared in Table 2.2, it should be the fungicide with the lowest percentage of isolates. As Table 2.2 shows, zero isolates showing sensitivity to boscalid and fluopyram and resistant to fluxapyroxad confirms this theory.

Only 1.2% of total isolates were confirmed to be resistant to boscalid and sensitive to fluopyram and fluxapyroxad. Fluopyram and fluxapyroxad have been on the market for some years now and are used in 100% of the commercial tart cherry orchards because they are the single-site fungicides available to control CLS. If these isolates were found all in one orchard, then that would prove the orchard had not transitioned to the newest SDHIs and would not be surrounded by other cherry orchards treated with these compounds (even though spores are dispersed by rain and wind, isolates from neighbor orchards could travel through the wind in infected leaves). The mutation of the gene that confers resistance to boscalid would not be, in this hypothetical case, the same as the mutation that confers resistance to fluopyram and/or fluxapyroxad. However, these isolates were found in different orchards, indicating that any application of either fluopyram or fluxapyroxad in the orchard did not achieve full coverage.

About 0.3% of isolates were resistant to boscalid and fluxapyroxad and sensitive to fluopyram. If these isolates were all from the same orchard, then it would be possible to conclude that fluopyram was not used in that location, but this is not the case. These few isolates were dispersed in different orchards, indicating that fluopyram spraying did not target all the leaves. Another hypothesis would be that these isolates have probably never been exposed to fluopyram or to fluxapyroxad and that resistance to fluxapyroxad was acquired by cross-resistance with boscalid.

Analyzing the number of isolates that are resistant to each of the fungicides and the number of these isolates that are resistant to more than one of these SDHIs, it is right to say that even though molecular work is still in progress to analyze the possible mutations that confer resistance to to the SDHIs, one or more mutations are for sure taking place in these populations. There is a very high chance of cross-resistance between these fungicides, especially when they become resistance to fluxapyroxad. The percentage of isolates that are resistant to fluxapyroxad at the same time they are resistant to boscalid and fluopyram is so high that tells that when they become resistant to fluxapyroxad they also become resistant to fluopyram and to boscalid.

Analysis of data has also been done by counties that show there is a significant interaction between counties and fungicides. This relationship could be due to the difference in the resistance levels between counties and between counties and years, which in turn could be due to the different orchards analyzed in 2016 and 2017 (Table 2.3).

It is difficult to determine, even with the results just mentioned, whether there is more or less resistance to SDHIs in a given county because the number of surveyed orchards varied significantly from county to county. However, it is possible to say that resistance to SDHIs has increased by year. Figure 2.8 shows that there is a significant difference between the number of orchards with resistance to SDHIs from 2016 to 2017. Red shows the number of orchards that have at least 30% of their isolates with MICs at or greater than 40 µg ml^{-1}, and blue shows the orchards that are considered to have populations of *B. jaapii* still sensitive to the respective fungicides.

Because analysis by county was not appropriate because did not provide a fair comparison, analysis by regions was done. The percentage of orchards considered resistant to boscalid greatly increased from 2016 to 2017 in the West Central region (Figure 2.5), and the same increase occurred to fluxapyroxad (Figure 2.7) in both regions analyzed (Northwest and West Central regions).   The number of orchards considered resistant to fluopyram showed a minimum difference between these years in the West Central region (Figure 2.6)

Then, to better understand the *in vitro* results, access to the fungicide application program of some orchards in both regions was obtained from growers and observations were made with the objective of interpreting the data. Some hypotheses were made and the most important conclusion is that a simple mistake like skipping an application, postponing application dates, not being careful about dosages, not knowing the route of the last application when doing the inside/outside method, or any other simple mistake can lead to an epidemic and result in great losses.

In analyzing the history of the application of fungicides to the BMTB Orchard, it is clear that fungicides were applied using the inside/outside method. Application of Merivon (pyraclostrobin and fluxapyroxad) has been the most efficient fungicide to control CLS in the BMTB Orchard because fungicide application at this orchard is done using the right rates of Merivon (4-6.7 fl oz/A). When tested in vitro for fluxapyroxad, most of the isolates for that orchard have MICs at 5 or 10 µg/ml, which means that the population of B. jaapii is still sensitive to this fungicide in this orchard. It also means that coverage was not done properly because otherwise it would not be possible to see infection throughout that orchard. Sensitivity to the fungicide is the risk that comes with the inside/outside method of applying the fungicides. The desired dosage of Luna Sensation (fluopyram and trifloxystrobin) to be applied on field rates is 5-7.6 fl oz/A, but the applied rate in the BMTB Orchard was only 2.5 fl oz./A. This mistake in the application of the fungicide can explain the differences seen in the MICs when isolates were tested for fluopyram sensitivity. MICs for fluopyram are high and show reduced sensitivity acquired by the population of B. jaapii to this fungicide by the selection of resistant isolates. Lower rates than what is prescribed can also be noted for the strobilurin Gem, and for the multisite fungicide Captan. This can be a problem and might be the cause of populations becoming resistant. Instead of fighting the pathogen, using lower dosages than required increases the risk for resistance of *B. jaapii* to the fungicide.

At VKOB Orchards, management of diseases, including CLS, has been done with good fungicide rotation, which includes the use of fluxapyroxad. Low MIC numbers have been found at this orchard when isolates were tested for this fungicide. The same thing did not happen when isolates were tested for fluopyram, which is not included in the fungicide rotation. *In vitro* results show high MICs, confirming resistance of *B. jaapii* to fluopyram and indicating that this fungicide was probably extensively used in the previous years.

At DNFB Orchards fungicides are also rotated. A total of four applications with Luna Sensation instead of the ideal two applications were done throughout the season using the inside/outside method. When using the inside/outside method, the number of inside applications should be the same as the number of outside applications. However, in this case, three of the applications were done inside and only one was done outside, which probably explains the mixed low and high MIC numbers for fluopyram at this orchard. The high MIC numbers are probably from the inside rows, where the selection of resistant isolates is probably happening. The low MIC numbers are probably from the outside rows where fluopyram was only applied once, indicating that there was not proper coverage with this fungicide.

Reports claim the use of SDHIs in the GFOB Orchard in the years of 2015, 2016 and 2017. The MICs at this orchard were really high when tested for fluopyram sensitivity, showing a reduced sensitivity to this fungicide. When tested for fluxapyroxad, the MICs showed a slight shift towards reduced sensitivity, even though Merivon had been applied in the right rates, with full coverage and in a rotation program. This shift towards resistance of the population could be the result of a cross-resistance between boscalid and fluxapyroxad or fluopyram and fluxapyroxad. It is still unknown if there are more mutations than the one found on the isolates resistant to boscalid, which proved not to confer resistance to fluopyram or fluxapyroxad, but it is likely to exist because *in vitro* results obtained from this study show so many isolates that are resistant to the three fungicides. CLS is still being controlled in the GFOB Orchard.

Data from field experiments with the purpose of analyzing infection and defoliation percentages has also been collected yearly on the NWMHRC with the purpose of testing the efficacy of the newest SDHI fungicides available for the control of CLS. Data from 2012, 2017 and 2018 can be compared to show the impressive effect of the vast use of the new SDHIs. Using the data from 2012 it is possible to conclude that CLS was not controlled by boscalid anymore. When Pristine was tested again in 2018, the results of the data showed even higher percentage numbers of infection and defoliation by the beginning of August when compared to 2012 data, proving that this fungicide is still not effective against CLS.

In 2012, fluopyram and fluxapyroxad were also tested and had a great impact on the control of CLS by decreasing the number of lesions and decelerating the defoliation process. Numbers of infection and defoliation were low, and trees were able to keep their leaves until necessary in September, assuring production of enough nutrients to enable the trees' survival through winter.

It is possible to conclude that the number of lesions and the percentage of defoliation were very high when compared to control trees and to results from 2012. One important observation about the 2017 data is the use of Merivon (pyraclostrobin and fluxapyroxad) and

Sercadis (fluxapyroxad). Both of these fungicides contain fluxapyroxad, but the number of lesions and the percentage of defoliation were a lot lower on trees treated with Merivon. This result is probably because Merivon also contains pyraclostrobin, which is most likely the effective component in this case. In 2018, the results of Sercadis and Merivon still differed, but it is possible to conclude that Merivon is also losing its efficacy.

In 2017, three different plots were treated with three different programs containing Luna Sensation at 5 fl oz. Trees that were sprayed with the minimum rate required of 5 fl oz/A, Luna Sensation (fluopyram and tryflosxytrobin), presented an acceptable defoliation percentage, but the percentage of infected leaves was really high by August 2, 2017. In 2018, that profile changed with even higher percentage of infection by that time in the season and triple the percentage of defoliation. At rates of 6 fl oz/A, Luna Sensation showed an acceptable percentage of infection and defoliation by August 30, 2018, meaning that dosages of this fungicide would have to be increased by growers.

CLS can be prevented with sprays before it becomes problematic. Once the disease is established, however, it is impossible to eradicate the disease in the orchard. To get their best return on time and investments, growers have to make sure the application of fungicides is done consistently. The FRAC has recommended the use of SDHI fungicides in mixtures with broad spectrum fungicides that are active against same pathogen populations, alternation of the mode of action in the fungicide spray program, a limit on the number of applications per season and preventative usage to avoid or delay resistance to this class of fungicides.

The next stage of this research is to determine whether cross-resistance is happening between boscalid and fluopyram, boscalid and fluxapyroxad, and between fluopyram and fluxapyroxad. Only the mutation that confers resistance to boscalid is currently known

(Outwater, Proffer, Rothwell, Peng, & Sundin, 2019), but the number of isolates resistant to the three fungicides is high and keeps growing, according to the data in this *in vitro* study, indicating there are other mutations.

BIBLIOGRAPHY

BIBLIOGRAPHY

Amiri, A., Heath, S. M., & Peres, N. A. (2014). Resistance to Fluopyram, Fluxapyroxad, and Penthiopyrad in Botrytis cinerea from Strawberry. *Plant Disease*, *98*(4), 532–539. https://doi.org/10.1094/PDIS-07-13-0753-RE.

Cherry Festival impact is $26.7 million | Business | record-eagle.com. (n.d.). Retrieved April 28, 2020, from https://www.record-eagle.com/news/business/cherry-festival-impact-is-26-7-million/article_8727cc4a-88b4-5a17-9e0d-7688f4ec2ceb.html

Dolores Fernández-Ortuño, Alejandro Pérez-García, Chamorro, M., Peña, E. de la, Vicente, A. de, & Torés, J. A. (2017). Resistance to the SDHI Fungicides Boscalid, Fluopyram, Fluxapyroxad, and Penthiopyrad in Botrytis cinerea from Commercial Strawberry Fields in Spain. *Plant Disease*, *Volume 101*(Number 7), 1306–1313.

Outwater, C. A. (2014). DETECTION OF BOSCALID RESISTANCE AND THE H272R MUTATION IN THE SDHB GENE OF BLUMERIELLA JAAPII By Submitted to Michigan State University for the degree of Plant Pathology – Master of Science.
Outwater, C., Proffer, T. J., Rothwell, N., Peng, J., & Sundin, G. W. (2019). Boscalid resistance in Blumeriella jaapii: distribution, effect on field efficacy, and molecular characterization. *Plant Disease*.

McGrath, M.T. 2004. What are Fungicides. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2004-0825-01. Updated 2016.

McManus, P. S., Proffer, T. J., Berardi, R., Gruber, B. R., Nugent, J. E., Ehret, G. R., … Ma, Z. (2007). Integration of Copper-Based and Reduced-Risk Fungicides for Control of Blumeriella jaapii on Sour Ch. *APS Journal*, *91*(3), 294–300.

Outwater, C. A. (2014). DETECTION OF BOSCALID RESISTANCE AND THE H272R MUTATION IN THE SDHB GENE OF BLUMERIELLA JAAPII By Submitted to Michigan State University for the degree of Plant Pathology – Master of Science.

Outwater, C., Proffer, T. J., Rothwell, N., Peng, J., & Sundin, G. W. (2019). Boscalid resistance in Blumeriella jaapii: distribution, effect on field efficacy, and molecular characterization. *Plant Disease*.

Proffer, T. J., Berardi, R., Ma, Z., Nugent, J. E., Ehret, G. R., McManus, P. S., … Sundin, G. W. (2006). Occurrence, Distribution, and Polymerase Chain Reaction-Based Detection of Resistance to Sterol Demethylation Inhibitor Fungicides in Populations of Blumeriella jaapii in Michigan. *Phytopathology*, *96*(7), 709–17. https://doi.org/10.1094/PHYTO-96-0709.

Quero-Garcia, J., Iezzoni, A., Pulawska, J., & Lang, G. (2017). Cherries: Botany, production and uses (first edition). CABI

CHAPTER 3

Effect of Temperature on the progression of American Brown Rot (*Monilinia fructicola*)

Literature Review

American brown rot (ABR) of *Prunus* species is a serious disease by the *Monilinia spp.* In Michigan, this disease is mainly caused by *Moninilia fructicola* (G. Winter) Honey, but worldwide brown rot can be caused by three species: *Monilinia laxa* (Aderhold and Ruhland), *Monilinia fructigena* (Aderhold and Ruthland), and *Monilinia fructicola*. The different varieties of fruit that can be affected include peaches, plums, nectarines, cherries, apricots, and other fruit species that can be found in the Genus *Prunus*. This pathogen infects fruits before, during, and post-harvest, causing rotting of the fruits and leading to severe yield losses. Fruits are more vulnerable to this disease after harvest because of the processes used to transport and store fruit. This disease also affects flowers and twigs, causing blossom and twig blights and cankers (Ritchie, 2000).

High humidity and warm temperatures combined with rainfall near or at harvest time form the ideal scenario for the development of ABR. Under these ideal conditions, ABR can become epidemic in about 24 hours. This disease can be found in most countries around the world where stone fruit production is popular, and in Michigan it is a big concern for stone fruits growers (Lizotte, et.al, 2011).

American brown rot is characterized by the discoloration of the fruit to a tan color, followed by the rapid decay of the fruit, which can oftentimes lead to the shriveling up and dehydration of the fruit until it looks almost unrecognizable from a healthy fruit crop. These shriveled masses are referred to as "mummies," and they stay attached to the branch of the trees throughout the winter. Any fruit that falls onto the ground will continue to rot while still producing new spores of *M. fructicola* ("PEACHES | MICHIGAN GROWN," n.d.). The fungus can also overwinter in cankers on twigs and branches left untreated from last season. Spores from the mummies or cankers emerge in spring to attack the flowers and twigs on the peach tree. Thousands of spore sacs appear on these mummies waiting for the ideal conditions to be spread and inhabit the peach trees (Elizabeth Bush & Keith Yoder, 2014).

Symptoms of ABR include blossom blight, twig blight, cankers, leaf short-hole, quiescent infection, and rotting of fruits. When infection occurs, small brown spots and gray masses of conidia appear on the surface of the rotten fruit. Infection first happens when new blossoms start to open on fruit trees; this is the first new tissue exposed that *M. fructicola* has a chance to infect. Infected blossoms will wilt, transform colors to tan brown, and cling to the twigs ("Brown-rot of stone fruits | Stone fruit diseases | Fruit and nuts | Plant diseases | Pests, diseases," 2010). This phase also affects twigs and branches when temperatures remain humid for an extended period. The second phase of infection occurs two to three weeks before the fruit harvest begins when the sugar content of the fruit is on the rise. ABR starts out as tan-brown spotting around the fruit and can quickly turn into the ash-like mummies found on the trees. The third phase is when fruits have been harvested and are exposed to the damages of transportation and storage (Holb, 2004).

In Michigan, the most important disease affecting the production of stone fruits, especially tart cherry and peaches (*Prunus persica* (L.) Batsch.). Peaches are produced close to Lake Michigan in the west central to southwest corner of Michigan. Although Michigan is not one of the largest producers of peaches in the U.S., it is famous throughout the country for the quality of the peaches it produces. Certain varieties such as Red Haven peaches, Flamin' Fury, and Stellar peach series are unique to Michigan and are especially known for their flavor ("PEACHES | MICHIGAN GROWN," n.d.).

Peaches are delicate fruits that have a short storage life of only two to four weeks and are very susceptible to infections of *M. fructicola*. They have to be stored at temperatures of 31°-32°F and 90-95% humidity in order to maintain their moisture. Peaches also can be easily damaged, increasing their chances of contamination, so handling, packaging, and transporting them are important parts of peach production ("Post-Harvest Cooling and Storage for Peaches," 2015).

Control methods

ABR on peaches is a very important disease that disseminates quickly and can greatly reduce the quality of the yield, or destroy the entire crop, in a matter of a few days. Although cultural methods such as orchard sanitation, removing mummies before flowers are out, minimizing damages during harvest, and bathing peaches in hot water after harvest are very important for the management of ABR, fungicides are the most effective and the primary option for controlling this disease. Despite the importance of fungicides, no post-harvest fungicides have been created since, and this fact makes the management of the disease difficult, especially because populations of *M. fructicola* have been acquiring resistance to the fungicides growers rely on, such as the DMIs (DeMethylation Inhibitors) (Al-haq, Seo, Oshita, & Kawagoe, 2001)

There are three stages that are especially important when controlling ABR: the first is during blossoming to prevent blossom and twig blights, the second is when fruits are maturing to prevent pre-harvest fruit rot, and the third is when fruits are in storage after being harvested to prevent post-harvest infection and, consequently, fruit rot (Holb, 2004).

In order to control ABR in the first stage, the removal of mummified fruits at the beginning of the season is essential. Removing mummified fruits and burning or burying them help avoid blossom and twig blights and reduces inoculum levels. It is impossible, however, to remove all mummified fruits in a commercial orchard because of the quantity of them and because of labor costs (Alan R. Biggs & Northover, 1985).

To control ABR in the second stage, growers must apply fungicides starting in early Spring. The application of these fungicides need to be done at the right time and in full dosages to preserve their efficacy and avoid the population of *M. fructicola* becoming resistant to them. It is also necessary that growers are aware of the effect of the fungicides on the environment. With the development of site-specific fungicides that are more prone to resistance development by the organisms, growers must also care about having a good fungicide application program in order to avoid creating fungicide resistance (Elizabeth Bush & Keith Yoder, 2014).

Pre-harvest fruit rot can be prevented by the application of as many as 30 fungicides throughout the peach harvest to prevent ABR(Al-haq et al., 2001). The application of calcium, magnesium, and titanium are also proved to help prolonging the storage life of the peaches for another two weeks without affecting the quality of these fruits (Trees & Sciences, 2008)

To control ABR in the third stage growers need to take certain precautions when storing fruits after the harvest. As many other fruits, peaches continue to ripen when exposed to heat and not properly stored. Hydrocooling of peaches is a method used to cool the fruits before storing them by running the peaches under cool water. It is also used to clean the fruits from chemical residues and debris, helping to decrease the number of spores in the yield. The process of hydrocooling needs to be done using chlorinated water that has hypochlorite ions added to it. If the process is done using unchlorinated water, then the method does not reduce brown rot but instead helps to disseminate the disease. Another method of cooling the peaches after harvest to better conserve them during the storage stage is using forced-air cooling. This process uses fans to rapidly move the cold air around the stored fruits ("Post-Harvest Cooling and Storage for Peaches," 2015).

Influence of Temperature and Humidity on Infection of Peaches by *Monilinia fructicola*

The ideal temperatures for the germination of *M. fructicola* spores is around 20-25°C. When compared with tart cherries (*Prunus cerasus*), peaches are less susceptible to infections probably because of the differences on the skin of the two fruits (A. R. Biggs & Northover, 1988). Sporulation and germination of conidia occur between temperatures of 5°C and 30°C, and spore production is greater in temperatures around 20°C (Philips, 1982).

The objective of this assay is to determine if there is a difference on the progression of disease and on the number of spores produced in different temperatures.

Materials and Methods

Inoculation Method

This study aims to understand if the differences in temperatures can affect the progression of disease and the number of spores produced on the lesions caused by ABR. Peaches were harvested in the beginning of August 2016 at the Southwest Research and Extension Center (SREC) located in the most important area of peach production in Michigan. After almost a two-hour trip, from SREC to MSU plant pathology farm where they were stored for several days in a cooler until everything was ready for the experiment. Plastic containers were washed with bleach and 70% ethanol and dried with sterile paper towels. Two strips of parafilm were stretched and placed at the bottom of the plastic bins as a surface to rest the peaches on. Each peach was sterilized, washed with 70% ethanol, and dried with Kim wipes to make sure none of the fruits were infected before inoculation. While washing each fruit, the stems were carefully pulled out for the purpose of keeping the peaches away from any infections caused by bacteria or fungus. A total of 12 peaches with their stem-end facing down were placed in bins lined with moist paper towels to keep the humidity as high as it could be and covered with lids.

A spore suspension was prepared with *M. fructicola* isolates. The colonies grew on peach slices for about 10 days. After this growing time in a covered tray inside a container with a lid, the spore solution was prepared with sterile distilled water and filtered through several layers of cheesecloth. The concentration of spores was measured with a hymacytometer under a microscope, and it was adjusted to $3x10^5$ cfu/ml of mature *M. fructicola* conidia. Using a pipette, 15µL of the solution was delivered on the top of each fruit. Immediately after the placement of the solution drop, each peach was punctured three times in a triangular pattern using a 30.5-gauge needle. The peaches were then misted with distilled water, and the containers were closed.

Following this treatment, fruits were held at a room temperature of 23.3°C and in incubators with temperatures of 28°C for some trials and 30°C for other trials. Disease incidence was observed for five days after inoculation and the percentage of fruits showing typical brown rot symptoms as well as the progression of disease and number of spores per lesion were recorded. Each isolate was inoculated at least two different times on a set of six or 12 peaches that were harvested at the same time and location. Every day when data was being collected, the peaches were misted with sterile distilled water to maintain humidity inside the plastic bins.

<u>Data Recording</u>

The fruits were observed daily to see whether they had developed any symptoms of ABR and to compare whether lesion size and number of spores differed between the peaches held at room temperature and those in the higher temperatures of the incubators. The relationship between temperature and lesion size and temperature and quantity of spores was analyzed through ANOVA test to find means for the factors, which included the 95% confidence intervals. As disease was detected, the percentage of peaches that became infected was recorded for five days consecutively at the same time each day. Any other lesions that did not originate from inoculation were considered natural infection and were not recorded.

Results

A total of six isolates were tested in two different temperatures, 23.3°C (low) and 28°C (high). These isolates were collected from peaches that had the typical symptoms such as lesions and spores. Disease incidence started to appear on day 2 after inoculation. Through daily analysis, Day 5 of observation was considered representative and, overall, none of the factors had a significant effect on the number of spores per lesion, even though some isolates such as SCHM-3 and WBWM-7 seemed to be more sensitive to the temperature differences (Figure 3.1). Also, neither temperature nor isolate had a significant effect on lesion size according to ANOVA, and only SCHM-3 seemed to be more sensitive to the temperature differences (Figure 3.2).

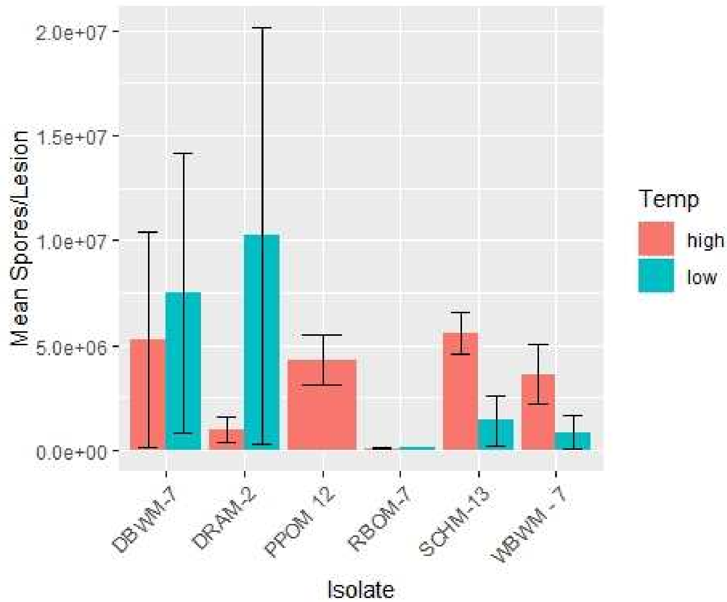

Figure 3.1 Density of spores per lesion
Brown rot mean spores per lesion after 5 days at temperatures of 23.3°C (low) and 28°C (high). Error bars represent standard error.

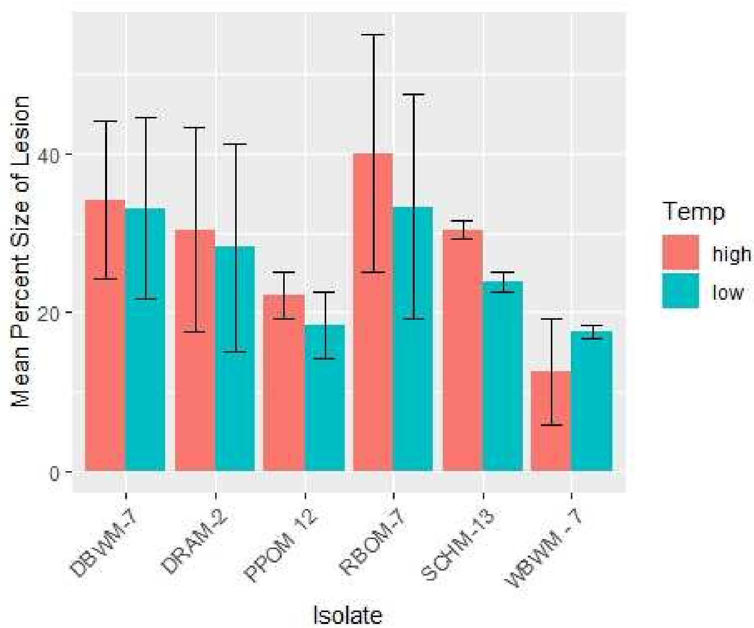

Figure 3.2 Mean size of lesion caused by *Monilinia fructicola*
Brown rot mean lesion size after 5 days at temperatures of 23.3°C (low) and 28°C (high). Error bars represent standard error.

Discussion

Even though literature says that temperature has an implication as a factor affecting the production of spores and the progression of disease, in this assay with these isolates temperature did not seem to be a significant factor when inoculations at 23°C and 28°C were compared. However, a few individual isolates did show a trend of reducing sporulation and lesion size at the colder temperature. Temperature response could be genotype dependent, which could explain the different responses or lack of a large response in some isolates.

One suggestion is that this study could be performed in peach potted trees because some existen*t in vitro* variances could be eliminated, and more accurate results could be obtained. In-vitro assay could also be improved and replicated more to reduce and possibly find temperature a significant factor.

BIBLIOGRAPHY

BIBLIOGRAPHY

Al-haq, M. I., Seo, Y., Oshita, S., & Kawagoe, Y. (2001). Fungicidal Effectiveness of Electrolyzed Oxidizing Water on Postharvest Brown Rot of Peach, *36*(7), 1310–1314.

Biggs, A. R., & Northover, J. (1985). (PDF) Inoculum sources for Monilinia fructicola in Ontario peach orchards. *Canadian Journal of Plant Pathology*.

Biggs, A. R., & Northover, J. (1988). Influence of temperature and wetness duration on Infection of peach and sweet cherry fruits by Monilinia fructicola. *Phytopathology*, *78*(10), 1352–1356.

Brown-rot of stone fruits | Stone fruit diseases | Fruit and nuts | Plant diseases | Pests, diseases. (2010).

Elizabeth Bush, & Keith Yoder. (2014). Monilinia (brown rot of stone fruit) - Bugwoodwiki.

Holb, I. J. (2004). The brown rot fungi of fruit crops (Monilinia spp.)-III. Important features of disease management .pdf. *International Journal of Horticultural Science*, 31–48.

PEACHES | MICHIGAN GROWN. (n.d.).

Philips, D. J. (1982). Changes in conidia of monilinia fructicola in response to incubation temperature. *Ecology and Epidemiology*, *72*, 1281–1283.Post-Harvest Cooling and Storage for Peaches. (2015).

Ritchie, D. F. (2000). Brown rot of stone fruits. *The Plant Health Instructor*. https://doi.org/DOI: 10.1094/PHI-I-2000-1025-01.Trees, F., & Sciences, W. P. (2008). Preharvest Factors Affecting Peach Quality.