AUGMENTING FUNGICIDAL ACTIVITY OF TETRACONAZOLE WITH

CHEMOSENSITIZATION AGENTS FOR CERCOSPORA LEAF SPOT MANAGEMENT

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

By

Nicholas James Metz

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Plant Pathology

April 2017

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Augmenting Fungicidal Activity of Tetraconazole with Chemosensitization
Agents for Cercospora Leaf Spot Management

By

Nicholas James Metz

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota

State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Gary Secor

Chair

Melvin Bolton

Mohamed Khan

Thomas Peters

Approved:

4/17/17	Jack Rasmussen
Date	Department Chair

ABSTRACT

Cercospora beticola (Sacc.) is the causal agent of Cercospora leaf spot (CLS). CLS is considered to be one of the most destructive foliar diseases of sugar beet in the world. CLS is managed in part through resistant cultivars, crop rotation, and cultural practices, but timely fungicide applications are necessary to manage disease effectively. Heavy reliance on fungicides to manage CLS has led to the development of resistance to multiple classes of fungicides. The most widely used class of fungicides is the demethylation inhibitors (DMIs). DMI-resistant *C. beticola* isolates have been increasing in incidence over the past decade. Chemosensitization agents (CAs) are compounds that have little to no antifungal activity, but may increase efficacy of commercial fungicides when co-applied. CAs could lead to better management of CLS and reduced production costs.

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to members of my graduate committee, Drs. Melvin Bolton, Gary Secor, Mohamed Khan, and Tom Peters for the advice and direction throughout my graduate studies.

I would like to thank Dr. Melvin Bolton and Dr. Gary Secor for giving me the opportunity of graduate work and all of the advice and guidance throughout my graduate program.

Finally, I would like to give thanks to my family members and friends for the encouragement and support throughout these studies.

TABLE OF CONTENTS

LIST OF TABLES

LITERATURE REVIEW

Introduction

Sugar has been part of the human diet for thousands of years. The first wild sugar beet ancestors were recorded around 1000 BC (Winner, 1993). Sugar beet leaves were originally used as herbs for cooking or as livestock fodder. Selection for higher sucrose content began, when it was discovered that fodder beet roots contained the same kind of sugar as sugar cane. Sugar beet ancestors were bred and selected for high sucrose content until the modern sugar beet (*Beta vulgaris* ssp. *vulgaris)* known today arose. The process of extracting and utilizing sugar from sugar beet took many decades to perfect. Although sucrose comes primarily from sugar cane and sugar beet, only sugar beet can be grown in temperate climates. This makes it an excellent crop for many regions of the United States as well as many European countries.

The United States is among the world's largest sugar producers. Unlike most other producing countries, the United States has large, well developed sugar cane and sugar beet industries. The Red River Valley region of western Minnesota and eastern North Dakota is the largest sugar beet production area in the United States. Since 2010, the region has averaged 275,000 hectares of sugar beet or about 57% of the total planted sugar beet acreage in the United States. The next largest region is in Idaho that averages 75,000 hectares or about 15% of the total United States acreage. In the 2016 crop year, sugar beet annual cash receipts were 2.9 billion dollars as compared to sugar cane that had 1.0 billion dollars (USDA-ERS).

Similar to other cropping systems, sugar beet has many pathogens that reduce quality and yield. Pathogens include fungi, bacteria, viruses, and nematodes that can cause disease from the roots to the leaves. *Cercospora beticola* is a fungal species that causes the disease Cercospora leaf spot (CLS) and can be very destructive when left unmanaged. Fungicides have been an

efficient management tool to reduce disease and economic loss. However, repeated application and extensive use of fungicide has given rise to a large population of resistant isolates of *C. beticola* to several fungicide groups. Consequently, alternative means of disease management need to be identified.

The Evolution and Domestication of the Host Species

The sugar beet known today is a member of the family Amaranthaceae. Ancestors of sugar beet were hexaploid and share several paralogous regions with grapevine, poplar, and cacao (Dohm, 2014). Sugar beet was likely domesticated from sea beet (*B. vulgaris* ssp. *maritima*). The center of origin of sea beet is believed to be the Middle East. From there, sea beet migrated up the coast of the Mediterranean and eventually around the Atlantic coast of Europe. Sea beet was primarily used as herbs for cooking and fodder before being a source of sugar. These plants had large seed balls, elongated-forked roots, and flowered early as compared to modern sugar beet. Ancestral *B. vulgaris* has been the source of many important characteristics to sugar beet including partial resistance to Cercospora leaf spot, Rhizomania, and other diseases (Biancardi et al., 2010). Many sub-species arose from sea beet such as spinach beet, Swiss chard, beetroot (red beet), yellow beet, fodder beet, weed beet and sugar beet.

Sugar beet has a unique growth habit compared to other species within the *Beta* genus. Sugar beet is biennial, but under certain conditions can be an annual. Sugar beets have large, glabrous, and dark green leaves that grow from the top of the root. Sugar beet has a conical, white, fleshy tap root that extends into the soil (Duke, 1983). During the first growing season, tap roots swell with sugars that the plant stores for overwintering. Cultivated sugar beet requires a vernalization period to begin their reproductive phase (Letschert el al., 1994). After vernalization, sugar beets bolt giving rise to tall stalks with small flowers that release pollen and

produce viable seed. Sugar beet that is used in the process of making sucrose for consumption is typically planted in the spring and harvested in the fall in the Red River Valley.

Commercialization of Sugar Beet

Many of the sugar beet varieties grown today originated from fodder beet grown in Germany called White Silesian (Biancardi et al., 2010). Early breeding efforts led to a lack of genetic diversity within the population resulting in hybrid sterility (Biancardi, 2012). Repeated selection and breeding for increased sugar content produced modern sugar beet lines (Dohm, 2014). The process of extracting sugar from sugar beet was discovered in 1747 by a German chemist named Andreas Sigismund Marggraf (Cooke and Scott, 2012). In 1799, another German chemist, Karl Achard the student of aforementioned Marggraf, invented a robust method of extracting sugar, which was the first time sucrose was extracted from sugar beet for commercial use (Draycott, 2006).

The first factory to be established in the United States was in Northampton, Massachusetts in 1838, but was closed in 1840 (Harveson, 2014). The first successfully operated sugar beet processing factory was founded in Alvarado, California in 1870. Imported sugar was taxed to protect the local sugar industry in the United States during the first half of the 20th century. Sugar beet production area was approximately 360,000 hectares in the 1950s (McGrath 2010). Currently there are five centers of the sugar beet cultivation in the United States including California, the Northwest, the Great Plains to east of the Rocky Mountains, the Red River Valley, and the Great Lakes area. In 2014, 28.4 million tons of sugar beet were produced from 464,260 hectares in ten states, including California, Colorado, Idaho, Michigan, Minnesota, Montana, Nebraska, North Dakota, Oregon, and Wyoming (Harvenson, 2014).

A new herbicide called Roundup with active ingredient glyphosate was developed by Monsanto in 1974. In 1988, Monsanto and KWS companies developed a new variety of sugar beet that was genetically modified to be glyphosate-resistant. The USDA and FDA approved glyphosate-resistant sugar beet in 2005. This trait became extremely popular among sugar beet growers and was estimated to be planted in 55% of sugar production fields in 2008-2009. The reason for the fast adaptation was that glyphosate-resistant sugar beet allowed farmers to achieve effective weed control with a wide application window while reducing the labor cost and use of other herbicides.

Molecular Characterization of the Species

Sugar beet has 18 chromosomes with an estimated genome size of 714-758 Mb. An estimated 63% of the genome is repetitive (Dohm et al., 2014). The total 27,421 protein-coding genes were predicted based on transcript data and annotated via sequence homology (Dohm et al., 2014). Mangin et al. (2015) collected 2246 worldwide beet accessions and another 1367 elite sugar beet lines, which were stored in three different international seed banks in US, Germany, and Greece. This collection not only covered a broad range of geographical origins of wild beet from the Atlantic coast of Morocco to Denmark to the Mediterranean coast of Algeria to Israel and Turkey, but also included representative regions of cultivated beet from Europe, Asia, and the United States. Single nucleotide polymorphisms developed from genic and intergenic sequences in a set of elite lines were mapped using three different F2 populations (Adetunji et al. 2014). Such a reference map may allow breeders to select favorable traits more easily. Disease tolerance, drought tolerance, increase in sucrose content, and increase in sucrose quality are traits that may be selected for with the reference genome.

Sugar Beet Diseases

Sugar beet and the pathogens infecting them have been co-existing for thousands of years. All parts of the sugar beet plant at every stage in the growing cycle are potentially susceptible to disease. Diseases range from seedling damping off in the beginning of the season to bacterial root rots in storage piles. Sugar beet pathogens are primarily fungi but also include bacteria, viruses, and nematodes.

Fungal diseases are usually split between two categories; foliar and root. Foliar diseases in sugar beet are most commonly caused by *C. beticola*, *Ramularia beticola*, *Alternaria alternata*, and *Uromyces betae*. Foliar diseases reduce the photosynthetic production of the leaf, thus reducing sucrose content in the tap root. Foliar diseases can be found wherever sugar beet is grown. The foliar disease beet rust, caused by *U. betae*, has a minor economic impact in the United States, but thrives in cooler climates in Europe. Powdery mildew (*Erysiphe betae*) and downy mildew (*Peronospora farinos*) cause minor disease on sugar beet. They tend to be found in growing areas with a mild, damp climate.

Fungal root diseases infect the taproot of sugar beet reducing sucrose content and quality. Symptoms of fungal root pathogens on sugar beet include root necrosis, wilting of foliage, crown rot and necrosis, and reduced size of the taproot. Fungal root rot pathogens include *Macrophomina phaseoline*, *Rhizoctonia solani*, and *Rhizopus stolonifera*. Not all fungal root pathogens causing rotting in the tap root. Fusarium yellows and Verticillium wilt, caused by *Fusarium oxysporum* and *Verticillium dahlia*, respectively, cause symptoms such as wilting, yellowing, and brittle leaves. Infected root tissue becomes brown and discolored in the vascular areas. One of the most important root diseases in sugar beet production is *Aphanomyces* root rot, caused by the soil borne oomycete *Aphanomyces cochlioides*. Symptoms include seedling

5

damping off and rotting of the taproot later in the growing season. *Phytophthora drechsleri* and

Pythium aphanidermatum are other oomycota that cause rot rotting diseases on sugar beet.

Diseases caused by viruses are very important to the sugar beet industry. These pathogens

are vectored by aphids, insects, fungi, or plasmodiophores. *Beet yellow virus*, *Beet yellow vein*

virus, *Beet mosaic virus*, and *Beet curly top virus* affect the crown and leaves of sugar beet

resulting in stunting and premature yellowing (Harveson et al., 2009). The most important viral

disease worldwide is Rhizomania caused by *Beet necrotic yellow vein virus* (BNYVV).

Polymyxa betae is a soil-borne plasmodium that vectors BNYVV, and without it the virus is

incapable of naturally infecting sugar beet. Rhizomania symptoms include root stunting and mild

yellowing of leaves, but the most recognizable symptom is the proliferation of lateral roots

giving the taproot a "bearded" look. These symptoms reduce sucrose yield of the plant and when

left unchecked can result in 100% yield loss.

Bacterial diseases and nematode parasites are also common in most sugar beet production

areas. Bacterial vascular necrosis and Pectobacterium root rot are highly destructive diseases in

North America, Chile, and Europe (Harveson 2009). Symptoms are not always present in the

canopy, but may occur on the taproot as a soft and dry rot that reduce root quality. Another

bacterial disease is bacterial leaf spot caused by the pathogen *Pseudomonas syringae*. The

symptoms are very similar to that of fungal leaf spots differing in shape and color of lesions on

leaves. The majority of yield loss caused by nematodes is from the sugar beet cyst nematode,

Heterodera schachtii (Kerry and Evans, 1996). The sugar beet cyst nematode can cause seedling

damping off and reduce stand counts resulting in well-defined circular areas of infection

(Harveson et al., 2009).

Cercospora Leaf Spot

Cercospora leaf spot is caused by the fungus *C. beticola* and is considered one of the most destructive foliar diseases of sugar beet. Cercospora leaf spot was first reported by Saccardo (1876). As time progressed, more and more descriptions of leaf spot disease on sugar beet were described that were probably *C. beticola* (Chupp, 1953). Regions with warm and humid growing seasons are often affected by Cercospora leaf spot outbreaks. Growers must use timely fungicide applications, good cultural practices, crop rotation, as well as plant genetically tolerant varieties to bring the crop to maturity (Windels, 1998). The sugar beet canopy may be destroyed resulting in sucrose and tonnage loss without these measures.

C. beticola is a hemibiotrophic fungus with no known sexual cycle observed in nature (Bolton et al., 2012). In the absence of a known sexual cycle, genetic variation in *C. beticola* may result from mutations, vegetative compatibility, or parasexual recombination. The lifestyle of *C. beticola* includes a biotrophic phase in which the fungus invades the host and attempts to avoid the host immune response while growing through the apoplast. Following the biotrophic phase, the necrotrophic phase ensues where toxins are secreted that result in host cell death (Koeck et al., 2011). *C. beticola* produces low-molecular-weight phytotoxins and hydrolytic enzymes that necrotize cells in advancing fungal growth (Weiland and Koch, 2004). One of these toxins is cercosporin, which is a photoactivated nonspecific toxin that generates reactive oxygen (Daub and Ehrenshaft 2000). Cercosporin produced by *C. beticola* is a virulence factor for infection (Weiland, 2004). Another toxin produced by *C. beticola* is beticolin. Beticolins destabilize host cells membranes resulting in leakage and ultimately death of the cell (Gapillout et al., 1996).

C. beticola infects the genus *Beta* and several other species in the Amaranthaceae family (Weiland and Koch, 2004). *C. beticola* has characteristic symptoms on *Beta* species that are random 0.2-0.5 cm in diameter leaf spots across the surface of mature leaves (Duffus and Ruppel, 1993). Cercospora leaf spot is unique from other leaf spot diseases because lesions do not start necrotizing at the point of contact and expand outward (Weiland et al., 2004). Instead, *C. beticola* causes plant cells to collapse in a near simultaneous manner resulting in lesions many millimeters wide (Steinkamp et al., 1979). After tissue collapse, characteristic red-brown ring of the lesion appears. Lesions are primarily on the leaf surface, but can occur on the leaf petioles. Lesions can expand after the initial collapse, but the expanding necrotic tissue area is primarily due to the increase of infection sites resulting in larger surface area of infection. As lesions expand and infection sites increase, the leaf may become completely necrotic.

Cercospora Leaf Spot Management

Managing Cercospora leaf spot has evolved over the years. Some of the earliest chemical control strategies relied on inorganic copper to manage *C. beticola* on sugar beet (Meriggi et al., 2000). Over 50 years later, the first fungicides with synthetic chemistries were developed that greatly reduced losses due to Cercospora leaf spot. Currently, there are a wide variety of both naturally derived and synthetic fungicides available for *C. beticola* management (Kahn et al., 2007).

C. beticola first showed signs of resistance to benzimidazoles in the late 1970s resulting in an epidemic in 1981 (Bugbee, 1982; Rupel and Scott, 1974). This led to the use of triphenyltin hydroxide for Cercospora leaf spot management. Agriculturists in the sugar beet industry began to promote fungicide rotations as a means to reduce resistance (Secor et al., 2010). Dependence on only two modes of action (benzimidizoles and triphenyltin hydroxide) led to the development

of resistance to triphenyltin hydroxide. Resistance was first reported in 1994 in the Red River Valley at levels where there was loss of disease control (Bugbee, 1995). The trend of increasing resistance incidence continued leading to the epidemic of 1998, which resulted in a large economic loss in Minnesota and North Dakota from reduced yield and increased fungicide application (Secor et al., 2010). The following year, the Environmental Protection Agency (EPA) granted an emergency exemption, allowing growers to use the sterol demethylation inhibitor (DMI) tetraconazole. The DMI fungicides difenoconazole and prothioconazole were registered in 2008 for Cercospora leaf spot management (Secor et al., 2010). Through repeated and widespread use of DMIs, resistant *C. beticola* isolates began to appear in the population (Bolton et al., 2012; Secor et al., 2010). The strobilurin class of fungicide is also popular in Cercospora leaf spot management. Resistance to strobilurin fungicides also occurred within nine years of their use (Bolton et al., 2013).

Mechanisms of Resistance

During the past decade, fungicide resistance mechanisms have been identified in *C. beticola*. Knowing how the pathogen is able to tolerate fungicides may provide insights on how to manage resistant isolates and develop molecular tools to identify resistant strains. Strobilurin or quinone outside inhibitor (QoI) fungicides have been a popular choice for management of Cercospora leaf spot. QoI mode of action is to bind to the quinone oxidation site of the cytochrome bc1 enzyme complex, which disrupts ATP production and ultimately leads to death of the fungus (Fisher and Meunier, 2008). The cytochrome b target is a membrane bound protein that forms the core of the mitochondrial bc1 complex and is encoded by the cytochrome b (*cytb*) gene. Point mutations that encode amino acid exchange from phenylalanine to leucine at position 129 (F129L), glycine to alanine at position 143 (G143A), and glycine to arginine at position 137

(G137R) occur within the gene and have been associated with resistance to QoI fungicides (Fisher and Meunier, 2008). Isolates with the F129L or G137R mutations express moderate resistance whereas isolates with the G143A mutation express high resistance (Gisi et al., 2002). The G143A mutation in *C. beticola* was first reported in Italy (Birla et al., 2012), followed by the United States (Bolton et al., 2013). *C. beticola* field isolates having this mutation have spread to other growing regions (Bolton et al., 2013). These mutations can be identified rapidly through real-time PCR (Bolton et al., 2013).

Benzimidazole fungicides have been used extensively in Cercospora leaf spot disease management. The benzimidazole class of fungicides act by binding to fungal tubulin. This mode of action interferes with mitosis and the fungal cytoskeleton (Sisler, 1988; Davidse et al., 1995). Benzimidazole resistance has developed in *C. beticola* and has been shown to be due to mutations in the β-tubulin gene which reduces the binding efficacy of the fungicide. A single point mutation in the β-tubulin gene is consistently found in all resistant isolates (Davidson et al., 2005). The mutation is found in the 198 codon where an amino acid substitution for glutamic acid to alanine (E198A) occurs (Reijo et al., 1994). This mutation may be used to rapidly identify resistant isolates (Luck et al., 1995).

Triphenyltin fungicides have been very effective for managing Cercospora leaf spot (Stallknecht and Calpouzos, 1968). The first strains of *C. beticola* that showed signs of resistance to triphenyltin hydroxide (TPTH) were in Greece (Giannopolitis, 1978). Resistance to TPTH in the United States was first reported in 1994, which spread rapidly throughout the Red River Valley area (Bugbee, 1995).

DMI fungicides became popular because of their broad-spectrum antifungal activities, protective and curative properties, and low levels of phytotoxicity. This class of fungicides acts

by inhibiting sterol P450 14α-demethylase (CYP51), which is a protein required for the ergosterol biosynthesis pathway in fungi (Karaoglanidis et al., 2010). DMI-resistant populations of *C. beticola* have been rapidly increasing from year to year (Karaoglanidis et al., 2000; Secor et al., 2010). DMI-resistance mechanisms in other fungi can include (i) mutations in the *Cyp51* gene that maintain protein function while avoiding inhibition by the DMI fungicide, (ii) overexpression of the *Cyp51* gene leading to excess targets for the DMI fungicide, and (iii) reduction of intracellular concentration by increasing ATP-binding cassette or major facilitator superfamily transporter efflux activity (Del Sorbo et al., 2000; Ma and Michailides 2005). *C. beticola* is tolerant to DMI fungicides due to over-expression of the *Cyp51* gene when exposed to tetraconazole (Bolton et al., 2012).

Chemosensitization Agents

The term "chemosensitization" was originally used in the medical community as a strategy to counter the development of resistant cancer cells to anticancer drugs. The resistance mechanisms to anticancer drugs are almost parallel to those developed by fungal pathogens against fungicides. Cancer cells develop resistance through mutations of target enzyme genes, over-expression of target enzymes, up-regulation of genes controlling efflux pumps, production of enzymes that detoxify the anticancer drugs, and DNA repair (Shabbits et al., 2003). When a chemosensitization agent is co-applied with an anticancer drug, it stresses or debilitates the cancer cells allowing for the anticancer drug to be effective again. Chemosensitization agents aid in lowering the dosages of the anticancer drug, overcoming resistant cancer cells, and lower the negative side-effects by avoiding the toxicity to non-target cells.

Similarly, chemosensitizing agents may be a useful strategy to manage plant pathogenic fungi. The use of chemosensitizing agents could help overcome resistance to commercial

fungicides. Chemosensitizing agents function by stressing or debilitating the defense of the fungus so that it would have increased sensitivity to commercial fungicides (Kim et al., 2015). A fundamental characteristic of a chemosensitizing agent is that while it aids in augmenting the fungicide efficacy, it presents little fungicidal activity alone (Campbell et al., 2012). With the limited number of fungicides available today, chemosensitization agents could play a role in plant disease management strategies.

Cinnamaldehyde is likely the most well-known natural plant product used as a chemosensitization agent against plant pathogenic fungi (Copping and Duke, 2007). The compound is a yellow oily liquid with a cinnamon odor and has been used to flavor foods and beverages. Cinnamaldehyde has been shown to have fungicidal properties against wood-decaying fungi (Yen and Chang, 2008) as well as antibacterial properties (Lee et al., 1998, Friedman et al. 2002). Cinnamaldehyde has inhibitory effects on ATPases, cell wall biosynthesis, and alteration of cell membrane integrity (Usta et al., 2003, Kyu-Ho et al., 2000, Xie et al., 2004). Cinnamaldehyde has been shown to reduce spore germination in *Aspergillus flavus* (Xie et al. 2004). Cinnamaldehyde has also been demonstrated to show chemosensitizing effects on human fungal pathogen *Candida spp.* (Shreaz et al., 2016).

Thymol, another natural plant product derived from *Thymus vulgaris* oil, is believed to disrupt fungal cell membrane integrity by reducing ergosterol content (Pinto et al., 2006). Thymol was used in a study as a chemosensitization agent co-applied with azoxystrobin in an effort to manage *Stagonospora nodorum* and *Phoma glomerata* (Dzhavakhiya et al., 2012). The results showed that the percentage of *S. nodorum* growth inhibition using thymol (10 ppm) alone was 1.1%, azoxystrobin alone was 14.8%, but when both compounds were co-applied the

inhibition was 40.9%. In the same study, this trend was observed for treatments on both *Phoma glomerata* and *A. alternata*.

Benzo derivatives and natural plant products have primarily been used successfully as chemosensitization agents to manage *Aspergillus* (Kim et al., 2014). The benzo analog used was octyl gallate, which has a mode of action that disrupts the fungal cell wall integrity (Kim et al., 2014). The minimum inhibitory concentration (MIC) was lowered from 0.35 mM to 0.05 mM when octyl gallate was co-applied with kresoxin methyl (strobulurin) to inhibit *A. fumigatus*. Importantly, the MIC of the chemosensitization agents that were applied alone resulted in minimal growth inhibition. Octyl gallate has been show to possess antifungal activity, but not enough to be a stand-alone fungicide (Kim et al. 2014).

Salicylaldehyde is another benzo analog which targets the mitogen-activated protein kinase (MAPK) pathway (Levin et al., 2005). It has been shown to be another promising candidate CA when co-applied with antimycin A or kresoxim-methyl against *Aspergillus spp.* (Kim et al., 2011). Chemosensitizing activity of salicylaldehyde combined with inhibitors of mitochondrial respiration anitmycin A or kresoxin-methyl, resulted in complete inhibition of fungal growth of *A. flavus* and *A. parasiticus* (Kim et al., 2011).

Natural compounds produced by fungi to inhibit growth of other encroaching fungi are also chemosensitizing agent candidates. Kojic acid is produced by *Aspergillus* and *Penicillium spp.* (Rodrigues et al. 2014). It acts by inhibiting the enzyme tyrosinase, which is an important enzyme in melanin biosynthesis (Chee et al., 2003). MIC was 1.3 to 2.4 times lower than when the compounds were applied independently when kojic acid was co-applied *in vitro* with H_2O_2MIC (Kim et al. 2014). Mutations in the *Z. tritici* gene encoding a polyketide synthase (*PKS1*) involved in melanin biosynthesis was linked to higher melanized colonies as well as

DMI resistance (Lendenmann et al. 2015). This finding suggests the melanin biosynthesis pathway may be another possible target for chemosensitization agents.

Conclusion

The advancement of agriculture relies on new research and technology to be developed. With the world population continuing to rise, new ways to supply higher yielding crops while reducing reliance on natural resources is necessary. Plant pathogens are still a major limiting factor of agricultural production. Fungicides that are readily available are becoming less efficient because of rapid resistance development. Cercospora leaf spot is a major issue for a majority of sugar beet growing regions. All of these contributing factors have led to research in new natural chemistries as well as new methods in disease management such as tank mixing and better disease forecasting (Zhou et al., 2014; Cioni et al., 2014).

Chemosensitization agents could potentially aid in Cercospora leaf spot management by increasing efficacy of DMI fungicides. Chemosensitization agents will be tested and combined with other adjuvants for the possibility of increasing the efficacy of tetraconazole. These new chemicals and compounds could lengthen the lifespan of already established fungicides that are losing efficacy all too quickly due to resistance. The best candidate chemosensitization agents that were discovered in the following research are being tested with a detached leaf assay and will eventually be tested *in planta* in a controlled greenhouse study. CAs would also need to be field tested to determine environmental fate/effects, such as the degradation caused by sunlight, metabolism, or possible volatilization. Chemosensitization agents may become available to growers in a shorter amount of time since many chemosensitization agents are natural compounds or are unregulated by the EPA.

References

Adetunji, I., Willems, G., Tschoep, H., Bürkholz, A., Barnes, S., Boer, M., Malosetti, M.,
Horemans, S., van Eeuwijk, F. (2014). Genetic diversity and linkage disequilibrium
analysis in elite sugar beet breeding lines and wild beet accessions. *Theoretical and
Applied Genetics*, 127:559-571.

Barbetti, M. J., & Nichols, P. G. H. (2005). Field performance of subterranean clover germplasm
in relation to severity of Cercospora disease. *Australasian Plant Pathology*, 34(2), 197-
201.

Biancardi, E., Panella, L. W., & Lewellen, R. T. (2011). *Beta maritima: the origin of beets*.
Springer Science & Business Media.

Biancardi, E., McGrath, J. M., Panella, L. W., Lewellen, R. T., & Stevanato, P. (2010). Sugar
beet. In *Root and tuber crops* (pp. 173-219). Springer New York.

Birla, K., Rivera-Varas, V., Secor, G. A., Khan, M. F., & Bolton, M. D. (2012). Characterization
of cytochrome b from European field isolates of Cercospora beticola with quinone
outside inhibitor resistance. *European journal of plant pathology*, 134(3), 475-488.

Bolton, M. D., Secor, G. A., Rivera, V., Weiland, J. J., Rudolph, K., Birla, K. Rengifo, J., and
Campbell, L. G. (2012). Evaluation of the potential for sexual reproduction in field
populations of Cercospora beticola from USA. *Fungal biology*, 116(4), 511-521.

Bolton, M. D., Rivera-Varas, V., del Río Mendoza, L. E., Khan, M. F., & Secor, G. A. (2012).
Efficacy of variable tetraconazole rates against Cercospora beticola isolates with differing
in vitro sensitivities to DMI fungicides. *Plant disease*, 96(12), 1749-1756.

Bolton, M., Rivera-Varas, V., Secor, G., Cattanach, A., & Metzger, M. (2013a). Identification of the G143A mutation in cytochrome b associated with QoI resistance in Cercospora beticola isolates from the Red River Valley. *Plant Health Progress*, 10.

Bolton, M. D., Rivera, V., & Secor, G. (2013b). Identification of the G143A mutation associated with QoI resistance in Cercospora beticola field isolates from Michigan, United States. Pest management science, 69(1), 35-39.

Bugbee, W. M. (1982). Sugar beet disease research – 1981. Sugarbeet Res. Ext. Rep. 12:155.

Bugbee, W. M. (1995). Cercospora beticola tolerant to triphenyltin hydroxide. Journal of sugar beet research, 32(4), 167-174.

Campbell, B. C., Chan, K. L., & Kim, J. H. (2012). Chemosensitization as a means to augment commercial antifungal agents. Frontiers in microbiology, 3, 79.

Chee, H. Y., & Lee, E. H. (2003). Fungistatic activity of kojic acid against human pathogenic fungi and inhibition of melanin-production in Cryptococcus neoformans. Mycobiology, 31(4), 248-250.

Chupp, C. (1953) A Monograph of the Fungus Genus Cercospora. Ithaca, NY.

Cioni, F., Collina, M., Maines, G., Khan, M. F., Secor, G. A., & Rivera, V. V. (2014). A New Integrated Pest Management (IPM) Model for Cercospora Leaf Spot of Sugar Beets in the Po Valley, Italy. *Sugar Tech*, 16(1), 92-99.

Copping, L. G., & Duke, S. O. (2007). Natural products that have been used commercially as crop protection agents. Pest management science, 63(6), 524-554.

Daub, M.E. and Ehrenshaft, M. (2000) The photoactivated cercospora toxin cercosporin: contributions to plant disease and fundamental biology. Annu. Rev. Phytopathol. 38, 461–490.

Davidse, L. C., & Ishii, H. (1995). Biochemical and molecular aspects of the mechanisms of

    action of benzimidazoles, N-phenylcarbamates and N-phenylformamidoximes and the

    mechanisms of resistance to these compounds in fungi. Modern selective fungicides, 305-

    322.

Davidson, R. M., Hanson, L. E., Franc, G. D., & Panella, L. (2006). Analysis of β-tubulin Gene

    Fragments from Benzimidazole-sensitive and-tolerant Cercospora beticola. Journal of

    Phytopathology, 154(6), 321-328.

Del Sorbo, G., Schoonbeek, H. J., & De Waard, M. A. (2000). Fungal transporters involved in

    efflux of natural toxic compounds and fungicides. Fungal Genetics and Biology, 30(1), 1-

    15.

Dohm, J.C., Minoche, A.E., Holtgräwe, D., Capella-Gutiérrez, S., Zakrzewski, F., Tafer, H.,

    Rupp, O., Sörensen, T.R., Stracke, R., Reinhardt, R., Goesmann, A., Kraft, T., Schulz,

    B., Stadler, P.F., Schmidt, T., Gabaldón, T., Lehrach, H., Weisshaar, B., and

    Himmelbauer, H.(2014). The genome of the recently domesticated crop plant sugar beet

    (*Beta vulgaris*). Nature, 505:546-549.

Draycott, A. P. (Ed.). (2008). Sugar beet. John Wiley & Sons.

Duffus, J.E. and Ruppel, E.G. (1993) Diseases. In The Sugarbeet Crop (Cooke, D.A. and Scott,

    R.K., eds). London: Chapman & Hall, pp. 346–427.

Duke, J. A. (1983). Handbook of energy crops: *Beta vulgaris* L.

Dzhavakhiya, V., Shcherbakova, L., Semina, Y., Zhemchuzhina, N., & Campbell, B. (2012).

    Chemosensitization of plant pathogenic fungi to agricultural fungicides. Frontiers in

    microbiology, 3.

Fisher, N., & Meunier, B. (2008). Molecular basis of resistance to cytochrome bc1 inhibitors. FEMS yeast research, 8(2), 183-192.

Friedman, M., Henika, P. R., & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica*. Journal of food protection, 65(10), 1545-1560.

Gapillout, I., Mikes, V., Milat, M. L., Simon-Plas, F., Pugin, A., & Blein, J. P. (1996). Cercospora beticola toxins. Use of fluorescent cyanine dye to study their effects on tobacco cell suspensions. Phytochemistry, 43(2), 387-392.

Giannopolitis, C. N. (1978). Occurrence of strains of Cercospora beticola resistant to triphenyltin fungicides in Greece. Plant Disease Reporter, 62(3), 205-208.

Gisi, U., Sierotzki, H., Cook, A., & McCaffery, A. (2002). Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest management science, 58(9), 859-867.

Harveson, M. R. (2014). History of sugarbeet production and use. *University of Nebraska CropWatch: Sugarbeets*.

Harveson, R. M., Hanson, L. E., & Hein, G. L. (2009). Compendium of beet diseases and pests (No. Ed. 2). American Phytopathological Society (APS Press).

Khan, J., Del Río, L. E., Nelson, R., & Khan, M. F. R. (2007). Improving the Cercospora leaf spot management model for sugar beet in Minnesota and North Dakota. *Plant Disease, 91*(9), 1105-1108.

Karaoglanidis, G. S., and Ioannidis, P. M. (2010). Fungicide resistance of Cercospora beticola in Europe. Pages 189-211 in: Cercospora Leaf Spotof Sugar Beet and Related Species. R. T.

Lartey, J. J. Weiland, L. Panella,and P. W. W. Crous, C. E., eds. The American

Phytopathological Society,St. Paul, MN.

Karaoglanidis, G. S., Ioannidis, P. M., & Thanassoulopoulos, C. C. (2000). Reduced sensitivity

of Cercospora beticola isolates to sterol-demethylation-inhibiting fungicides. Plant

Pathology, 49(5), 567-572.

Kerry, B. R., & Evans, K. (1996). New strategies for the management of plant parasitic

nematodes. Principles and practice of managing soil borne plant pathogens, APS Press,

St. Paul, Minnesota, USA, 134-152.

Kim, J. H., Mahoney, N., Chan, K. L., Campbell, B. C., Haff, R. P., & Stanker, L. H. (2014). Use

of benzo analogs to enhance antimycotic activity of kresoxim methyl for control of

aflatoxigenic fungal pathogens. Global health issues of aflatoxins in food and agriculture:

Challenges and opportunities, 96.

Kim, J. H., & Chan, K. L. (2014). Augmenting the antifungal activity of an oxidizing agent with

kojic acid: Control of Penicillium strains infecting crops. Molecules, 19(11), 18448-

18464.

Kim, J. H., Campbell, B. C., Mahoney, N., Chan, K. L., & Molyneux, R. J. (2011).

Chemosensitization of aflatoxigenic fungi to antimycin A and strobilurin using

salicylaldehyde, a volatile natural compound targeting cellular antioxidation system.

Mycopathologia, 171(4), 291-298.

Kim, J. H., Chan, K. L., & McConnell, L. L. (2015). Augmenting the efficacy of fungal and

mycotoxin control via chemosensitization. Outlooks on Pest Management, 26(4), 171-

175.

Koeck, M., Hardham, A. R., & Dodds, P. N. (2011). The role of effectors of biotrophic and hemibiotrophic fungi in infection. Cellular microbiology, 13(12), 1849-1857.

Kyu-Ho, B. A. N. G., Dong-Won, L. E. E., Hee-Moon, P. A. R. K., & Young-Ha, R. H. E. E. (2000). Inhibition of fungal cell wall synthesizing enzymes by trans-cinnamaldehyde. Bioscience, biotechnology, and biochemistry, 64(5), 1061-1063.

Lee, H. S., & Ahn, Y. J. (1998). Growth-inhibiting effects of Cinnamomum cassia bark-derived materials on human intestinal bacteria. Journal of Agricultural and Food Chemistry, 46(1), 8-12.

Lendenmann, M. H., Croll, D., & McDonald, B. A. (2015). QTL mapping of fungicide sensitivity reveals novel genes and pleiotropy with melanization in the pathogen Zymoseptoria tritici. Fungal Genetics and Biology, 80, 53-67.

Letschert, JPW, Lange W, Frese L, Van Den Berg RG. (1994). Taxonomy of *Beta* Section *Beta*. Journal of Sugar Beet Research 31: 69-85.

Levin, D. E. (2005). Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiology and molecular biology reviews, 69(2), 262-291.

Luck, J. E., & Gillings, M. R. (1995). Rapid identification of benomyl resistant strains of Botrytis cinerea using the polymerase chain reaction. Mycological Research, 99(12), 1483-1488.

Ma, Z., and Michailides, T. J. (2005). Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.

Mangin, B., Sandron, F., Henry, K., Devaux, B., Willems, G., Devaux, P., and Goudemand, E. (2015). Breeding patterns and cultivated beets origins by genetic diversity and linkage disequilibrium analyses. Theoretical and Applied Genetics, 128:2255-2271.

Meriggi, P., Rosso, F., Ioannides, P.M. and Ayala Garcia, J. (2000) Fungicide treatments against Cercospora leaf spot in sugar beet (Beta vulgaris L.). Adv. Sugar Beet Res. IIRB, 2, 77–102.

Pinto, E., Pina-Vaz, C., Salgueiro, L., Gonçalves, M. J., Costa-de-Oliveira, S., Cavaleiro, C.& Martinez-de-Oliveira, J. (2006). Antifungal activity of the essential oil of Thymus pulegioides on Candida, Aspergillus and dermatophyte species. *Journal of medical microbiology*, *55*(10), 1367-1373.

Reijo, R. A., Cooper, E. M., Beagle, G. J., & Huffaker, T. C. (1994). Systematic mutational analysis of the yeast beta-tubulin gene. Molecular biology of the cell, 5(1), 29-43.

Rodrigues, A. P. D., Farias, L. H. S., Carvalho, A. S. C., Santos, A. S., do Nascimento, J. L. M., & Silva, E. O. (2014). A novel function for kojic acid, a secondary metabolite from Aspergillus fungi, as antileishmanial agent. PloS one, 9(3), e91259.

Rupel, E. G., and Scott, P. R. (1974). Strains of Cercospora resistant to benomyl in the U.S.A. Plant Dis. Rep. 58:434-436.

Saccardo, P.A. (1876) Fungi Veneti novi vel critici. Series V. Nuovo GiornaleBot. Italiano, 8, 162–211.

Secor, G. A., Rivera, V. V., Khan, M. F. R., & Gudmestad, N. C. (2010). Monitoring fungicide sensitivity of Cercospora beticola of sugar beet for disease management decisions. Plant Disease, 94(11), 1272-1282.

Shabbits, J. A., Hu, Y., & Mayer, L. D. (2003). Tumor Chemosensitization Strategies Based on

    Apoptosis Manipulations1. Molecular cancer therapeutics, 2(8), 805-813.

Shreaz, S., Wani, W. A., Behbehani, J. M., Raja, V., Irshad, M., Karched, M. & Hun, L. T.

    (2016). Cinnamaldehyde and its derivatives, a novel class of antifungal agents.

    Fitoterapia, 112, 116-131.

Sisler, H. D. (1988). Fungicidal action and fungal resistance mechanisms.

Stallknecht, G. F., & Calpouzos, L. (1968). Fungicidal action of triphenyl tin hydroxide toward

    Cercospora beticola on sugar beet leaves. Phytopathology, 58(6), 788-+.

Steinkamp, M.P., Martin, S.S., Hoefert, L.L. and Ruppel, E.G. (1979) Ultrastructure of lesions

    produced by Cercospora beticola in leaves of Beta vulgaris. Physiol. Plant Path. 15, 13–

    26.

Usta, J., Kreydiyyeh, S., Barnabe, P., Bou-Moughlabay, Y., & Nakkash-Chmaisse, H. (2003).

    Comparative study on the effect of cinnamon and clove extracts and their main

    components on different types of ATPases. Human & experimental toxicology, 22(7),

    355-362.

United States Department of Agriculture Economic Research Service

Weiland, J. J. (2001). Light-induced secretion of esterase by Cercospora beticola Sacc. In 10th

    International Congress of Molecular Plant Microbe Interaction. St Paul MN: IS-MPMI.

Weiland, J., & Koch, G. (2004). Sugarbeet leaf spot disease (Cercospora beticola Sacc.).

    Molecular plant pathology, 5(3), 157-166.

Windels, C.E., Lamey, H.A., Hilde, D., Widner, J. and Knudsen, T. (1998) A Cercospora leaf

    spot model for sugar beet: in practice by an industry. Plant Dis. 82, 716–726.

Winner, C. (1993). Genetics and breeding. In: D.A. Cooke, R.K. Scott (Eds.), The Sugar Beet
Crop (pp.1-32). Chapman & Hall, London.

Xie, X. M., Fang, J. R., & Xu, Y. (2004). Study of antifungal effect of cinnamaldehyde and citral
on Aspergillus flavus. Food Science, 9(3).

Yen, T.B., and Chang, S.T.(2008).Synergistic effects of cinnamaldehyde in combination with
eugenol against wood decay fungi. Bioresour. Technol. 99, 232–236.

Zhou, R., Kaneko, S. I., Tanaka, F., Kayamori, M., & Shimizu, M. (2014). Robust Detection of
Cercosporo Leaf Spot of Sugar Beet in Real Field. In *2014 Montreal, Quebec Canada
July 13–July 16, 2014* (p. 1). American Society of Agricultural and Biological Engineers.

AUGMENTING FUNGICIDAL ACTIVITY OF TETRACONAZOLE WITH CHEMOSENSITIZATION AGENTS FOR CERCOSPORA LEAF SPOT MANAGEMENT

Abstract

Cercospora leaf spot (CLS), caused by *Cercospora beticola*, is one of the most destructive foliar diseases of sugar beet. Management strategies for CLS rely on timely fungicide application including fungicides in the sterol demethylation inhibitor (DMI) class. Reliance on DMIs has led to the onset of reduced sensitivity in *C. beticola* populations. New strategies to manage DMI-resistant strains of *C. beticola* are necessary for disease management. Co-application of certain natural compounds may increase efficacy of DMIs, through a process called chemosensitization. Chemosensitization agents (CAs) alter susceptibility of target cells that have become resistant to a therapeutic agent. In agricultural settings, CAs have been shown to increase the susceptibility of fungal pathogens to fungicides. In this study, thymol, octyl gallate, cinnamaldehyde, salicylaldehyde, kojic acid, carpropamid, fenoxanil, 2-hydroxy-4-methoxybenzaldehyde (2H4), and 3,5- dimethoxy-4-hydroxy-acetophenone (3,5 DH) were tested for chemosensitization activity. Seven strains of *C. beticola* with high resistance (EC$_{50}$ >10.0 μg ml^{-1}) to the DMI fungicide tetraconazole and three tetraconazole-sensitive strains (EC$_{50}$ <0.1 μg ml^{-1}) collected in ND and MN were used in this study. Strains were grown in non-amended media, media amended with 1.0 μg ml^{-1} tetraconazole , and media amended with 1.0 μg ml^{-1} tetraconazole and a CA at varying concentrations (10.0, 1.0, 0.1, and 0.01 mM). The addition of fenoxanil, kojic acid, and 3,5 DH did not significantly reduce growth of tetraconazole-resistant strains when co-applied with tetraconazole. However, octyl gallate (10.0, 1.0, and 0.1 mM), thymol (10.0 and 1.0 mM), salicylaldehyde (10.0 and 1.0 mM), cinnamaldehyde (10.0 and 1.0

mM), and 2H4 (10.0 and 1.0) significantly reduced growth of tetraconazole-resistant strains when co-applied with tetraconazole. Taken together, this study suggests that certain CAs are potential management tools for CLS.

Introduction

Cercospora leaf spot, caused by the fungus *Cercospora beticola* (Sacc.), is one of the most destructive foliar disease of sugar beet in the world. The fungus over-winters as stroma in leaf debris (Weiland, 2004). Sporulation occurs from overwintered stromata and spores are delivered to leaf surfaces by wind and water splash. Spores germinate and enter through stomata where hyphae grow intracellularly. Conidia are produced in the center of the necrotic tissue and can be dispersed by rain splash or wind to initiate another disease cycle as long as conditions are conducive. Hot and humid conditions are favorable for disease. Management strategies for Cercospora leaf spot include crop rotation and use of resistant varieties, but the disease is managed most effectively when combined with timely fungicide applications (Kaiser, 2010).

The sterol demethylation inhibitors (DMIs) are one of the most important chemistries in the fungicide repertoire. This group of fungicides act by inhibiting cytochrome P450-dependent sterol 14α-demthylase (CYP51), which is required for biosynthesis of ergosterol in the cell membrane (Brent, 1995). DMIs gained popularity due to their protective and curative nature along with low phytotoxicity (Sanssene, 2011). However, DMI-resistant isolates have been increasing in field populations (Bolton et al., 2012; Secor et al., 2010). The most common resistance mechanisms to DMI fungicides include (i) mutations in the *CYP51* gene that avoid inhibition while maintaining function, (ii) overexpression of the *Cyp51* gene leading to an excess of DMI target, and (iii) reduction of concentration within the cell by means of increased ATP-binding cassette or major facilitator superfamily transporter efflux activity (Ma, 2005). DMI-

resistant *C. beticola* isolates exhibit a higher native expression of *Cyp51* compared to sensitive strains, which is further induced in the presence of the DMI fungicide tetraconazole (Bolton et al., 2012; Bolton et al., 2016).

With resistance on the rise, new management practices are necessary. One potential strategy to augment fungicide efficacy is through the use of chemosensitization agents (CAs). CAs were originally introduced as a means to render resistant cancer cells more sensitive to anticancer drugs. The resistance mechanisms to anticancer drugs are almost parallel to those developed by fungal pathogens against fungicides. For example, cancer cells develop resistance through mutations of target enzyme genes, over-expression of target enzymes, up-regulation of genes controlling efflux pumps, production of enzymes that detoxify the anticancer drugs, and DNA repair (Shabbits et al., 2003). In agriculture settings, CAs could function by rendering a resistant isolate to be sensitive by debilitating or stressing the pathogen so that the primary fungicide is once again effective. CAs may have minimal antifungal activity alone, but when co-applied with commercial fungicides, they function as synergists that affect the target pathogen in a way that it becomes more sensitive to the commercial fungicide (Campbell et al, 2012). This may increase efficacy of commercial fungicides and possibly reduce the selection pressure for resistant isolates in field populations. Chemosensitization has been tested on different pathogens with several different CAs. For example, the CA octyl gallate (OG) was co-applied with kresoxim methyl to enhance the sensitivity of *Aspergillus parasiticus* and *A. flavus* (Kim et al., 2014). The CAs thymol and salicylaldehyde increased efficacy of azoxystrobin, kresoxim-methyl, difenoconazole, and tebuconazole for management of *Stagonospora nodorum* and *A. flavus* (Dzhavakhiya et al., 2012; Kim et al., 2011). Natural compounds such as alkaloid, berberine, and several phenolic compounds have been shown to enhance antifungal activity of

fludioxonil against *A. flavus* (Kim et al., 2007). In this study, CAs were screened for their ability to increase tetraconazole efficacy to better manage DMI-resistant and DMI-sensitive strains of *C. beticola*.

Materials and Methods

Sample Collection, Fungal Isolation, and Clonal Correction

Sugar beet leaves with CLS symptoms were received from commercial fields of American Crystal Sugar Company, Minn-Dak Farmers Cooperative, and Southern Minnesota Beet Sugar Cooperative, which represent all sugar beet production areas in Minnesota and eastern North Dakota in 2015. Leaves were processed immediately upon arrival at the laboratory. Conidia were harvested by applying 50 µl of Tween water (T-water; 0.06% (vol/vol) Tween 20 (Sigma-Aldrich, St. Louis, MO) and 0.02% (wt/vol) filter-sterilized ampicillin added after the solution had been autoclaved) to a *C. beticola* leaf spot. The leaf spot was gently scraped with a pipette tip to dislodge conidia into the T-water. The conidia-laden T-water was collected and transferred to water agar plates (1.5% (w/v) agar (BD, Franklin Lakes, USA), 0.02% (w/v) filter-sterilized ampicillin added after the solution had been autoclaved) for 24 h at 22°C (±1°C) to initiate conidia germination. One germinated conidium was transferred to a clarified V8-medium plate (10% (v/v) clarified V8 juice (Campbell's Soup Co., Camden, USA), 0.5% (w/v) $CaCO_3$, and 1.5% (w/v) agar (BD)) and was incubated at 25°C for two weeks. This single spore-derived colony was the source inoculum plate for subsequent fungicide sensitivity assays.

EC_{50} calculations were carried out as described by Secor and Rivera (2012). Briefly, agar plugs (5 mm each) were taken from the leading edge of colony growth of the original non-amended CV8-agar plates described above and place on CV8-agar plates amended with serial ten-fold dilutions of tetraconazole from 0.001 to 1.0 µg ml^{-1}. After 15 days, inhibition of radial

growth was measured and compared to growth on non-amended medium and was used to calculate an EC_{50} value for each isolate.

Field isolates were clone-corrected using simple sequence repeat (SSR) markers to insure that selected isolates were not clonal. Fifty *C. beticola* isolates with a range of EC_{50} values to tetraconazole were selected for further analysis. Eight polymorphic markers were amplified in two multiplex reactions as described by Vaghefi et al. (2017). Briefly, multiplex mixes 1 and 2 were pooled together into one bin for capillary electrophoresis. The eight polymorphic markers were divided into two multiplex PCR sets each containing four primer pairs. Multiplex reaction were performed in 15 µl volumes consisting of 10 ng template DNA, 3µL of the Multiplex PCR 5x Master Mix (New England BioLabs Inc. Ipswich, MA), and the corresponding fluorescent-labeled primers. Fragment analysis and allele calling were performed to identify clones. In total, seven unique *C. beticola* genotypes that exhibited EC_{50} values greater than 10.0 µg ml^{-1} were selected and deemed DMI-resistant. Likewise, three unique genotypes with EC_{50} value less than 0.1 µg ml^{-1} were selected and deemed DMI-sensitive.

The selected ten isolates were grown on CV8 agar plates for 10 days at 25˚C under constant light. Colonies were scraped with a microscope slide to dislodge the spores allowing them to become suspended in the T-water. The spore suspension was poured onto a fresh CV8 plate. Spore plates were incubated for three days at 25˚C with 24 hours of light to induce sporulation. After the incubation period, 3 ml of liquid Fries media (Friesen and Faris, 2012) was added to the plates and were gently scraped with a slide to liberate spores from the media. The spore suspension was then adjusted to 10^3 ml^{-1} for the chemosensitization assay.

Fungicide and Candidate Chemosensitization Agents

Technical grade tetraconazole, the active ingredient in Minerva (Sipcam Agro, Durham, NC), was used in the chemosensitization assay as the CA partner (Sigma-Aldrich).

The chemosensitization agents used in this experiment were cinnamaldehyde (3-phenylprop-2-enal), salicylaldehyde (2-hydroxybenzaldehyde), kojic acid (2-hydroxymethyl-5-hydroxy-Ɣ- pyrone), octyl gallate, thymol (2-isopropyl-5-methylphenol), carpropamid (2,2-Dichloro-N-[1-(4-chlorophenyl)ethyl]-1-ethyl-3-methylcyclopropanecarboxamide), fenoxanil (N-(1-Cyano-1,2-dimethylpropyl)-2-(2,4-dichlorophenoxy)propionamide), 3-5-dimethoxy-4-hydroxy-acetophenone (3,5 DH), and 2-hydroxy-4-methoxybenzaldehyde (2H4) were obtained from Sigma-Aldrich.

Chemosensitization Bioassay

To study the influence of CAs on isolates of *C. beticola* and on their sensitivity to tetraconazole, the fungus was grown in fries media supplemented with different concentrations of the CAs, fungicide alone, or in combination of one another. To test whether candidate CAs increase tetraconazole efficacy, approximately 10^3 spores were collected from each isolate and 98 μL of spore suspension was transferred to 12 individual wells (one row) of a Nunc 96 well flat-bottom plate (Fisher Scientific, Hampton, NH) using Fries media as the growth medium. The top row contained media with no spores and subsequent rows contained spores of appropriate *C. beticola* isolates. CAs were dissolved in ethanol at concentrations of 1.0 M, 100.0 mM, 10.0 mM, and 1.0 mM before being added to wells. Each well was amended with tetraconazole (0.1 μg ml^{-1}), a CA to a final concentration of 10.0, 1.0, 0.1, and 0.01 mM, or each of the components combined. A water control of unamended media was included in each assay. The plates were

sealed with SealPlate (Excel Scientific, Victorville, CA) before absorbance measurements were

taken. Cultures were incubated at 21°C under 24 h light conditions for 5 days.

Growth was quantified daily by collecting absorbance values from each well generated

from a GENios plate reader (Tecan) using XFLUOR4 software (V 4.51) using a measurement

wavelength of 595 nm (OD_{595}) and 2x2 reads per well after shaking the plate briefly. The

absorbance value and associated standard deviation and error for each isolate was determined by

taking the average of 12 wells (replications) per time point. Each experimental run consisted of

five time points, which were taken every 24 h starting with day zero up to and including day

four. Day zero OD_{595} measurements were taken immediately after treatments were added to each

well. Fungal growth was calculated by subtracting the absorbance value of day zero and fries

values from each time point.

Statistical Treatment

Each experiment on fungal growth in the presence of CAs, tetraconazole, or their

combinations was performed in replications of 12 wells per isolate. Each experiment was

repeated once. Growth reduction was determined as a percentage of growth of treated fungi

based on average fungal growth absorbance values compared to growth in unamended media.

Means of different treatments, SE and SD, and significant differences ($p < 0.1$) of means among

treatments and controls were determined using a *t*-test for independent variables.

Results

Clone Correction

Isolates were clone corrected using SSR markers and only non-clonal isolates were

selected for further analysis. Seven DMI-resistant and three DMI-sensitive isolates were used in

determining which co-applications of CAs and tetraconazole resulted in substantial growth

reduction. The differences in growth reduction among the seven tetraconazole-resistant isolates were not significant ($p < 0.1$). Likewise, the differences in growth reduction among the three tetraconazole-sensitive isolates were not significant ($p < 0.1$). There were no significant differences between experimental runs ($p < 0.1$). Therefore, data from one representative experiment is shown.

CAs Targeting Oxidative Stress Response Pathways

CAs were divided into three functional groups based on their putative modes of action. The first group of CAs (octyl gallate, salicylaldehyde, 2H4, and 3,5 DH) are thought to target oxidative stress response pathways (Kim et al., 2014; Dzhavakhiya et. al., 2012). The second functional group of CAs (cinnamaldehyde and thymol) are suggested to target the integrity of the fungal cell wall (Shreaz et al., 2016; Pinto et al., 2006). The last group of CAs (kojic acid, fenoxanil, and carpropamid) have been shown to target melanin biosynthesis in the cell wall (Bentley et al., 2006; Kurahashi 2001).

When octyl gallate was applied alone, it generally caused reduced growth in both tetraconazole-sensitive and -resistant strains concomitant with increasing concentrations of octyl gallate (Table 1). When octyl gallate was co-applied with tetraconazole to resistant strains, there was no increased growth reduction compared to tetraconazole alone until it was applied at concentrations of 1.0 and 10.0 mM where it caused a 100% growth reduction (Table 1). When octyl gallate was co-applied with tetraconazole to sensitive strains, there was 100% growth reduction in all concentrations tested (Table 1).

31

Table 1. Fungicidal effects of octyl gallate, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

Treatment[1]	% Growth reduction (St. dev.)[2]	
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 μg ml^{-1})	81.6 (4.0)	54.0 (3.7)
OG (0.01 mM)	25.5 (5.2)	20.5 (7.6)
OG (0.1 mM)	56.5 (7.5)	44.5 (8.9)
OG (1.0 mM)	55.0 (5.5)	45.3 (6.3)
OG (10.0 mM)	58.2 (5.6)	50.4 (7.8)
Tet + OG (0.01 mM)	100.0 (6.6)	40.0 (5.7)
Tet + OG (0.1 mM)	100.0 (8.1)	54.5 (1.2)
Tet + OG (1.0 mM)	100.0 (5.5)	100.0 (5.5)
Tet + OG (10.0 mM)	100.0 (5.6)	100.0 (5.6)

[1] Treatments used in this study: tetraconazole (Tet) and octyl gallate (OG).
[2] Standard deviation of means among treatments and controls were determined using *t*-test for independent variables.

There was no growth reduction in tetraconazole-sensitive or -resistant isolates when salicylaldehyde was applied at 0.01 mM (Table 2). Increasing concentrations of salicylaldehyde caused a corresponding and similar increase in growth reduction in both tetraconazole-sensitive and -resistant strains when applied alone (Table 2). When salicylaldehyde was co-applied with tetraconazole, there was no increase in growth reduction compared to tetraconazole alone until it was applied at concentrations of 1.0 and 10.0 mM in tetraconazole-resistant strains (Table 2). When salicylaldehyde was co-applied with tetraconazole to sensitive strains, there was a 100% growth reduction in all concentrations tested (Table 2).

Table 2. Fungicidal effects of salicylaldehyde, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

Treatment[1]	% Growth reduction (St. dev.)[2]	
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 µg ml^{-1})	81.6 (4.0)	54.0 (3.7)
SA (0.01 mM)	0.0 (2.4)	0.0 (3.2)
SA (0.1 mM)	25.5 (2.3)	27.7 (4.6)
SA (1.0 mM)	30.0 (2.5)	36.2 (1.5)
SA (10.0 mM)	65.3 (5.4)	61.9 (6.7)
Tet + SA (0.01 mM)	100.0 (3.9)	32.7 (5.2)
Tet + SA (0.1 mM)	100.0 (4.7)	46.7 (6.0)
Tet + SA (1.0 mM)	100.0 (2.3)	97.3 (1.9)
Tet + SA (10.0 mM)	100.0 (5.4)	100.0 (2.6)

[1] Treatments used in this study: tetraconazole (Tet) and salicylaldehyde (SA),
[2] Standard deviation of means among treatments and controls were determined using t-test for independent variables.

When 2H4 was applied alone, it caused an increase in growth reduction in both tetraconazole-sensitive and -resistant strains concomitant with increasing concentrations of 2H4 (Table 3). When 2H4 was co-applied with tetraconazole to resistant strains, there was no increase in growth reduction compared to tetraconazole alone until it was applied at concentrations of 1.0 and 10.0 mM (Table 3). All concentrations of 2H4 when co-applied with tetraconazole caused a 100% reduction in growth in tetraconazole-sensitive strains (Table 3).

Table 3. Fungicidal effects of 2H4, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

Treatment[1]	% Growth reduction (St. dev.)[2]	
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 µg ml^{-1})	81.6 (4.0)	54.0 (3.7)
2H4 (0.01 mM)	10.5 (9.8)	12.8 (7.8)
2H4 (0.1 mM)	15.8 (8.8)	18.6 (7.9)
2H4 (1.0 mM)	22.6 (6.5)	20.6 (6.8)
2H4 (10.0 mM)	65.5 (6.9)	60.5 (7.8)
Tet + 2H4 (0.01 mM)	100.0 (6.0)	16.7 (8.4)
Tet + 2H4 (0.1 mM)	100.0 (8.3)	15.0 (7.5)
Tet + 2H4 (1.0 mM)	100.0 (6.1)	83.6 (2.0)
Tet + 2H4 (10.0 mM)	100.0 (6.1)	86.7 (5.8)

[1] Treatments used in this study: tetraconazole (Tet) and 2-hydroxy-4- methoxy benzaldehyde (2H4).
[2] Standard deviation of means among treatments and controls were determined using t-test for independent variables.

The candidate CA 3,5 DH was not soluble at 10.0 mM, so only data from 0.01 to 1.0 mM is presented. Increasing concentrations of 3,5 DH caused a corresponding and similar increase in growth reduction in both tetraconazole-sensitive and -resistant strains when applied alone (Table 4). When 3,5 DH was co-applied with tetraconazole, there was no increase in growth reduction compared to tetraconazole alone (Table 4). There was a 100% reduction in growth among sensitive isolates when 3,4 DH was applied at all concentrations with tetraconazole (Table 4).

Table 4. Fungicidal effects of 3,5 DH, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

Treatment[1]	% Growth reduction (St. dev.)[2]	
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 µg ml^{-1})	81.6 (4.0)	54.0 (3.7)
3,5 DH (0.01 mM)	20.6 (6.7)	18.7 (6.6)
3,5 DH (0.1 mM)	27.8 (3.4)	30.2 (7.4)
3,5 DH (1.0 mM)	26.5 (4.5)	25.3 (7.2)
Tet + 3,5 DH(0.01mM)	100.0 (6.8)	21.6 (1.8)
Tet + 3,5 DH (0.1 mM)	100.0 (7.1)	19.8 (6.7)
Tet + 3,5 DH (1.0 mM)	100.0 (5.5)	25.0 (5.5)

[1] Treatments used in this study: tetraconazole (Tet) and 3-5-dimethoxy-4-hydroxy-acetophenone.
[2] Standard deviation of means among treatments and controls were determined using t-test for independent variables.

CAs Targeting Cell Wall Integrity

When cinnamaldehyde was applied alone, it caused no growth reduction at 0.01 mM but generally caused an increase in growth reduction in both tetraconazole-sensitive and -resistant strains with increasing cinnamaldehyde concentrations from 0.1 to 10.0 mM (Table 5). When cinnamaldehyde was co-applied with tetraconazole to resistant strains, there was no increase in growth reduction compared to tetraconazole alone until it was applied at concentrations of 1.0 and 10.0 mM (Table 5). When cinnamaldehyde was co-applied with tetraconazole to sensitive strains, there was 100% growth reduction in all concentrations tested (Table 5).

Table 5. Fungicidal effects of cinnamaldehyde, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

	% Growth reduction (St. dev.)[2]	
Treatment[1]	**Tetraconazole-sensitive**	**Tetraconazole-resistant**
Tet (0.1 μg ml^{-1})	81.6 (4.0)	54.0 (3.7)
CN (0.01 mM)	0.0 (7.4)	0.0 (8.7)
CN (0.1 mM)	15.9 (10.1)	0.0 (9.9)
CN (1.0 mM)	40.9 (9.4)	45.5 (7.6)
CN (10.0 mM)	65.1 (9.5)	60.7 (6.5)
Tet + CN (0.01 mM)	100.0 (5.1)	46.8 (8.3)
Tet + CN (0.1 mM)	100.0 (6.0)	42.2 (6.8)
Tet + CN (1.0 mM)	100.0 (5.2)	100.0 (3.8)
Tet + CN (10.0 mM)	100.0 (5.0)	100.0 (2.9)

[1]Treatments used in this study: tetraconazole (tet) and cinnamaldehyde (CN).
[2]Standard deviation of means among treatments and controls were determined using t-test for independent variables.

There was a general increase in growth reduction in both tetraconazole-sensitive and -resistant strains with increasing concentrations of thymol (Table 6). When thymol was co-applied with tetraconazole to resistant strains, growth reduction was not more than tetraconazole alone until it was applied at concentrations of 1.0 and 10.0 mM (Table 6). Thymol co-applied with tetraconazole to sensitive strains resulted in complete growth reduction (Table 6).

Table 6. Fungicidal effects of thymol, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

Treatment[1]	% Growth reduction (St. dev.)[2]	
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 μg ml^{-1})	81.6 (4.0)	54.0 (3.7)
Thy (0.01 mM)	18.7 (5.1)	14.0 (6.7)
Thy (0.1 mM)	35.3 (7.1)	34.9 (4.6)
Thy (1.0 mM)	59.1 (6.9)	49.6 (3.6)
Thy (10.0 mM)	67.9 (3.6)	65.2 (5.8)
Tet +Thy (0.01 mM)	100.0 (5.4)	35.3 (6.0)
Tet + Thy (0.1 mM)	100.0 (6.7)	31.1 (9.5)
Tet + Thy (1.0 mM)	100.0 (8.0)	87.3 (6.3)
Tet + Thy (10.0 mM)	100.0 (8.4)	96.6 (2.8)

[1]Treatments used in this study: tetraconazole (Tet) and thymol (Thy).
[2]Standard deviation of means among treatments and controls were determined using *t*-test for independent variables.

CAs Targeting Melanin Biosynthesis

The candidate CA kojic acid would not go into solution to test at 10.0 mM, so only data from 0.01 to 1.0 mM is presented. When kojic acid was applied alone, it generally caused an increase in growth reduction in both tetraconazole-sensitive and resistant strains as concentrations increased (Table 7). When kojic acid was co-applied with tetraconazole to resistant strains, no concentration tested caused an increase in growth reduction compared to tetraconazole alone (Table 7). Kojic acid co-applied with tetraconazole to sensitive strains caused 100% growth reduction at concentrations higher than 0.01 mM (Table 7).

Table 7. Fungicidal effect of kojic acid, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

| Treatment[1] | % Growth reduction (St. dev.)[2] | |
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 µg ml^{-1})	81.6 (4.0)	54.0 (3.7)
KA (0.01 mM)	12.3 (7.4)	15.1 (4.5)
KA (0.1 mM)	10.9 (5.5)	12.4 (6.7)
KA (1.0 mM)	27.6 (10.1)	25.9 (9.9)
Tet + KA (0.01 mM)	100.0 (7.6)	42.5 (8.4)
Tet + KA (0.1 mM)	100.0 (2.9)	52.7 (4.7)
Tet + KA (1.0 mM)	100.0 (7.0)	50.3 (3.6)

[1]Treatments used in this study: tetraconazole (Tet) and kojic acid (KA)
[2]Standard deviation of means among treatments and controls were determined using *t*-test for independent variables.

The candidate CA fenoxanil would not go into solution to test at 10.0 mM, so only data from 0.01 to 1.0 mM is presented. When fenoxanil was applied alone, it generally caused an increase in growth reduction in both tetraconazole-sensitive and resistant strains as concentrations increased (Table 8). When fenoxanil was co-applied with tetraconazole to resistant or sensitive strains, no concentration tested caused an increase in growth reduction compared to tetraconazole alone (Table 8).

Table 8. Fungicidal effect of fenoxanil, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

| Treatment[1] | % Growth reduction (St. dev.)[2] | |
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 µg ml^{-1})	81.6 (4.0)	54.0 (3.7)
FN (0.01 mM)	12.3 (7.8)	17.1 (5.6)
FN (0.1 mM)	26.9 (7.0)	30.2 (8.9)
FN (1.0 mM)	30.9 (7.2)	25.3 (5.2)
Tet + FN (0.01 mM)	45.5 (6.4)	27.6 (4.0)
Tet + FN (0.1 mM)	58.5 (8.7)	33.1 (6.0)
Tet + FN (1.0 mM)	60.5 (6.6)	39.1 (3.8)

[1]Treatments used in this study: tetraconazole (Tet) and fenoxanil (FN).
[2]Standard deviation of means among treatments and controls were determined using *t*-test for independent variables.

The candidate CA carpropamid would not go into solution to test at 10.0 mM, so only data from 0.01 to 1.0 mM is presented. When carpropamid was applied alone, it generally caused an increase in growth reduction in both tetraconazole-sensitive and resistant strains as concentrations increased (Table 9). When carpropamid was co-applied with tetraconazole to resistant strains, no concentration tested caused a significant increase in growth reduction compared to tetraconazole alone (Table 9). When carpropamid was co-applied with tetraconazole to sensitive strains, only carpropamid applied at 0.01 and 0.1 mM caused an increase in growth reduction greater than tetraconazole alone (Table 9).

Table 9. Fungicidal effect of carpropamid, tetraconazole, and their combination on *C. beticola* growth *in vitro*.

Treatment[1]	% Growth reduction (St. Dev.)[2]	
	Tetraconazole-sensitive	Tetraconazole-resistant
Tet (0.1 μg ml^{-1})	81.6 (4.0)	54.0 (3.7)
Carp (0.01 mM)	16.5 (2.0)	10.5 (1.9)
Carp (0.1 mM)	19.7 (2.9)	13.9 (7.7)
Carp (1.0 mM)	32.1 (5.5)	40.4 (4.9)
Tet + Carp (0.01 mM)	94.9 (8.7)	33.7 (4.0)
Tet + Carp (0.1 mM)	89.5 (11.3)	30.4 (3.8)
Tet + Carp (1.0 mM)	71.1 (6.6)	33.6 (7.8)

[1]Treatments used in this study: tetraconazole (Tet) and carpropamid (Carp).
[2]Standard deviation of means among treatments and controls were determined using *t*-test for independent variables.

Discussion

Crop protection using timely fungicide applications is a powerful tool for effective management of plant pathogens. With extensive and repeated use of fungicides belonging to the same class however, reduced sensitivity has become an increasing problem in disease management. DMI fungicides have been widely used to manage Cercospora leaf spot. Reduced sensitivity to DMI fungicides have been documented in *C. beticola* field isolates (Secor et al., 2010; Bolton et al., 2012). New practices are necessary to improve disease management. Some

of these tactics may include breeding for enhanced disease resistance, using beneficial microbes, or using fungicide tank mixes to better manage *C. beticola*.

In this study, we sought to identify CA/tetraconazole combinations that would render tetraconazole-resistant *C. beticola* strains more sensitive to the fungicide. To achieve this, we tested CAs that targeted different pathways of the pathogen identified from previous studies on plant and human fungal pathogens (Kim et al., 2010; Faria et al., 2011). We identified three CAs that improved tetraconazole efficacy that target the oxidative stress response pathways. The CAs salicyladehyde, octyl gallate, and 2H4 significantly increased the efficacy of tetraconazole in resistant strains (Table 1-3). In addition, the two CAs cinnamaldehyde and thymol that target cell wall integrity were identified that increased tetraconazole efficacy (Tables 5 and 6). Thymol, octyl gallate, cinnamaldehyde, 2H4, and salicylaldehyde caused nearly 100% growth reduction of both sensitive and resistant isolates at 1.0 mM concentration when co-applied with tetraconazole. These five CAs were shown to increase efficacy of the fungicides difenoconazole and tebuconazole against *Alternaria sp.*, *Fusarium culmorum*, and *Phoma glomerata* in previous studies (Dzhavakhiya et al., 2012; Kim et al., 2008, 2011, 2014).

Interestingly, thymol and cinnamaldehyde are naturally produced plant compounds that have been EPA-approved for food consumption. This may mean that not only are these compounds promising commercial CAs, but they may also be safe and have little environmental impact. Additionally, salicylaldehyde has been shown to mimic the action of salicylic acid, which is a key plant signaling molecule involved in plant immunity (Jones and Dangl, 2006). Consequently, this compound may not only make *C. beticola* more sensitive to tetraconazole, it may also induce plant immune response pathways. Salicylaldehyde was shown to have a

chemosensitization effect when tested on aflatoxigenic fungi *Aspergillus flavus* (Kim et al., 2010).

Not all CAs tested in this study had synergistic effects with tetraconzole. Fenoxinil, kojic acid, carpropamid, and 3,5 DH were compounds that had little to no effect on the efficacy of tetraconazole to reduce the growth of resistant *C. beticola* isolates. Kojic acid, fenoxanil, and carpropamid are melanin biosynthesis inhibitors (MBI). MBIs have been used to manage the pathogen *Magnaporthe oryzae* that causes rice blast disease (Kurahashi, 2001). Lendenmann et al. (2015) recently showed that increased melanin in the cell wall was associated with DMI resistance in *Zymoseptoria tritici*. Nonetheless, no MBI tested in our study enhanced tetraconazole efficacy. In fact, carpropamid had an antagonistic effect with tetraconazole where increasing concentrations concomitantly reduced tetraconazole efficacy (Table 9). MBIs may not have been effective on *C. beticola* because of different cellular components in the cell wall.

3,5 DH was also not effective as a CA when tested against *C. beticola*. However, other benzaldehydes have been shown to be promising CAs to manage *A. fumigatus* (Kim et al., 2011). Screening other benzaldehydes may result in positive chemosensitization in DMI-resistant isolates of *C. beticola*.

With no new fungicides on the horizon and efficacy of current fungicides decreasing, the discovery of new CAs may be important for the sugar beet industry. Co-application of CAs could result in a reduction of the number of fungicide applications. Another potential advantage of CAs is better management of fungicide-resistant mutants of *C. beticola*. A common way to manage resistance is to increase dosage of fungicides. However, this has likely increased the selection of highly resistant isolates. In our study, we demonstrated that CAs such as cinnamaldehyde and salicylaldehyde reduced growth by 100% when combined with tetraconazole. This could lead to

lowering tetraconazole dosage and making current application rates more effective. Increased efficacy could lead to reducing the number of applications in a growing season. In addition, the relatively low cost of CAs that may reduce the overall cost of chemical treatments would be a very attractive management strategy from the agricultural economic viewpoint.

Future research will be directed towards identifying adjuvants for *in planta* CA applications that maintain or promote the chemosensitization activity identified in this study. The identification of the appropriate surfactant may increase the solubility of CAs that are not soluble at the highest concentrations attempted in this study. In addition, studies directed towards identifying combinations of CAs that enhance tetraconazole efficacy will be carried out. Finally, CAs will be tested for synergistic activity for other fungicide classes that currently have resistance issues. The ultimate outcome would be to find the optimal mixture of fungicide, CA, and adjuvant to allow for the best management of both resistant and sensitive *C. beticola* strains. Finding this mixture may lead to better management of Cercospora leaf spot at a lower economic and environmental impact.

References

Bentley, R. (2006). From miso, sake and shoyu to cosmetics: a century of science for kojic acid. *Natural product reports*, 23(6), 1046-1

Bolton, M. D., Rivera-Varas, V., del Río Mendoza, L. E., Khan, M. F., & Secor, G. A. (2012). Efficacy of variable tetraconazole rates against Cercospora beticola isolates with differing in vitro sensitivities to DMI fungicides. *Plant disease*, 96(12), 1749-1756.

Bolton, M. D., Ebert, M. K., Faino, L., Rivera-Varas, V., de Jonge, R., Van de Peer, Y., ... & Secor, G. A. (2016). RNA-sequencing of Cercospora beticola DMI-sensitive and-

resistant isolates after treatment with tetraconazole identifies common and contrasting pathway induction. *Fungal Genetics and Biology*, 92, 1-13.

Brent, K. J., & Hollomon, D. W. (1995). *Fungicide resistance in crop pathogens: How can it be managed?*. Brussels: GIFAP.

Campbell, B. C., Chan, K. L., & Kim, J. H. (2012). Chemosensitization as a means to augment commercial antifungal agents. Frontiers in microbiology, 3, 79.

Dzhavakhiya, V., Shcherbakova, L., Semina, Y., Zhemchuzhina, N., & Campbell, B. (2012). Chemosensitization of plant pathogenic fungi to agricultural fungicides. Frontiers in microbiology, 3.

Faria, N. C. G., Kim, J. H., Gonçalves, L. A. P., Martins, M. D. L., Chan, K. L., & Campbell, B. C. (2011). Enhanced activity of antifungal drugs using natural phenolics against yeast strains of Candida and Cryptococcus. *Letters in applied microbiology*, 52(5), 506-513.

Friesen, T. L., & Faris, J. D. (2012). Characterization of plant-fungal interactions involving necrotrophic effector-producing plant pathogens. *Plant Fungal Pathogens: Methods and Protocols*, 191-207.

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. *Nature*, 444(7117), 323-329.

Kaiser, U., Kluth, C., & Märländer, B. (2010). Variety-specific Epidemiology of Cercospora beticola Sacc. and Consequences for Threshold-based Timing of Fungicide Application in Sugar Beet. *Journal of phytopathology*, 158(4), 296-306.

Kim, J. H., Mahoney, N., Chan, K. L., Molyneux, R. J., May, G. S., & Campbell, B. C. (2008). Chemosensitization of fungal pathogens to antimicrobial agents using benzo analogs. *FEMS microbiology letters*, 281(1), 64-72.

Kim, J. H., Chan, K. L., Mahoney, N., & Campbell, B. C. (2011). Antifungal activity of redox-active benzaldehydes that target cellular antioxidation. *Annals of clinical microbiology and antimicrobials*, 10(1), 23.

Kim, J. H., Campbell, B. C., Mahoney, N., Chan, K. L., & Molyneux, R. J. (2011). Chemosensitization of aflatoxigenic fungi to antimycin A and strobilurin using salicylaldehyde, a volatile natural compound targeting cellular antioxidation system. Mycopathologia, 171(4), 291-298.

Kim, J. H., Mahoney, N., Chan, K. L., Campbell, B. C., Haff, R. P., & Stanker, L. H. (2014). Use of benzo analogs to enhance antimycotic activity of kresoxim methyl for control of aflatoxigenic fungal pathogens. Global health issues of aflatoxins in food and agriculture: Challenges and opportunities, 96.

Kurahashi, Y. (2001). Melanin biosynthesis inhibitors (MBIs) for control of rice blast. *Pesticide Outlook*, 12(1), 32-35.

Lendenmann, M. H., Croll, D., & McDonald, B. A. (2015). QTL mapping of fungicide sensitivity reveals novel genes and pleiotropy with melanization in the pathogen Zymoseptoria tritici. Fungal Genetics and Biology, 80, 53-67.

Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.

Pinto, E., Pina-Vaz, C., Salgueiro, L., Gonçalves, M. J., Costa-de-Oliveira, S., Cavaleiro, & Martinez-de-Oliveira, J. (2006). Antifungal activity of the essential oil of Thymus pulegioides on Candida, Aspergillus and dermatophyte species. *Journal of medical microbiology*, 55(10), 1367-1373.

Shabbits, J. A., Hu, Y., & Mayer, L. D. (2003). Tumor Chemosensitization Strategies Based on Apoptosis Manipulations1. Molecular cancer therapeutics, 2(8), 805-813.

Shreaz, S., Wani, W. A., Behbehani, J. M., Raja, V., Irshad, M., Karched, M. & Hun, L. T. (2016). Cinnamaldehyde and its derivatives, a novel class of antifungal agents. Fitoterapia, 112, 116-131.

Sanssené, J., Selim, S., Roisin-Fichter, C., Djerroud, L., Deweer, C., & Halama, P. (2011). Protective and curative efficacy of prothioconazole against isolates of Mycosphaerella graminicola differing in their in vitro sensitivity to DMI fungicides. *Pest management science*, 67(9), 1134-1140.

Secor, G. A., Rivera, V. V., Khan, M. F. R., & Gudmestad, N. C. (2010). Monitoring fungicide sensitivity of Cercospora beticola of sugar beet for disease management decisions. *Plant Disease*, 94(11), 1272-1282.

Secor, G. A., & Rivera, V. V. (2012). Fungicide resistance assays for fungal plant pathogens. *Plant fungal pathogens: Methods and protocols*, 385-392.

Steinkamp, M.P., Martin, S.S., Hoefert, L.L. and Ruppel, E.G. (1979) Ultrastructure of lesions produced by Cercospora beticola in leaves of Beta vulgaris. Physiol. Plant Path. 15, 13–26.

Vaghefi, N., Kikkert, J. R., Bolton, M. D., Hanson, L. E., Secor, G. A., & Pethybridge, S. J. (2017). De novo genome assembly of Cercospora beticola for microsatellite marker development and validation. *Fungal Ecology*, 26, 125-134.

Weiland, J., & Koch, G. (2004). Sugarbeet leaf spot disease (Cercospora beticola Sacc.). *Molecular plant pathology*, 5(3), 157-166.

OVERALL CONCLUSION

Overall the discovery of CAs that, when co-applied with a fungicide, increase the ability to manage *C. beticola* may be an important step in disease management. Cercospora leaf spot is the most destructive foliar pathogen and causes economic losses in the millions. Fungicides are the primary solution for managing this disease. DMI fungicides are widely used for Cercospora leaf spot management. Resistance to this fungicide in the *C. beticola* population has been increasing. Increased resistance has led to lower efficacy of tetraconazole and higher disease pressure. This has led researchers to discover new compounds that can help with efficacy of current fungicides and better control resistance.

CAs are co-applied with a fungicide to increases the fungicide efficacy. This idea was taken and applied to *C. beticola* and tetraconazole. Nine CAs were tested at various concentrations in combination with tetraconazole at a constant concentration *in vitro*. Thymol, cinnamaldehyde, salicylaldehyde, 2H4, and octyl gallate were compounds that increased the efficacy of tetraconazole when applied to both sensitive and resistant *C. beticola* isolates. Kojic acid, 3,5 DH, and fenoxinil were CA compounds that showed minimal chemosensitization properties. Carpropamid displayed antagonistic effects when combined with tetraconazole. The efficacy of tetraconazole decreased when carpropamid was co-applied. Discovering new CAs with diverse modes of action would greatly benefit the sugar beet industry.

CAs could be a potential tool to the ever changing disease management strategies in agriculture. The compounds are applied with pre-existing fungicides and work synergistically to increase the efficacy of the fungicide resulting in better disease management. Higher fungicide efficacy could lead to lower dosages and less applications of fungicides within the growing season. CAs are relatively inexpensive and less toxic than fungicides making them both

economic and environmentally safe. Fungicide resistance is another problem that CAs could possibly answer. This could result in lowering the resistant population and reducing the emergence of new fungicide resistance. Mixing other adjuvants (wetters, stickers, etc.) with the mix of CA and fungicides could also lead to higher fungicide efficacy. Finding the optimal mixture of all of these different components could substantially impact the agriculture industry.

Future work with this study would be to apply treatments in greenhouse studies to determine how the compounds react on the leaves surface. The final step would be to do field trials with large tank mixes and exposure to the natural environment. This study was just the first step to identify potential CA candidates and with more time and effort these compounds could lead to a better disease management for Cercospora leaf spot in sugar beet.