2021

# CHARACTERISTICS OF *CERCOSPORA NICOTIANAE* WITH REDUCED SENSITVITY TO AZOXYSTROBIN

William Barrett Barlow
*University of Kentucky*, wbarlow93@gmail.com
Author ORCID Identifier:
https://orcid.org/0000-0002-2422-2666
Digital Object Identifier: https://doi.org/10.13023/etd.2021.433

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

William Barrett Barlow, Student

Dr. Emily Pfeufer, Major Professor

Dr. Rick Bennett, Director of Graduate Studies

# CHARACTERISTICS OF *CERCOSPORA NICOTIANAE* WITH REDUCED SENSITIVITY TO AZOXYSTROBIN

---

THESIS

---

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky


By

William Barrett Barlow

Lexington, Kentucky

Co- Directors: Dr. Emily Pfeufer, Professor of Plant Pathology

and Dr. Kiersten Wise, Professor of Plant Pathology

Lexington, Kentucky


2021



https://orcid.org/0000-0002-2422-2666

ABSTRACT OF THESIS

Burley and dark tobacco production are important to agriculture in Kentucky, worth $145.1 and $94.4 million in 2020, respectively. The price received for a tobacco crop is influenced by leaf quality, determined by multiple characteristics including leaf damage. Frogeye leaf spot (FLS), caused by *Cercospora nicotianae,* has historically been a minor disease of tobacco. However, when FLS infections reach the upper canopy of tobacco, lesions are seen as damage that negatively influence leaf quality. Fungicides are regularly used to manage foliar diseases of tobacco, and the only systemic fungicide active ingredient labeled for tobacco is azoxystrobin. Azoxystrobin belongs to the quinol outside inhibitor (QoI) class of fungicides, named for the outer quinol binding site of the cytochrome $bc_1$ complex of the inner mitochondrial matrix. When azoxystrobin binds to the Qo site of cytochrome b (cytb), it stops the flow of electrons and halts the production of adenosine triphosphate (ATP). Azoxystrobin was first released for use in 1996, and in 1998 resistance was detected in *Erysiphe graminis* f.sp. *tritici* in Europe. Resistance to QoI fungicides has now been reported in over 50 phytopathogens. Resistance to QoI fungicides occurs as the result of single nucleotide polymorphisms (SNPs) in cytb that result in the substitution of phenylalanine for leucin at codon 129 (F129L), glycine for arginine at position 137 (G137R), and glycine for alanine at position 143 (G143A). The F129L and G137R mutations confer partial resistance, while G143A confers complete resistance to QoI fungicides. In 2017, QoI-resistant *C. nicotianae* was reported in Kentucky, with both F129L and G143A mutations detected in the population.

This research looks at the selectivity of azoxystrobin in *C. nicotianae* populations with mixed azoxystrobin sensitivity. By sampling FLS infected leaves we were able to identify cytochrome b mutations from recovered *C. nicotianae* isolates. In inoculation mixtures containing wild type, F129L and G143A cytochrome b, there was significant selection for the G143A mutation in one season. This strong selection could result in *C. nicotianae* populations that do not respond to azoxystrobin treatment. We also compared the biological fitness of *C. nicotianae* with wild type, F129L or G143A cytb mutations to determine if there is any fitness cost associated with QoI resistance. *In vitro* fitness was determined as the conidial viability or mycelial growth rate. We also looked at the survival of azoxystrobin-resistant *C. nicotianae* over winter. We found that there were no *in vitro* fitness penalties associated with azoxystrobin resistance and no difference in the survival of mutated individuals. Conventional FLS fungicide programs alternate azoxystrobin with mancozeb to manage QoI resistance development. We explored biological or organic fungicides, already labeled in tobacco, for efficacy against FLS. The biological fungicide programs were not as effective

against FLS compared to the conventional regimen, but treatments using only biological products did not shift the populations toward QoI resistance. An integrated pest management approach is the best way to control FLS and manage resistance development. Crop rotation and sanitation will minimize the introduction of *C. nicotianae* in field production. Identification of QoI resistance mutations in *C. nicotianae* populations will allow producers to make informed fungicide applications and minimize resistance development. Further, there is a need to label new or existing chemicals for use in tobacco to rotate modes of action and slow QoI resistance development.

KEYWORDS: Tobacco, Fungicide Resistance, QoI Fungicides, Fitness Penalties, *Cercospora nicotianae*

William Barrett Barlow
*(Name of Student)*

12/10/2021
Date

CHARACTERISTICS OF *CERCOSPORA NICOTIANAE* WITH REDUCED SENSITIVITY TO AZOXYSTROBIN

By
William Barrett Barlow

Dr. Emily Pfeufer
Co-Director of Thesis

Dr. Kiersten Wise
Co-Director of Thesis

Dr. Rick Bennett
Director of Graduate Studies

12/10/2021
Date

# DEDICATION

This thesis is dedicated to my family and friends, whose love, guidance, support, and encouragement has helped me through life and graduate school.

ACKNOWLEDGMENTS

There are several people who I would like to thank for helping me during my time at the University of Kentucky and as a graduate student. First, I would like to thank Dr. Emily Pfeufer, without her I would have never had the opportunity to continue my education and training as a plant pathologist. Working with Dr. Pfeufer was instrumental in the development of the technical and critical thinking skills required for success. I would also like to thank Dr. Robert Pearce and members of his tobacco research program. It was Dr. Pearce who introduced me to agricultural research, almost 10 years ago, and he has continued to support me on every step of my professional journey. Next, I would like to thank Dr. Kiersten Wise, who stepped up, without hesitation, to finish my thesis and prepare me for graduation. It was Dr. Wise's insights and knowledge that helped me produce a final thesis I am proud to publish. I would like to thank the other members of my committee, Dr. Paul Vincelli and Dr. Mark Farman, whose insights helped me improve my final thesis. Finally, I would like to thank the technical staff who helped me develop the skills and methodologies that made this research possible. It takes a village.

TABLE OF CONTENTS

# LIST OF TABLES

LIST OF FIGURES

# CHAPTER 1. LITERATURE REVIEW

## 1.1 Tobacco production overview

In 2019, the largest tobacco producing nations, in tonnes of unmanufactured tobacco, were Brazil, China, India, the United States, and Zimbabwe (FAO, 2019). In 2020, the United States produced 176,635,000 kg of tobacco worth $819.1 million (USDA, 2020b). Kentucky is the second largest tobacco-producing state in the United States, behind North Carolina. Kentucky produced 48,641,000 kg of tobacco on 20,800 hectares, worth a total of $237.1 million(USDA, 2020a). The two main tobacco types produced in Kentucky are burley and dark tobacco. Dark tobacco is then divided by curing method into dark fire-cured or dark air-cured. While significantly more land area in Kentucky is in burley tobacco production (14,973 hectares vs. 5,827 dark tobacco hectares), the two types are similar in farmgate value, with burley worth $145.7 million and dark tobacco worth $91.4 million(USDA, 2020a).

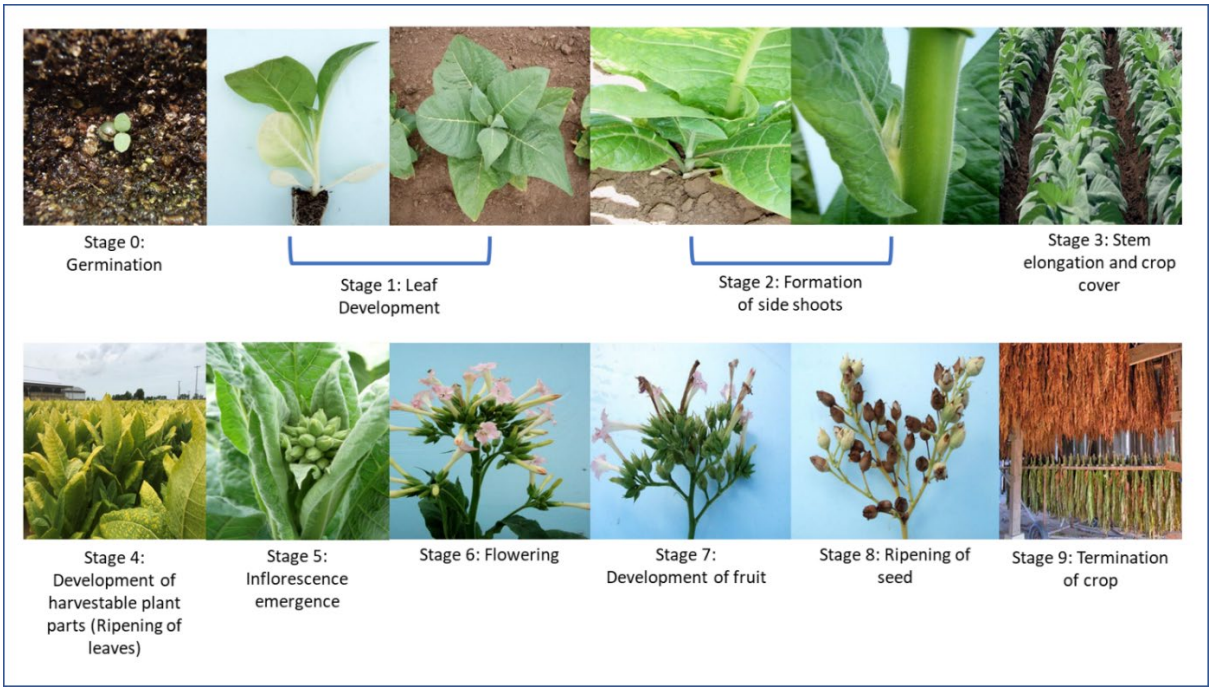
The light burley tobacco type is a mutant of a dark tobacco type, discovered in Kentucky in 1864. It quickly gained popularity in the tobacco market and became the predominant tobacco type grown in Kentucky (Shew & Lucas, 1991). Burley tobacco was historically grown throughout the entire state of Kentucky but is now concentrated in central Kentucky. Burley tobacco is primarily used in cigarette production, where it is combined with flavorings and blended with the flue-cured tobacco type. Dark tobacco is mostly used to produce smokeless tobacco products such

as chewing tobacco or moist snuff. Most dark tobacco production occurs in western Kentucky, western Tennessee, and western Virginia.

There are 10 principal growth stages in tobacco production (figure 1) (CORESTA, 2019). Growth stage (0), germination, occurs in greenhouse production systems in expanded polystyrene (EPS) trays filled with soilless media floated on beds of water amended with fertilizer and pesticides. The leaf development stage (1) encompasses leaf development of seedlings and field plants, the substages describe the number of fully expanded leaves present. The formation of side shoots (2) describes the development of both ground suckers and upper stalk suckers. These suckers compete for resources with the main leaves and are removed during production. Stem elongation and crop cover (3) describe the vertical growth of the stalk and horizontal growth and leaf expansion in and between rows. During stage three weeds are controlled with mechanical cultivation, either by tractor or hand, or with graminicides that specifically target grassy weeds. Development of harvestable plant parts (4) describes the degree of leaf ripening. Inflorescence emergence (5), flowering (6), development of fruit (7), and seed ripening (8) stages involve the development and maturation of the reproductive organs. The reproductive organs are removed during topping and plant growth inhibitors are sprayed over the tobacco plant to stop any additional sucker growth. The final stage, termination of crop (9), includes the harvest and curing of tobacco leaves (CORESTA, 2019). During the final stage in burley tobacco production the plants are cut by hand and speared onto a

long slender wooden tobacco stick. The sticks are left in the field for 1-7 days to wilt the tobacco. Field wilting tobacco reduces weight due to water, develops more elasticity and reduces leaf loss during handling. The harvested tobacco sticks are then hung in an air-curing structure until the leaves are stripped and bailed for sale in early winter.

Figure 1.1 Tobacco growth stages. Modified from (CORESTA, 2019, Bailey, 2006).




From 2000-2015, the total consumption of combustible tobacco products decreased by 33.5,% but the consumption of non-cigarette combustible and smokeless tobacco products increased by 117.1% and 23.1%, respectively (Wang et al., 2016). In 2017, approximately 50.6 million American adults used tobacco

products, 40.8 million used any combustible tobacco product, and 9.4 million used multiple tobacco products (Cornelius et al., 2020). The change in tobacco consumer habits could be a response to the ongoing efforts of tobacco prevention and control strategies, advertising and promotion of smokeless tobacco products, or reduced consumer cost of non-cigarette combustible tobacco products (Wang et al., 2016). Another explanation for the shift in tobacco consumer habits might be the impact of increased regulations imposed on tobacco products by the Food and Drug Administration since the Family Smoking Prevention and Control Act of 2009 (FDA, 2020).

## 1.2 Frogeye leaf spot

Frogeye leaf spot (FLS) is a foliar disease of tobacco that produces small lesions with parchment like centers, brown-red margins, and pinhead sized clusters of black conidiophores under humid conditions (figure 1.2). Frogeye leaf spot and the causal pathogen, *Cercospora nicotianae* Ellis & Everh., were first described by Ellis and Everhart in North Carolina in 1893 (Shew & Lucas, 1991). *C. nicotianae* could overwinter, similarly to *C. beticola,* on crop debris from previous growing seasons as dormant mycelium or stromata (Windels et al., 1998). *C. nicotianae* could also persist on other solanaceous hosts such as *Lycopersicon esculentum* (tomato), *Solanum tuberosum* (potato), and *Capsicum annuum* (pepper), or on non-solanaceous hosts like *Glycine max* (soybean) (Farr & Rossman). Infection usually occurs after strong winds, rain, or insects blow, splash, or carry conidia, the asexual spores of *C. nicotianae,* onto the

leaf surface of a susceptible host. With free water on the leaf, the conidia germinate, penetrate through the leaf stomates, and cause infection by producing phytotoxins, hydrolytic enzymes and reactive oxygen species by cercosporin (Swiderska-Burek et al., 2020). FLS is polycyclic, with multiple infection cycles occurring when environmental conditions are conducive for disease, high humidity and dew in the morning followed by dry periods in the afternoon (Shew & Lucas, 1991).

*C. nicotianae* is a member of the Mycosphaerellaceae family, one of the largest groups of pt pathogenic fungi. Conidia produced by *C. nicotianae* are long, slender, straight, or slightly curved, multi-septate, and vary greatly in size (25-370 μm X 3.4-6.1 μm) depending on environmental conditions. Conidia form from dark brown, septate conidiophores that erupt through the plant leaf surface during alternating periods of humidity and dryness (Shew & Lucas, 1991).

Stripped tobacco leaves are assigned a leaf grade depending on the size, texture, color, and properties determined by experienced graders (Zhang & Zhang, 2011). FLS lesions can cause serious problems in dark tobacco, where blemishes are an important consideration for leaf grade, directly affecting the market value. Historically, FLS in burley tobacco production was of minor economic importance, and in some instances was seen as an indicator of maturity in cured leaf, producing better burning agents for cigarette blends (B. Ratliff, *Personal Communication*; Li et al., 2021). However, late season infections of FLS can create green spots that appear during curing, and negatively affect quality (Bailey et al., 2021). Most recently, burley tobacco growers in several south-central Kentucky counties have had price dockage or tobacco crops rejected due to high levels of FLS on their crop (Li et al., 2021).

## 1.3 Frogeye leaf spot management

The management of plant disease requires a combination of strategies to optimize disease control. The basic objectives of a disease management strategy are to reduce the amount of initial inoculum present in the field, reduce the rate of infection, and to reduce the duration of the disease epidemic (Arneson, 2001). The general approaches to accomplish these objectives are: avoid sites where inoculum is present or where there are favorable environmental conditions for disease, prevent the introduction of inoculum, destroy inoculum already present, and use resistant cultivars (Isleib, 2013).

### 1.3.1 Cultural practices

Cultural practices are important for frogeye leaf spot management in tobacco. The first consideration when managing for disease is site selection. Sites that have a history of disease problems or that have environmental conditions favorable for disease, such as high humidity, poor air movement, prolonged periods of shade, or close proximity to bodies of water, should be avoided. One of the most important considerations for tobacco disease management is crop rotation. Crop rotation reduces disease pressure in the field by lowering the initial level of pathogen inoculum in the environment. It is recommended to rotate to non-solanaceous crops for three to five years following a tobacco production season (Bailey et al., 2021).

Sanitation practices prevent the introduction of new inoculum from other sites where disease is already present (Bailey et al., 2021). In 2017, frogeye

leaf spot was first identified on greenhouse transplants from three counties in south-central Kentucky (Dixon et al., 2018). It is important that producers use healthy transplants when setting tobacco fields in the spring to ensure that they are not introducing *C. nicotianae* inoculum. Research on *Cercospora beticola*, the causal agent of Cercospora leaf spot in sugar beet, suggests that spore dispersal occurs primarily by wind (Khan et al., 2008). Maximizing the distance from curing structures to greenhouse float bed production areas reduces the introduction of inoculum from previous tobacco crops into tobacco transplant production. Sanitization of mowing equipment used in the greenhouse float-bed production system, used for clipping transplants, minimizes the risk of spreading frogeye inoculum throughout the transplant greenhouse. The clippings from the mowing equipment should be removed from the greenhouse area and disposed of to eliminate potential reservoirs of inoculum.

Proper control of weeds also helps reduce the amount of inoculum present in the field. Heavy weed cover reduces airflow, increases shade and humidity, directly competes for nutrients, and may serve as alternative hosts for disease. Pre-emergence herbicide applications applied prior to transplanting are used for initial weed control. After transplanting, weeds are controlled mechanically by cultivation until the tobacco leaf canopy closes or with graminicides that directly target weedy grasses. Weed pressure is reduced after canopy closure limits the amount of light reaching the soil (Bailey et al., 2021).

### 1.3.2 Chemical control

Fungicides are another important tool for managing disease in tobacco production. There were very few proprietary fungicides available prior to 1940 (Latin, 2011). The earliest fungicides largely consisted of inorganic chemical mixtures that were prepared at home for use on horticultural crops or as seed treatments (Brent & Hollomon, 2007a). Between 1940 and 1970 the discovery and use of modern chemical pesticides for crop protection began to emerge (Morton & Staub, 2008). The first fungicides developed were contact fungicides considered non-selective general cell toxicants and were just as toxic to plant cells as they were fungal cells (Latin, 2011). Contact fungicides do not penetrate plant surfaces and are only mobile with precipitation, irrigation, and dew (Latin, 2011). Adequate spray coverage is essential for effective disease control with contact fungicides. Two of the most widely used contact multi-site fungicides, mancozeb and chlorothalonil, were both developed in the 1960s. After 1970, the agrochemical industry began to develop systemic, site-specific fungicide classes. Site-specific fungicides target a biological process within the fungal pathogen (Latin, 2011). Systemic fungicides are absorbed into the plant and can be redistributed to other parts of the plant (Salamanca & Saalu, 2021). The mobility of systemic fungicides vary from locally mobile to full systemic activity throughout the entire plant (Latin, 2011). The cost associated with developing site-specific fungicides has led the agrochemical industry to focus on large acreage crops like corn, soybean, wheat etc. and put less emphasis

on small acreage specialty crops. The limited the number of fungicides currently labeled for tobacco production, and the prospect of new chemistries being labeled to control foliar fungal phytopathogens in tobacco creates a unique challenge for tobacco producers.

The dithiocarbamate mancozeb is used in many crops for the management of plant pathogenic fungi including ascomycetes, oomycetes, basidiomycetes, and imperfect fungi (Gullino et al., 2010). Mancozeb is labelled to control many foliar fungal or fungal-like diseases in tobacco, such as blue mold, frogeye leaf spot, target spot, and brown spot. Mancozeb is a contact, protectant fungicide that belongs to the Fungicide Resistance Action Committee (FRAC) Group M (multiple site) mode of action. Since mancozeb acts on multiple target sites in fungal pathogens it is considered low risk for fungicide resistance development (FRAC, 2021a). However, the EPA determined that a by-product of mancozeb breaking down, ethylene thiourea (ETU) is a probable human carcinogen (McDaniel et al., 2005). Therefore, mancozeb residues in tobacco production are a concern. To reduce mancozeb residue in cured tobacco leaf, it is recommended that tobacco producers apply mancozeb no later than seven weeks after transplanting and alternate with fungicides with different modes of action (Bailey et al., 2021).

Strobilurin fungicides were first identified and isolated in 1977 from the wood-rotting basidiomycete fungus *Strobilurus tenacellus* infecting pinecones from *Pinus silverstris*. Two compounds, strobilurin A and strobilurin B, were isolated from *S. tenacellus* and showed very good anti-microbial activity against fungi and

yeast (Anke et al., 1977). The natural strobilurin compounds are sensitive to UV degradation and therefore unstable in the environment but provided researchers a molecular guide to develop the first synthetic strobilurins. The first synthetic strobilurins, azoxystrobin and kresoxim-methyl, were developed in 1992 by Syngenta and BASF, respectively. Azoxystrobin and kresoxim-methyl were commercially available in 1996, and since then eight chemical groups with fifteen active ingredients have been introduced with the same mode of action (Fernández-Ortuño et al., 2010).

FRAC classifies fungicides into classes based upon their sites of action. Strobilurin fungicides belong to a class known as quinone outside inhibitors (QoI), because they bind to the quinone outside (Qo) binding site in the cytochrome $bc_1$ complex (Complex III) of the inner fungal mitochondrial matrix. All QoI fungicides possess a similar toxophore that contains a carbonyl oxygen moiety responsible for binding to the Qo site in cytochrome b. Natural strobilurin compounds, azoxystrobin, and picoxystrobin are classified as methoxyacrylates because they contain a ß-methoxyacrylate moiety (Gisi et al., 2002). QoI fungicides inhibit respiration by disrupting electron transfer in the electron transport chain and ultimately halting the production of adenosine triphosphate (ATP). This mode of action, respiration inhibition, is especially effective at inhibiting fungal growth during developmental stages in the fungal life cycle. Energy-intensive growth phases, such as spore germination and zoospore motility, are very susceptible to this mode of action. Consequently, QoI fungicides

are most effective when applied prior to infection or early in the disease cycle (Fernández-Ortuño et al., 2010).

## 1.4 Pathogen resistance to QoI fungicides

Early fungicides targeted multiple sites of action, and pathogens rarely develop resistance to these broad-spectrum compounds. With the introduction of compounds that target more specific sites of action the late 1960s, fungicide resistance has become a major concern (Ma & Michailides, 2005). QoI fungicides were first introduced to Europe in 1996, within two years resistance was reported in the wheat downy mildew pathogen *Erysiphe graminis* f.sp. *triciti* (Chin et al., 2001). Because of the single-site specificity of the QoI fungicide class, and resistance problems arising soon after their introduction, FRAC classifies QoI fungicides (FRAC Group 11) as high risk for development of resistance (Brent & Hollomon, 2007b).

The primary mechanism for resistance to QoI fungicides is due to single nucleotide polymorphisms (SNPs) in the mitochondrial cytochrome b gene (*cytb)* which results in amino acid substitutions. The most common mutation that confers high resistance, resulting in a complete loss of control, to QoI fungicides is a substitution that replaces the glycine at codon position 143 for an alanine (G143A) (figure 1.3) (Fernández-Ortuño et al., 2010). The two less common mutations result from amino acid substitutions phenylalanine for leucine at codon position 129 (F129L) (figure 1.3) and glycine for arginine at position 137 (G137R) (figure 1.3), respectively. The F129L and G137R mutations confer moderate resistance to QoI fungicides

(FRAC, 2021b). These amino acid substitutions reduce QoI fungicides' affinity for the Qo target site in fungal mitochondria (Fernández-Ortuño et al., 2010). As of 2012, there are 36 species of fungi, from 20 distinct genera, listed with field resistance to QoI fungicides (FRAC, 2012).

Sensitivity to QoI fungicides is defined by the effective concentration of fungicide that inhibits germination or growth in 50% of the individual ($EC_{50}$) compared to germination or growth in the absence of fungicide (Russell, 2002). The $EC_{50}$ value indicates half of the fungicide rate that produces maximal control. Another metric that defines pathogen sensitivity to QoI fungicides is the resistance factor (RF), which is calculated as quotient of the $EC_{50}$ of resistant isolate over the $EC_{50}$ of a sensitive or wild-type isolate (FRAC, 2021b).

The soybean frogeye leaf spot pathogen, *C. sojina,* is reported to have resistance to QoI fungicides due to the G143A mutation. Isolates of *C. sojina* with this mutation have $EC_{50}$ values 100 times greater than baseline *C. sojina* $EC_{50}$ values (Zeng et al., 2015). The baseline is an established reference point for fungal sensitivity to a fungicide. Pathogen populations with fungicide sensitivity outside of the baseline range are considered less-sensitive or resistant (Russell, 2002). Some plant pathogens contain a type I intron directly following the codon at position 143 in *cytb*. Species that possess the intron directly following codon position 143 cannot properly splice the intron when the G143A mutation is present, producing respiratory deficient mutants in the presence of selection by QoI fungicides (FRAC, 2011). In species with this intron, it is unlikely they will develop complete

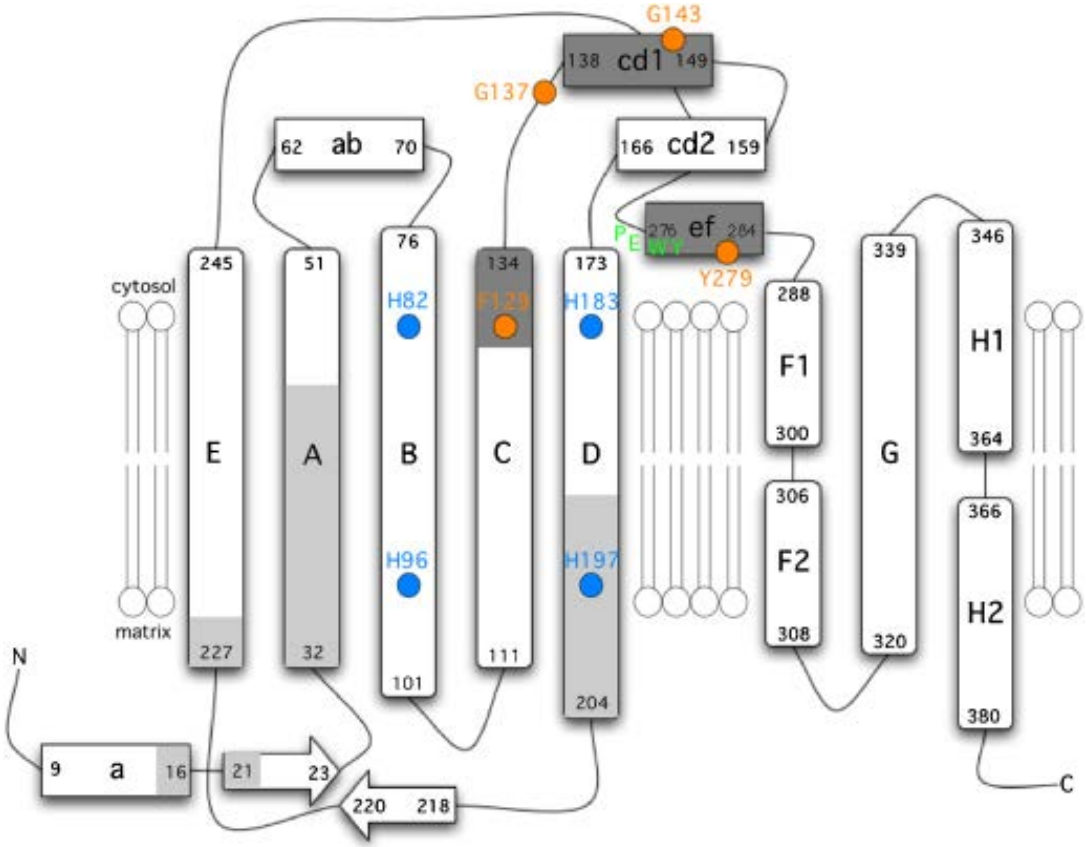
QoI resistance due to the G143A mutation. The G143A mutation is not found in species, *Alternaria solani, Puccinia* spp., *Plasmopara vitivola,* and *Pyrenophora teres,* that contain the intron following codon position 143 (Grasso et al., 2006). *Botrytis cinerea* was found to contain two copies of the *cytb* gene. One copy contained three introns designated Bcbi-67/68, Bcbi-131/132, and Bcbi-164. The second copy of *cytb* discovered in *B. cinerea* contained four introns, the three previously mentioned, and Bcbi-143/144. *B.* cinerea that contained the G143A mutation and Bcbi-143/144 intron are unable properly splice the intron and produce a functioning *cytb* (Banno et al., 2009).

The substitution of leucine for phenylalanine at position 129, mutation F129L, confers moderate resistance to QoI fungicides (Fernández-Ortuño et al., 2010). This mutation is less common than the G143A mutation, occurring in 9 of the 36 species with field resistance to QoI fungicides (FRAC, 2012). Phytopathogens that possess both the G143A and F129L mutations are less common, only reported to occur in *P. viticola, Puccinia tritici-repentis,* and *Pyricularia grisea* (FRAC, 2012).

A second, incomplete mechanism for resistance development to QoI fungicides is the activation of the alternative oxidase (AOX) pathway. When primary respiration is suppressed by QoI fungicides, AOX is activated to rescue ATP synthesis. In this mechanism, fungi bypass complex III and IV in the electron transport chain through AOX by accepting electrons directly from ubiquinol to produce low levels of ATP (Wood & Hollomon, 2003). AOX likely has a limited role *in planta*, as a

rescue mechanism, due to reduced ATP production and sensitivity to plant flavones (Hawkins & Fraaije, 2018). At high concentrations salicylhydroxamic acid (SHAM), a competitive inhibitor of AOX, AOX will be inactive (Murphy et al., 1999, Wood & Hollomon, 2003). SHAM is used in fungicide sensitivity assays to inhibit AOX when studying QoI resistant isolates *in vitro* to ensure that differences are due to QoI resistance mutations (Zhang et al., 2012b).

Figure 1.3 Schematic of cytochrome b, with the quinone inside (Qi) binding site in light grey and the quinone outside (Qo) binding site in dark grey. The QoI resistance mutations are shown with orange dots (Fisher & Meunier, 2008).



## 1.5 Pathogen fitness

Biological fitness is an indication of an organism's ability to successfully reproduce and pass its genetic information into the next generation. The biological fitness of fungicide resistance mutations plays an important role in the prevalence of fungicide resistance in plant pathogen populations (Brent & Hollomon, 2007b). Genetic mutations that are responsible for reduced sensitivity to fungicides may also reduce the fitness of the mutated individual. Mutated individuals could produce less reproductive spores or have slower vegetative growth. In this case, individuals would be at a disadvantage in the absence of fungicide applications, and therefore less likely to persist in the population without selection. It is also possible that the mutation will not affect the activity of the target, which would give those mutated individuals an advantage in a pathogen population selected on by QoI fungicides.

The G143A mutation reportedly does not negatively impact cytochrome b activity and is unlikely to impact the fitness of mutated individuals (Gisi et al., 2002, Zhang & Bradley, 2017). Several plant pathogenic fungi with QoI resistance associated with the G143A mutation have been evaluated for fitness cost. In *C. sojina*, QoI-resistant isolates were compared with QoI-sensitive isolates for conidial production, conidial germination, and aggressiveness to determine if the QoI-resistant isolates had a fitness penalty. Conidia production and germination did not differ between QoI- resistant *C. sojina* isolates and baseline *C. sojina* isolates (Zhang & Bradley, 2017).

Similar conclusions were observed with *Alternaria solani,* the potato early blight pathogen. Populations of *A. solani,* collected from 2002-2006, were examined for the presence for the F129L mutation. The frequency of the F129L mutation remained at high levels in *A. solani* populations despite few applications of QoI fungicides during this period. Likewise, populations from states that had limited applications of QoI fungicides also contained moderate levels of individuals with the F129L mutation. The F129L mutation remains stable in populations without selection from QoI fungicides. This suggests that there is unlikely a fitness cost associated with the F129L mutation in isolates with reduced sensitivity to QoI fungicides (Pasche et al., 2004).

Another study performed from 1996 to 1999, soon after the registration of QoI fungicides in Europe, looked at pathogen fitness in the absence of selection for *Eryisphe graminis* f.sp. *tritici*, the causal agent of powdery mildew on cereal grains (Chin et al., 2001). A baseline sensitivity for *E. graminis* f.sp. *triciti* to QoI fungicides was established from isolates originating in fields that had never been treated with QoI fungicides. Then isolates were obtained from fields treated or not treated with QoIs or from airborne spore collections. Dramatic increases in the rate of resistant isolates was observed in airborne spore collections from 1997 to 1998, which might suggest that there is not a significant cost to fitness in resistance mutations (Chin et al., 2001). The authors then examined competition between QoI sensitive and resistant isolates. A mixture of two isolates, one sensitive to QoIs and one resistant to QoIs, was grown on non-fungicide treated host tissue. The resistant isolate remained stable after three generations. On host

tissue treated with 0.1 μg·ml$^{-1}$ trifloxystrobin, the resistant isolate was heavily selected for, making up over 80% of the recovered isolates (Chin et al., 2001). In locations that were untreated, resistance was not detected but where QoI fungicides were used the resistant individuals comprised 42% and 57% of the populations, indicating that significant selection could occur in a single growing season (Chin et al., 2001).

## 1.6 Summary

Tobacco is an important crop for agricultural production in Kentucky. The limited availability of fungicides with differing sites of action creates a challenge for producers faced with foliar fungal pathogens. Resistance to any of the available fungicide products could severely limit the tobacco producer's profitability. The work complied in this thesis characterizes resistance attributes of *C. nicotianae* to the QoI fungicide azoxystrobin, the only site-specific systemic fungicide labeled for use in tobacco production. A better understanding of the resistance profile of FLS will help create informed management decisions for resistant *C. nicotianae* populations.

# CHAPTER 2. AZOXYSTROBIN SELECTIVITY IN *CERCOSPORA NICOTIANAE* POPULATIONS WITH MUTATIONS CONFERRING RESISTANCE TO QOI FUNGICIDES

## 2.1 Introduction

Tobacco producers need to sell leaves free of blemishes and disease to receive the highest price for their crop (Bailey et al., 2021). Frogeye leaf spot (FLS), caused by *Cercospora nicotianae*, has historically been a disease of minor importance and even seen as a desirable quality, producing better burning agents for cigarette blends, in leaves from the lowest stalk position. A recent increase in disease incidence and severity across tobacco production regions has resulted in FLS on higher stalk positions, leading to price dockage and crop rejection a result of damage (B. Ratliff, *Personal communication*; Dixon et al., 2020). FLS is traditionally managed using an integrated approach that includes crop rotation, disease free transplants, and fungicide spray programs. Conventional fungicide spray programs start around the first mechanical cultivation, two to three weeks post-transplant, or when environmental conditions are conducive for disease (Bailey et al., 2021). Azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC) is currently the only systemic fungicide labeled for use in tobacco to control foliar fungal pathogens. Azoxystrobin is sprayed in alternation with the contact fungicide mancozeb (Manzate Pro-Stick; United Phosphorus INC, King of Prussia, PA). Recently, *C. nicotianae* populations have been identified that have reduced sensitivity to azoxystrobin, and the occurrence of FLS in greenhouse transplant production was recently reported (Dixon et al., 2020, Dixon et al., 2018). These findings mean that FLS can be more difficult to

manage for tobacco producers that have QoI-resistant strains of *C. nicotianae* in the population.

Azoxystrobin's mode of action (MOA) is respiration inhibition. It belongs to the quinone outside inhibitor (QoI) group of fungicides classified by the Fungicide Resistance Action Committee (FRAC) group 11 (FRAC, 2021b). QoI fungicides act as competitive inhibitors of the quinone outside binding site on cytochrome b (cytb) in complex III of the inner mitochondrial matrix. This stops the transfer of electrons from cytb to cytochrome $c_1$ and ultimately halts the production of adenosine triphosphate (ATP) (Fernández-Ortuño et al., 2010). QoI fungicides were first commercially released for use in Europe in 1996 and QoI-resistant individuals of *Erysiphe graminis* f.sp. *triciti* were detected from Germany as soon as 1998 and from Belgium, Denmark, France, Germany and the United Kingdom in 1999 (Chin et al., 2001). Resistance to QoI fungicides is the result of single nucleotide polymorphisms (SNPs) that result in amino acid substitutions at the quinone outside binding site. The substitution from phenylalanine to leucine at codon position 129 (F129L) and glycine to arginine (G137R) confer moderate or partial resistance to QoI fungicides. These mutations result in sensitivity to QoI fungicides that is 10-50 times less than wild-type sensitivity (FRAC, 2021b). The substitution from glycine to alanine at codon position 143 (G143A) results in complete resistance to QoI fungicides, meaning isolates with this mutation are over 100 times less sensitive than wild-type individuals (FRAC, 2021b).

The occurrence of phytopathogens containing both mutations for resistance to QoI fungicides is rare, only occurring in *Plasmopara viticola, Pyrenophora tritici-repentis,* and *Pyricularia grisea* (FRAC, 2012). Conidial germination assays and molecular methods confirmed that *C. nicotianae* possesses both the F129L and G143A QoI resistance mutations (Dixon et al., 2020, Li et al., 2021). *C. nicotianae* populations that contain both partial and complete resistance to QoI fungicides creates a challenge for tobacco producers with limited fungicides in their arsenal. An experiment looking at fungicide mixtures containing QoIs with mancozeb, folpet, cymoxanil, fosetyl-aluminum or copper found that mixtures containing mancozeb had the least effect on preventing selection for QoI resistance (Genet et al., 2006). It has been reported in other pathogens with the F129L or G137A mutations that spraying fungicides at the maximum labeled rate is sufficient to control disease epidemics but pathogens with the G143A resistance mutation cannot be controlled with QoI fungicides(FRAC, 2021b). Greenhouse experiments with *Pyrenophora triticii-repentis* showed that QoI fungicides were not effective at controlling disease in plants inoculated with isolates with the G143A mutation, but disease was managed in plants inoculated with isolates with the F129L mutation (Sierotzki et al., 2007). In experiments that look at the effect of fungicide dose on QoI resistance selection, the selection pressure increases as the fungicide dose increases. In *Plasmopara viticola* there was a significant reduction in QoI sensitivity after the first generation (Genet et al., 2006). It is unknown how *C. nicotianae* populations containing the F129L and G143A mutations respond after selection from the producer's standard fungicide program (Bailey et al., 2021). There is potential for resistance selection in

populations comprised of individuals with different sensitivities to QoI fungicides. Since both F129L and G143A mutations have been identified in *C. nicotianae,* it is important to see if azoxystrobin alternated with mancozeb will provide adequate control of FLS and determine the selection pressure it exerts on populations with both QoI resistance mutations over a single growing season. If there is no fitness cost associated with these mutations it is likely the QoI mutations will persist in the environment in the absence of QoI selection (Hawkins & Fraaije, 2018). The objective of this work is to examine changes in *C. nicotianae* populations with different cytb mutations (F129L and G143A) after exposure to a tobacco producer's standard fungicide spray program (Bailey et al., 2021) throughout a growing season.

## 2.2 Materials and methods

### 2.2.1 Field trial design

A field trial was conducted in 2019 at the University of Kentucky Spindletop Research Farm in Lexington, KY. Land preparation, fertility, weed management, and insect control were managed following the University of Kentucky Cooperative Extension Service guidelines outlined in the Burley and Dark Tobacco Production Guide (UK ID-160)(Bailey et al., 2021). A 0.142 hectare field was set with burley tobacco variety KT-212LC on May 23, 2019. The field location was selected to reduce inoculum introduction from outside sources. The field selected had been out of tobacco research for over three years and was 0.4 kilometers away from other tobacco research fields. Experimental plots were 9.14 meters long, separated by a 1.52-meter plant free break, and consisted of two

treatment rows flanked on both sides by a non-treated border row. The experiment was arranged as a randomized complete block design with four replications of each treatment. Treatments consisted of different inoculation treatments, using inoculum mixtures of *C. nicotianae* with previously identified QoI-resistance mutations at the F129L or G143A locations in the cytb gene (Table 2.1)(Dixon et al., 2020). Isolates of *C. nicotianae* with no resistance mutations (wild-type or fungicide sensitive) were also included. A total of nine treatments consisting of different ratios of isolates were used (Table 2.2). Each treatment, except treatment one, was sprayed with the grower's standard fungicide program (Table 2.3)(Bailey et al., 2021).

Table 2.1 Cercospora nicotianae isolates selected for use in field inoculation experiments based on their azoxystrobin sensitivity group, determined by EC50 effective concentration to inhibit conidial germination by 50% (EC50).

| Isolate | Year Collected | County and State of Origin | $EC_{50}$ µg/ml | Known cytb mutations [a] | GenBank accessions |
|---|---|---|---|---|---|
| 16Cn008 | 2016 | Green County, KY | 2.879 | G143A | MK369753 |
| 16Cn009 | 2016 | Green County, KY | 0.292 | F129L | MK369754 |
| 16Cn056 | 2016 | Scott County, KY | 0.036 | no mutation | MK369759 |

[a] Cytochrome b (cytb) genotype was confirmed using mut4 PCR primers (Zeng et al., 2015) and Sanger sequencing (Dixon et al., 2020).

Table 2.2 Field inoculation treatments used to determine Cercospora nicotianae population compositions after exposure to azoxystrobin in a production setting at a 2019 trial in Lexington, KY. The isolates selected were confirmed to possess the wild-type cytochrome b, or F129L or G143A mutations that confer resistance to QoI Fungicides (Dixon et al., 2020).

| | | Ratio of inoculum by *C. nicotianae* isolate with known sensitivity to azoxystrobin | | |
|---|---|---|---|---|
| Inoculum Treatment [a] | Fungicide program | Wild Type | F129L | G143A |
| Non-inoculated | No fungicide | | | |
| Non-inoculated | Fungicide applied | | | |
| WT only | Fungicide applied | 100% | 0% | 0% |
| WT and F129L | Fungicide applied | 50% | 50% | 0% |
| WT, F129L, G143A | Fungicide applied | 33% | 33% | 33% |
| F129L and G143A | Fungicide applied | 0% | 50% | 50% |
| F129L only | Fungicide applied | 0% | 100% | 0% |
| WT and G143A | Fungicide applied | 50% | 0% | 50% |
| G143A only | Fungicide applied | 0% | 0% | 100% |

[a.] The nine treatments consisted of four replications in a randomized complete block design. Inoculum were titrated to 4.1 x $10^4$ conidia/ml and then combined according to treatment inoculation mixture.

### 2.2.2 Inoculum preparation and application

Isolates with previously known sensitivity to azoxystrobin and known cytb gene mutations (Dixon et al., 2020) were revived from -80°C, transferred onto clarified V8 (CV8) media, and grown under fluorescent and blue-black light (370-400nm) for 10-14 days to induce sporulation. CV8 media is produced by combining V8 juice with 1.5% $CaCO_3$, stirring for 30 minutes, the centrifuging for 10 mins at 3000 rpm. The clarified V8 was combined with DI water and bacteriological agar (Difco, Becton Dickinson and Company, MD, U.S.A) at 19 g/l, autoclaved until molten, and poured into plastic petri dishes. Agar plugs were cut out of the plates with a sterilized #5 cork borer (5.25mm x 5.25mm) and added to 10 ml of sterile deionized water in a 15 ml centrifuge tube (USA Scientific, Orlando, FL). The mixture was vortexed with a fixed speed mini-vortex (Fisher Scientific, Hampton, NH) for 30 seconds and strained through sterile cheese cloth to remove agar chunks. New CV8 Petri plates received 75 µl of the spore suspension evenly spread over the plate. The CV8 plates were grown on the lab bench under fluorescent and blue to black (370-400nm) light for 10-14 days. The inoculum plates were irrigated with sterile deionized water and scraped with a sterile plastic scraper to dislodge the conidia. The Petri plates for each isolate were combined and the concentration of conidia was determined with a hemocytometer. All inoculation suspensions were titrated to equal the lowest concentration obtained from an isolate, $4.1x10^4$ conidia/ml. The inoculation suspensions were then combined in mixtures depending on treatment (Table 2.2) and sprayed from a 1.5 l handheld pump sprayer (Tolco, Toledo, OH) at a rate of

5 ml/s over the top of each treatment row for 6 seconds. The walking speed was 5.6 kph and the total amount of inoculum applied was 60 ml/plot. The inoculation was applied on June 26, 2019, during the leaf development stage.

### 2.2.3 Fungicide applications

All treatments, except the non-inoculated no spray, were sprayed using the recommended foliar fungicide program (Table 2.3) (Bailey et al., 2021). Fungicide applications followed a 10-14 day spray interval when weather conditions permitted. Fungicide applications were made using a Lee Spider DP sprayer (Lee Agra, Lubock, TX) fitted with a two-row spray boom consisting of three 360 Undercover drop nozzle bodies (360 Yield Center, Morton, IL) fitted with flat fan XR 80015 and Turbo TeeJet Induction 110015 nozzles (TeeJet Technologies, Glendale Heights, IL) and two TeeJet Conjet VisiFlo TX-VK8 nozzles over top of the rows (TeeJet Technologies, Glendale Heights, IL). Drop nozzle bodies were spaced one meter apart, and the two overhead nozzles were one meter apart. The spray volume was 280.63 L/ha when the plants were below waist height. The spray volume was increased to 467.7 L/ha as the plants got larger. All sprays were applied at 413.69 kPa.

Table 2.3 The tobacco producer's standard fungicide program to control frogeye leaf spot, caused by Cercospora nicotianae, including dates the fungicide was applied in the 2019 trial.

| Fungicide | Active ingredient | Application rate | Volume | Date |
|---|---|---|---|---|
| Quadris | azoxystrobin | 95.74 ml/ha | 280.62 L/ha | July 10, 2019 |
| Manzate Pro-Stick | mancozeb | 2.4 g/l | 467.7 L/ha | July 25, 2019 |
| Quadris | azoxystrobin | 95.74 ml/ha | 467.7 L/ha | August 8, 2019 |

### 2.2.4 FLS disease severity ratings

FLS foliar disease ratings were taken three times throughout the season to determine FLS disease severity. The disease severity ratings were taken after each fungicide application, during stem elongation and crop cover, and before crop termination on July 19, 2019, July 31, 2019, and August 28, 2019. Disease severity was determined on a scale from 0-100% based on leaf area affected by FLS. Seven plants were chosen arbitrarily from each plot to be rated. The lower and upper canopy were rated separately, for two ratings per selected plant. Lower canopy ratings were taken from leaves one meter above soil level and upper canopy ratings were taken from the top meter of the plant. Upper and lower canopy ratings were analyzed separately, disease severity rating means were tested for equal variances, then subjected to One-Way ANOVA to determine significance ($\alpha = 0.05$). Means were separated using Tukey's Honestly Significant Difference (HSD) (Minitab 19, Minitab LLC, State College, PA).

### 2.2.5 Field sample collection and isolate preparation

Leaf samples with visible *C. nicotianae* conidia were collected three times per growing season before fungicide applications, and prior to trial termination on July 9, 2019, July 19, 2019, and August 28, 2019. One leaf with visible FLS symptoms was sampled per plant, from seven separate tobacco plants per plot. Leaf samples were stored in brown paper bags and then transferred to a walk-in cooler for storage at 4°C until they could be processed. The *C. nicotianae*-infected leaves were surface sterilized in 10% v/v bleach solution for 1 minute before being transferred into two sterilized deionized water rinses. The leaf samples were layered between sterilized paper towels to partially dry while the other leaf samples were sterilized. Once all the leaves from a plot were sterilized, *C. nicotianae* lesions were excised and transferred into Petri dishes (Falcon, Corning Life Sciences, Tewksbury, MA). The leaf excisions were bagged in small clear plastic bags and left for 72 hours under fluorescent and blue to black (370-400 nm) light. After 72 hours, the plates were examined under a dissecting microscope. Single conidia were lifted from the conidiophores with a sterilized scalpel and transferred into ¼ strength acidified potato dextrose agar (1/4 aPDA) media (Difco, Becton Dickinson and Company, MD, U.S.A) amended with 1 ml/l lactic acid (1/4 aPDA). When single conidia could not be lifted from the excised lesions, mycelial colonies were collected by scraping the FLS lesions and grown for 7-10 days on 1/4 aPDA. A plug from the mycelial colony was transferred to CV8 to induce sporulation. A plug from the CV8 was added to 10 ml sterile deionized water, vortexed in a fixed speed mini-vortex and 0.1 ml of the conidial

solution was transferred to ¼ aPDA. A single spore was collected the next day and transferred to CV8. All single spore colonies were incubated at room temperature, on CV8, under fluorescent white and blue to black (370-400 nm) light on a 12 hr day/night cycle, to induce sporulation, for 10-14 days. Small squares were cut from the leading edge of the single spore colonies with a sterile scalpel, transferred into 1.5 mL microfuge tubes (USA Scientific, Orlando, FL) containing 0.5 ml of 15% v/v glycerol, and stored at -80°C to be revived as needed.

### 2.2.6 DNA extraction

Stored *C. nicotianae* isolates were revived from -80°C storage and plated on clarified V8 media covered with a sterilized disk of cellophane. The cultures were grown at room temperature under fluorescent white and blue-black light for 10-14 days. Mycelium was scraped from half of the clarified V8 plate and transferred into 1.5 mL Beadbeater (Biospec Product, Bartlesville, OK) tubes containing two 6.35 mm glass beads. The tubes were covered with Parafilm (Beemis Company Inc, Neenah, WI) then a small hole was poked in the center of the parafilm. The tubes were covered with a Kimwipe (Kimtech, Kimberly-Clark, Irvine, TX) and bound together with a rubber band. The fungal mycelia were freeze dried using a VirTis Freezemobile 25ES (VirTis, Los Angeles, CA) for 24-48 hours. DNA was extracted using a modified version of an SDS/CTAB extraction method (Niu et al., 2008). For the initial grinding step, the BeadBeater tubes of freeze-dried mycelium were chilled in liquid nitrogen for 30 seconds, then pulverized with a

Mini-BeadBeater 2.0 cell disrupter (Biospec Product, Bartlesville, OK). After the addition of the extraction buffer, RNase A, mercaptoethanol and sodium dodecyl sulfate (SDS) the initial water bath stage was extended from 5 to 30 minutes at 65°C. Before the precipitation step, the samples were incubated at 4°C for 30 minutes. An additional DNA purification step was added after the DNA pellet was dissolved in Milli Q (Millipore Corporation, Beford, MA). The DNA pellet and Milli Q solution was incubated at 55° C for 30 minutes, vortexed briefly, and the debris was precipitated in a centrifuge at 10,000 RPM for one minute and the supernatant with DNA was transferred into a new 1.5 ml micro-centrifuge tube.

DNA quantity and quality was determined using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wimington, DE). Some of the stock DNA was transferred to 0.5mL microfuge tube and diluted to 10ng/ul for use in PCR identification of resistance mutations. The remaining DNA stock was stored at -80°C.

### 2.2.7 Molecular identification of QoI mutations in *C. nicotianae* isolates

*C. nicotianae* isolates collected from the experiment were screened for mutations conferring resistance to QoI-fungicides using a polymerase chain reaction (PCR) protocol utilizing primers developed and optimized by (Li et al., 2021). Each reaction was prepared in 25 µl volume using a Titanium Taq PCR kit (TaKaRa Bio USA, Mountain View, CA) with 10X Titanium Taq buffer, 0.1 µM forward and reverse primers, 0.1 mM deoxynucleoside triphosphate, 1X Titanium Taq polymerase, and 50 ng of DNA template. Thermal cycling parameters were an

initial denaturation step at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 68.3°C for wild-type and F129L primers or 68.8°C for G143A primers, and extension at 72°C for 30 seconds. A final extension at 72°C for 10 minutes completed the reaction.

The PCR amplicons were read on 2% SeaKem LE agarose gel (Lonza, Bend, OR) using EZ-Vision (AMRESCO; VMR Life Science, Solon, OH) DNA staining dye. The amplicons were separated in 20-well gels using gel electrophoresis at 90V for 35 min. The gels were imaged on a Bio-Rad Gel Doc XR (Bio-Rad, Hercules, CA).

*C. nicotianae* isolates were classified into azoxystrobin resistance groupings (wild-type, moderately resistant; F129L, and high resistance G143A) if an amplicon was identified for the complimentary primer set. These groupings were analyzed for each sampling date by Pearson's Chi-Square (Minitab 20, Minitab LLC, State College, PA).

## 2.3 Results

### 2.3.1 Frogeye leaf spot development

The first foliar disease ratings taken on July 19th showed a difference in disease severity by treatment. As expected, the non-inoculated treatments had the lowest disease in the upper and lower canopy ratings. The wild type (WT) only, WT and F129L, WT, F129L and G143A, F129L and G143A, and F129L only inoculation mixes had the highest FLS disease severity in the lower leaf canopy. Treatments with inoculation mixes containing WT and G143A and G143A only had disease severity ratings that grouped in between the other treatments. The upper canopy

FLS severity ratings on July 19, 2019 were lowest in the non-inoculated treatments. The WT only and F129L and G143A had the highest FLS disease severity (Table 2.4).

The second foliar disease rating on July 31st produced similar disease severity ratings in the lower canopy. Both non-inoculated treatments had the least severe FLS. Inoculation mixtures WT and F129L, F129L and G143A, F129L only, and WT and G143A grouped together with the highest FLS severity. Treatment had no effect on upper canopy ratings (Table 2.4).

The final disease rating on August 28th showed no significant differences among treatments in the lower or upper canopy (Table 2.4).

Table 2.4 The mean disease severity ratings as a percentage of the leaf affected by frogeye leaf spot for seven plants divided into lower and upper canopy.

| | | July 19, 2019 | | July 31, 2019 | | August 28, 2019 | |
|---|---|---|---|---|---|---|---|
| Treatment | Fungicide program | Lower Canopy[a] | Upper Canopy | Lower Canopy | Upper Canopy | Lower Canopy | Upper Canopy |
| Non-inoculated | No fungicide | 2.25a | 0.01a | 5.68a | 0.46 | 27.32 | 6.78 |
| Non-inoculated | Fungicide applied | 2.04a | 0.18a | 5.25a | 0.75 | 28.14 | 7.57 |
| WT only | Fungicide applied | 18.04b | 3.46b | 33.82ab | 2.39 | 46.96 | 8 |
| WT and F129L | Fungicide applied | 20.11b | 1.82ab | 35b | 2.18 | 43.79 | 7.18 |
| WT, F129L, G143A | Fungicide applied | 18.07b | 2.57ab | 27.96ab | 1.64 | 42.6 | 10.89 |
| F129L and G143A | Fungicide applied | 22.79b | 3.18b | 41.32b | 2.21 | 47.43 | 8.75 |
| F129L only | Fungicide applied | 18.43b | 2.5ab | 36.9b | 2.54 | 48.29 | 8.43 |
| WT and G143A | Fungicide applied | 16.61ab | 2.61ab | 40.79b | 2.5 | 44.39 | 9.43 |
| G143A only | Fungicide applied | 12.54ab | 2.11ab | 32.93ab | 1.86 | 55.11 | 10.86 |
| *P*-Value | | 0.000 | 0.002 | 0.001 | 0.078 | 0.141 | 0.204 |

[a] Means were tested for equal variance, analyzed by One-Way ANOVA, and separated by Tukey's Honestly Significant Difference ($\alpha$=0.05). Means that do not share a letter are significantly different.

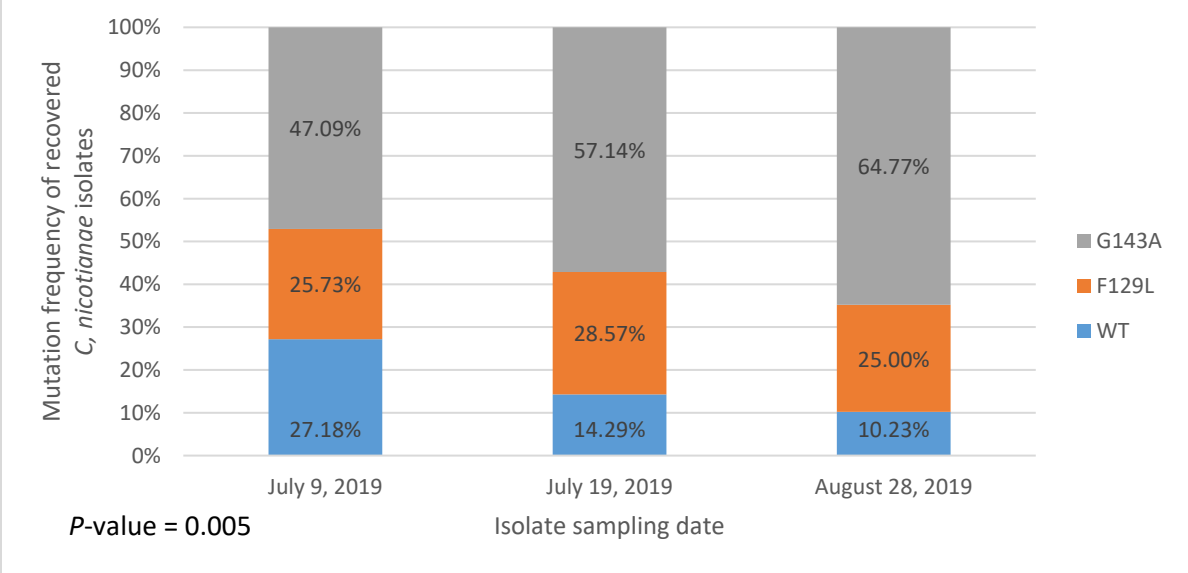
### 2.3.2 Identification of QoI-resistance mutations in *C. nicotianae* samples

There were 204, 84, and 88 individual isolates recovered on July 9, 2019, July 19, 2019, and August 28, 2019, respectively. Across all treatments, the frequency of isolates with a wild type cytb decreased with each sample date (Figure 2.1).

Across all treatments, the overall percentage of *C. nicotianae* with the F129L mutation remained relatively stable, representing 25.7, 28.6, and 25% of the population at each sampling date. However, the overall population of isolates with the G143A mutation increased from 47.1 to 64.8% from the first sampling date to the last sampling date, resulting in a 17% increase in the isolates recovered that contained a G143A mutation.

(Figure 2.1). The ratio of sensitivity groups for every treatment were significantly different ($P$=0.005) across the sampling dates.

Figure 2.1 The mutation frequency of isolates recovered from all treatments of *Cercospora nicotianae* populations comprised of individuals with wild-type, F129L, and G143A cytochrome b, identified using PCR primers optimized to detect QoI resistance mutations (Li et al., 2021). Pearson's Chi-Square analysis shows a significant difference ($P$=0.005) in mutation frequencies between sampling dates (α=0.05).

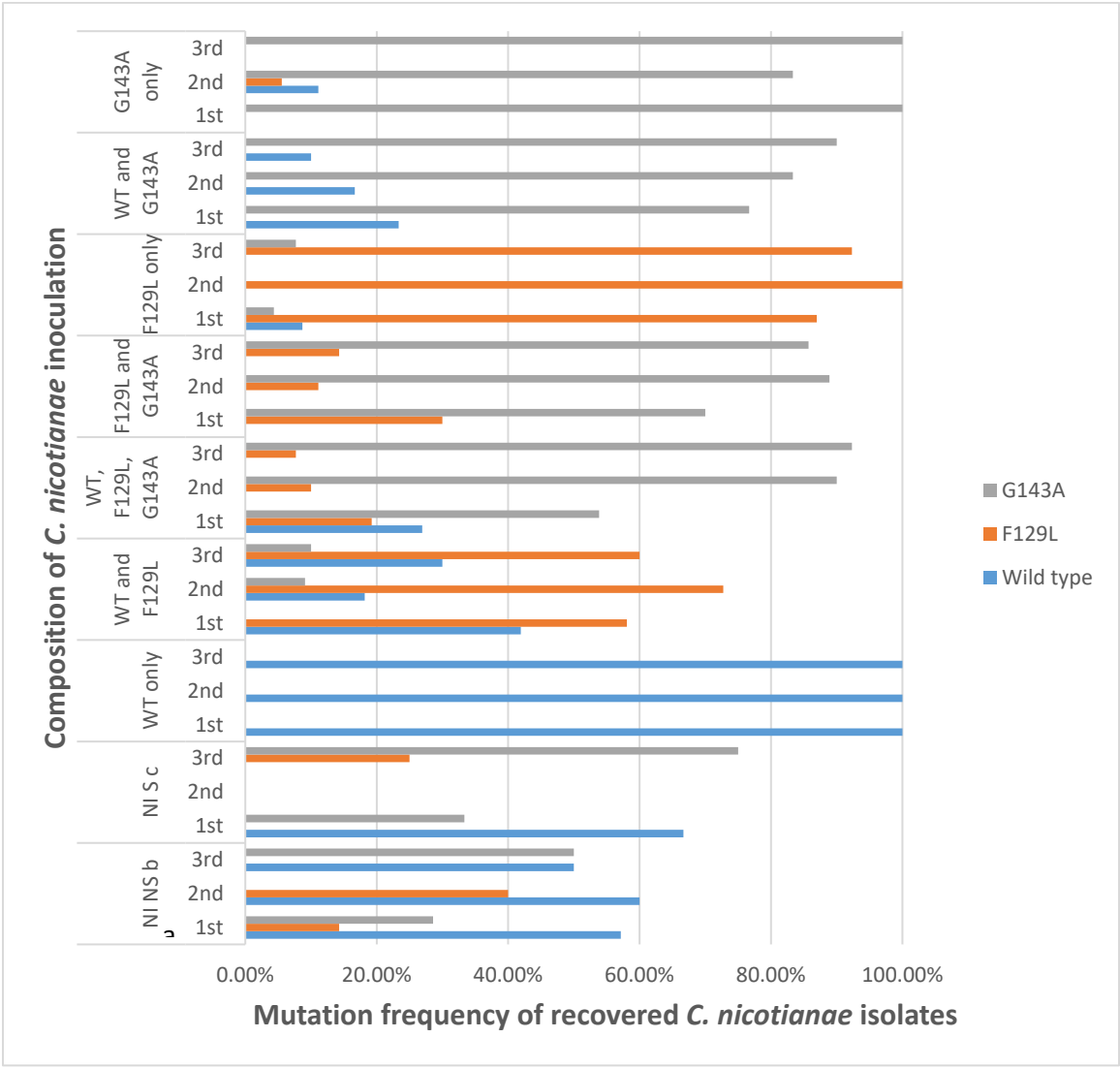



We compared detected mutations by treatment for samples collected at the final rating date, August 28, 2019. In the non-inoculated, non-sprayed treatment, there were 4 isolates recovered, and the mutation frequencies of these isolates were split evenly between wild-type and G143A. The non-inoculated sprayed treatment consisted of 8 isolates, with 25% containing the F129L mutation and 75% with the G143A mutation. We recovered 100% WT isolates from the WT only inoculum treatment at the end of the season.

The WT and F129L mix showed a shift towards the F129L and G143A mutations, with 30, 60, and 10% of the recovered isolated being identified as WT, F129L, and G143A, respectively. The WT, F129L and G143A mix showed strong selection towards the G143A mutation, with 12 of the 13 isolates recovered possessing the G143A mutation and one isolate having the F129L mutation. Sampling of the F129L and G143A inoculation mixture showed more selection for isolates with the F129L mutation over isolates with G143A. Of the 21 recovered isolates, 13 were F129L mutants and 8 were G143A mutants, but these were not significantly different ($P$ = 0.207). The F129L only inoculation produced mostly F129L mutants at the end of the season, 12 out of 13, and one G143A mutant. Isolates recovered from the WT and G143A inoculation mixture were only 10% WT and 90% of the isolates were highly resistant. The G143A only inoculation treatment had 7 recovered isolates, 100% of them were the highly resistant genotype.

Mutation frequencies from each sample date for every treatment were compared using Pearson's Chi-square analysis. The only treatment with enough data points to determine significance was the full mixture treatment ($P$=0.048). The non-inoculated no spray, non-inoculated sprayed, WT only, WT and F129L, F129L only, and G143A only treatments lacked enough data points to produce a $P$-value. The F129L and G143A, and WT and G143A inoculation mixtures were not significantly different, $P$=0.207 and $P$=0.643, respectively.

Figure 2.2 Cytochrome b (cytb) mutations conferring resistance to QoI fungicides recovered from field tobacco inoculated with mixtures *Cercospora nicotianae* with known cytb mutations. Isolates were sample three times through a single growing season and mutations were identified using polymerase chain reaction (PCR). Mutation frequencies were analyzed with Pearson's Chi-Square (α=0.05).




[a] 1st, 2nd , and 3rd sample dates were on July 9, 2019, July 19, 2019 and August 28, 2019, respectively.

[b]NI NS= non-inoculated non-sprayed

[c] NI S= non-inoculated sprayed with azoxystrobin and mancozeb

## 2.4 Discussion

There are few pathogens with multiple known mutations conferring QoI resistance. These pathogens are particularly concerning in field scenarios, since farmers may not be aware of what the dominant mutation in the population is until chemical failures occur. In greenhouse azoxystrobin efficacy trials with *P. tritici-repentis,* isolates with wild type cytb were fully controlled and isolates with the F129L and G143A mutation were controlled 80 and 40%, respectively (Sierotzki et al., 2007). In our research, we examined the change in *C. nicotianae* QoI mutation frequency when treatments with known levels of F129L and G143A mutations experienced selection pressure through fungicide applications.

The FLS severity ratings unearthed an interesting pattern. The non-inoculated treatments had significantly lower levels of FLS through the second disease rating, which reinforces the importance of restricting the introduction of *C. nicotianae* inoculum in the field by following production guidelines (Bailey et al., 2021). Crop rotation, disease free transplants, and fungicide spray programs should be utilized to ensure *C. nicotianae* conidia aren't introduced into the tobacco field to limit damage from FLS. Among the treatments that were inoculated with mixtures containing the isolate with the G143A mutation conferring high resistance to azoxystrobin, the average FLS severity was lower in the first two rating dates than treatments inoculated with wild-type isolates or isolates with the F129L mutation. This might be an indication that individuals with the G143A mutation could be less aggressive. Aggressiveness has been evaluated in *A. solani* with resistance to succinate dehydrogenase inhibitor (SDHI) fungicides, QoIs and anilinopyrimidines (AP). There was no change in

aggressiveness in QoI-resistant mutants alone, but an increased aggressiveness was associated with SDHI resistance (Bauske & Gudmestad, 2018). However, in our study, at the final disease severity rating there were no significant differences in FLS severity among any of the treatments. This final rating was taken 20 days after the last fungicide application. Residual fungicide activity, and consequently disease control, would be minimal at this time since QoI fungicides have approximately 14 days of activity after application (Latin, 2011).

*Cercospora nicotianae* isolates recovered from the treatments inoculated with equal parts of isolates of all three azoxystrobin sensitivity groups, had significantly higher levels of the G143A mutation compared to the initial inoculum concentration. Over 90% of the isolates recovered from treatments inoculated with equal parts of wild-type and isolates with the G143A mutation contained the G143A mutation by the end of the trial, however so few isolates were collected that statistical analysis was not able to determine if this was a significant shift in sensitivity from the initial inoculum concentration. The increase in overall percentage of isolates containing the G143A mutation throughout the season, despite treatment, indicates that regardless of the composition of *C. nicotianae* populations, strong selection could occur in a single season when fungicides are applied. If selection for QoI-resistant isolates occurs within a single season, QoI fungicides could fail to control FLS if the population is comprised of individuals with the G143A mutation. This was found to be the case in *E. graminis* f.sp. *tritici* isolates sampled from locations in Germany that had received QoI applications (Chin et al., 2001) However, the threshold mutation

frequency for loss-of-control with QoI fungicides has not been established for *C. nicotianae*.

If QoI resistance mutations carry a fitness cost, theoretically, efficacy of QoI fungicides may return after break in use. However, if there is no fitness cost associated with QoI resistance mutations it is unlikely that *C. nicotianae* populations will return to baseline sensitivity. Competition assays have been performed for QoI-resistant *Colletotrichum acutatum,* containing both F129L and G143A. Fitness was evaluated as mycelial growth rate, aggressiveness without QoI selection pressure, conidial production, and competition. Researchers observed there were no obvious fitness cost associated with either mutation, and removing selection pressure from QoI fungicides may not reduce populations of QoI-resistant *C. acutatum* (Forcelini et al., 2018).

In our study, we attempted to control the introduction of outside sources of *C. nicotianae* inoculum by geographically isolating the field where the research was conducted. However, without the use of genetic markers to confirm the source of *C. nicotianae* isolated from sampled treatments, we cannot definitively say that changes in the ratio of QoI mutations observed was a direct result of our inoculations. To my knowledge, no work has been done on the spore survival and dispersal of *C. nicotianae*. Future studies that examine changes in mutation frequencies in the *C. nicotianae* population under selection pressure could be done in the greenhouse or use genetic markers to help confirm results. However, spore dispersal and survival has been explored in *C. beticola* and *C. sojina*. Spore trapping studies found that dispersal in *C. beticola* was found to occur primarily

by wind (Khan et al., 2008) and over relatively short distances (Imbusch et al., 2021), though it is possible they could travel further. The most severe disease symptoms are likely the result of secondary infections originating from lesions within the field (Imbusch et al., 2021). Spore survival studies of *C. beticola* and *C. sojina* looked at conidial viability of infected leaf debris on the soil surface or buried at depth intervals. In *C. beticola* they found that conidia present at the soil surface remained viable longer but in *C. sojina* there was no difference in conidial viability at any soil level. In both trials they found that conidia are viable up to 22 or 24 months, respectively (Khan et al., 2008, Zhang & Bradley, 2014).

Azoxystrobin is still the only labeled foliar fungicide with systemic activity in tobacco, and with the potential for QoI-resistant isolates to dominate the *C. nicotianae* population and result in loss of FLS control, it is important to actively manage FLS against resistance development. Fungicide spray programs that utilize azoxystrobin should alternate with other modes of action to reduce selection pressure and lower the overall *C. nicotianae* population. Understanding the current sensitivity of the *C. nicotianae* population to azoxystrobin is equally important for tobacco producers. Using azoxystrobin on *C. nicotianae* populations with high frequencies of the G143A mutation will likely have little effect on disease control and shift the population further towards complete resistance.

# CHAPTER 3. SURVIVAL AND FITNESS OF *CERCOSPORA NICOTIANAE* WITH MUTATIONS THAT CONFER RESISTANCE TO AZOXYSTROBIN

## 3.1 Introduction

Frogeye leafspot (FLS), caused by *Cercospora nicotianae* Ellis & Everh., is one of the oldest described diseases of tobacco (Shew & Lucas, 1991). Symptoms of FLS include small circular lesions (2-15mm) with a chlorotic halo, red-brown necrotic ring, and parchment like centers. Under humid conditions, black psuedostromata with conidiophores and conidia form, giving the lesion the appearance of a small eye (Shew & Lucas, 1991, Dixon et al., 2020). FLS is a polycyclic disease, with multiple infection cycles occurring in one growing season. Historically, FLS has not been a major concern to tobacco producers, but since 2015 disease severity has increased to the point of price dockage and crop rejection. FLS on the lowest stalk position was seen as desirable by graders, creating better burning agents for cigarette blends. FLS in any of the upper leaf canopy is seen as damage, reducing the quality of the leaf. Leaf loss or weight reduction can also occur when infections are severe and during late infections green spots appear on the cured leaf (B. Ratliff, *personal communication*; Dixon et al., 2020; Bailey et al., 2021). FLS also has been reported in greenhouse tobacco transplant production systems (Dixon et al., 2018). With FLS present in the greenhouse, *C. nicotianae* individuals are exposed to one additional treatment of azoxystrobin which could increase the frequency of azoxystrobin resistance in field populations. Any *C. nicotianae*-infected greenhouse transplants planted in the field could serve as an initial inoculum source for FLS.

Cultural practices, such as crop rotation, tillage, and greenhouse sanitation are important to reduce the amount of inoculum present for tobacco production. Crop rotation, tillage, and greenhouse sanitation are used in conjunction with fungicide applications as an integrated pest management approach to control FLS in tobacco production. Azoxystrobin (Quadris, Syngenta Crop Protection, Greensboro, NC) is the only systemic fungicide labeled in tobacco production for FLS. Tobacco producers manage FLS with applications of azoxystrobin in rotation with mancozeb (Manzate Pro-Stick, United Phosphorus Inc., King of Prussia, PA) starting three to four weeks after transplanting or when conditions are conducive for disease (Bailey et al., 2021). Azoxystrobin belongs to the quinone outside inhibitor (QoI) group of fungicides, which is the Fungicide Resistance Action Committee (FRAC) group 11. Fungicides in FRAC group 11 inhibit fungal respiration by limiting the production of adenosine triphosphate (ATP) by binding to the outer quinol oxidation site on cytochrome b (cytb), blocking the transfer of electrons through the cytochrome bc1 enzyme complex in the inner mitochondrial membrane (Fernández-Ortuño et al., 2008).

Shortly after QoI fungicides were approved for use in Europe in 1996, fungicide resistant *Erysiphe graminis* f.sp. *triciti* isolates were identified in Germany in 1998. In 1999, fungicide resistant isolates were confirmed in Belgium, Denmark, France, Germany and the United Kingdom (Chin et al., 2001). The mechanism responsible for reduced sensitivity to azoxystrobin in fungi are three single nucleotide polymorphisms (SNPs) in the mitochondrial cytb gene that result in the substitution from phenylalanine to leucine at codon position 129 (F129L),

glycine to arginine at codon position 137 (G137R) and from glycine to alanine at codon position 143 (G143A) (FRAC, 2021b). The level of resistance associated with the F129L and G137R mutations is considered moderate (partial), while the level of resistance associated with the G143A mutation is high (complete) (FRAC, 2021b). Resistance to azoxystrobin due to the F129L and G143A mutations has already been documented in *C. nicotianae* collected from multiple sites in Kentucky (Dixon et al., 2020). Another mechanism fungi use to overcome QoI fungicides is the alternative oxidase pathway (AOX). Described as a rescue mechanism, the alternative oxidase pathway bypasses the cytochrome bc1 enzyme complex by directly accepting electrons from ubiquinol, producing a fractional amount of ATP (Wood & Hollomon, 2003).

Occasionally mutations that result in reduced sensitivity to fungicides also renders these fungi less biologically fit than wild-type individuals sensitive to the fungicide, which is known as a fitness penalty (Hawkins & Fraaije, 2018). Examples of *in vitro* and *in planta* fitness parameters includes spore germination, mycelial growth, and disease severity (Hawkins & Fraaije, 2018, Pasche & Gudmestad, 2008, Zhang & Bradley, 2017). Fitness evaluations in other pathogens with resistance to QoI fungicides have had mixed results. Some QoI-resistant phytopathogens are reported to have no fitness costs (Zhang & Bradley, 2017), while some QoI-resistant phytopathogens are reported to have lower fitness but increased aggressiveness than their QoI-sensitive counterparts (Pasche & Gudmestad, 2008). Pasche and Gudmestad compared wild-type *Alternaria solani* isolates collected from potato fields for percent germination

and disease severity. They found that the wild-type *A. solani* had a higher percentage of germinating spores but caused lower disease severity in greenhouse inoculations on tomato (Pasche & Gudmestad, 2008). Zhang and Bradley found that QoI-sensitive and QoI-resistant isolates did not differ *in vitro* in terms of germination rate, mycelial growth, or *in planta* regarding initial disease severity on a susceptible soybean cultivar (Zhang & Bradley, 2017). In a scenario where the resistant individual is less fit than the sensitive individual, resistant populations decrease over time when fungicide selection pressure is removed, and fungicide efficacy can be restored over time. This has been documented in *C. beticola* with resistance to triphenyltin hydroxide (Hawkins & Fraaije, 2018, Secor et al., 2010). To date fitness comparisons between QoI-sensitive and QoI-resistant isolates of *C. nicotianae* have not been reported, and it is unknown if a period without QoI fungicide selection will restore fungicide sensitivity.

Pathogen survival is another fitness trait. Research on how QoI-resistant fungi survive over multiple years has occurred on other Cercospora pathogens. In survival trials of other *Cercospora sp*. researchers used infected leaf material on the soil surface or buried and certain depths to see if conidial viability was inhibited. There was no difference in soil depth and the viability of *C. sojina* but the viability of conidia from *C. beticola* decreased and deeper soil depths. This could inform agronomic management decisions to bury infected crop debris with tillage after a season. Conidia of *C. beticola* and *C. sojina* were viable for up to 22 and 24 months, respectively (Zhang & Bradley, 2014, Khan et al., 2008). The tobacco production guide recommends producer's rotate fields out of tobacco for

a minimum of three years to reduce disease pressure but that isn't always possible (Bailey et al., 2021). The ability of QoI-resistant isolates of *C. nicotianae* to persist in the environment between production years is unknown. The absence or persistence of QoI-resistant individuals between growing seasons could also infer a fitness penalty (Hawkins & Fraaije, 2018).

With limited fungicide options available in tobacco to control FLS it is important to understand the characteristics of QoI-resistant isolates of *C. nicotianae*. The objectives of this research are (i) to determine if *C. nicotianae* conidial germination and mycelial growth is impacted in isolates with the F129L and G143A mutations, and (ii) observe how QoI sensitivity affects field survival over a winter season.

## 3.2 Materials and methods

### 3.2.1 *Cercospora nicotianae* isolate collection

*C. nicotianae* isolates were collected from seven tobacco farms in 2016 and 2017 (Appendix 1). In 2016, the isolates were collected from farms with reduced azoxystrobin efficacy against FLS and from organic production farms without azoxystrobin applications for at least three years prior. Samples collected in 2016 were collected within one month of harvest. In 2017, samples were collected at the first incidence of FLS from greenhouse plants, Kentucky Plant Disease Diagnostic Laboratories, or visits from extension agents and specialists (Dixon et al., 2020). Isolates were surfaced sterilized in 10%v/v bleach for one minute then rinsed in two sterile de-ionized water baths for 30 seconds each. Sterilized leaf

samples were patted dry with sterile paper towels. FLS lesions were excised from the leaves and transferred into 60mm Petri dish (Falcon, Corning Life Sciences, Tewksbury, MA). The Petri dishes placed in clear plastic bags and left on a lab bench under fluorescent and black to blue (370-400nm wavelength) light on a 12-hr day/night cycle for 3-5 days. A single conidium was collected from each lesion, using a sterilized scalpel, and transferred onto clarified V8 (CV8) media. CV8 media is produced by combining V8 juice with 1.5% $CaCO_3$, stirring for 30 minutes, the centrifuging for 10 mins at 3,000 rpm. The clarified V8 was combined with de-ionized water and bacteriological agar (Difco, Becton Dickinson and Company, MD, U.S.A) at 19 g/liter, autoclaved until molten, and poured into plastic petri dishes. Where single conidia couldn't be collected a mycelial colony was grown on ¼ strength acidified potato dextrose agar (¼ aPDA) (Difco, Becton Dickinson and Company, MD, U.S.A) amended with 1ml/liter lactic acid for 7-10 days and transferred to CV8 to induce sporulation. A plug was cut from the sporulating plate, added to 10 ml sterile de-ionized water, vortexed with a fixed speed mini-vortex (Fisher Scientific, Waltham, MA) and 0.1 ml of the conidial suspension transferred to ¼ aPDA. A single spore was collected 24 hrs later and transferred to CV8. All isolates were grown under fluorescent and black to blue light on a 12-hr day/night cycle to induce sporulation. Plugs were cut with a scalpel and transferred into 1.5 ml microfuge tubes (USA Scientific, Orlando, FL) filled with 15% v/v glycerol and stored at -80°C to be revived as needed (Dixon et al., 2020).

### 3.2.2 Evaluating isolate sensitivity to azoxystrobin

Sensitivity testing was determined and reported in (Dixon et al., 2020). Briefly, sensitivity of *C. nicotianae* to azoxystrobin was defined as the effective concentration of azoxystrobin to inhibit germination by 50% ($EC_{50}$) (Russell, 2002). $EC_{50}$ values were determined by counting the number of germinating conidia, on PDA amended with azoxystrobin and salicylhydroxamic acid (SHAM) to inhibit AOX, out of 100 or 50, for 2016 and 2017, respectively (Dixon et al., 2020). Each isolate was tested three times. A conidium was considered germinating if the germ tube was at least half the length of the conidium. Conidial suspensions were created by suspending a CV8 plug, from revived stock, in 10 ml of deionized water and briefly vortexed. The conidial suspension was transferred in 0.1 ml aliquots on potato dextrose agar (PDA) media amended with serial dilutions from 0 to 10 µg/ml of technical grade azoxystrobin (99.5% pure) (Syngenta Crop Protection, Greensboro, NC) and 70 µg/ml SHAM (Acros Organics, Geel, Belgium) to inhibit the alternative oxidase pathway (AOX) (Dixon et al., 2020). The plated isolates were incubated for 20-28 hours in the dark and then germinating conidia were counted under 100X magnification with a compound microscope (Leica Microsystems Inc., Buffalo Grove, IL).

### 3.2.3 *In vitro* fitness comparisons

#### 3.2.3.1 Mycelial growth

*C. nicotianae* isolates collected in 2016 and 2017 (Table 3.2) were selected for fitness testing based on their sensitivity group determined by $EC_{50}$ values (Dixon

et al., 2020). Twenty-four isolates were selected for conidial germination comparisons, eight isolates representing three sensitivity groupings, from the sensitive, moderately resistant, and highly resistant sensitivity groups (Table 3.2).

*In vitro* fitness was evaluated based on mycelial growth rate and conidial viability of *C. nicotianae* isolates collected from 2016 and 2017 on non-fungicide-amended PDA media plates. Isolates were revived from -80°C, a single PDA plug was added to sterile deionized water, mixed in a fixed speed mini-vortex, and 0.1 ml was transferred onto a PDA media plate. Mycelial growth rate was determined by cutting 5.25 mm plugs with a #5 cork borer, from a PDA plate with *C. nicotianae* growing on it and transferring the plug into the center of a new PDA plate. Mycelial growth rate was determined from measurements taken with digital calipers every 2-3 days, the colony area was determined from two perpendicular diameter measurements, minus the area of the inserted plug, divided by 14 days. There were three replicate sets of Petri plates for each isolate and the mycelial growth assay was completed twice using the same isolates. The average mycelial growth rate was combined for each sensitivity group, wild-type, moderately resistant, and highly resistant. The mean mycelial growth rates were tested for equal variance, analyzed by One-Way ANOVA, and means were separated using Tukey's Honestly Significant Difference (HSD) (Minitab 20, Minitab LLC, State College, PA).

#### 3.2.3.2 Conidial germination

Conidial viability was assessed for the same isolates screened for mycelial growth (Table 3.2). Viability was measured as the percentage of germinating conidia out of 50 (2017) or 100 (2016) (Table 3.2). Conidia grown on CV8 media, to induce sporulation, were dislodged by scraping, suspended in sterile de-ionized water, then homogenized with a fixed speed mini vortex. The conidial suspension was transferred onto PDA amended with 70 µg/ml SHAM to inhibit the AOX pathway in *C. nicotianae*. After 20-28 hours of incubation at room temperature in the dark, germination of the conidia was observed under a compound microscope. The conidia were considered germinating if it possessed a germ tube that was at least half the length of the conidia. Data were subjected to Analysis of Variance (ANOVA) with groups separated using Tukey's HSD ($\alpha$=0.05).

### 3.2.4 Survival of *C. nicotianae* populations with mixed azoxystrobin sensitivity groups

Field trials to examine survival of *C. nicotianae* occurred during 2019 and 2020 at the University of Kentucky's research farm at Spindletop in Lexington Kentucky. The 2019 and 2020 survival trials were conducted in separate field locations. The field preparation, fertility, weed management, and insect control were accomplished using guidelines published in the University of Kentucky Extension publication Burley and Dark Tobacco Production Guide (UK ID-160) (Bailey et al., 2021). The 2019 experiment was in a field inoculated with a 1:1:1 mixture of *C. nicotianae* with one isolate from all three azoxystrobin sensitivity

groups in 2018. The 2020 experiment was in a different field location on the Lexington farm that was inoculated in 2019 with *C. nicotianae* mixture ratios of 1:0:0, 1:1:0, 1:1:1. 0:1:1, 0:1:0, 1:0:1, and 0:0:1 representing wild-type, moderately resistant (F129L), and highly resistant (G143A), respectively. The goal was to determine if this inoculum persisted in the environment and could infect tobacco the following year and retain QoI-sensitivity profiles. The experiment was set with burley tobacco variety KT-212LC on May 23rd and June 5th for the 2019 and 2020 field trials, respectively. To my knowledge, varietal resistance to FLS in commercial lines of burley tobacco has not been reported. The experiment was designed as a randomized complete block with four replications per treatment. The 2019 experimental design accounted for inoculation treatments performed in 2018, with the non-treated control placed where no inoculation occurred in 2018. The 2020 experimental design accounted for inoculation treatments performed in 2019 with the non-treated non-sprayed control and non-treated sprayed control were no inoculation occurred in 2019 and the plot either did or did not receive fungicide treatment.

Treatments for the 2019 overwintering trial (Table 3.1) were either the plot in the previous field trial was a non-inoculated non-fungicide sprayed control or it had been inoculated with a 1:1:1 mixture of isolates with WT, F129L, and G143A cytb.

In 2020, treatments for the 2020 survival trial (Table 3.1) were if the previous field trial plots had been non-inoculated non-fungicide treated, non-inoculated, sprayed, or inoculated with a 1:1:1 sensitivity group mixture. The 2020 overwintering treatments were sprayed the previous year with the grower's

standard fungicide program as follows: azoxystrobin (Quadris) 8oz/A, mancozeb (Manzate Pro-Stick) 2lb/100gal, azoxystrobin (Quadris) 8oz/A.

Table 3.1 Treatments for the 2019 and 2020 Cercospora nicotianae survival trials, showing the inoculation isolate ratios applied the previous year and the ratio of azoxystrobin sensitivities of the isolates used in the current trial year.

| 2019 Treatments | 2018 Inoculation Ratio | WT | F129L | G143A |
|---|---|---|---|---|
| | Non-inoculated | | | |
| | WT, F129L, and G143A | 33.33% | 33.33% | 33.33% |
| 2020 Treatments | 2019 Inoculation Ratio | | | |
| | Non-inoculated no spray | | | |
| | Non-inoculated sprayed | | | |
| | WT, F129L, and G143A | 33.33% | 33.33% | 33.33% |

### 3.2.5 Disease severity ratings

In 2019, the trial was rated three times for mean disease severity (Table 3.3). The 2020 trial was rated four times for mean disease severity (Table 3.4). Disease severity ratings were divided into two stalk positions, lower canopy, and upper canopy. Lower canopy ratings were taken from the soil to roughly the first meter of the tobacco plant, the upper canopy ratings were taken from the top meter of the tobacco plant. Disease severity was calculated as the percentage of leaf area with FLS symptoms. Seven plants were chosen to be rated arbitrarily each rating

date. The average of the seven plant ratings was tested against rep for equal variance, treatments compared with One-Way ANOVA, and means were separated using Tukey's Honestly Significant Difference ($\alpha$=0.05) (Minitab 20, Minitab LLC, State College, PA).

### 3.2.6 Isolate collection

Isolates were collected from leaf samples gathered during the trial season. In 2019 samples were collected on July 12, 2019, and August 19, 2019, starting at the onset of disease and ending before trial termination. In 2020, the trial was only sampled once, before termination on September 1, 2020. Leaf samples were collected arbitrarily from seven leaves throughout each plot with visible FLS symptoms in each plot. The leaves were stored at 4°C until they could be processed.

Leaves were surface sterilized for one minute in 10% v/v bleach, then rinsed twice in sterile deionized water. Leaf tissue was partially dried on sterilized paper towels, then FLS lesions were excised, excisions were then transferred into plastic petri dishes. Petri dishes were covered in clear plastic bags and placed under fluorescent and blue to black (370-400 nm) light on 12-hour day/night cycle for two to four days to induce sporulation. Leaf tissue was then examined under a dissecting microscope. If there was *C. nicotianae* conidia present, single conidia were removed with a sterile scalpel and transferred onto clarified V8 media. If the FLS lesions were not sporulating, the lesion was scraped with the scalpel and the collected conidium was plated to start mycelial colonies on ¼ strength acidified

potato dextrose agar (1/4aPDA). Mycelial colonies were grown for 7-10 days. A plug was cut from these plates, suspended in de-ionized water, vortexed, then transferred to another ¼ aPDA plate. After 24 hours a single spore was collected and transferred onto a clarified V8 media plate.

Single spore cultures were grown under fluorescent and UV light for another 10-14 days. Agar squares (3-5 mm) were cut from the leading edge of mycelial growth with a sterile scalpel and transferred into a 1.5 ml micro centrifuge tube (USA Scientific, Orlando, FL) filled with 0.5 ml of 15% v/v glycerol. The isolates were stored in a -80°C freezer and revived as needed.

### 3.2.7 DNA extraction

In preparation for molecular characterization of field-collected isolates, isolates were revived from -80°C storage and plated on clarified V8 media covered with a sterile disk of cellophane. The plated isolates were grown under fluorescent and UV light on a 12-hour day/night cycle for 10-14 days. The mycelium and conidia were scraped from half of the plate into a BeadBeater (Biospec Product, Bartlesville, OK) tube with two 6.35 mm glass beads. The BeadBeater tube was covered with Parafilm (Beemis Company Inc, Neenah, WI) and a small hole was poked in the center. The BeadBeater tubes were bound together with a rubber band and covered with a Kimwipe (Kimberly-Clark, Irvine, TX). The samples were then freeze dried for 24-48 hours.

The DNA was extracted using a modified SDS/CTAB method (Niu et al., 2008). The initial grinding step was accomplished with the BeadBeater tubes, chilled in liquid nitrogen, and pulverized in a Mini-BeadBeater 2.0 cell disrupter (Biospec

Product, Bartlesville, OK). The after the addition of the extraction buffer, mercaptoethanol, RNase A, and SDS the initial water bath incubation stage was extended from 5 min to 30 min. After the addition of 2-propanol before the precipitation step a cold incubation at 4°C for 30 min was added. After the DNA pellet was dissolved in Milli Q (Millipore Corporation) an additional purification step was added to remove debris. The DNA solution was warmed to 55°C, vortexed briefly, and spun at 10,000RPM in a centrifuge. The supernatant was transferred into a new 1.5 ml centrifuge tube.

The DNA quantity and quality was checked with a Nano-Drop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The stock DNA solution was used to make 0.1 ml microfuge tubes of concentration 10 ng/µl for PCR sensitivity group identification.

### 3.2.8 Molecular identification of mutations conferring resistance to QoI fungicides

Samples were analyzed using for mutations conferring resistance to azoxystrobin using a PCR method designed and optimized by (Li et al., 2021). Each reaction was prepared in 25 µl volume using a Titanium Taq PCR kit (TaKaRa Bio USA, Mountain View, CA) with 10X Titanium Taq buffer, 0.1 µM forward and reverse primers, 0.1 mM deoxynucleoside triphosphate, 1X Titanium Taq polymerase and 50 ng of DNA template. The thermal cycling parameters were an initial denaturation step at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 30 s, annealing at 68.3°C for 30 s, and extension at 72°C. A final extension at 72°C completed the reaction.

The PCR amplicons were read on 2% SeaKem LE agarose gel (Lonza, Bend, OR) using EZ-Vision (AMRESCO; VMR Life Science, Solon, OH) DNA staining dye. The amplicons were separated in 20-well gels using gel electrophoresis at 90V for 35 min. The gels were imaged on a Bio-Rad Gel Doc XR (Bio-Rad, Hercules, CA).

## 3.3 Results

### 3.3.1 *In vitro* fitness comparison

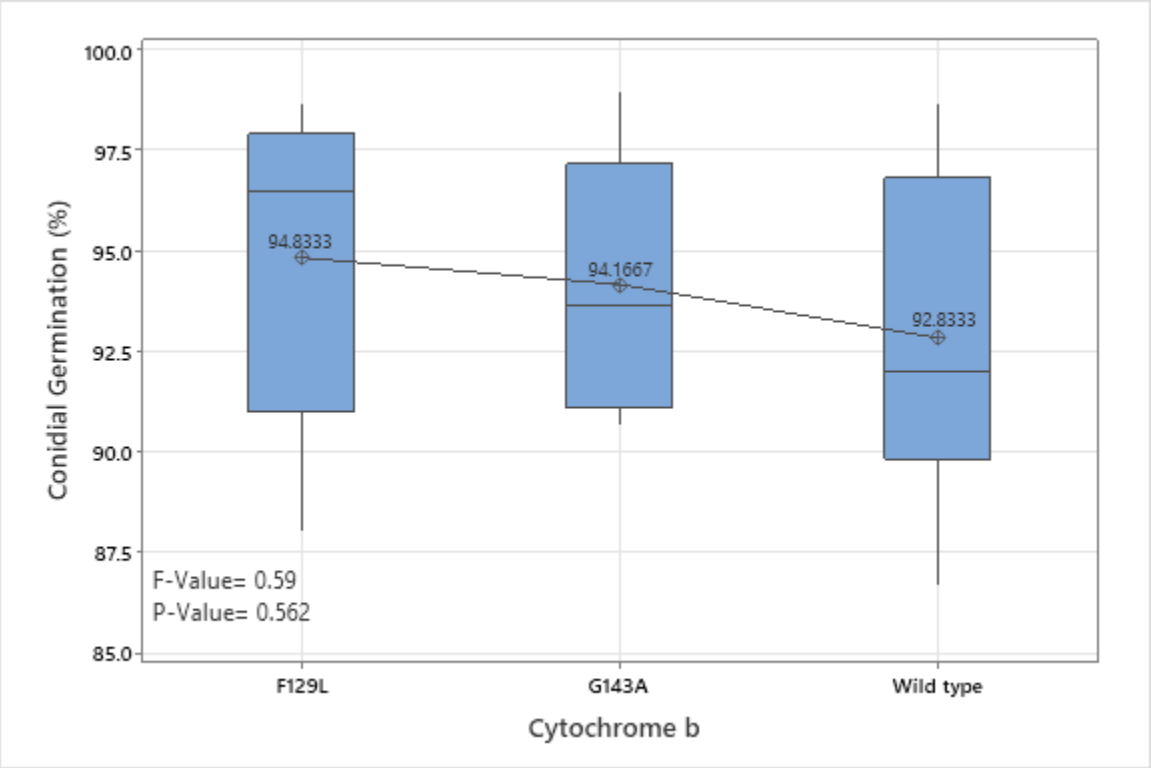
#### 3.3.1.1 Conidial germination

The mean conidial germination rate for the sensitive, moderately resistant, and highly resistant sensitivity groups were 92.83%, 94.83%, and 94.16%, respectively (Table 3.2). There were no significant differences in the conidial viability with respect to the isolate's sensitivity to azoxystrobin ($P$=0.5623) (Fig. 3.1).

Table 3.2 Cercospora nicotianae isolates collected from several farms across central Kentucky in 2016 and 2017 selected for conidial germination and mycelial growth rate fitness comparisons (Dixon et al., 2020). Conidial viability was determined as germinating conidia on potato dextrose agar (PDA) amended with 70 µg/mL salicylhydroxamic acid (SHAM).

| Isolate | Year Collected | $EC_{50}$ | Azoxystrobin Sensitivity Group | Mycelial Growth Rate ($mm^{2/day}$) | Conidial Viability |
|---|---|---|---|---|---|
| H2A | 2017 | 0.015 | Sensitive | 113.967 | 91.333 |
| 17H1405a | 2017 | 0.025 | Sensitive | 110.686 | 95.333 |
| 17CN016 | 2017 | 0.025 | Sensitive | 120.030 | 91.333 |
| 16Cn058 | 2016 | 0.030 | Sensitive | 129.386 | 98.667 |
| L407 | 2017 | 0.030 | Sensitive | 114.782 | 86.667 |
| 16Cn056 | 2016 | 0.036 | Sensitive | 96.505 | 97.333 |
| 17D1 | 2017 | 0.038 | Sensitive | 91.124 | 89.333 |
| 16cn044 | 2016 | 0.045 | Sensitive | 115.193 | 92.667 |
| 17P753d | 2017 | 0.177 | Moderate | 107.089 | 94.000 |
| 16Cn026 | 2016 | 0.245 | Moderate | 110.235 | 96.667 |
| 16Cn004 | 2016 | 0.283 | Moderate | 105.667 | 98.333 |
| 16Cn009 | 2016 | 0.292 | Moderate | 119.593 | 96.333 |
| 17JJ501e | 2017 | 0.313 | Moderate | 110.502 | 90.000 |
| 17P751a | 2017 | 0.332 | Moderate | 116.032 | 88.000 |
| 16Cn043 | 2016 | 0.328 | Moderate | 110.562 | 96.667 |
| 16Cn006 | 2016 | 0.535 | Moderate | 100.622 | 98.667 |
| 17JJ614c | 2017 | 1.212 | High | 113.984 | 90.667 |
| 17Cn003 | 2017 | 1.888 | High | | 97.333 |
| 16Cn035 | 2016 | 1.891 | High | 113.160 | 91.000 |
| 17Cn013 | 2017 | 2.510 | High | | 91.333 |
| 17Cn018 | 2017 | 2.531 | High | 120.435 | 96.667 |
| 17P846 | 2017 | 2.763 | High | 96.929 | 92.667 |
| 16Cn008 | 2016 | 2.879 | High | 131.127 | 99.000 |
| 17Cn006 | 2017 | 3.021 | High | 122.954 | 94.667 |
| *P*-Value | | | | 0.502 | 0.562 |

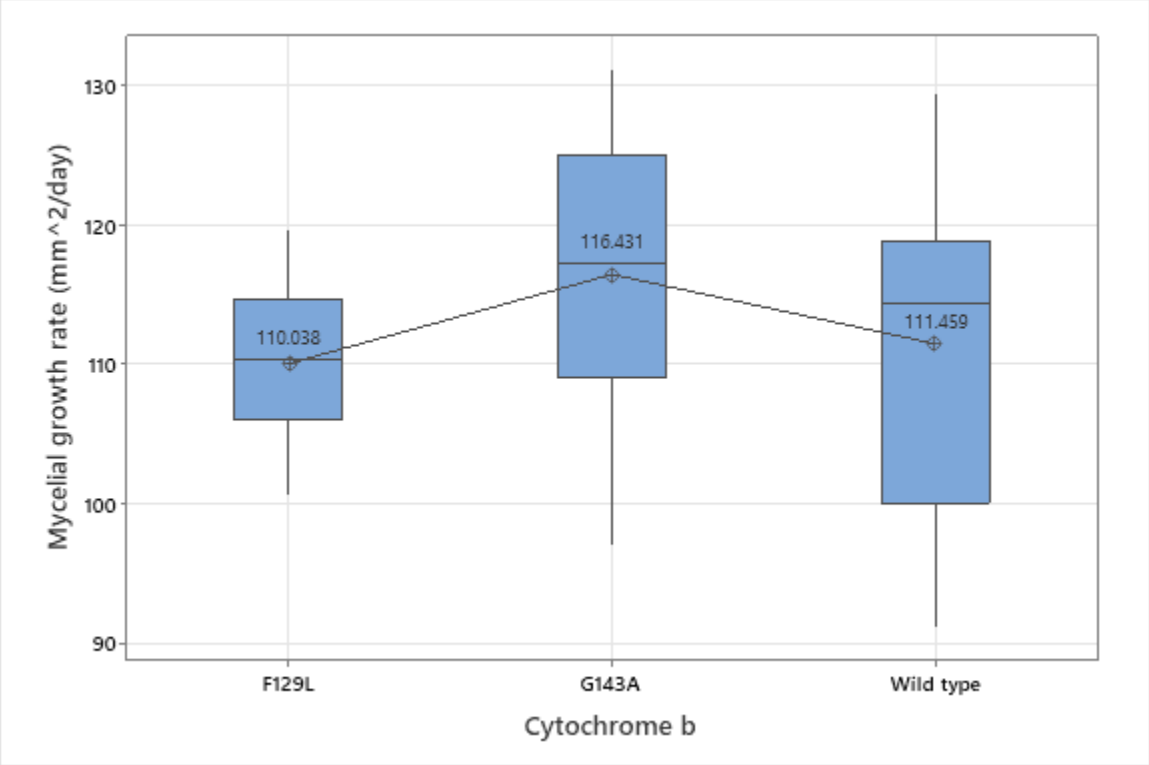
Figure 3.1 Boxplot of the Cercospora nicotianae conidial germination assay, showing the distribution of means for the sensitive, moderately resistant, and highly resistant azoxystrobin sensitivity groups. Conidial germination was determined on PDA amended with 70 µg/ml SHAM as the number of germinating conidia per 50 or 100 for 2017 and 2016, respectively. Conidia were considered germinating if they possessed a germ tube at least half the length of the conidia.




### 3.3.1.2 Mycelial growth rate

Due to contamination developing after isolates 17Cn003 and 17Cn013 were revived, they were dropped from the mycelial growth assay. The remaining isolates were measured for 14 days and grouped by azoxystrobin sensitivity. The sensitive, moderately, and highly resistant groups had mean mycelial growth rates of 111.46, 110.04, 116.43 $mm^2$/day (Table 3.2). The mycelial growth rate between sensitivity groups was not significantly different ($P$=0.502) (Fig 3.2).

Figure 3.2 Distribution of Cercospora nicotianae mycelial growth rate averages between azoxystrobin sensitivity groups. A 5.25mm circular plug of growing C. nicotianae was transferred to the center of a non-amended PDA plate. Mycelial growth rate was determined by taking two perpendicular measurements of diameter every 2 days for 14 days. Growth rates were averaged in sensitivity groups and analyzed by One-Way ANOVA.




### 3.3.2 Field survival disease severity ratings

Treatment differences were only observed for FLS disease severity ratings in 2019 in the upper canopy at the first rating date. The non-inoculated treatment had average FLS severity of 0.33% in the upper canopy and the full mixed population treatment had 1.5% FLS disease severity. No other treatment effects on FLS severity were observed (Table 3.3).

Table 3.3 Mean frogeye leaf spot disease severity ratings for the 2019 trial. Disease severity was determined as the percentage of leaf covered by FLS symptoms. Ratings are separated by date and stalk position.

| | July 12, 2019 | | July 31, 2019 | | August 19, 2019 | |
|---|---|---|---|---|---|---|
| | Lower | Upper | Lower | Upper | Lower | Upper |
| Non-inoculated | 8.70 | 0.33 b | 11.05 | 1.76 | 13.91 | 5.29 |
| Full mix 1:1:1 [a] | 12.78 | 1.56 a | 9.86 | 1.19 | 9.86 | 3.81 |
| *P*-Value [b] | 0.19 | 0.02 | 0.74 | 0.30 | 0.06 | 0.11 |

[a] A 1:1:1 mixture of isolates with wild type, F129L or G143A cytochrome b.

[b] Ratings were compared using One-Way ANOVA and means were separated with Tukey's Honestly Significant Difference. Means that do not share a letter are significantly different.

Table 3.4 Mean frogeye leaf spot disease severity ratings for the 2020 trial. Disease severity was determined as the percentage of leaf covered by FLS symptoms. Ratings are separated by date and upper or lower stalk position.

| | July 15, 2020 | | July 28, 2020 | | August 12, 2020 | | August 26, 2020 | |
|---|---|---|---|---|---|---|---|---|
| | Lower | Upper | Lower | Upper | Lower | Upper | Lower | Upper |
| Non-inoculated non-sprayed | 0.71 | 0 | 1.07 | 0 | 5 | 1.14 | 4.35 | 1.21 |
| Non-inoculated sprayed | 1.36 | 0 | 1 | 0.07 | 1.57 | 0.36 | 3.64 | 0.86 |
| Full mix 1:1:1 [a] | 1.64 | 0 | 1 | 0 | 2.71 | 0.86 | 2.5 | 0.79 |
| *P*-value | 0.369 | | 0.976 | 0.47 | 0.32 | 0.68 | 0.36 | 0.42 |

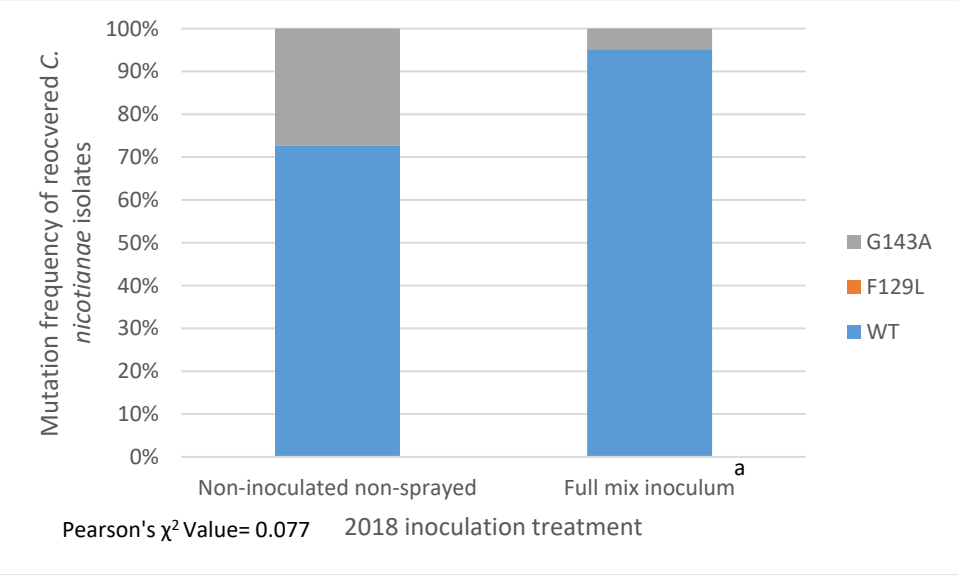
[a] A 1:1:1 mixture of isolates with wild type, F129L or G143A cytochrome b.

[b] Ratings were compared using One-Way ANOVA and means were separated with Tukey's Honestly Significant Difference. Means that do not share a letter are significantly different.

### 3.3.3 Molecular identification of mutation frequency

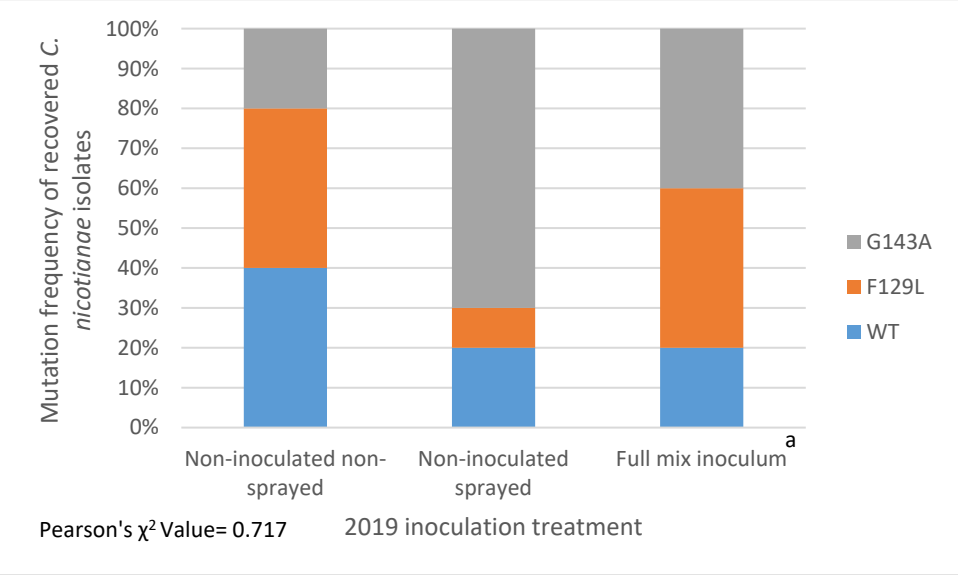
The PCR primers were able to identify mutations in the *C. nicotianae* cytb gene associated with QoI resistance. However, the total number of isolates recovered for both years was low. In 2019, only 31 isolates were recovered across both sampling dates. In 2020, only 20 isolates were recovered for all three treatments. Isolates with identified mutations were grouped, and frequencies were compared using Pearson's Chi-Square test, but there was no significant difference between mutation frequencies in 2019 or 2020 (Figure 3.3)(Figure 3.4). In an attempt to account for the small sample size, mutation frequencies were analyzed in in paired comparisons using Fisher's Exact Test (Hazra & Gogtay, 2016). There were no significant differences detected with this method, (P=1) for every pair.

Figure 3.3 Mutation frequency of Cercospora nicotianae isolates recovered from the 2019 trial. Cytochrome b (cytb) mutations were identified using PCR with primers developed by (Li et al., 2021). These three primer sets amplified wild type cytb and cytb with SNPs that result in F129 or G143A mutations. Mutation frequencies were compared using Pearson's Chi-Square analysis.




[a] A 1:1:1 inoculation mixture of isolates containing wild type, F129L or G143A mutations in cytochrome b.

Figure 3.4 Mutation frequency of Cercospora nicotianae isolates recovered from the 2020 trial. Cytochrome b (cytb) mutations were identified using PCR with primers developed by (Li et al., 2021). These three primer sets amplified wild type cytb and cytb with SNPs that result in F129 or G143A mutations. Mutation frequencies were compared using Pearson's Chi-Square analysis.




[a] A 1:1:1 inoculation mixture of isolates containing wild type, F129L or G143A mutations in cytochrome b.

## 3.4 Discussion

Biological fitness is a measure of an organism's ability to survive and propagate it's genetic material into the next generation (Orr, 2009). If *C. nicotianae* individuals with the F129L or G143A mutation are as fit as the wild-type it is not likely that the population would return to azoxystrobin sensitivity in the absence of selection (Hawkins & Fraaije, 2018). There were no significant differences

observed for the conidial germination or mycelial growth rate of tested *C. nicotianae* isolates from the wild-type, moderately resistant (F129L), or highly resistant (G143A) azoxystrobin sensitivity groups. This could indicate the absence of any major fitness penalties associated with either the F129L or G143A mutations. Similar findings were reported in other fitness evaluations performed with *Alternaria solani, C. beticola, Magnaporthe grisea,* and *Pyricularia grisea* with resistance to QoI fungicides ((Zhang & Bradley, 2017, Pasche & Gudmestad, 2008, Avila-Adame & Köller, 2003, Kim et al., 2003). However, smaller fitness penalties could be present and only detectable in competition assays (Hawkins & Fraaije, 2018).

Previous inoculation with mixed populations of QoI-resistant *C. nicotianae* did not impact FLS ratings in field experiments in 2019 or 2020. Comparisons for aggressiveness in both *C. sojina* and *A. solani*, occurring in greenhouse trials, concluded that F129L and G143A mutants were more aggressive than sensitive isolates and could cause more severe infections (Pasche & Gudmestad, 2008, Zhang & Bradley, 2017), but *Magnaporthe oryzae* G143A mutants were less aggressive then the wild type individuals (Ma & Uddin, 2009). In *C. sojina* the comparisons were performed on two soybean (*Glycine max*) cultivars, cv. Blackhawk and cv. Davis. Soybean cultivar Davis possesses a resistance gene (*Rcs3*) that confers resistance to FLS, while cv. Blackhawk is susceptible to FLS. To my knowledge there are no commercial varieties of burley tobacco with resistance to FLS. Increased aggressiveness was not observed in our *C. nicotianae*

field populations, where disease severity was similar in *C. nicotianae* populations with mixed azoxystrobin sensitivity.

Isolate populations recovered from 2019 and 2020 varied greatly in the frequency of detected QoI resistance mutations. The isolate population recovered from 2019 was mostly comprised of wild-type individuals, while the population recovered from 2020 sampling was comprised mostly of isolates that contained the G143A mutation conferring high resistance to QoI fungicides. Statistical analysis on these data was difficult because we failed to recover a large sample of isolates. A monitoring program, collecting and identifying isolates from multiple locations, would be useful to determine if *C. nicotianae* with wild type or mutated cytb persists in the environment over the winter.

Field isolates recovered from the survival trials are assumed to have originated from the previous year and overwinter on crop debris or in the soil. The selection of field location attempted to geographically isolate the trials from other tobacco trials close enough to spread *C. nicotianae* through wind dispersal. It cannot be said for certain that all inoculum originated from inoculations applied the previous year without identification by microsatellite markers or similar tracking. There is a possibility that inoculum was carried by wind from neighboring tobacco crops. A spore trapping study done on the dispersal of *C. beticola* conidia found that the majority of infections start from aerial spore dispersal. This occurs primarily over short distances, but long-distance spore dispersal is still possible (Imbusch et al., 2021).

Determining fitness costs associated with azoxystrobin resistance in *C. nicotianae* is crucial in understanding the implications of management strategies. In *C. beticola* resistant to triphenyltin hydroxide, fungicide efficacy returned after a decrease in fungicide applications. This is likely caused by reduced competitive ability in resistant isolates compared to sensitive individuals (Secor et al., 2010). If azoxystrobin resistant isolates of *C. nicotianae* were less fit than sensitive isolates, reducing azoxystrobin applications should return *C. nicotianae* populations back to sensitivity with a break in use (Hawkins & Fraaije, 2018). From the data collected from 2019 and 2020 *in vitro* and in field experiments, it seems unlikely that there are any major fitness disadvantages in *C. nicotianae* with the F129L or G143A cytb mutations. A large-scale monitoring program to track population based changes in resistance levels would provide better insight to resistance development (Brent & Hollomon, 2007a). Current azoxystrobin resistance management strategies should be focused on reducing the total population of *C. nicotianae* by alternating fungicides with different modes of action or mixtures, crop rotation, and greenhouse sanitation.

# CHAPTER 4. FUNGICIDE ALTERNATIVES FOR FROGEYE LEAF SPOT CONTROL IN AREAS WITH POPULATIONS OF *CERCOSPORA NICOTIANAE* WITH REDUCED SENSITIVITY TO AZOXYSTROBIN

## 4.1 Introduction

Fungicides have been used to control plant pathogens for over 200 years. Some of the very earliest fungicides, copper and sulfur compounds, are still utilized in agriculture today (Brent & Hollomon, 2007a). By the 1940s new chemistries were developed that were more efficacious , requiring smaller application quantities and with better control of phytopathogens (Morton & Staub, 2008). These new chemistries were protective contact fungicides with multiple target site modes of action. These fungicides remain on the plant surface, disrupting pathogen colonization and infection. In the 1970s, systemic fungicide chemistries emerged. These chemistries are taken up by the plant and redistributed to non-treated parts of the plant. In the 1990s, new chemical classes with novel modes of action were developed. These novel modes of action target pathogen specific physiological processes, such as respiration, nucleic acid metabolism, cell wall formation, signal transduction, membrane integrity and microtubule assembly (Morton & Staub, 2008, FRAC, 2021a, Latin, 2011). As of 2021 there are 50 unique novel modes of action along with chemicals that have multi-site, host defense induction, and unknown modes of action (FRAC, 2021a).

Fungicide resistance in plant pathogens was a rare occurrence before 1970 but became a problem soon after the introduction of site-specific systemic fungicides (Brent & Hollomon, 2007a). Resistance development is now a major consideration in the development and marketability of new novel fungicide

modes of action. The likelihood of resistance development is dependent on several factors in the fungicide chemistry, application frequency, and the target pathogen's biology and reproduction (Hahn, 2014). Mechanisms of resistance often involve the mutation of the fungicide's target protein as long as the mutated protein maintains adequate function and does not reduce pathogen fitness (Hahn, 2014).

Azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC) is currently the only systemic fungicide labeled for use in tobacco to control fungal foliar pathogens. Azoxystrobin belongs to the quinone outside inhibitor (QoI) chemical class, the Fungicide Resistance Action Committee (FRAC) group 11. Fungicides in FRAC group 11 inhibit adenosine triphosphate (ATP) production by binding to the outer quinol oxidation site of the cytochrome *bc1* enzyme complex (cytb), blocking the transfer of electrons in the inner mitochondrial matrix (Fernández-Ortuño et al., 2010). Azoxystrobin was first commercialized in 1996, but soon after its release, reduced sensitivity to azoxystrobin was reported in several plant pathogenic fungi (Fernández-Ortuño et al., 2008). The mechanisms for resistance to QoI fungicides results from single nucleotide polymorphisms (SNPs) in the cytb gene that result in amino acid substitutions at codon positions 129, 137 and 143. The substitution of phenylalanine to leucine at position 129 (F129L) and glycine to arginine at position 137 (G137R) confer moderate or partial resistance. The substitution of glycine for alanine at position 143 (G143A) confers high or complete resistance (Fernández-Ortuño et al., 2010).

Azoxystrobin was first labeled for use in tobacco in 2005 to control *Peronospora tabacina* (blue mold), *Alternaria solani* (target spot), and *Cercospora nicotianae* (frogeye leaf spot). In 2015, the tobacco pathologist at the University of Kentucky received reports of increased frogeye leaf spot (FLS) disease severity and incidence. *C. nicotianae* isolates were collected from tobacco farms in central and south-central Kentucky, then evaluated for sensitivity to azoxystrobin, and screened for mutations conferring resistance to QoI fungicides. The isolates separated into three sensitivity groups, sensitive (wild type), moderately resistant (containing the F129L mutation), and highly resistant (containing the G143A mutation) (Dixon et al., 2020, Li et al., 2021). The rate of azoxystrobin required to reduce conidial germination by 50% ($EC_{50}$ value) for these groups were 0.03, 0.3, and 2.0 μg/ml, for the sensitive, moderately resistant, and highly resistant groups, respectively (Dixon et al., 2020). The $EC_{50}$ values for *C. nicotianae* are consistent with azoxystrobin sensitivities in *C. sojina* in soybean, *Ascochyta rabiei* in chickpea, *Pyricularia grisea* in turfgrass, *Alternaria solani* in potato and *Erysiphe graminis* in wheat (Zhang et al., 2012a, Wise et al., 2009, Kim et al., 2003, Pasche et al., 2004, Chin et al., 2001).

Due to concerns about synthetic pesticide's impact on human and environmental health, there has been a reduction in the synthetic chemicals available for use. While records from the Roman Empire indicate that natural pesticides have been around for a long time, interest in pesticides made from natural products has recently increased as a result of tighter regulations and health concerns (Dayan et al., 2009). Some of the synthetic chemicals available today are derived from

natural compounds. Strobilurins were first discovered in a wood rotting basidiomycete *Strobilurus tenacellus* (Anke et al., 1977). Other natural pesticides utilize bacterial species, such as *Bacillus amyloliquefaciens*, that have been shown induce plant defense, inhibit fungal growth and reproduction, and influence gene expression in phytopathogens (Liu et al., 2019). These natural pesticides will likely be instrumental in managing the development of resistance in synthetic pesticides (Dayan et al., 2009).

A key tool in the delaying fungicide resistance development is utilizing fungicides with differing modes of action. Fungicide mixtures with multiple modes of action or spray schedules with alternating modes of action will reduce selection of fungicide-resistant individuals and overall populations of pathogens (Hollomon, 2015). Current recommendations for controlling *C. nicotianae* are to use alternating sprays of azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC) and mancozeb (Manzate Pro-Stick; United Phosphorus Inc, King Of Prussia, PA) at the first cultivation or when conditions are conducive for disease (Bailey et al., 2021). However, because of industry concerns with mancozeb residue, the application of mancozeb is limited to seven weeks post-transplant. The limited availability of fungicides from different modes of action creates added difficulty in managing QoI-resistance in *C. nicotianae*. The objectives of this work are to (i) examine fungicides labeled for use in tobacco for their efficacy against *C. nicotianae* with known QoI resistance (ii) compare organic and biological fungicide alternatives to the recommended fungicide

program, and (iii) determine if there is better control using the maximum labeled rate of azoxystrobin.

## 4.2 Materials and methods

### 4.2.1 Greenhouse screenings of fungicides for efficacy against FLS

Nine biological or organic fungicides were screened in a greenhouse trial to identify two chemistries with comparable efficacy to azoxystrobin on FLS (Table 4.1). These fungicides were selected because they were already labeled for use in burley tobacco. The treatments were arranged in the greenhouse in a randomized complete block design with eight fungicide treatments, and a water treated control. There was only one replication for each treatment. Expanded polystyrene (EPS) tobacco trays with 288 cells were cut into smaller five by seven cell (35 total cells), miniature trays. The miniature EPS trays were filled with Carolina Choice (Carolina Soil Company, Kinston, NC) soilless tobacco media and seeded by hand on March 26, 2019, with tobacco variety KT 212LC (Workman Tobacco, Murray KY), which is known to be susceptible to FLS. The filled and seeded trays were then floated in small grey plastic tubs filled with 3,350 mL water amended with 1.7 g of 20-10-20 fertilizer. Water was added as needed and 8.5 g fertilizer was added every week post germination. On May 20$^{th}$, one tray was sprayed with one of the candidate fungicides for each treatment. Trays were separated so that no leaves from other trays were contacting another tray. Sprays were applied with an amber bullet spray bottle with black micro sprayer (Tolco, Toledo, OH) at the field rate scaled down to the area of the 35-cell tray, 284 cm$^2$, in a carrier volume of 3 ml water (Table 4.1).

Within 24 hours the trays were then sprayed with a three-way mixture of *C. nicotianae* representing all three azoxystrobin sensitivity groups. The trays were covered with clear plastic bags for three days, then the plastic bags were removed. The fungicides were then evaluated for efficacy by counting the total number of FLS lesions on every plant for each tray on June 7, 2019, June 11, 2019 and June 17, 2019. Two products were then selected to be incorporated into the fungicide spray programs used in the 2019 and 2020 field trials

Table 4.1 List of fungicides, active ingredients, their manufacturers, and mode of action against fungal pathogens included in the greenhouse fungicide screen for efficacy against frogeye leaf spot. Direct modes of action target physiological processes in the pathogen. Indirect modes of action induce plant defense by reactive oxygen burst, nitrous oxide (NO) production, callose deposition and lignification (Liu et al., 2019). The per hectare rate equivalent was scaled down to 284 cm2, the area of a 7 cell by 5 cell expanded polystyrene (EPS) tray cut from a 288 cell EPS tray.

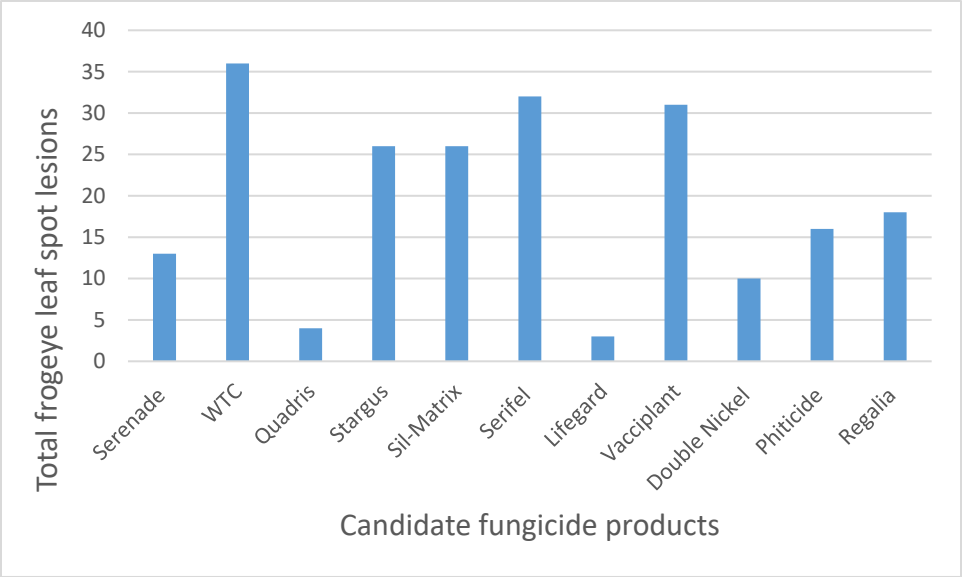
| Product | Active Ingredient | Manufacturer, Location | Mode of Action | | Rate equivalent |
|---|---|---|---|---|---|
| | | | Direct | In-direct | |
| Double Nickel LC | *Bacillus amyloliquefaciens* strain D747 | Certis USA, Columbia, MD | x | x | 2.34 L/ha |
| Lifegard WG | *Bacillus mycoides* isolate J | Certis USA, Columbia, MD | | x | 0.32 kg/ha |

| | | | | | |
|---|---|---|---|---|---|
| Phiticide | mono- and dibasic sodium, potassium, and ammonium phosphites | Drexel Chemical Company, Memphis, TN | | x | 5.85 L/ha |
| Vacciplant | Laminarin | Helena Agri-Enterprises LLC, Collierville, TN | | x | 1.46 L/ha |
| Regalia | *Reynoutria sachalinensis* | Marrone Bio Innovations, Davis, CA | | x | 9.35 L/ha |
| Serenade ASO | *Bacillus subtilis* strain QST 713 | Bayer Crop Science, Creve Coeur, MO | x | x | 3.36 kg/ha |
| Serifel | *Bacillus amyloliquefaciens* strain MBI 600 | BASF, Florham Park, NJ | x | x | 1.17 L/ha |
| Sil-Matrix | Potassium silicate | Certis USA, Columbia, MD | | x | 2.81 L/ha |
| Stargus (MBI-110) | *Bacillus amyloliquefaciens* strain F727 | Marrone Bio Innovations, Davis, CA | x | x | 9.35 L/ha |
| Quadris | Azoxystrobin | Syngenta Crop Protection, Greensboro, NC | x | | 0.58 L/ha |

The biological fungicide products that had the fewest number of lesions per tray of tobacco were Lifegard (Certis USA LLC, Columbia, MD) and Double Nickel

(Certis USA LLC, Columbia, MD) with 3 and 10 lesions, respectively (Figure 4.1 ). The standard fungicide treatment, azoxystrobin (Quadris), had 4 FLS lesions. Based on this data we elected to use Lifegard and Double Nickel as our biological fungicide treatments in field trials.

Figure 4.1 Results of the 2019 greenhouse fungicide efficacy screening showing the total number of FLS lesions per 35 cell tray 14 days post inoculation.




### 4.2.2 Field trials to evaluate product efficacy against FLS

Field trials were conducted in 2019 and 2020 at the University of Kentucky Spindletop Research Farm, in Lexington, KY. Land preparation, fertility, weed management and insect control were managed following the University of Kentucky Cooperative Extension Service guidelines outlined in the Burley and Dark Tobacco Production Guide (ID-160) (Bailey et al., 2021).

The fields were set with burley tobacco variety KT 212-LC, known to be susceptible to FLS, on May 23, 2019, and June 12, 2020, for 2019 and 2020, respectively. The plots were set in a randomized complete block design, with each treatment replicated four times. The plots were 9.14 meters long by 2.13 wide, with approximately 30-35 plants per plot. The plots consisted of two treated rows bordered on both sides by non-treated rows and were separated on both ends by 1.5 meter plant free breaks. The plots were all treated the same following agronomic guidelines outlined in ID-160 (Bailey et al., 2021) except for the fungicide spray program treatments.

### 4.2.3 Inoculation

In 2019, the trial was inoculated with a three-way mixture of inoculum. The isolates used for inoculation were 16Cn008, 16Cn009, and 16Cn056 with known azoxystrobin sensitivity levels and confirmed wild type cytb, F129L, and G143A mutations. The isolates were sampled, isolated and stored from samples collected in from multiple locations in Kentucky in 2016 (Dixon et al., 2020). The inoculum was prepared from isolates revived from -80°C storage by transferring an agar plug into sterile deionized water, briefly vortexed with a fixed speed mini-vortex (Fisher Scientific, Hampton, NH) then adding 0.1 ml of the conidial suspension on clarified V8 plates. Clarified V8 is made from V8 juice amended mixed with 1.5% $CaCO_3$, stirred for 30 minutes, and centrifuged at 3,000 RPM. The 100 ml of the supernatant was combined with 400 ml sterilized deionized water and bacteriological agar (Difco, Becton Dickinson and Company, MD, U.S.A) at 19 g/l, autoclaved until molten and poured into plastic Petri dishes

(Falcon, Corning Life Sciences, Orlando, Fl). The CV8 plates were grown under fluorescent and blue to black light for 10-14 days. The plates were flooded with sterile de-ionized water and conidia were dislodged with a sterile plastic scraper. The irrigated plate was poured into a sterile glass bottle with all the plates of the same isolate. The inoculum concentration was quantified using a hemocytometer and the three isolates were titrated to equal the lowest concentration obtained from an isolate, $4.17x10^4$ conidia/ml. All three inoculation isolates were combined in equal parts. The inoculation mixture was applied on June 18, 2019 and June 30, 2020 with a concentration of $4.17x10^4$ conidia/ml and $1.2x10^4$ conidia/ml, respectively. The inoculation mixture was sprayed over top of every plant in the treatment row from a 1.5 l handheld pump sprayer (Tolco, Toledo, OH) at a rate of 5ml/s for 6 seconds per row. The walking speed was 5.6 kph and the total amount of inoculum applied per plot was 60 ml. Inoculation was applied during stage 1, the field leaf development stage.

Table 4.2 Cercospora nicotianae isolates selected for use in field inoculation experiments based on their azoxystrobin sensitivity group, determined by the effective concentration to inhibit conidial germination by 50% (EC50)(Russell, 2002). Cytochrome b genotype was confirmed using mut4 PCR primers (Zeng et al., 2015) and Sanger sequencing (Dixon et al., 2020).

| Isolate | Year Collected | County and State of Origin | $EC_{50}$ µg/ml | Known cytb mutations | GenBank accessions |
|---|---|---|---|---|---|
| 16Cn008 | 2016 | Green County, KY | 2.879 | G143A | MK369753 |
| 16Cn009 | 2016 | Green County, KY | 0.292 | F129L | MK369754 |
| 16Cn056 | 2016 | Scott County, KY | 0.036 | no mutation | MK369759 |

### 4.2.4 Fungicide applications

Treatments for the 2019 and 2020 field trials are listed below and based on greenhouse fungicide screening results. Final selected treatments for 2019 are listed in (Table 4.3) and final treatments for 2020 are listed in (Table 4.4), respectively.

Table 4.3 Fungicide treatments, application timing, and applied rate for the 2019 field trial.

| Treatment description | Chemical [a] | Application Timing [b] | Rate per hectare |
|---|---|---|---|
| Non-treated Control | N/A | | |
| Grower's Standard | azoxystrobin | 1 | 0.58 L |
| | mancozeb | 2 | 2.4 g/L |
| | azoxystrobin | 3 | 0.58 L |
| Alternative Standard | azoxystrobin | 1 | 0.58 L |
| | Cuprous oxide | 2 | 1.36 kg |
| | azoxystrobin | 3 | 0.58 L |
| Increased Rate | azoxystrobin | 1 | 0.88 L |
| | mancozeb | 2 | 2.4 g/L |
| | azoxystrobin | 3 | 0.88 L |
| | azoxystrobin | 1 | 0.58 L |

| | | | |
|---|---|---|---|
| Alternative Biological One | *Bacillus amyloliquefaciens* strain D747 | 2 | 2.34 L |
| | azoxystrobin | 3 | 0.58 L |
| Alternative Biological Two | azoxystrobin | 1 | 0.58 L |
| | *Bacillus mycoides* isolate J | 2 | 0.32 kg |
| | azoxystrobin | 3 | 0.58 L |
| Biological Only | *Bacillus amyloliquefaciens* strain D747 | 1 | 2.34 L |
| | *Bacillus mycoides* isolate J | 2 | 0.32 kg |
| | *Bacillus amyloliquefaciens* strain D747 | 3 | 2.34 L |

[a] Trade names for the fungicides are Quadris (azoxystrobin), Manzate Pro-Stick (mancozeb), Nordox 75WG (cuprous oxide), Double Nickel (*Bacillus amyloliquefaciens* strain D747), and Lifegard (*Bacillus mycoides* isolate J).

[b] Application Timing dates are July 25, 2019 (1), August 8, 2019 (2), and August 19, 2019 (3).

Table 4.4 Fungicide treatments, application timing, and applied rate for the 2020 field trial.

| Treatment Description | Chemical a | Application Timing b | Rate per hectare |
|---|---|---|---|
| Untreated Control | N/A | | |
| Grower's Standard | azoxystrobin | 1 | 0.58 L |
| | mancozeb | 2 | 2.4 g/L |
| | azoxystrobin | 3 | 0.58 L |
| Alternative Biological One | azoxystrobin | 1 | 0.58 L |
| | *Bacillus amyloliquefaciens* strain D747 | 2 | 2.34 L |
| | azoxystrobin | 3 | 0.58 L |
| Alternative Biological Two | azoxystrobin | 1 | 0.58 L |
| | *Bacillus mycoides* isolate J | 2 | 0.32 kg |
| | azoxystrobin | 3 | 0.58 L |
| Biological Only | *Bacillus mycoides* isolate J | 1 | 0.32 kg |
| | *Bacillus amyloliquefaciens* strain D747 | 2 | 2.34 L |

| | | | |
|---|---|---|---|
| | *Bacillus mycoides* isolate J | 3 | 0.32 kg |
| Alternative Standard | azoxystrobin | 1 | 0.58 L |
| | Cuprous oxide | 2 | 1.36 kg |
| | azoxystrobin | 3 | 0.58 L |
| Increased Rate | azoxystrobin | 1 | 12 oz |
| | mancozeb | 2 | 2.4 g/L |
| | azoxystrobin | 3 | 12 oz |

[a]Trade names for the fungicides are Quadris (azoxystrobin), Manzate Pro-Stick (mancozeb), Nordox 75WG (cuprous oxide), Double Nickel (*Bacillus amyloliquefaciens* strain D747), and Lifegard (*Bacillus mycoides* isolate J).

[b] Application timing dates are 7/15/2020 (1), 7/29/2020 (2), and 8/13/2020 (3).

In 2019 and 2020, the fungicide treatments were applied at a carrier volume of 280.62 l/ha for the first application when the tobacco plants were below waist height. The carrier volume was raised to 467.7 l/ha for the second and third application timings when tobacco plants were above waist height. Fungicides were sprayed on a 10-to-14-day schedule depending on acceptable spraying conditions. All sprays were applied with a Lee Spider DP sprayer (Lee Agra, Lubock, TX) fitted with a two-row spray boom with three 360 Undercover drop nozzles (360 Yield Center, Morton, IL) fitted with flat fan XR 80015 and Turbo TeeJet Induction 110015 nozzles (TeeJet Technologies, Glendale Heights, IL), and two over the top TeeJet Conjet VisiFlo TX-VK8 nozzles (TeeJet Technologies, Glendale Heights, IL). Drop nozzle bodies were spaced one meter apart and the

two overhead nozzles were one meter apart. All sprays were applied at 413.69 kPa.

### 4.2.5 Field trial FLS disease severity ratings

Foliar disease ratings were taken three and four times throughout the season for 2019 and 2020, respectively. FLS disease severity 2019 ratings were taken on July 24, 2019, August 1, 2019, and August 19, 2019. FLS disease severity ratings for 2020 were recorded on July 15, 2020, July 28, 2020, August 12, 2020, and August 26, 2020. The ratings were taken before each fungicide application during the stem elongation and crop cover growth stage and once after all applications had been applied before trial termination. Ratings were taken from seven tobacco plants, chosen arbitrarily, from each plot. The ratings per plant were separated into two groups, one rating for the lower leaf canopy and one rating for the upper leaf canopy. The lower leaf canopy rating was taken from leaves from the soil to mid-plant, and the upper canopy ratings were taken from mid-plant to the top of the plant. Disease severity was determined as the percentage of leaf infected with FLS on a scale from 0-100%. The lower and upper canopy ratings were analyzed separately. Disease severity ratings were tested for equal variance, analyzed with one-way ANOVA and means were separated by Tukey's honestly significant difference (HSD) (Minitab 19, Minitab LLC, State College, PA).

### 4.2.6 Field sample collection and isolate preparation

Leaf samples were collected twice during the growing season in 2019 and 2020. The first leaf sampling occurred on July 19, 2019, and July 15, 2020, before the

first fungicide application to determine mutation frequencies in the starting population of *C. nicotianae*. The final leaf sample collection was on August 29, 2019, and August 27, 2020, after all the fungicide applications were made, to determine mutation frequencies after exposure to different fungicide programs. *C. nicotianae* isolates were collected from each plot, twelve leaf samples with actively sporulating FLS lesions were collected. Infected leaf samples were stored until they could be processed in a walk in 4° C cooler. To process the samples, the leaves were surface sterilized in a 10% v/v bleach solution for one minute, then transferred twice into a sterile deionized water rinse. The sterilized leaves were then partially dried on sterile paper towels. FLS lesions were excised from the leaves, transferred into a Petri plate (Falcon, Corning Life Sciences, Tewksbury, MA) wrapped in a clear plastic bag, and moved onto the lab bench under fluorescent and blue-black light (370-400 nm) on a 12hr day/night cycle to induce sporulation. After 48 to 72 hours the samples were examined under a dissecting microscope for conidia. When present, single conidia were collected with a sterile scalpel and transferred into a plate with clarified V8 (CV8) agar (Dixon et al., 2020). When conidia were not present mycelial cultures were started on ¼ acidified potato dextrose agar (¼ aPDA). A small agar square with mycelium, cut from ¼ aPDA, was added to 10 ml sterile deionized water, briefly vortexed, then transferred onto another ¼ aPDA plate. After 24 hours a single spore was collected and transferred onto CV8 agar as a single conidial isolate(Dixon et al., 2020). The CV8 plates were moved back under fluorescent and blue-black light for seven to ten days. Small agar plugs were cut from the leading edge of mycelial growth, transferred into 1.5 ml micro-centrifuge (USA

Scientific, Orlando, FL) tubes filled with 0.5 ml of 15% v/v glycerol, and stored in a -80°C freezer.

### 4.2.7 DNA extraction

Stored *C. nicotianae* isolates were revived from the -80°C freezer by removing a single plug from the storage tube and transferring it onto a plate with clarified V8 agar covered with sterilized cellophane. The cultures were grown under fluorescent and blue to black light for 10-14 days. Half of the mycelium and conidia from the cellophane covered V8 plates were scraped then transferred into a BeadBeater tube (Biospec Product, Bartlesville, OK) with two 6.35 mm glass beads. The BeadBeater tubes were covered with a piece of Parafilm (Beemis Company Inc, Neenah, WI) with a small hole poked in the center. The BeadBeater tubes were then covered with a Kimwipe (Kimberly-Clark, Irvine, TX). The filled BeadBeater tubes were freeze-dried in a VirTis Freezemobile 25 ES (VirTis, Los Angeles, CA) for 24 to 48 hours. DNA extraction was accomplished using a modified SDS/CTAB method (Niu et al., 2008). The initial grinding step was replaced, the lyophilized samples were chilled in liquid nitrogen for 30 seconds and pulverized in a Mini-BeadBeater 2.0 cell disrupter (Biospec Product, Bartlesville, OK). After the addition of the extraction buffer, RNase A, mercaptoethanol, and SDS, incubation at 65°C was extended to 30 minutes. An incubation stage was added after the lysis stage, the supernatant was transferred to new microfuge tubes, an equal part of isopropanol was added, and the samples were stored at -4°C for 30 minutes. A final purification stage was added, the DNA pellet was suspended in 0.5 mL of sterile Milli Q (Millipore

Corporation) water, heated to 55°c in a dry bath incubator, vortexed briefly with a mini-vortex and debris was precipitated in a centrifuge at 10,000 rpm for one minute. Then 0.5mL of the supernatant was transferred into new microfuge tubes.

DNA quantity and quality was determined using a Nano-Drop ND1000 (Nanodrop Technologies LLC, Wilmington, DE). The extracted stock DNA was used to make a 0.1 ml micro-centrifuge tube 10 ng/µl dilution for use in polymerase chain reaction (PCR) identification of resistance mutaitons. The stock DNA was then stored in a -80°C freezer.

### 4.2.8 Molecular identification of QoI-resistance mutations

*C. nicotianae* isolates collected from the field experiments were screened for cytb mutations conferring resistance to QoI-fungicides using a polymerase chain reaction (PCR) method developed and optimized by (Li et al., 2021). Each PCR was prepared in 25-µl volumes with a Titanium Taq PCR kit (TaKaRa Bio USA, Mountain View, CA) with 1x Titanium Taq Buffer, 0.1 µM forward and reverse primers, 0.1 mM deoxynucleoside triphosphate, and 1X Titanium Taq polymerase with 50 ng of extracted DNA template. The thermal cycling parameters were an initial denaturation at 95°C for 5 min, then 40 cycles of denaturing at 95°C for 30 s, annealing at 68.3°C for 30 s, and extension at 72°C for 30 s. A final extension at 72°C for 10 minutes completed the PCR.

The PCR amplicons were read using gel electrophoresis on 2% SeaKem LE agarose gel (Lonza, Bend, OR) using EZ-Vison (AMRESCO; VMR Life Science, Solon, OH) DNA staining dye. The amplicons were separated by electrophoresis

at 90v for 30 minutes. Gels were imaged with a Bio-Rad Gel Doc XR (Bio-Rad, Hercules, CA).

The *C. nicotianae* isolates were assigned azoxystrobin sensitivity groupings (wild-type, moderately resistant; F129L, and high resistance; G143A) if an amplicon was identified for the complimentary primer set. Mutation frequencies between sample dates were organized in two-way tables and compared using Pearson's Chi-Square (Minitab 20, Minitab LLC, State College, PA).

## 4.3 Results

### 4.3.1 Disease severity ratings

In 2019, treatments did not affect FLS severity in the lower or upper canopy across all ratings dates when compared with Tukey's honestly significant difference (Table 4.5). The lower canopy severity ratings for the first date were significantly different when compared using Fisher's least significant difference (LSD).

Table 4.5 2019 mean disease severity ratings as determined as the percentage of leaf with FLS symptoms from 0-100% for seven plants per plot. Ratings were divided into two groups, the lower and upper leaf canopy. The mean disease severity was tested for equal variance, analyzed by One-Way ANOVA, and means were separated using Tukey's Honestly Significant Difference (HSD) ($\alpha$=0.05).

| | Disease Severity | | | | | |
|---|---|---|---|---|---|---|
| | July 24, 2019 | | August 1, 2019 | | August 19, 2019 | |
| | Canopy Position | | | | | |
| Active Ingredients [a] | Lower [b] | Upper | Lower | Upper | Lower | Upper |
| Non-Treated Control | 8.32 ab | 3.79 | 24.82 | 6.32 | 46.5 | 11.57 |
| Azoxystrobin [c], mancozeb, azoxystrobin | 8.82 ab | 3.57 | 23.25 | 4 | 29.71 | 12.32 |
| Azoxystrobin, cuprous oxide, azoxystrobin | 10.61 ab | 3.82 | 23.68 | 3.46 | 33.86 | 8.07 |
| Azoxystrobin (0.88 L/ha), mancozeb, azoxystrobin (0.88 L/ha) | 11.96 a | 3.85 | 21.64 | 4.75 | 41.82 | 14.25 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Azoxystrobin, *Bacillus amyloliquefaciens* D747, azoxystrobin | 8.71 ab | 3.86 | 23.64 | 4.18 | 35.04 | 11.25 |
| Azoxystrobin, *Bacillus mycoides* J, azoxystrobin | 9.29 ab | 3.14 | 20.25 | 4.96 | 37.57 | 9.46 |
| *Bacillus amyloliquefaciens* D747, *Bacillus mycoides* J, *Bacillus amyloliquefaciens* D747 | 7.68 b | 3.68 | 20.86 | 4.5 | 41.14 | 14.21 |
| *P*-value | 0.022 | 0.989 | 0.584 | 0.258 | 0.273 | 0.305 |

[a] Trade names for the fungicides are Quadris (azoxystrobin), Manzate Pro-Stick (mancozeb), Nordox 75WG (cuprous oxide), Double Nickel (*Bacillus amyloliquefaciens* strain D747), and Lifegard (*Bacillus mycoides* isolate J).

[b] Means that do not share letters are significantly different

[c] azoxystrobin was applied at a rate of 0.58 L/ha unless specified

Treatment did affect FLS severity in the lower canopy in 2020. The standard fungicide spray program of azoxystrobin and mancozeb had significantly less FLS compared to the non-treated control at the last two rating timings. The final disease severity rating on August 26, 2020 had the lowest FLS severity with the grower's standard, alternative standard, and increased rate having 13.89, 13.46, and 12.75% disease severity, respectively. All other treatments grouped in

between the non-treated control and the standard fungicide treatments (Table 4.6).

Table 4.6 2020 mean disease severity ratings as determined as the percentage of leaf with FLS symptoms from 0-100% for seven plants per plot. Ratings were divided into lower and upper leaf canopy ratings. The mean disease severity was tested for equal variance, analyzed by One-Way ANOVA, and means were separated using Tukey's Honestly Significant Difference (HSD) (α=0.05).

| | FLS Disease Severity Ratings | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | July 15, 2020 | | July 28, 2020 | | August 12, 2020 | | August 26, 2020 | |
| | Canopy Position | | | | | | | |
| Active Ingredients [a] | Lower | Upper | Lower | Upper | Lower [b] | Upper | Lower | Upper |
| Non-Treated Control | 15.68 | 0.57 | 17.79 | 0.11 | 20.57[a] | 0.86 | 22.79[a] | 0.38 |
| Azoxystrobin [c], mancozeb, azoxystrobin | 17.57 | 1.19 | 15.64 | 0.21 | 8.393[c] | 0.43 | 13.89[b] | 0.14 |
| Azoxystrobin, *Bacillus amyloliquefaciens* D747, azoxystrobin | 17.82 | 0.286 | 12.19 | 0.52 | 12.39[bc] | 0.61 | 15.18[ab] | 0.34 |
| Azoxystrobin, *Bacillus mycoides* J, azoxystrobin | 19.5 | 0.95 | 12.11 | 0.61 | 12.04[bc] | 0.5 | 15.64[ab] | 0.62 |
| *Bacillus mycoides* J, *Bacillus amyloliquefaciens* D747, *Bacillus* | 22.14 | 1 | 13.25 | 0.36 | 16.57[ab] | 0.86 | 19.71[ab] | 0.32 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *mycoides* J | | | | | | | | |
| Azoxystrobin, cuprous oxide, azoxystrobin | 16.18 | 0.48 | 9.48 | 0.29 | 11.43[bc] | 0.61 | 13.46[b] | 0.18 |
| Azoxystrobin(0.88 L/ha), mancozeb, azoxystrobin (0.88 L/ha) | 18.18 | 0.67 | 11.43 | 0.18 | 11.32[bc] | 0.43 | 12.75[b] | 0.14 |
| *P*-value | 0.597 | 0.221 | 0.259 | 0.230 | 0.000 | 0.577 | 0.004 | 0.469 |

[a] Trade names for the fungicides are Quadris (azoxystrobin), Manzate Pro-Stick (mancozeb), Nordox 75WG (cuprous oxide), Double Nickel (*Bacillus amyloliquefaciens* strain D747), and Lifegard (*Bacillus mycoides* isolate J).

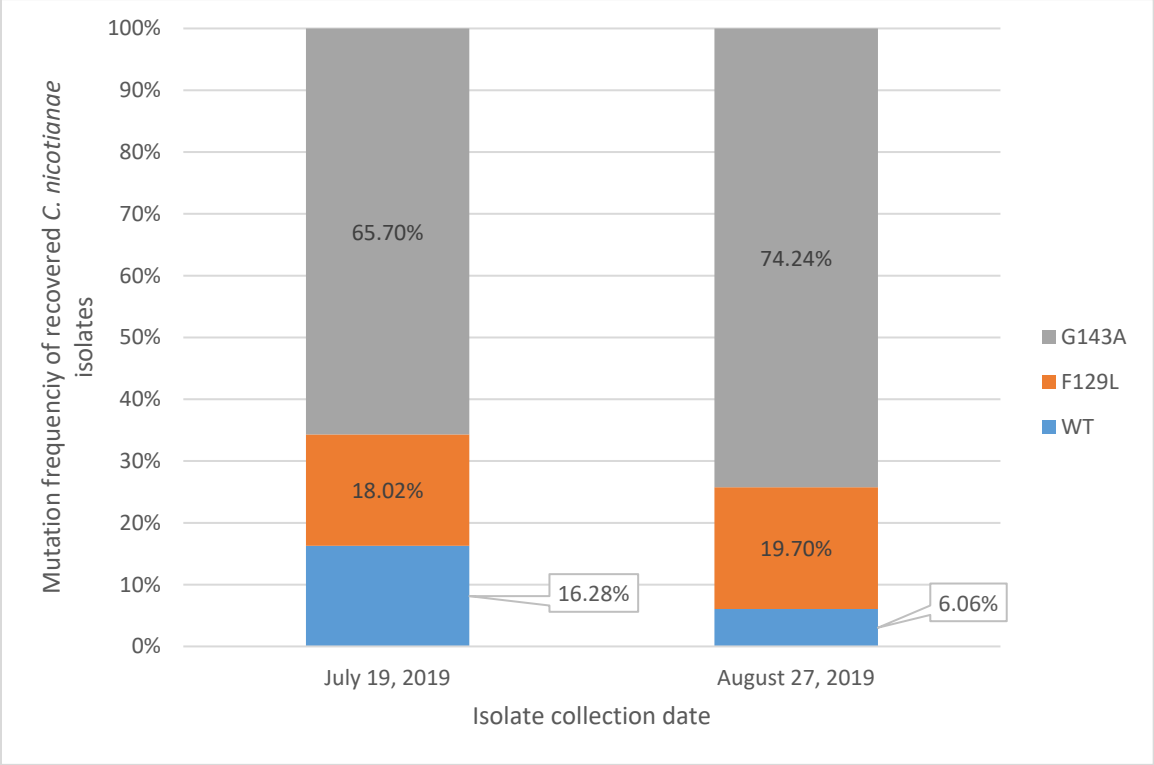
[b] Means that do not share letters are significantly different

[c] azoxystrobin was applied at a rate of 0.58 L/ha unless specified

### 4.3.2 Isolation and molecular identification of isolates with mutations conferring azoxystrobin resistance

In 2019, the first and second sample date yielded 172 and 66 isolates, respectively (Figure 4.2). Of the 172 isolates collected from the first sample date, over 65% of the isolates had the G143A mutation, while the remaining isolates recovered had almost equal frequencies of isolates with the F129L mutation (18%) and no mutations (wild type; 16%) The second sampling date was mostly comprised of individuals with the G143A mutation, accounting for 74.24% of the recovered isolates. The frequencies of isolates recovered with the F129L mutation slightly increased from 18.02% to 19.7%. There was a 10% decrease in isolates recovered with wild type cytb.

Figure 4.2 Mutation frequencies of *Cercospora nicotianae* isolates recovered from the 2019 trial separated by cytochrome b mutations that confer resistance to azoxystrobin.




The total mutation frequencies for the 2019 isolates were not significantly different ($P$=0.117) between sample dates when compared by Chi-Square analysis. By treatment, the only significant difference ($P$=0.028) in mutation frequency was in the alternative biological one, where was a 43% increase in the number of individuals with the G143A mutation. The alternative standard, alternative biological two, and biological only treatments did not have enough data points to perform Chi-Square analysis.

The total mutation frequency for all treatments in 2020 was significantly different between sample dates ($P$=0.000) (Figure 4.3). The mutation frequencies of *C. nicotianae* isolates recovered and identified on July 15, 2020

from the alternative standard, increased rate, alternative biological one, and alternative biological two treatments were significantly ($P$=0.028, 0, 0.004, and 0.003, respectively) different than the mutation frequency of the isolates recovered on August 27, 2020. All treatments saw an increase in individuals with the F129L or G143A mutations. Every other fungicide treatment had a significant shift in mutation frequency towards the F129L and G143A mutations (Table 4.7).

Figure 4.3 Mutation frequencies of Cercospora nicotianae isolates recovered from the 2020 trial separated by cytochrome b mutations that confer resistance to azoxystrobin.

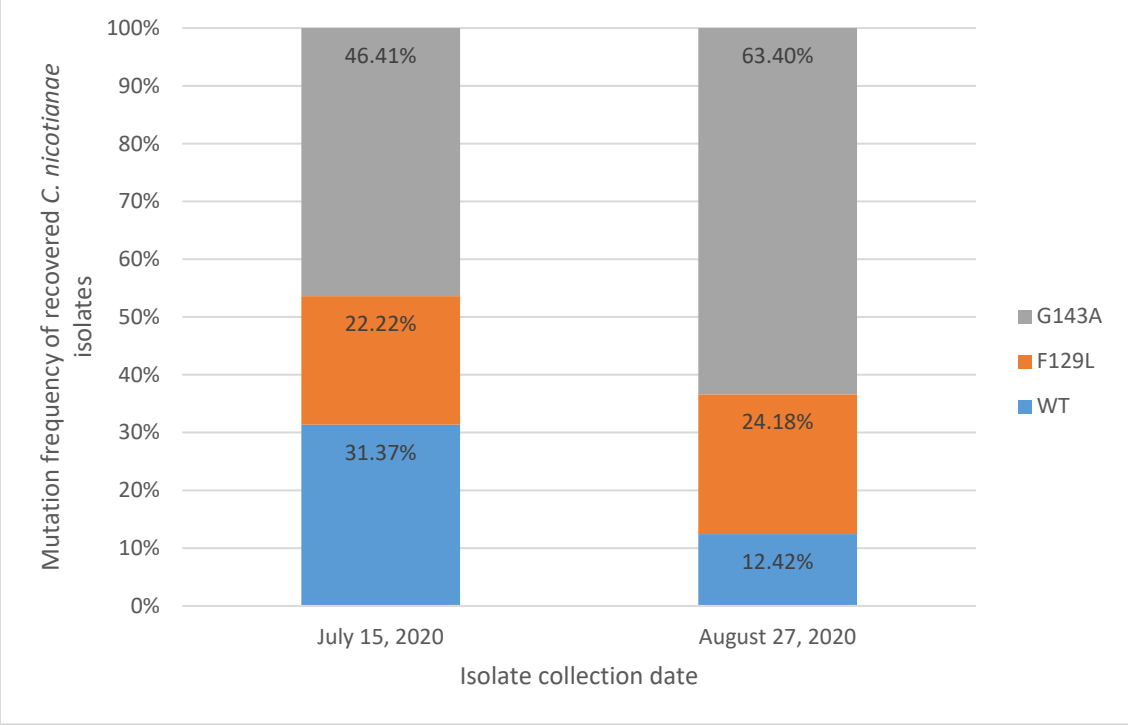



Table 4.7 Cytochrome b mutation frequencies of Cercospora nicotianae isolates recovered from the 2019 and 2020 alternative fungicide trial by corresponding treatment. Mutation frequencies were compared using Pearson's Chi-Square test (α=0.05).

| Treatments [a] | Mutation Frequencies |
|---|---|
| | Sampling Date |

| Non-Treated Control | July 19, 2019 | August 29, 2019 | Non-treated Control | July 15, 2020 | August 27, 2020 |
|---|---|---|---|---|---|
| WT | 70.00% | 36.36% | | 21.74% | 37.50% |
| F129L | 10.00% | 27.27% | | 21.74% | 16.67% |
| G143A | 20.00% | 36.36% | | 56.52% | 45.83% |
| *P*-value | 0.295 | | | 0.497 | |
| Producer's Standard [b] | | | Producer's Standard [b] | | |
| WT | 0.00% | 0.00% | | 20.00% | 0.00% |
| F129L | 40.91% | 27.27% | | 16.00% | 27.78% |
| G143A | 59.09% | 72.73% | | 64.00% | 72.22% |
| P-value | 0.443 | | | 0.111 | |
| Alternative Standard [c] | | | Alternative Bio One [e] | | |
| WT | 4.76% | 0.00% | | 27.27% | 5.26% |
| F129L | 19.05% | 50.00% | | 13.64% | 47.37% |
| G143A | 76.19% | 50.00% | | 59.09% | 47.37% |
| P-value | | | | 0.028 | |
| Increased Rate [d] | | | Alternative Bio Two [f] | | |
| WT | 14.81% | 0.00% | | 30.00% | 8.33% |
| F129L | 33.33% | 8.33% | | 45.00% | 8.33% |
| G143A | 51.85% | 91.67% | | 25.00% | 83.33% |
| P-value | 0.053 | | | 0 | |
| Alternative Bio One [e] | | | Biological Only [g] | | |

| | | | | | |
|---|---|---|---|---|---|
| WT | 19.35% | 0.00% | | 35.00% | 26.09% |
| F129L | 38.71% | 15.38% | | 35.00% | 26.09% |
| G143A | 41.94% | 84.62% | | 30.00% | 47.83% |
| P-value | 0.028 | | | 0.491 | |
| Alternative Bio Two [f] | | | Alternative Standard [c] | | |
| WT | 7.14% | 0.00% | | 36.84% | 0.00% |
| F129L | 3.57% | 0.00% | | 10.53% | 29.17% |
| G143A | 89.29% | 100.00% | | 50.00% | 70.83% |
| P-value | | | | 0.004 | |
| Biological Only [g] | | | Increased Rate [d] | | |
| WT | 25.00% | 0.00% | | 50.00% | 4.76% |
| F129L | 15.63% | 0.00% | | 16.67% | 19.05% |
| G143A | 59.38% | 100.00% | | 33.33% | 66.67% |
| P-value | | | | 0.003 | |

[a] Treatments consisted of two fungicides applied in alternation.

[b] Producer's standard = azoxystrobin, mancozeb, azoxystrobin

[c] Alternative standard = azoxystrobin, cuprous oxide, azoxystrobin

[d] Increased rate = max label rate azoxtstrobin, mancozeb, max rate azoxystrobin

[e] Alternative bio one = azoxystobin, *Bacillus amyloliquefaciens* D747, azoxystrobin or azoxystrobin, *Bacillus mycoides* Isolate J, azoxystrobin for 2019 and 2020, respectively.

[f] Alternative bio two = azoxystrobin, *Bacillus mycoides* Isolate J, azoxystrobin or azoxystrobin, *Bacillus amyloliquefaciens* D747, azoxystrobin for 2019 and 2020, respectively

[g] Biological only = *Bacillus amyloliquefaciens* D747, *Bacillus mycoides* isolate J, *Bacillus amyloliquefaciens* D747 or *Bacillus mycoides* isolate J, *Bacillus amyloliquefaciens* D747, *Bacillus mycoides* isolate J for 2019 and 2020, respectively.

## 4.4 Discussion

In both years of this study, all fungicide and biological treatments resulted in less FLS than the non-treated control, however in 2019, treatment did not significantly affect FLS. This could be the result of higher than average rainfall during July and August 2019 (KY Mesonet) washing away fungicide deposits, limiting the time fungicide deposits are present on the leaf surface (Latin, 2011). There are many factors that influence the induction of host resistance by organic or biological control agents. The individual host genome varies between plants and gene expression could be greater or less depending. The host may also already be expressing a level of resistance during infection and applications of bio control agents might not induce resistance to a higher level. Abiotic factors such as drought, temperature, or photoperiod could also influence the degree of resistance expression (Walters et al., 2013). The application timing of the biologicals could play a role in the establishment of the organism on the leaf surface. Applying biological components during intense sunlight and high temperatures might degrade or desiccate the biological control organism, reducing the efficacy of biological fungicides. In the future we could examine if biological fungicide applications during dawn or dusk, when weather conditions are more hospitable, allow for better establishment and control.

In 2020, final FLS severity was significantly lower in treatments that had conventional protectant fungicides alternated with azoxystrobin. This is likely due to rotating the modes of action, incorporating multiple target site (group M)

fungicides, reducing *C. nicotianae* populations after the first azoxystrobin selection. In fungicide programs with biological active ingredients, FLS severity was reduced compared to the non-treated control, but still higher than fungicide programs with conventional fungicides. Lifegard alternated with Double Nickel had the second highest disease severity and was not significantly different than the non-treated control. In *C. nicotianae* populations with reduced sensitivity to azoxystrobin, the spray programs with azoxystrobin still outperform programs with only biological fungicides.

When the active ingredient of Double Nickel, *B. amyloliquefaciens,* was used to control the oomycete *Phytophthora sojae* in soybean (*Glycine max)*, there was direct inhibition of *P. sojae* and the activation of host defense (Liu et al., 2019). Transcriptomic analysis of the interaction between *B. amyloliquefaciens* and *P. sojae* or *B. amyloliquefaciens* and *G. max* showed the dynamic expression of genes involved in metabolism, large and small ribosomal subunits, and protein transport. The authors also used staining assays and fluorescent probe kits to determine that a *B. amyloliquefaciens* fermentation broth (BAFB) induced a hypersensitive response and increasing levels of reactive oxygen species (ROS), nitrous oxide (NO), callose deposition and lignification (Liu et al., 2019). To my knowledge, similar studies have not been performed on commercial cultivars of tobacco (*Nicotianae tabacum*).

In 2019, azoxystrobin alternated with Double Nickel resulted in a significant shift towards *C. nicotianae* isolates containing the G143A mutation. In isolates that were recovered from the 2020 field trial, every treatment with

azoxystrobin, except the grower's standard, resulted in a significant shift in mutation frequency towards azoxystrobin resistance. In 2020, there was no significant difference in the mutation frequencies from the non-treated control or biological only treatment, likely because there was no azoxystrobin applications applied to these populations. Likewise, when Lifegard or Double Nickel was used in alternation with azoxystrobin there were significant shifts towards azoxystrobin resistance mutations F129L and G143A. The likely cause of this is the ineffectiveness of these products in reducing the population of azoxystrobin-resistant isolates selected after the first azoxystrobin application. This indicates that there is a significant selection that shift *C. nicotianae* towards azoxystrobin resistance in a single growing season. Overwintering and survival data gathered and reported in the previous chapter is inconclusive on whether these mutations will persist through the winter and cause *C. nicotiane* infections the following year.

With the development of azoxystrobin resistance in *C. nicotianae,* the only systemic foliar fungicide labeled for use in tobacco, it is increasingly important to utilize an integrated pest management approach to manage FLS. Identification of the mutation frequencies will help producers make more informed management decisions. Stopping the introduction and establishment of inoculum and proactively spraying fungicides is important in areas where the population already contains resistant individuals. In areas where *C. nicotianae* has not developed resistance to azoxystrobin, alternating azoxystrobin with multi-site fungicides like mancozeb and copper will slow resistance development

(Hollomon, 2015). It has been reported that tank mixing the at-risk mode of action with a separate mode of action is more effective at slowing the rate of resistance development and could prolong the usefulness of the at-risk mode of action (Hollomon, 2015). Industry should support labeling new or existing chemistries to control FLS in tobacco production, adding more modes of action to mix or rotate with azoxystrobin and slowing azoxystrobin resistance development.

# APPENDICES

## [APPENDIX 1] *Cytochrome b* Mutations F129L and G143A Confer Resistance to Azoxystrobin in *Cercospora nicotianae*, the Frogeye Leaf Spot Pathogen of Tobacco. (Dixon et al., 2020) https://apsjournals.apsnet.org/doi/abs/10.1094/PDIS-02-19-0382-RE

Azoxystrobin is the only synthetic, systemic fungicide labeled in the United States for management of frogeye leaf spot (FLS) of tobacco (Nicotiana tabacum L.), caused by Cercospora nicotianae. Though traditionally considered a minor disease in the United States, FLS has recently become yield and quality limiting. In 2016 and 2017, 100 C. nicotianae isolates were collected from symptomatic tobacco from eight counties in Kentucky, United States. Prior to azoxystrobin sensitivity testing, some C. nicotianae isolates were found to utilize the alternative oxidase pathway and, after assay comparisons, conidial germination was utilized to evaluate sensitivity in C. nicotianae as opposed to mycelial growth. Azoxystrobin sensitivity was determined by establishing the effective concentration to inhibit 50% conidial germination (EC50) for 47 (in 2016) and 53 (in 2017) C. nicotianae isolates. Distributions of C. nicotianae EC50 values indicated three qualitative levels of sensitivity to azoxystrobin. Partial cytochrome b sequence, encompassing the F129L and G143A mutation sites, indicated single-nucleotide polymorphisms (SNPs) conferring the F129L mutation in C. nicotianae of moderate resistance (azoxystrobin at $0.177 \leq EC50 \leq 0.535$ µg/ml) and the G143A mutation in isolates with an azoxystrobin-resistant phenotype (azoxystrobin EC50 > 1.15 µg/ml). Higher frequencies of resistant isolates were identified from greenhouse transplant (4 of 17) and conventionally produced (58 of 62) tobacco samples, as compared with field-grown tobacco (<4 weeks prior to harvest; 4 of 62) or organically produced samples (1 of 7), respectively. Together, these results suggest that resistance to azoxystrobin in C. nicotianae occurs broadly in Kentucky, and generate new hypotheses about selection pressure affecting resistance mutation frequencies.

## [APPENDIX 2] **Molecular Identification of Mutations Conferring Resistance to Azoxystrobin in Cercospora nicotianae. (Li et al., 2021)** https://apsjournals.apsnet.org/doi/abs/10.1094/PDIS-02-20-0441-RE

Cercospora nicotianae, the causal agent of frogeye leaf spot (FLS) of tobacco, has been exposed to quinone outside inhibitor (QoI) fungicides for more than a decade through azoxystrobin applications targeting other major foliar diseases. From 2016 to 2018, a total of 124 isolates were collected from tobacco fields throughout Kentucky. Sensitivity of these isolates to azoxystrobin was previously characterized by determining the effective concentration to inhibit 50% conidial germination (EC50). Based on azoxystrobin EC50, isolates were categorized into three discrete groups: high sensitivity (<0.08 µg/ml), moderate sensitivity (0.14 to 0.64 µg/ml), and low sensitivity (>1.18 µg/ml). Variability in sensitivity in a limited number of C. nicotianae isolates was previously shown to be a result of

resistance mutations in the fungicide target gene. To improve understanding of C. nicotianae cytochrome b (cytb) structure, the gene was cloned from three isolates representing each EC50 group, and sequences were compared. Our analysis showed that cytb gene in C. nicotianae consists of 1,161 nucleotides encoding 386 amino acids. The cytb sequence among the cloned isolates was identical with the exception of the F129L and G143A point mutations. To more rapidly determine the resistance status of C. nicotianae isolates to azoxystrobin, a polymerase chain reaction (PCR) assay was developed to screen for mutations. According to this assay, 80% (n = 99) of tested C. nicotianae isolates carried an F129L mutation and were moderately resistant to azoxystrobin, and 7% (n = 9) carried the G143A mutation and were highly resistant. A receiver operating characteristic curve analysis suggested the PCR assay was a robust diagnostic tool to identify C. nicotianae isolates with different sensitivity to azoxystrobin in Kentucky tobacco production. The prevalence of both the F129L and G143A mutations in C. nicotianae from Kentucky differs from that of other pathosystems where resistance to QoI fungicides has been identified, in which the majority of sampled isolates of the pathogen species have a broadly occurring cytb mutation.

# BIBLIOGRAPHY


Anke T, Oberwinkler F, Steglich W, Schramm G, 1977. STROBILURINS - NEW ANTIFUNGAL ANTIBIOTICS FROM BASIDIOMYCETE STROBILURUS-TENACELLUS (PERS EX FR) SING. *Journal of Antibiotics* **30**, 806-10.

Arneson PA, 2001. Plant Disease Management Strategies. In. *Plant Disease Epidemiology: Temporal Aspects.* The Plant Health Instructor: The American Phytopathological Society. (2021.)

Avila-Adame C, Köller W, 2003. Characterization of spontaneous mutants of Magnaporthe grisea expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. *Curr Genet* **42**, 332-8.

Bailey A, 2006. *Harvesting, Curing, and Preparing Dark Air-Cured Tobacco for Market*

Bailey A, Pearce B, Bush L*, et al.*, 2021. *Burley and Dark Tobacco Production Guide*. (UK ID-160.)

Banno S, Yamashita K, Fukumori F*, et al.*, 2009. Characterization of QoI resistance in Botrytis cinerea and identification of two types of mitochondrial cytochrome b gene. *Plant Pathology* **58**, 120-9.

Bauske MJ, Gudmestad NC, 2018. Parasitic Fitness of Fungicide-Resistant and -Sensitive Isolates of Alternaria solani. *Plant Disease* **102**, 666-73.

Brent KJ, Hollomon DW, 2007a. *Fungicide Resistance In Crop Pathogens: How Can It Be Managed*. Fungicide Resistance Action Committee.

Brent KJ, Hollomon DW, 2007b. *Fungicide Resistance: The Assessment of Risk*. Fungicide Resistance Action Committee.

Chin KM, Chavaillaz D, Kaesbohrer M, Staub T, Felsenstein FG, 2001. Characterizing resistance risk of Erysiphe graminis f.sp tritici to strobilurins. *Crop Protection* **20**, 87-96.

Coresta, 2019. No. 7- A Scale for Coding Growth Stages in Tobacco Crops. In.: Cooperation Centre for Scientific Research Relative to Tobacco, 17.

Cornelius ME, Wang TW, Jamal A, Loretan CG, Neff LJ, 2020. Tobacco Product Use Among Adults - United States, 2019. *Mmwr-Morbidity and Mortality Weekly Report* **69**, 1736-42.

Dayan FE, Cantrell CL, Duke SO, 2009. Natural products in crop protection. *Bioorganic & Medicinal Chemistry* **17**, 4022-34.

Dixon E, Barlow W, Walles G*, et al.*, 2020. Cytochrome b Mutations F129L and G143A Confer Resistance to Azoxystrobin in Cercospora nicotianae, the Frogeye Leaf Spot Pathogen of Tobacco. *Plant Disease* **104**, 1781-8.

Dixon E, Kennedy B, Pearce R, Pfeufer E, 2018. Occurrence of Frogeye Leaf Spot, Caused by Cercospora nicotianae, on Greenhouse Tobacco Transplants in Kentucky. *Plant Disease* **102**, 1036.

Fao, 2019. FAOSTAT. In. Food and Agriculture Organization of the United Nations. (2021.)

Farr DF, Rossman AY. Fungal Databases. In. U.S. National Fungal Collections: ARS USDA.

Fda, 2020. Family Smoking Prevention and Tobacco Control Act - An Overview. In. FDA.gov: Food and Drug Administration. (2021.)

Fernández-Ortuño D, Torés JA, De Vicente A, Pérez-García A, 2008. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *International Microbiology* **11**, 1.
Fernández-Ortuño D, Torés JA, Vicente A, Pérez-García A. The QoI Fungicides, the Rise and Fall of a Successful Class of Agricultural Fungicides, *2010*.
Fisher N, Meunier B, 2008. Molecular basis of resistance to cytochrome bc1 inhibitors. *Fems Yeast Research* **8**, 183-92.
Forcelini BB, Rebello CS, Wang NY, Peres NA, 2018. Fitness, Competitive Ability, and Mutation Stability of Isolates of Colletotrichum acutatum from Strawberry Resistant to QoI Fungicides. *Phytopathology* **108**, 462-8.
Frac, 2012. List of pathogens with field resistance towards QoI fungicides. In. *QoI Working Group Introduction and General Information.* Fungicide Resistance Action Committee.
Frac, 2021a. FRAC Code List 2021: Fungal control agents sorted by cross resistance pattern and mode of action (including coding for FRAC Groups on product labels). In.: Fungicide Reistance Action Committee.
Frac, 2021b. Introduction and General Information. In: Sierotzki H, ed. Fungicide Resistance Action Committee: QoI Working group: Fungicide Resistance Action Committee. (2021.)
Genet J-L, Jaworska G, Deparis F, 2006. Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations of Plasmopara viticola. *Pest Management Science* **62**, 188-94.
Gisi U, Sierotzki H, Cook A, Mccaffery A, 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science* **58**, 859-67.
Grasso V, Palermo S, Sierotzki H, Garibaldi A, Gisi U, 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag Sci* **62**, 465-72.
Gullino ML, Tinivella F, Garibaldi A, Kemmitt GM, Bacci L, Sheppard B, 2010. Mancozeb: Past, Present, and Future. *Plant Disease* **94**, 1076-87.
Hahn M, 2014. The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *Journal of chemical biology* **7**, 133-41.
Hawkins NJ, Fraaije BA, 2018. Fitness Penalties in the Evolution of Fungicide Resistance. In: Leach JE, Lindow SE, eds. *Annual Review of Phytopathology, Vol 56.* 339-60. (Annual Review of Phytopathology; vol. 56.)
Hazra A, Gogtay N, 2016. Biostatistics Series Module 4: Comparing Groups - Categorical Variables. *Indian J Dermatol* **61**, 385-92.
Hollomon D, 2015. Fungicide Resistance: Facing the Challenge. *Plant Protection Science* **51**, 170-6.
Imbusch F, Liebe S, Erven T, Varrelmann M, 2021. Dynamics of cercospora leaf spot disease determined by aerial spore dispersal in artificially inoculated sugar beet fields. *Plant Pathology* **70**, 853-61.
Isleib J, 2013. Strategies for disease control in crops. In.: Michigan State University Extension
Khan J, Rio LED, Nelson R, Rivera-Varas V, Secor GA, Khan MFR, 2008. Survival, Dispersal, and Primary Infection Site for Cercospora beticola in Sugar Beet. *Plant Disease* **92**, 741-5.

Kim YS, Dixon EW, Vincelli P, Farman ML, 2003. Field resistance to strobilurin (Q(o)I) fungicides in Pyricularia grisea caused by mutations in the mitochondrial cytochrome b gene. *Phytopathology* **93**, 891-900.
Latin R, 2011. *A Practical Guide to Turfgrass Fungicides*. The American Phytopathological Society.
Li H, Barlow W, Dixon E, Amsden BF, Hirsch RL, Pfeufer EE, 2021. Molecular Identification of Mutations Conferring Resistance to Azoxystrobin in Cercospora nicotianae. *Plant Disease* **0**, PDIS-02-20-0441-RE.
Liu D, Li K, Hu J, Wang W, Liu X, Gao Z, 2019. Biocontrol and Action Mechanism of Bacillus amyloliquefaciens and Bacillus subtilis in Soybean Phytophthora Blight. *International Journal of Molecular Sciences* **20**, 2908.
Ma B, Uddin W, 2009. Fitness and Competitive Ability of an Azoxystrobin-Resistant G143A Mutant of Magnaporthe oryzae from Perennial Ryegrass. *Plant Disease* **93**, 1044-9.
Ma ZH, Michailides TJ, 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection* **24**, 853-63.
Mcdaniel PA, Solomon G, Malone RE, 2005. The tobacco industry and pesticide regulations: case studies from tobacco industry archives. *Environmental health perspectives* **113**, 1659-65.
Morton V, Staub T. A Short History of Fungicides, *2008*.
Murphy AM, Chivasa S, Singh DP, Carr JP, 1999. Salicylic acid-induced resistance to viruses and other pathogens: a parting of the ways? *Trends in Plant Science* **4**, 155-60.
Niu C, Kebede H, Auld DL, Woodward JE, Burow G, Wright RJ, 2008. A safe inexpensive method to isolate high quality plant and fungal DNA in an open laboratory environment. *African Journal of Biotechnology* **7**.
Orr HA, 2009. Fitness and its role in evolutionary genetics. *Nature reviews. Genetics* **10**, 531-9.
Pasche J, Gudmestad N, 2008. Prevalence, competitive fitness and impact of the F129L mutation in Alternaria solani from the United States. *Crop Protection* **27**, 427-35.
Pasche JS, Wharam CM, Gudmestad NC, 2004. Shift in sensitivity of Alternaria solani in response to Q(o)I fungicides. *Plant Disease* **88**, 181-7.
Russell PE, 2002. *Sensitivity baselines in fungicide resistance research and management*. Crop Life International Brussels, Belgium.
Salamanca LR, Saalu E, 2021. Fungicides and How to Use Them Effectively. In. Iowa State University Extension and Outreach Horticulture and Home Pest News: Iowa State University Extension and Outreach. (2021.)
Secor GA, Rivera VV, Khan MFR, Gudmestad NC, 2010. Monitoring Fungicide Sensitivity of Cercospora beticola of Sugar Beet for Disease Management Decisions. *Plant Disease* **94**, 1272-82.
Shew HD, Lucas GB, 1991. *Compendium of tobacco diseases*. St. Paul, Minn.: APS Press, the American Phytopathological Society.
Sierotzki H, Frey R, Wullschleger J*, et al.*, 2007. Cytochrome b gene sequence and structure of Pyrenophora teres and P. tritici-repentis and implications for QoI resistance. *Pest Manag Sci* **63**, 225-33.

Swiderska-Burek U, Daub ME, Thomas E, Jaszek M, Pawlik A, Janusz G, 2020. Phytopathogenic Cercosporoid Fungi-From Taxonomy to Modern Biochemistry and Molecular Biology. *International Journal of Molecular Sciences* **21**, 18.
Usda N, 2020a. 2020 State Agricultural Overview: Kentucky. In. National Agricultural Statistics Service: United States Department of Agriculture. (2021.)
Usda N, 2020b. Statistics by Subject. In. USDA National Agricultural Statistics Service: United States Department of Agriculture. (2021.)
Walters DR, Ratsep J, Havis ND, 2013. Controlling crop diseases using induced resistance: challenges for the future. *Journal of Experimental Botany* **64**, 1263-80.
Wang TW, Kenemer B, Tynan MA, Singh T, King B, 2016. Consumption of Combustible and Smokeless Tobacco - United States, 2000-2015. *Mmwr-Morbidity and Mortality Weekly Report* **65**, 1357-63.
Windels CE, Lamey HA, Hilde D, Widner J, Knudsen T, 1998. A Cerospora Leaf Spot Model for Sugar Beet: In Practice by an Industry. *Plant Disease* **82**, 716-26.
Wise KA, Bradley CA, Pasche JS, Gudmestad NC, 2009. Resistance to QoI Fungicides in Ascochyta rabiei from Chickpea in the Northern Great Plains. *Plant Disease* **93**, 528-36.
Wood PM, Hollomon DW, 2003. A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. *Pest Manag Sci* **59**, 499-511.
Zeng F, Arnao E, Zhang G*, et al.*, 2015. Characterization of Quinone Outside Inhibitor Fungicide Resistance in Cercospora sojina and Development of Diagnostic Tools for its Identification. *Plant Disease* **99**, 544-50.
Zhang F, Zhang X, 2011. Classification and quality evaluation of tobacco leaves based on image processing and fuzzy comprehensive evaluation. *Sensors (Basel, Switzerland)* **11**, 2369-84.
Zhang G, Bradley CA, 2014. Survival of Cercospora sojina on Soybean Leaf Debris in Illinois. *Plant Health Progress* **15**, 92-6.
Zhang GR, Bradley CA, 2017. Comparison of quinone outside inhibitor fungicide-resistant and -sensitive isolates of Cercospora sojina. *Crop Protection* **94**, 59-63.
Zhang GR, Newman MA, Bradley CA, 2012a. First Report of the Soybean Frogeye Leaf Spot Fungus (Cercospora sojina) Resistant to Quinone Outside Inhibitor Fungicides in North America. *Plant Disease* **96**, 767-.
Zhang GR, Pedersen DK, Phillips DV, Bradley CA, 2012b. Sensitivity of Cercospora sojina isolates to quinone outside inhibitor fungicides. *Crop Protection* **40**, 63-8.

# VITA

1. Educational institutions attended and degrees already awarded
    - B.S. in Biology, December 2015. University of Kentucky
2. Professional positions held
    - Undergraduate research assistant, University of Kentucky, College of Agriculture, Food, and Environment, Kentucky Tobacco Research and Development Center, May 2012-August 2012
    - Undergraduate research assistant, University of Kentucky, College of Agriculture, Food, and Environment, Department of Plant and Soil Science, May 2013-Decemeber 2015
    - Temporary paraprofessional, University of Kentucky, College of Agriculture, Food, and Environment, Department of Plant Pathology, April 2016-July 2017
    - Research analyst, University of Kentucky, College of Agriculture, Food, and Environment, Department of Plant Pathology, July 2017-present
3. Scholastic and professional honors
    - Association of Plant Pathology Scholars, president 2019-2020
4. Publications
    - Extension publication (refereed):
        - **Barlow, W.,** Pfeufer, E., Pearce, R. C. 2018. PPFS-AG-T-8: Fungicide Guide for Burley and Dark Tobacco, 2018. Lexington, KY: College of Agriculture, Food, and Environment, Cooperative Extension Service. http://plantpathology.ca.uky.edu/files/ppfs-ag-t-08.pdf
    - Journal articles:
        - Dixon, E., **Barlow, W.**, Walles, G., Amsden, B., Hirsch, R. L., Pearce, R., Pfeufer, E. 2020. Cytochrome b mutations F129L and G143A confer resistance to azoxystrobin in *Cercospora nicotianae*, the frogeye leaf spot pathogen of tobacco. Plant Disease; https://doi.org/10.1094/PDIS-02-19-0382-RE .
        - Li, H., **Barlow, W**., Dixon, E., Amsden, B., Hirsch, R. L., Pfeufer, E. 2020. Molecular Identification of Mutations Conferring Resistance to Azoxystrobin in *Cercospora nicotianae*. Plant Disease; https://doi.org/10.1094/PDIS-02-20-0441-RE .
    - Plant Disease Management Reports:

- Fungicide programs for cucumber downy mildew management, 2018. Plant Disease Management Reports. Pending publication.

- Foliar fungicides for management of frogeye leaf spot in burley tobacco, 2018. Plant Disease Management Reports. Pending publication.

- Oomycete specific fungicides combined with varietal resistance for management of black shank in burley tobacco, 2017. Plant Disease Management Reports. Pending publication.

- **Barlow, W.,** Pearce, R., Pfeufer, E. 2018. Fungicides for frogeye leafspot management in burley tobacco, 2017. Plant Disease Management Reports. Vol. 12.

- **Barlow, W.,** Pfeufer, E. Fungicide delivery equipment for early blight management in staked determinate tomato, 2017. Plant Disease Management Reports. Vol. 12.

- **Barlow, W.,** Dixon, E., Pfeufer, E. Evaluations of fungicides for management of anthracnose in pickling cucumber, 2016. Plant Disease Management Reports. Vol. 12.

- **Barlow, W.,** Pfeufer, E., Pearce, R. Evaluation of oomycete-specific fungicides for management of black shank in burley tobacco, 2016. Plant Disease Management Reports. Vol. 11.

- Published abstract:
  - **Barlow, W.,** Dixon, E., Pearce, R., Pfeufer, E. 2017. Sensitivity to azoxystrobin in selected Kentucky populations of *Cercospora nicotianae*, the frogeye leaf spot pathogen of tobacco. Phytopathology 107: S5.65.

- Bulletins, conference proceedings, and project updates (nonrefereed):
  - Strang, J., Walsh, J., Smigell, C., Snyder, J., Pfeufer, E., **Barlow, W**. 2017. Pumpkin Fungicide and Cultivar Evaluation. *Annual Fruit and Vegetable Report, 2017.* University of Kentucky PR-739: http://www2.ca.uky.edu/agcomm/pubs/PR/PR739/PR739.pdf .

- Pfeufer, E., **Barlow, W.,** Fealko, E. 2017. Small Scale Equipment to Optimize Tomato Disease Management. *Annual Fruit and Vegetable Report, 2017.* University of Kentucky PR-739: http://www2.ca.uky.edu/agcomm/pubs/PR/PR739/PR739.pdf .

- Grants
  - **Barlow, W.,** Pfeufer, E. "Practical disease management with reduced-sensitivity tobacco pathogens: frogeye leaf spot in burley tobacco and angular leaf spot in dark tobacco." $14,500. Altria Client Services, January 2018.

- National conference and meetings

  - **Barlow, W.,** Dixon, E., Pearce, R., Pfeufer, E. "Azoxystrobin sensitivity in the frogeye leaf spot pathogen of tobacco, *Cercospora nicotianae*." Tobacco Workers Conference. Myrtle Beach, SC. (January 2018).

  - **Barlow, W.,** Davis, D., Pfeufer, E. "Fungicide Application for Small Scale Tomato Production." Kentucky Fruit and Vegetable Conference, 2018. Lexington, KY. (January 2018).

  - **Barlow, W.,** Walles, G., Dixon, E., Pfeufer E. "*In vitro* fitness comparisons among *Cercospora nicotianae* of varying sensitivity to azoxystrobin." APS Plant Health Conference, 2019. Cleveland, OH. (August 2019).

  - **Barlow, W.,** Dixon, E., Pfeufer E. "Composition of Mixed Sensitivity *Cercospora nicotianae* Populations After Multiple Azoxystrobin Applications." Tobacco Workers Conference. Louisville, KY. (January 2020).

William Barrett Barlow