ASSESSMENT OF QUINONE OUTSIDE INHIBITOR SENSITIVITY AND FROGEYE LEAF SPOT RACE OF *CERCOSPORA SOJINA* IN GEORGIA SOYBEAN

by

BENNETT C. HARRELSON

(Under the Direction of James Buck)

ABSTRACT

Frogeye leaf spot (FLS), caused by the fungal pathogen *Cercospora sojina* K. Hara, is a foliar disease of soybean (*Glycine max* L. (Merr.)) responsible for yield reductions throughout the major soybean producing regions in the world. In the United States, management of FLS relies heavily on the use of resistant cultivars and in-season fungicide applications, specifically within the class of quinone outside inhibitors (QoIs), which has resulted in the development of fungicide resistance in many states. In 2018 and 2019, 80 isolates of *C. sojina* were recovered from 6 counties in Georgia and were screened for QoI fungicide resistance using molecular and in vitro assays, with resistant isolates confirmed from three counties. Additionally, 40 isolates were used to evaluate pathogen race on six soybean differential cultivars by assessing susceptible or resistant reactions. Isolate reactions suggested 12 races of *C. sojina* present in Georgia, four of which have not been previously described.

INDEX WORDS: Frogeye leaf spot, FLS, *Cercospora sojina*, Soybean, Fungicide, Quinone outside inhibitors, QoI, Fungicide resistance, Pathogen race

ASSESSMENT OF QUINONE OUTSIDE INHIBITOR SENSITIVITY AND FROGEYE LEAF SPOT RACE OF *CERCOSPORA SOJINA* IN GEORGIA SOYBEAN

by

BENNETT C. HARRELSON

B.S., Clemson University, 2018

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

ASSESSMENT OF QUINONE OUTSIDE INHIBITOR SENSITIVITY AND FROGEYE LEAF SPOT RACE OF *CERCOSPORA SOJINA* IN GEORGIA SOYBEAN

by

BENNETT C. HARRELSON

Major Professor:	James W. Buck
Committee:	Albert K. Culbreath
	Robert C. Kemerait Jr.

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
December 2020

DEDICATION

This work is dedicated to my family.

ACKNOWLEDGEMENTS

I have been blessed and honored to work with many tremendous people who have all had an incredible impact on shaping me as both a scientist but more importantly as a person during this chapter of my life. I would first like to thank Dr. James Buck for the opportunity to pursue a master's degree in his program. The guidance, unwavering support, and wisdom provided throughout this journey will be taken with me as I embark on the next chapters of my life. I would also like to express my great appreciation to Dr. Steven Jeffers for the opportunities placed upon me as an undergraduate to work and learn under his direction and experience the field of plant pathology. His belief in me allowed me to pursue challenges and a career I would have never imagined, for that I am forever grateful.

I would also like to thank my committee members Dr. Albert Culbreath and Dr. Bob Kemerait. The guidance, support, and helpful suggestions you both have provided has been instrumental not only to the completion to my research, but to my growth as a scientist and for that I thank you. I would like to thank the other faculty members at the University of Georgia who have broadened my knowledge of agriculture and plant pathology, the instruction and lessons learned from each of you will be invaluable as I go forward in my career. I would also like to specifically thank the faculty, staff, and graduate students at the University of Georgia Griffin Campus; Mondays and Thursdays will no longer be the same without a lunchtime soccer match.

Many people are to thank for the countless hours of their time given to see this project come to completion. First, I would like to thank members of Dr. Buck's lab along with Dr.

Alfredo Martinez's lab; Bikash Ghimire, Thomas Buck, John Youmans, Brian Vermeer and, especially, Carol Picard. The assistance provided by each of you, from planting and rating soybean to numerous hours spent in the lab, your help, support, and friendship, will never be forgotten, thank you. I would also like to thank the members of Dr. Babu Srinivasan's lab, Pin-chu Lai and Yi-Ju Chen, for the molecular and statistical support, along with the friendship provided throughout this journey.

Finally, I would like to thank my family. Mom, Dad, Jarett, Brandon, Addison, and especially Lindsay, you each have provided me with the strength and courage to pursue challenges, no matter how daunting, and to push myself to accomplish my dreams. Without each one of you and the constant and unwavering love and support you have given to me throughout my time at the University of Georgia, none of this would have been possible.

Jeremiah 29:11

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

1 LITERATURE REVIEW 1

Soybean 1

Frogeye Leaf Spot 4

Fungicides 14

Research Objectives 21

Literature Cited 22

2 ASSESSMENT OF QUINONE OUTSIDE INHIBITOR FUNGICIDE SENSITIVITY OF *CERCOSPORA SOJINA* FROM GEORGIA SOYBEAN 29

Abstract 29

Introduction 30

Materials and Methods 34

Results 40

Discussion 42

Literature Cited 47

3 EVALUATION OF PATHOGEN RACE OF *CERCOSPORA SOJINA* RECOVERED FROM GEORGIA SOYBEAN 59

Abstract 59

Introduction 60

Materials and Methods 63

Results 68

Discussion 69

Literature Cited 74

LIST OF TABLES

Page

Table 2.1: Location, year, and number of QoI resistant *Cercospora sojina* isolates recovered from Georgia soybean in 2018 and 2019.....51

Table 2.2: Analysis of variance of percent reduction of radial growth of *Cercospora sojina* isolates to two fungicides, azoxystrobin and pyraclostrobin, at six concentrations.....52

Table 3.1: Proposed races of *Cercospora sojina* recovered from Georgia soybean based on their reaction on 6 soybean differential cultivars.....79

Table 3.2: Location, year, and number of *Cercospora sojina* races identified within each location. 80

Table 3.3: Resistance index of 6 soybean differential cultivars to 80 isolates of *Cercospora sojina* from Georgia.....81

LIST OF FIGURES

Page

Figure 1.1: **A.** *Cercospora sojina* growing in culture on V8 agar. **B.** *C. sojina* hyaline conidia observed at 100x. 6

Figure 1.2: Soybean foliage displaying symptoms associated with frogeye leaf spot: circular to angular lesions with a reddish to brown margin and light brown to white center. 8

Figure 2.1: County map of Georgia, with *Cercospora sojina* isolates recovered from counties highlighted in red. 53

Figure 2.2: Results from PCR-RFLP of the 238-bp product of the cytochrome *b* region using restriction enzyme *AluI*. Cleavage of the band fragment, indicating the presence of the guanine to cytosine nucleotide base change resulting in QoI resistance, is shown in two band fragment sizes approximately 78- and 160-bp, respectively. Sensitive isolates, not containing the mutation responsible for the G143A mutation, display a single band fragment approximately 238-bp in length. 54

Figure 2.3: Sensitivity of three *Cercospora sojina* isolates: one QoI sensitive (middle), one QoI resistant (right), and one baseline isolate with no prior exposure to QoI fungicides (left) to 6 concentrations of both azoxystrobin (left column) and pyraclostrobin (right column) 7 days after plating. 55

Figure 2.4: Sensitivity of three *Cercospora sojina* isolates: one QoI sensitive (middle), one QoI resistant (right), and one baseline isolate with no prior exposure to QoI fungicides (left)

to 6 concentrations of both azoxystrobin (left column) and pyraclostrobin (right column) 14 days after plating. 56

Figure 2.5: Sensitivity of three *Cercospora sojina* isolates: one QoI sensitive (middle), one QoI resistant (right), and one baseline isolate with no prior exposure to QoI fungicides (left) to 6 concentrations of both azoxystrobin (left column) and pyraclostrobin (right column) 21 days after plating. 57

Figure 2.6: Percent reduction of mycelia growth, compared to nonamended control, of quinone outside inhibitor resistant and sensitive isolates of *Cercospora sojina* recovered from soybean in Georgia in 2018 and 2019 to azoxystrobin at 0.0001, 0.001, 0.01, 0.1, 1 and 10 µg ml^{-1}. 58

Figure 2.7: Percent reduction of mycelia growth, compared to nonamended control, of quinone outside inhibitor resistant and sensitive isolates of *Cercospora sojina* recovered from soybean in Georgia in 2018 and 2019 to pyraclostrobin at 0.0001, 0.001, 0.01, 0.1, 1 and 10 µg ml^{-1}. 59

Figure 3.1: Number of Georgia isolates of *Cercospora sojina* within each pathotypes designation. 82

Figure 3.2: Frequency of susceptible reactions of each differential cultivar to 80 isolates of *Cercospora sojina* recovered from Georgia. 83

Figure 3.3: Soybean differential cultivars inoculated with *Cercospora sojina* displaying different reaction phenotypes: **A.** incompatible or resistant reaction on 'Davis' resulting in no lesions, or **B.** compatible or susceptible reactions on 'Blackhawk' resulting in symptoms associated with *C. sojina* infection. 84

CHAPTER I

LITERATURE REVIEW

SOYBEAN

Soybean, *Glycine max* L. (Merr.), first emerged as a domesticated plant in the northeastern region of China between 1046 and 256 BCE, during the time of the Zhou Dynasty. However, the domestication of the soybean through trial and error was thought to occur earlier, between 1550 and 1027 BCE during the Shang Dynasty (Hartman et al. 2015). China was the world's largest producer and exporter of soybeans until the middle of the 20th century. Since the 1950's, the United States has been the leading soybean producing country in the world (Qui and Chang 2010). However, soybean was not introduced to the U.S. until 1765, yet took nearly 175 years before becoming a widely planted and cultivated crop (Hartman et al. 2015). In 1929, U.S. soybean production was around 245,000 metric tons and by 1940, U.S. soybean production increased to roughly 2 million metric tons. During this time period, soybean meal was recognized for its low cost and high protein content for use in livestock and poultry feed, causing a drastic increase in soybean cultivation (US Soy 2006).

Soybean is the largest source of animal protein feed and the second largest source of vegetable oil in the world (USDA-ERS 2018). Soybean seeds are made up of approximately 20% oil content and 40% protein content and are typically crushed for oil extraction as the remaining meal is used as a high protein source for animal feed (Shurtleff and Aoyagi 2016). The top three producers of soybean are the United States, Brazil, and Argentina (Hartman et al. 2015). In 2017,

over 89 million metric tons were harvested in the U.S., worth over 41 billion dollars (NASS 2018). In the U.S., soybean accounts for 90% of the total oilseed produced (US-ERS 2018). Oil derived from soybean is a major component of cooking oils, margarine, salad oils, and shortenings. Soybean meal, known for its high protein content, is used in animal feed, as well as the base of many soy products including soy flour, soy concentrate, soy isolates, and textured soy protein (US Soy 2006).

Soybean, a member of the family Fabaceae and genius *Glycine*, is extensively adapted to its environment (Hartman et al. 2015). From germination, to the onset of flowering, and into maturation, soybean growth and development are controlled at all stages by photoperiod and temperature (Johnson et al. 1960; Major et al. 1975). Soybean cultivars are classified based on their regional adaptation into maturity groups (MG), with 13 MG present ranging from MG 000 to MG X. The earliest maturing varieties are designated MG 000 and are adapted to higher latitudes, such as in Canada, whereas the latest maturing varieties are designated X and are adapted to tropical and sub-tropical regions (Hartman et al. 2015). Soybean cultivars in MGs 000 through IV are typically classified as indeterminate due to their continued production of vegetative apical meristems even after flowering. In contrast, cultivars in MGs V through X are classified as determinate due to conversion of vegetative meristems to floral meristems in response to shorter day lengths, thus ceasing vegetative growth and beginning reproductive growth (Bernard 1972). In Georgia, MGs V and VI are recommended; however, adapted early and late MGs IV and VII can be grown, respectively (Freeman et al. 2019).

In the past 40 years, the geographic range of soybean production in the U.S. has changed. The total planted hectares in the southern U.S. has declined due to historically lower yields and increased competition with more profitable crops. Soybean production in the northern and mid-

western states has increased with the development of faster growing varieties adapted to drier climatic conditions (US Soy 2006). In Georgia, soybeans accounted for over 150,000 harvested acres worth over $61 million to Georgia's economy in 2018 (NASS 2018). Soybeans are produced throughout the state of Georgia with the northwestern region of the state historically having greatest production. However, depending on year and soybean prices, parts of central and southwest Georgia also see production increases (NASS 2017a).

Soybean diseases and nematodes accounted for losses of over 2 billion bushels between 2010 and 2014 in the U.S and Canada. Of these losses, the top three maladies were soybean cyst nematode (*Heterodera glycines*), seedling diseases (*Rhizoctonia*, *Pythium*, *Fusarium*, and/or *Phomopsis* spp.), and charcoal rot (*Macrophomina phaseolina*), respectively (Allen et al. 2017). However, soybean production in the southern U.S. states (Alabama, Arkansas, Delaware, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, and Virginia) occurs with high relative humidity and warm temperatures which are conducive to fungal disease presence and prevalence. In 2015, frogeye leaf spot (FLS) (*Cercospora sojina*) accounted for over 11.5 million bushels in yield losses of soybean, ranking second behind soybean cyst nematode at over 13.4 million bushels, in the southern-most states (Bradley et al. 2017).

In Georgia, pest and diseases associated with soybean yield losses often differ from surrounding southern states. The most yield-limiting pest throughout the soybean production region in the United States is generally *H. glycines*; however, due to soils present in Georgia containing low fertility and organic matter, *H. glycines* does not typically cause significant yield loss (Freeman et al. 2019). In Georgia, southern root-knot nematode *(Meloidogyne incognita*) is often considered the most yield limiting pest of soybean, yet other species of root-knot nematode

such as peanut root-knot (*M. arenaria*) and Javanese root-knot nematode (*M. jayanica*) can also cause yield reductions (R. Kemerait, *personal communication*). In 2017, *Meloidogyne* spp. caused an estimated $3.9 million in damage in Georgia ranking first in soybean disease and nematode associated damage. Yet, in terms of fungal diseases, two pathogens within the genus *Cercospora*, *Cercospora kikuchii* which causes Cercospora leaf blight (CLB), and *Cercospora sojina* which causes frogeye leaf spot (FLS), were reported to cause losses of over $200,000 in 2017, which ranked tied for second behind nematodes in Georgia (Little 2019).

FROGEYE LEAF SPOT

Frogeye leaf spot is a foliar disease caused by the Ascomycete fungal pathogen *Cercospora sojina* K. Hara (Hartman et al. 2015; Mian et al. 2008). FLS was first reported in Japan in 1915, and then first in U.S. in South Carolina in 1924. However, specimens of the first report in South Carolina were never collected and the true identity of the organism is unknown. In 1925, specimens collected from soybean leaf samples from South Carolina, Mississippi, and Louisiana provided the first confident report of *C. sojina* in the U.S. (Lehman 1928). FLS is reported to be present wherever soybean is grown in the world, but is more destructive in regions with warm temperatures and high relative humidity (Hartman et al. 2015).

In the U.S., FLS has historically been predominantly present in the southeastern region, but has more recently been reported affecting soybean in more northern mid-western states such as Iowa, Ohio, and Wisconsin (Cruz and Dorrance 2009; Mengistu et al. 2002; Yang et al. 2001). Reports of FLS in northern regions of the U.S. is thought to be due to warmer temperatures, increased planting of susceptible soybean cultivars, and the use of conservation tillage practices,

resulting in infested plant debris serving as sources of over-wintering material for inoculum (Mian et al. 2008).

The causative agent, *C. sojina*, produces hyaline conidia when young and elongate to fusiform conidia when mature. Asexual conidia serve as both primary and secondary inoculum and vary in septation from 0 to 10. Conidia also vary in length and width; however, on infected leaves, conidia are 6-8 x 40-60 µm in size. *C. sojina* conidia are produced by conidiophores and are present in fascicles of two to 25. Conidiophores vary in color and size, ranging from light to dark brown and between 4-6 x 52-120 µm. A single conidiophore produces one to three conidia (Hartman et al. 2015).

Within the over 3,000 species of *Cercospora*, many species produce the light activated toxin cercosporin as a pathogenicity factor, such as the other soybean pathogen within the genus, *C. kikuchii* (Goodwin et al. 2001; Soares et al. 2015). Although, *C. sojina* is not known to be able to produce cercosporin, previous phylogenetic analyses place both *C. kikuchii* and *C. sojina* within a cercosporin-producing clade (Chupp 1954; Goodwin et al. 2001). Moreover, all species within the cercosporin producing clade lack a known sexual stage (Goodwin et al. 2001). No sexual stage has been confirmed for *C. sojina*; although, relatively equal distributions of mating types have been observed in the field, suggesting that sexual reproduction is likely occurring (Kim et al. 2013).

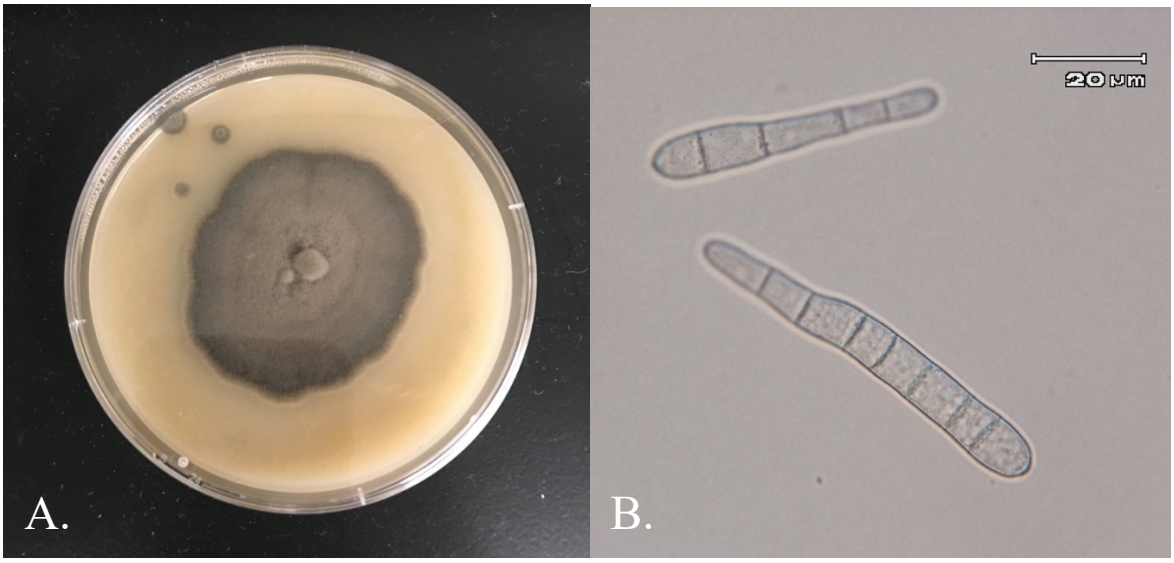

Figure 1.1. **A.** *Cercospora sojina* growing in culture on V8 agar. **B.** *C. sojina* hyaline conidia observed at 100x.

Symptomology

FLS primarily infects soybean foliage, yet seeds, pods, and stems can also become infected (Grau et al. 2004; Hartman et al. 2015). Foliar infections manifest as circular to angular lesions that range from 1 to 5 mm in diameter. Lesions start out as dark, water-soaked spots and then turn brown and develop reddish to brown margins. When lesions begin to sporulate, the undersides of leaves become darker and fascicles of conidiophores arise from dark fungal stromata. As lesions mature, the dark brown centers give way to light brown to white non-sporulating centers (Hartman et al. 2015). As foliar infection increases, lesions may coalesce to form large diseased spots. Once the lesions and large diseased spots reach approximately 30% of the leaf surface area, a premature blighting of the leaf may occur, resulting in leaf senescence (Hartman et al. 2015).

Stem, pod, and seed lesions resulting from FLS often occur later in the growing season and are less common than foliar lesions. Stem lesions are dark brown to black with flattened to sunken centers. As the lesions mature, the centers become light to dark gray. *C. sojina* lesions on stems are long and narrow in shape, often two to four times as long as they are wide. Soybean pod lesions appear sunken and circular to elongate in shape, with a reddish to brown color. From pod lesions, *C. sojina* moves through the pod wall and then is able to infect maturing seeds (Hartman et al. 2015). Once seeds within the pod are infected, infection can then spread from seed to seed as they mature (Laviolette et al. 1970). Soybean seeds infected with *C. sojina* develop brown to gray circular spots to large blotches covering the entire seed coat. Infected seeds usually cause cracking and flaking of the seed coat (Singh and Sinclair 1985).

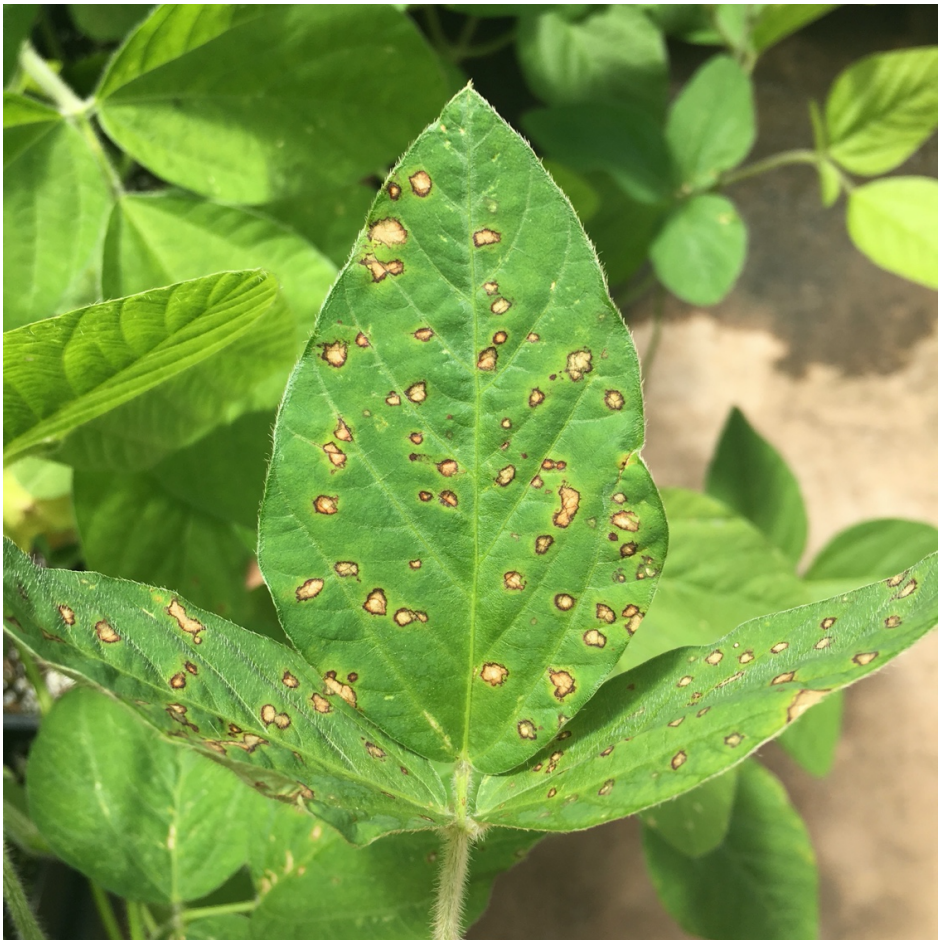

Figure 1.2. Soybean foliage displaying symptoms associated with frogeye leaf spot: circular to angular lesions with a reddish to brown margin and light brown to white center.

Disease Cycle and Spread

C. sojina primarily survives and overwinters as mycelium in infected seeds and in infected soybean residue in fields, (Hartman et al. 2015; Heatherly and Hodges 1998; Mian et al. 2008) with infected soybean residue harboring *C. sojina* in the field for up to two years (Zhang 2012). Soybean seeds infected with *C. sojina* can germinate and will produce weak, stunted seedlings with lesions present on the cotyledons. Sporulation of lesions on cotyledons and infested soybean residue in the field serve as primary inoculum to infect other seedlings. Seedlings are often highly susceptible before leaves have fully expanded. Once leaves become fully expanded, soybean leaves are more resistant to *C. sojina* infection (Mian et al. 2008). After infection, lesions may take up to two weeks to develop (Hartman et al 2015). FLS disease spread is favored by warm (25-30°C) and humid (>90%) environmental conditions (Cruz and Dorrance 2009; Mian et al. 2008). If warm, moist conditions continue, sporulation can occur as soon as 48-hours post-infection; however, sporulation can be sporadic in seasons without frequent dews or rainfall (Phillips 1999). Conidia are spread relatively short distances by wind and/or rain, and in seasons with frequent rainfall, sporulation and infection can occur as long as conditions are favorable (Hartman et al. 2015).

Soybean yield losses due to FLS are predominantly caused by reductions in photosynthetic leaf area from necrotic lesions and/or premature defoliation of leaves (Dashiell and Akem 1991; Mian et al. 2008). Yield reductions ranging between 10-60% have been reported from FLS (Akem and Dashiell 1994; Dashiell and Akem 1991; Mian et al. 1998). Infection by *C. sojina* is dependent on environmental conditions and the fungus can infect soybean throughout most of its vegetative and reproductive growth stages. However, yield losses associated with FLS tend to be higher when infection occurs prior to flowering by reducing the

photosynthetic capacity of the plant before pod fill (Dashiell and Akem 1991). Yield reductions due to infection prior to flowering are manifested as reductions in seed size and weight. These reductions can be attributed to a decrease in movement of metabolites to developing seeds, with seed weight being reduced up to 27% (Mwase and Kapooria 2001). After pod fill, at or beyond growth stage R5, soybean plants can still become infected with FLS, though there is very little impact on yield (Dorrance and Mills 2010).

Pathogen Races

Throughout the world numerous races of *C. sojina* have been identified, with 22 races reported in Brazil (Yorinori 1992), 11 in China (Huo et al. 1998), and 12 in the U.S. (Grau et al. 2004). These races produce differing reactions, from immune to susceptible, on varying soybean cultivars (Hartman et al. 2015; Phillips 1999). Determining the similarity between races identified in the U.S. and races identified in other countries is difficult because of the varying sets of differential cultivars used to determine races (Grau et al. 2004). To combat this inconsistency in race identification, various sets of soybean differentials have been proposed in the last 50 years (Yorinori and Sinclair 1982; Pace et al. 1992). Still, none of these sets of differential cultivars have been formally recognized because of lack of knowledge of inheritance and allelic relationships of resistant cultivars and the variability of *C. sojina* (Baker et al. 1999).

In order to facilitate future studies and comparison of existing *C. sojina* isolates, Mian et al. (2008) proposed a core set of 12 soybean differential cultivars to determine the race of *C. sojina* isolates. To select the core set of 12 differentials, 93 isolates of *C. sojina* collected in the U.S., Brazil and China were screened against 38 soybean cultivars and scored for a reaction of "resistant" or "susceptible". Based on these reactions, differentials were clustered by similarity

and a representative differential cultivar was selected from each cluster, 10 in total. Differentials 'Lincoln', 'Kent', and 'Davis', containing *Rcs* genes, were chosen, along with ancestral cultivars developed for the southern region to include 'Peking', 'CNS', 'Palmetto', 'Tracy', 'S-100', and universal susceptible 'Blackhawk'. In addition to the 10 differentials selected based on the cluster analysis, two more soybean cultivars, 'Hood' and 'Lee', were also added to the proposed differential set (Mian et al. 2008). Since their proposal, studies conducted in Ohio using the core set of soybean differentials have revealed 20 races of *C. sojina*, 9 of which had not been previously described (Cruz and Dorrance 2009).

Other studies have been conducted assessing *C. sojina* pathogen race structure using the proposed set of differential cultivars. To better understand the diversity and varying aggressiveness present among 83 *C. sojina* isolates from Brazil, China, and the U.S. when screened on the 12 differential cultivars, Mengistu et al. (2020) proposed grouping isolates based on reaction and virulence into 5 pathogenicity groups (PG1, PG2, PG3, PG4, PG5). Disease severity of each isolate on each differential was scored on a 0 to 9 scale, where 0 = no disease and 9 ≥ 90% of leaf surface area was diseased. Based on these, an average disease severity rating was created and then converted to a 0 to 5 resistance classification scale: 0 = 0 (immune), 1 to 2 = 1 (resistant), 3 to 4 = 2 (moderately resistant), 5 to 6 = 3 (moderately susceptible), 7 to 8 = 4 (susceptible), and 9 = 5 (very susceptible) (Mengistu et al. 2020).

Issues have arisen when screening *C. sojina* isolates on the set of 12 soybean differentials proposed by Mian et al. (2008). Of the proposed set, only three of the 12 differentials contain known resistance genes, *Rcs1* (Athow and Probst 1952), *Rcs2* (Athow et al. 1962), and *Rcs3* (Phillips and Boerma 1982). Other differentials have been found to contain genes non-allelic to the *Rcs3* gene, yet some are tightly linked to the *Rcs3* locus and thus produce the same reactions

as differential ‘Davis’, which contains the *Rcs3* gene (Baker et al. 1999; Mian et al. 2008; Pace et al. 1993). To account for these similar reactions among the proposed set of 12 differentials, Fagundes et al. (2018) used 6 of the 12 differentials, which allowed for unique reactions among cultivars, and reported 6 races of *C. sojina* in Arkansas.

Genes for Resistance

Until the later part of the 1900s, the use of resistant soybean cultivars provided adequate control of FLS in the U.S. After the 1950s, the introduction of new physiological races of *C. sojina*, starting with race 2 in the late 1950s, races 3 and 4 in the 1960s, and finally race 5 in late 1970s created great concern for the management of FLS (Athow et al. 1962; Phillips and Boerma 1981; Ross 1968; Mian et al. 2008). During the 1980s, race 5 was thought to be a great threat to soybean production in the southern U.S. due to widespread planting of susceptible cultivars such as ‘Bragg’ (Phillips and Boerma 1981).

To combat these races, three single genes were identified that conveyed resistance to FLS:, *Rcs1*, found in ‘Lincoln,’ conferring resistance to race 1 (Athow and Probst 1952); *Rcs2*, found in ‘Kent,’ to race 2 (Athow et al. 1962), and *Rcs3*, found in ‘Davis,’ conferring resistance to race 5 and to all other known races in the U.S., as well as isolates from Brazil (Boerma and Phillips 1983; Mian et al. 2008a; Phillips and Boerma 1981). Other inherited genes, non-allelic to *Rcs3*, that conveyed resistance to race 5 were also found in cultivars ‘Lee’, ‘Ransom’, ‘Stonewall’ and later ‘Peking’ (Baker et al. 1999; Pace et al. 1993). Moreover, in 2011 two additional single, dominant genes conveying resistance were discovered on chromosome 13, different from the *Rcs3* gene on chromosome 18, in PI lines 594891 and 594774 and were denoted as *Rcs*(PI594891) and *Rcs*(PI594774), respectively (Hoskin 2011; Pham et al. 2015).

Rcs4 and *Rcs7* were designated as resistant to race 4 in Brazil and race 7 in China, respectively (Buzzell 1988; Zou et al. 1999); however, neither gene showed significant evidence to being non-allelic to *Rcs1*, *Rcs2*, and *Rcs3*, thus only *Rcs1*, *Rcs2*, *Rcs3, Rcs*(PI594891) and *Rcs*(PI594774) are recognized by the Soybean Genetics Committee (Baker et al. 1999; Buzzell 1988; Hoskin 2011; Pham et al. 2015). Cultivars containing *Rcs* genes are still able to become infected with FLS, but lesions are usually small and non-sporulating (Athow and Probst 1952).

Disease Management

To prevent the development of FLS, specific cultural practices are recommended, such as planting high-quality pathogen-free soybean seed and crop rotation with a non-host crop (Grau et al. 2004; Mian et al. 2008). In regions where resistant cultivars are adapted and high yielding in the given environment, commercially available seed containing either the *Rcs3* gene or a combination of the *Rcs1* and *Rcs2* genes are recommended (Hartman et al. 2015). In studies conducted in Illinois, *C. sojina* was shown to be viable in soybean residue for up to two years. Depending on depth of residue, at least two years is advised for a rotation with a non-host crop to reduce pathogen inoculum (Zhang 2012). Historically, tillage has been recommended to limit pathogen inoculum levels present in fields by removing or burying infected residue which serves as over-wintering material and primary inoculum going into the next season (Grau et al. 2004; Phillips 1999). However, with increased acreage of soybeans being grown in no-till systems to reduce production costs and increase soil health, a study conducted in Tennessee showed no significant difference in FLS disease severity between tilled and no-tilled fields without the presence of a fungicide. However, when applied, a fungicide significantly decreased disease severity and improved yield up to 17% in tilled fields compared to no-till (Mengistu et al. 2014).

Fungicide applications can provide adequate control of FLS, with foliar applications typically recommended between soybean growth stages R2 and R5, or full bloom and beginning pod, respectively (Hartman et al. 2015; Mian et al. 2008a). Various fungicide classes are labeled for use in soybean for control of FLS, including products such as methyl benzimidazole carbamates (MBCs, FRAC Group 1), demethylation inhibitors (DMIs, FRAC Group 3), succinate dehydrogenase inhibitors (SDHIs, FRAC Group 7), and quinone outside inhibitors (QoIs, FRAC Group 11) (Wise 2015). Many studies have been conducted throughout the soybean producing regions of the U.S., including Indiana, Kentucky, Louisiana, Mississippi, Ohio, Tennessee, and Virginia, have shown that a foliar fungicide application can reduce FLS incidence and severity when applied prior to infection (Chappell and Phipps 2005; Cochran 2016; Hershman et al. 2001; Mills and Dorrance 2008; Price et al. 2014; Sciumbato et al. 2006; Shaner and Buechly 2006). In Georgia, it is recommended that fungicides be applied as a combination product or a tank-mix of two or more chemistries to provide a broader spectrum of activity and longer protection window against soybean diseases (Freeman et al. 2019). Fungicide seed treatments are also recommended when planting lower quality seed to control disease spread by infected seeds, with applications shown to provide effective control of FLS by improving seed germination, reducing seed infection, and improving yields by up to 15% (Akem 1995; Blackman et al. 1979; Hartman et al. 2015; Mian et al. 2008).

FUNGICIDES

For hundreds of years, humans have utilized products with fungicidal activity to limit and control fungal diseases. These products were often discovered through observations, such as grain recovered from shipwreck's lacked bunt (*Tilletia tritici* T. laevis), leading to the brining of

grain with salt and lime to reduce the disease in the middle of the 1600s (Morton and Staub 2008). Also, the discovery in the late 1800s that grape vines sprayed with a combination of copper sulfate and lime, now known as the Bordeaux mixture, did not lose their leaves to downy mildew (*Plasmopara viticola*) (Klittich 2008). However, these inorganic products, effective over the non-treated option, were often used at high rates and had the potential to damage plants (Morton and Staub 2008).

Through the 1920s, antifungal compounds were limited to inorganic compounds such as sulfur, copper, mercury, and arsenic. These compounds, applied at high rates (10 to 20 kg/hectare), did not always provide great control and at times were phytotoxic due rate and frequency of use (Morton and Staub 2008). Yet, the introduction of the first organic fungicide, Thiram, (fungicide class: dithiocarbamate) introduced in 1942, resulted in a significant breakthrough in synthetically produced fungicides, sparking a drastic increase in the development of crop protection products over the next 50 years (Latin 2011). By 2000, over 11 new fungicide classes had been discovered, causing an increased emphasis on disease control. These new synthetically produced fungicides were more effective, less phytotoxic, and required lower application rates than previous inorganic products (Morton and Staub 2008).

Fungicides, unlike insecticides and some herbicides that control established insects and weeds, are primarily are used to protect healthy plants from infection (Damicone and Smith 2009). To be effective, most fungicides have to be applied before infection occurs, providing a protective barrier that prevents pathogen infection, known as preventative fungicides. However, some fungicides also have curative properties, in which the fungicide is present in plant tissues and is able to control pathogen growth after infection, typically during the early infection stage (Mueller and Robertson 2008).

Fungicides are typically classified based on their biochemical modes of action. Mode of action (MOA) refers to the specific biochemical target of a specific active ingredient. From these MOAs, fungicides are split into two broad groups based on their MOA, single-site and multi-site. These terms refer only to one aspect of the MOA and describe only the general mechanism of the inhibitor or active ingredient (Latin 2011). Fungicides such as respiration inhibitors, sterol biosynthesis inhibitors, nucleic acid inhibitors, and cell division disruptors are some of the most commercially successful, due to their single-site MOA and systemic activity, allowing them to move within the plant, offering both curative and preventative properties (Damicone and Smith 2009). Multi-site MOA fungicides are less understood than single-site MOA fungicides yet have been available commercially longer. These products often interfere with enzymatic activity disrupting metabolism and cell integrity (Gisi and Seirotzki 2008). Current commercially available multi-site MOA fungicides do not have systemic activity, although some may have locally systemic properties, allowing them to move from the plant's surface into the plant tissue, but once in the plant, are unable to relocate within the plant (Schumann and D'Arcy 2010).

Fungicide use in soybean has increased drastically in the U.S. from approximately 1,200 g/ha in 2005 to over 2,400 g/ha in 2015 (Bandara et al. 2020). Increases are thought to be due to increased availability of products labeled for use on soybean, increased awareness of soybean diseases, and the introduction of soybean rust into the U.S. in 2004 (Mueller et al. 2013). The increased use of foliar fungicides during that time frame differed between the southern states of AL, AR, DE, FL, GA, KY, LA, MD, MO, MS, NC, OK, SC, TN, TX, and VA which combined to apply over 17,000 g/ha compared to the northern states of IL, IN, IA, KS, MI, MN, NE, ND, OH, PA, SD, and WI which applied ~2,800 g/ha (Bandara et al. 2020). Increased use of foliar fungicides in the southern states could be due to more conducive environmental conditions, such

as high humidity and temperature, for development of foliar diseases. In Georgia, the use of fungicides on soybean was greatly influenced by the presence of soybean rust (R. Kemerait, *personal communication*) and during 2005 through 2015 Georgia ranked second in foliar fungicide use (approximately 2,200 g/ha) only to Louisiana (Bandara et al. 2020).

Quinone Outside Inhibitor Fungicides

Quinone outside inhibitor (QoIs) or strobilurin fungicides are one of the most widely used fungicide classes in current use. This class of fungicides has been an effective tool since they were first brought to market in 1996, due to their broad spectrum of activity (Bartlett et al. 2002; Sauter et al. 1999). Strobilurin fungicides contain many different active ingredients including pyraclostrobin, kresoxim-methyl, metominostrobin, and azoxystrobin. Azoxystrobin (AZ), initially announced in 1992 and released in 1996, was the first QoI fungicide commercially available and by 2002 was registered in 72 countries for use on 84 different crops (Bartlett et al. 2002). By 1999, azoxystrobin sales reached over $400 million, making it the world's greatest selling fungicide. Moreover, within the same year, the sales of strobilurin and related fungicides reached approximately $620 million, representing over 10% of the global fungicide market (Bartlett et al. 2002).

The active ingredients used in strobilurin fungicides were first discovered from naturally occurring anti-fungal compounds produced by numerous wood-rotting fungi within the Basidiomycota, including the species *Strobilurus tenacellus*, which the class of fungicides was named after (Bartlett et al. 2002). However, these naturally occurring compounds were unsuitable for agricultural use, due to their rapid degradation when exposed to ultraviolet light. These natural chemicals served as the starting point for the production of synthetic derivatives by

researchers at Syngenta and BASF, resulting in some of the most effective and successful fungicides commercially available (Fernández-Ortuño et al. 2010).

Part of the effectiveness and success of fungicides within the strobilurin class is their broad-spectrum of activity. Products within this class have been demonstrated to have activity against all four major groups of plant pathogenic fungi: Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycetes (Bartlett et al. 2002). The fungicidal activity of strobilurin fungicides is due to their ability to bind to the outer quinol oxidation (Qo) site of the cytochrome bc_1 protein complex (complex III) in the inner membrane of the mitochondria, thus inhibiting mitochondrial respiration (Becker et al. 1981; Vincelli 2012). The mitochondrial cytochrome *b* has two quinol binding locations, outside (Q_o) and inside (Q_I), and during the mitochondrial respiration process, a series of oxidation and reduction reactions occur transferring two protons across the mitochondrial membrane. In addition, complex III aids in the shuttling of electrons down the electron transport chain (ETC), thus assisting in the generation of proton gradient used for ATP production (Fisher and Meunier 2008). The binding of the fungicide inhibits the transfer of electrons between the cytochrome *b* and cytochrome c_1 subunits, causing a reduction in cellular energy through halting the production of ATP and ultimately killing the fungal cell (Becker et al. 1981; Fernández-Ortuño et al. 2010).

Besides their fungicidal activity, fungicides in the strobilurin class have also shown to have yield associated benefits with applications in the absence of disease in several crops (Bartlett et al. 2002). Two hypotheses have been presented to explain what is termed, the strobilurin "greening effect" in which delayed leaf senescence and increases in biomass and yield have been attributed QoI applications. The first hypothesis states that applications of strobilurins directly affect plant physiological processes such as leaf senescence, ethylene biosynthesis,

photosynthetic activity, and plant antioxidant enzyme activity that aid in yield benefits (Bartlett et al. 2002; Beck et al. 2002; Grossmann and Retzlaff 1997) The second hypothesis states that the use of strobilurin fungicides show increase yields over other fungicide classes due to the inhibition of spore germination from pathogenic, non-pathogenic, and saprophytic fungi, thus stopping energy-demanding plant defense responses (Bartlett et al. 2002; Schöfl and Zinkernagel 1997).

Quinone Outside Inhibitor Fungicide Resistance

Due to their effectiveness and success, fungicides in the QoI class have often been overused in many agricultural systems. Four years after their introduction to the market, QoI resistant pathogens were first reported in 2000. These reports included *Blumeria graminis* f. sp. *tritici* on wheat in France, Germany, and in the U.K.; *Pseudoperonospora cubensis* on cucumber in Spain and Japan; and *Plasmopara viticola* on grape in Italy (Gisi et al. 2002). Overuse and continued use of a single-site MOA have now led to resistance development in over 40 fungal species (FRAC 2018) as a result of mutations in the cytochrome bc_1 (Fernández-Ortuño et al. 2008). The Fungicide Resistance Action Committee (FRAC), which groups all fungicides based on their biochemical mode of action and determines each groups risk of resistance development, assessed fungicides within the strobilurin class (FRAC group 11) to have high risk to the development of resistance, with cross-resistance reported between all members of the fungicide class (FRAC 2019).

The mitochondrial cytochrome bc_1 is a protein complex located within the inner membrane of the mitochondria and is encoded by a mitochondrial gene rather than a nuclear encoded DNA gene. Genes encoded by the mitochondrial genome often have a greater chance

for mutations due to lack of precision of DNA repair mechanisms (Fisher and Meunier 2008; Gisi et al. 2002). Moreover, the mitochondrial genome has a lower genetic diversity due to being uniparentally inherited compared to the nuclear genome, which is biparentally inherited. This lack of biparental inheritance is thought to allow the mitochondrial genome to be able to withstand rearrangement and is believed to be more prone to mutation (Gisi et al. 2002).

Three-point mutations, resulting in amino acid substitutions, have been identified to convey resistance by preventing the binding of the fungicide to the Qo site. The main mechanism of resistance regarding QoI resistance is a nucleotide-base change from a guanine to cytosine at amino acid position 143 (Fernández-Ortuño et al. 2008). This nucleotide-base change causes an amino acid substitution from a glycine to an alanine and is commonly referred to as the G143A mutation. Fungal isolates containing the G143A mutation express complete resistance, or qualitative resistance, and are always associated with control failures of QoIs to manage disease (Fernández-Ortuño et al. 2008). Isolates have also been found to have mutations at position 129, resulting in an amino acid substitution from phenylalanine to leucine, known as F129L, and at position 137, causing a change from glycine to arginine, known as G137R. However, isolates containing these mutations, F129L and G137R, only express partial or quantitative resistance to QoI fungicides and are usually overcome by a recommended field level rate of the fungicide (Gisi et al. 2002; Fernández-Ortuño et al. 2008).

The first report of quinone outside inhibitor fungicide resistant *C. sojina* occurred in western Tennessee in 2010. Soybeans from the western Tennessee field showed continual increases in FLS severity after multiple applications of pyraclostrobin and were determined to have developed cross resistance to QoI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin (Zhang et al. 2012). Further studies concluded that isolates obtained from the field

possessed the G143A mutation, conveying complete QoI resistance (Zeng et al. 2015). Since 2010, QoI resistant *C. sojina* isolates have been recovered from Alabama, Arkansas, Delaware, Illinois, Indiana, Iowa, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Ohio, Tennessee, and Virginia (Standish et al. 2015; Zeng et al. 2015; Zhang 2012; Zhang et al. 2018). Between 2016 and 2017, several commercial fields from counties in the northwestern part of Georgia experienced increased FLS severity after applications of QoI fungicides, leading to suspected QoI resistance (R. Kemerait, *personal communication*). In order to accurately determine the sensitivity of *C. sojina* isolates recovered from Georgia to products within the QoI fungicide class, and to assess how resistant cultivars can be used to manage FLS, the following research objectives were created:

RESEARCH OBJECTIVES

1. Determine quinone outside inhibitor fungicide sensitivity present in *Cercospora sojina* from Georgia by conducting an extensive state-wide survey of commercial and state variety testing locations containing frogeye leaf spot symptomatic soybean.

2. Evaluate pathogen race of *Cercospora sojina* recovered from Georgia soybean by screening isolates on soybean differential cultivars ‘Davis’, ‘Hood’, ‘Lee’, ‘Lincoln’, ‘Tracy’ and ‘Blackhawk’.

LITERATURE CITED

Akem, C. N. 1995. The effect of timing of fungicide applications on control of frogeye leaf spot and grain yield of soybeans. Eur. J. Plant Pathol. 101:183-187.

Akem, C. N., and Dashiell, K. E. 1994. Effect of planting date on severity of frogeye leaf spot and grain yield of soybeans. Crop Prot. 13:607–610.

Allen, T. W., Bradley, C. A., Sisson, A. J., Byamukama, E., Chilvers, M. I., Coker, C. M., Collins, A. A., Damicone, J. P., Dorrance, A. E., Dufault, N. S., Esker, P. D., Faske, T. R., Giesler, L. J., Grybauskas, A. P., Hershman, D. E., Hollier, C. A., Isakeit, T., Jardine, D. J., Kelly, H. M., Kemerait, R. C., Kleczewski, N. M., Koenning, S. R., Kurle, J. E., Malvick, D. K., Markell, S. G., Mehl, H. L., Mueller, D. S., Mueller, J. D., Mulrooney, R. P., Nelson, B. D., Newman, M. A., Osborne, L., Overstreet, C., Padgett, G. B., Phillips, P. M., Price, P. P., Sikora, E. J., Smith, D. L., Spurlock, T. N., Tande, C. A., Tenuta, A. U., Wise, K. A., and Wrather, J. A. 2017. Soybean Yield Loss Estimates Due to Diseases in the United States and Ontario, Canada, from 2010 to 2014. Plant Health Prog. 18:19-27. Online. http://dx.doi.org/10.1094/PHP-RS-16-0066

Athow, K. L., and Probst, A. H. 1952. The inheritance of resistance to frogeye leaf spot of soybeans. Phytopathology 42:660-662.

Athow, K. L., Probbst, A. H., Kartzman, C. P., and Laviolette, F. A. 1962. A newly identified physiological race of *Cercospora sojina* on soybean. Phytopathology 52:712-714.

Baker, W. A., Weaver, D. B., Qiu, J., and Pace, P. F. 1999. Genetic analysis of frogeye leaf spot resistance in PI54610 and Pecking soybean. Crop Sci. 39:1021-1025.

Bandara, A. Y., Weerasooriya, D. K., Conley, S. P., Bradley, C. A., Allen, T. W., Esker, P. D. 2020. Modeling the relationship between estimated fungicide use and disease-associated yield losses of soybean in the United States: Foliar fungicides vs foliar disease. PLoS ONE 15(6): e0234390. https://doi.org/10.1371/journal.pone.0234390.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. Pest. Manag. Sci. 58:649-662.

Beck, C., Oerke, E. C., and Dehne, H. W. 2002. Impact of strobilurins on physiology and yield formation of wheat. Mededelingen 62: 181-187.

Becker, W. F., von Jagow, G., Anke, T., and Steglich, W. 1981. Oudemansin, strobilurin A, strobilurin B and myxothizaol: new inhibitors of the cytochrome bc1 segment of the respiratory chain with an E-ß-methoxyacrylate system as a common structural element. FEBS Letters 132:329-333.

Bernard, R. L. 1972. Two genes affecting stem termination in soybeans. Crop Sci. 12:235-239.

Backman, P. A., Rodriguez-Kabana, R., Hammond, J. M., and Thurlow, D. L. 1979. Cultivar, environment, and fungicide effects on foliar disease losses in soybeans. Phytopathology 69:562-564.

Boerma, H. R., and Phillips, D. V. 1983. Genetic implications of the susceptibility of Kent soybeans to *Cercospora sojina*. Phytopathology 74:1666-1668.

Bradley, C., Allen, T., Chilvers, M., Giesler, L., Mehl, K., Mueller, D., Tenuta, A., Sisson, A., and Wise, K. 2017. Soybean Disease Loss Estimates From the United States and Ontario, Canada—2015. Crop Protection Network. Online. https://crop-protection-network.s3.amazonaws.com/publications/cpn-1018-soybean-disease-loss-estimates-from-the-united-states-and-ontario-canada.pdf

Buzzell, R. I. 1988. Soybean genetics committee report. Soybean Genet. Newsl. 15:2-8.

Chappell, G. F., and Phipps, P. M. 2006. A comparison of fungicide spray programs for soybean disease management. F&N Tests 1:FC056.

Chupp, C. 1954. A monograph of the fungus genus *Cercospora*. New York: The Ronald Press.

Cochran, A. M. 2016. Evaluation of fungicide efficacy against *Cercospora sojina* and selection for QoI-fungicide resistance in soybean. MS Thesis. University of Tennessee-Knoxville. Knoxville, Tennessee.

Cruz, C. D., and Dorrance, A. E. 2009. Characterization and survival of *Cercospora sojina* in Ohio. Plant Health Prog. doi: 10.1094/php-2009-0512-03-rs

Damicone, J., and Smith, D. 2009. Fungicide resistance management. Oklahoma State cooperative extension service. F-7663. Oklahoma State University. Stillwater, OK. http://pods.dasnr.okstate.edu/docushare/dsweb/Get/Version-6953/EPP-7663web.pdf

Dashiell, K. E., and Akem, C. N. 1991. Yield losses in soybeans from frogeye leaf spot caused by *Cercospora sojina*. Crop Prot. 10:465-468.

Dorrance, A. E., and Mills, D. 2010. Frogeye leaf spot of soybean. Ohio State University Extension Fact Sheet AC 53-10. Ohio State University Extension, Columbus, OH.

Fernández-Ortuño, D., Torés, J. A., De Vicente, A., and Pérez-García, A. 2008. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. Int. Microbiol. 11:1-9.

Fernández-Ortuño, D., Torés, J. A., De Vicente, A., and Pérez-García, A. 2010. The QoI Fungicides: the rise and fall of a successful class of agricultural fungicides. Pages 203-220 in: Fungicides. Odille Carisse, ed.

Fisher, N., and Meunier, B. 2008. Molecular basis of resistance to cytochrome bc1 inhibitors.

FEMS Yeast Res. 8: 183-192.

Freeman, M., Harris, G., Kemerait, R., Prostko, E., Roberts, P., Smith, A., Rabinowitz, A., and Porter, W. 2019. 2019 Georgia Soybean Production Guide, GA: University of Georgia Cooperative Extension Service Publ., Athens, GA.

Fungicide Resistance Action Committee. 2018. List of Plant Pathogenic organisms resistant to disease control agents. Online. http://www.frac.info/frac/index.htm.

Fungicide Resistance Action Committee. 2019. FRAC Code List. Online. http://www.frac.info/frac/index.htm.

Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manage. Sci. 58:859-867.

Gisi, U., and Sierotzki, H. 2008. Fungicide modes of action and resistance in downy mildews. Eur. J. Plant Pathol. 122:157-167.

Goodwin, S. B., Dunkle, L. D., and Zismann, V. L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91: 648-658.

Grau, C. G., Dorrance, A. E., Bond, J., and Russin, J. S. 2004. Fungal diseases. p. 679-763, *In*: H. R. Boerma, J. E. Specht (Eds.), Soybean: Improvement, production, and uses, third edition. Am So Agron, Crop Sci Soc Amer, and Soil Sci Soc Amer. Madison, WI.

Grossmann, K., and Retzlaff, G. 1997. Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). Pest. Sci. 50:11-20.

Hartman, G. L., Rupe, J. C., Sikora, E. J., Domier, L. L., Davis, J. A., and Steffey, K. L. 2015. Compendium of soybean diseases. Fifth Edition. American Phytopathological Society, St. Paul, MN.

Heatherly, L. G., and Hodges, H. F. 1998. Soybean Production in the Midsouth. CRC Press. Boca Raton, FL.

Hershman, D., Kennedy, B., Yielding, T., and Bachi, P. 2011. Evaluation of selected fungicides for control of foliar diseases of soybean in Kentucky. Plant Dis. Manag. Rep. 5:FC005.

Hoskin, A. 2011. Genetic mapping of soybean resistance genes to frogeye leaf spot in five Chinese Plant Introductions and efficiency of early generation selection for low phytate soybean lines. PhD Thesis. Institute of Plant Breeding, Genetics, and Genomics. University of Georgia.

Huo, H., Ma, S. M., and Lu, G. Z. 1988. The study of *Cercospora sojina* Hara races in Heilongjiang Province. (In Chinese.) Soybean Sci. 7:315-318.

Johnson, H. W., Borthwick, H. A., and Leffel, R. C. 1960. Effects of photoperiod and time of planting on rates of development of the soybean in various stages of the life cycle. Bot. Gaz. 122:77-95.

Kim, H., Newell, A., Cota-Sieckmeyer, R., Rupe, J., Fakhoury, A., and Bluhm, B. 2013. Mating-type distribution and genetic diversity of Cercospora sojina populations on soybean from Arkansas: evidence for potential sexual reproduction. Phytopathology 103: 1045-1051.

Klittich, C. J. 2008. Milestones in fungicide discovery: Chemistry that changed agriculture. Online. Plant Health Progress doi: 10.1094/PHP-2008-0418-01-RV.

Latin, R. 2011. A Practical Guide to Turfgrass Fungicides. American Phytopathological Society, St. Paul, MN.

Laviolette, F. A., Athow, K. L., Probst, A. H., Wilcox, J. R., and Abney, T. S. 1970. Effect of bacterial pustule and frogeye leaf spot on yield of Clark soybean. Crop Sci. 10:418-419.

Lehman, S.G. 1928. Frog-eye leaf spot of soybean caused by *Cercospora diazu* Miura. J. Agric. Res. 35:811–833.

Little, E. 2019. 2017 Georgia Plant Disease Loss Estimates, GA: University of Georgia Cooperative Extension Service Publ., Athens, GA.

Major, D. J., Johnson, D. R., Tanner, J. W., and Anderson, I. C. 1975. Effects of daylength and temperature on soybean development. Crop Sci. 15:174-179.

Mengistu, A., Kelly, H. M., Bellaloui, N., Arelli, P. R., Reddy, K. N., and Wrather, A. J. 2014. Tillage, fungicide, and cultivar effects on frogeye leaf spot severity and yield in soybean. Plant Dis. 98:1476-1484.

Mengistu, A., Kurtzweil, N.C., and C.R. Grau. 2002. First report of frogeye leaf spot (*Cercospora sojina*) in Wisconsin. Plant Dis. 86:1272.

Mengistu, A., Ray, J. D., Kelly, H. M., Lin, B., Yu, H., Smith, J. R., Arelli, P. R., and Bellaloui, N. 2020. Pathotype grouping of *Cercospora sojina* isolates on soybean and sensitivity to QoI fungicides. Plant Dis. 104:373-380.

Mian, M. A. R., Boerma, H. R., Phillips, D. V., Kenty, M. M., Shannon, G., Shipe, E. R., Soffes Blount, A. R., and Weaver, D. B. 1998. Performance of frogeye leaf spot resistant and susceptible near isolines of soybean. Plant Dis. 82:1017-1021.

Mian, M. A. R., Missaoui, A. M., Walker, D. R., Phillips, D. V., and Boerma, H. R. 2008. Frogeye leaf spot of soybean: a review and proposed race designations for isolates of *Cercospora sojina* Hara. Crop Sci. 48:14-24.

Mian, R., Bond, J., Joobeur, T., Mengistu, A., Weibold, W., Shannon, G., Wrather, A. 2008a. Identification of soybean genotypes resistant to *Cercospora sojina* by field screening and molecular makers. Plant Disease 93:408-411.

Mills, D. R., and Dorrance, A. E. 2008. Evaluation of soybean fungicides for yield response and frogeye leaf spot control in Ohio. Plant Dis. Manag. Rep. 2:FC043.

Morton, V., and Staub, T. 2008. A Short History of Fungicides. Online, APS*net* Features. doi:10.1094/APSnetFeature-2008-0308.

Mueller, D., and Robertson, A. 2008. Preventative vs. Curative Fungicides. Iowa State University Extension and Outreach. Iowa State University. Ames, IA. https://crops.extension.iastate.edu/cropnews/2008/07/preventative-vs-curative-fungicides

Mueller, D. S., Wise, K. A., Dufault, N. S., Bradley, C. A., and Chilvers, M. A. 2013. Fungicides for field crops. American Phytopathological Society Press, St. Paul, MN.

Mwase, W. F., and Kapooria, R. G. 2001. Incidence and severity of frogeye leaf spot and associated yield losses in soybeans in agroecological zone II of Zambia. Mycopathology 149:73-78.

NASS. 2015. United States Department of Agriculture National Agricultural Statistics Service. 2015 Georgia Soybean Production. Online. http://www.nass.usda.gov/

NASS. 2016. United States Department of Agriculture National Agricultural Statistics Service. 2016 Georgia Soybean Production. Online. http://www.nass.usda.gov/

NASS. 2017. United States Department of Agriculture National Agricultural Statistics Service. 2017 Georgia Soybean Production. Online. http://www.nass.usda.gov/

NASS. 2017a. United States Department of Agriculture National Agricultural Statistics Service. Georgia County Estimates Soybean 2016-2017. Online. http://www.nass.usda.gov/

NASS. 2018. United States Department of Agriculture National Agricultural Statistics Service. National Statistics for Soybeans. Online. http://www.nass.usda.gov/

Pace, P. F., Weaver, D. B., and Ploper, L. D. 1993. Additional genes for resistance to frogeye leaf spot race 5 in soybean. Crop Sci. 33:1144-1145.

Pace, P. F., Weaver, D. B., Ploper, L. D., and Gabe, H. L. 1992. Physiologic specialization in frogeye leaf spot; sources of resistance, and implications in a breeding program. P. 109. *In* Agronomy abstracts. ASA, Madison, WI.

Pham, A-T., Harris, D. K., Buck, J., Hoskins, A., Serrano, J., Abdel-Haleem, H., Cregan, P., Song, Q., Boerma, H. R., and Li, Z. 2015. Fine mapping and characterization of candidate genes that control resistance to *Cercospora sojina* K. Hara in tow soybean germplasm accessions. PLoS ONE 10(5): e0126753. doi:10.137/journal.pone.0126753

Phillips, D. V. 1999. Frogeye leaf spot. Pages 20-21, in: G. L. Hartman, J. B. Sinclair, J. C. Rupe (Eds.), Compendium of Soybean Diseases, Fourth Edition. American Phytopathological Society, St. Paul, MN.

Phillips, D. V., and Boerma, H. R. 1981. *Cercospora sojina* race 5: A threat to soybean in the southeastern United States. Phytopathology 71:334-336.

Price, P., Padgett, G. B., and Purvis, M. A. 2014. The effect of selected fungicides and application timings on frogeye leaf spot. Plant Dis. Manag. Rep. 8:FC223.

Qui, L. J., and Chang, R. Z. 2010. The Origin and History of Soybean. Pages 1-23 in: Soybean: Botany, Production, and Uses. G. Singh, ed. CABI, Wallingford, Oxfordshire, U.K.

Ross, J. T. 1968. Additional physiologic races of *Cercospora sojina* on soybean in North Carolina. Phytopathology 58:708-709.

Sauter, H., Steglich, W., and Anke, T. 1999. Strobilurins: evolution of a new class of active substances. Angew Chem. Internat. Edn. 38:1328-1349.

Schöfl, U. A., and Zinkernagel, V. 1997. A test method on microscopic assessments to determine curative and protectant fungicide properties against *Septoria tritici*. Plant Pathol. 46:545-556.

Schumann, G. L., and D'arcy, C. J. 2010. How can we prevent or manage plant disease epidemics? Pages 282-284 in: Essential Plant Pathology. Schumann, G. L., and D'arcy, C. J. Second edition. American Phytopathological Society. St. Paul, MN.

Sciumbato, G. L., Blessitt, J. B., and Poston, D. H. 2006. Fungicides for control of late season foliar diseases. F&N Tests 1:FC066.

Shaner, G., and Buechley, G. 2006. Control of diseases of soybean in central Indiana with foliar fungicides. F&N Tests 1:FC034.

Shurtleff, W. and Aoyagi, A. 2016. History of Soybean Crushing – Soy Oil and Soybean Meal (980-2016). Soyinfo Center, Lafayette, CA.

Singh, T., and Sinclair, J. B. 1985. Histopathology of *Cercospora sojina* in soybeans. Phytopathology 75:185-189.

Soares, A. P. G., Guillin, E. A., Borges, L. L., Silva, A. C. T., Almeida, A. M. R., Grijalba, P. E., Gottlieb, A. M., Bluhm, B. H., Oliveria, L. O. 2015. More *Cercospora* species infect soybean across the Americas than meets the eye. PLoS ONE 10(8): e0133495. doi: 10.1371/journal.pone.0133495.

Standish, J. R., Tomaso-Peterson, M., Allen, T. W., Sabanadzovic, S., and Aboughanem-Sabanadzovic, N. 2015. Occurrence of QoI fungicide resistance in *Cercospora sojina* from Mississippi soybean. Plant Dis. 99:1347-1352.

USDA-ERS. 2018. United States Department of Agriculture Economic Research Service. Soybean & Oil Crops. Online. https://www.ers.usda.gov/

United States Soybean Export Council. 2006. U.S. Soy: International Buyers' Guide.

www.ussec.org/resources/buyers-guide/

Vincelli, P. 2012. QoI (Strobilurin) Fungicides: Benefits and Risks. APSnet Features. doi:10.1094/PHI-I-2002-0809-02.

Wise, K. 2015. Diseases of soybean: Fungicide efficacy for control of foliar soybean diseases. Purdue University Cooperative Extension. West Lafayette, Indiana.

Yang, X.B., Uphoff, M. D., and Sanogo, S. 2001. Outbreaks of soybean frogeye leaf spot in Iowa. Plant Dis. 85:443.

Yorinori, J. T. 1992. Management of foliar fungal diseases in Brazil. p. 185-193. *In* L.G. Copping et al (ed.) Pest management in soybean. Elsevier Applied Science, London.

Yorinori, J. T., and Sinclair, J. B. 1982. *Cercospora sojina*: a set of differential cultivars for race identification. Phytopathology 72:173 (abstr.).

Zeng, F., Arnao, E., Zhang, G., Olaya, G., Wullschleger, J., Sierotzki, H., Ming, R., Bluhm, B. H., Bond, J. P., Fakhoury, A. M., and Bradley, C. A. 2015. Characterization of quinone outside inhibitor fungicide resistance in *Cercospora sojina* and development of diagnostic tools for its identification. Plant Dis. 99:544-550.

Zhang, G. 2012. *Cercospora sojina*: Overwinter survival and fungicide resistance. Department of Crop Sciences. PhD Thesis. University of Illinois at Urbana-Champaign. Urbana, Illinois.

Zhang, G., Allen, T. W., Bond, J. P., Fakhoury, A. M., Dorrance, A. E., Weber, L., Faske, T. R., Giesler, L. J., Hershman, D. E., Kennedy, B. S., Neves, D. L., Hollier, C. A., Kelly, H. M., Newman, M. A., Kleczewski, N. M., Koenning, S. R., Thiessen, L. D., Mehl, H. L., Zhou, T., Meyer, M. D., Mueller, D. S., Kandel, Y. R., Price III, P. P., Rupe, J. C., Sikora, E. J., Standish, J. R., Tomaso-Peterson, M., Wise, K. A., and Bradley, C. A. 2018. Widespread occurrence of Quinone outside inhibitor fungicide-resistant isolates of *Cercospora sojina*, causal agent of frogeye leaf spot of soybean, in the United States. Plant Health Prog. 19:295-302.

Zhang, G. R., Newman M. A., and Bradley, C. A. 2012. First report of the soybean frogeye leaf spot fungus (*Cercospora sojina*) resistant to quinone outside inhibitor fungicides in North America. Plant Dis. 96: 767-767.

Zou, J., Dong, W., Yang, Q., Cao, Y., and Chen, S. 1999. Inheritance of resistance to race 7 of *Cercospora sojina* in soybeans and RAPD tagging of the resistance gene. Chin Sci. Bull. 44:452-455.

CHAPTER II

ASSESSMENT OF QUINONE OUTSIDE INHIBITOR FUNGICIDE SENSITIVIY OF *CERCOSPORA SOJINA* RECOVERED FROM GEORGIA SOYBEAN[1]

[1] Harrelson, B. C., Culbreath, A. K., Kemerait, Jr., R. C., and Buck, J. W. To be submitted to *Plant Disease*.

ABSTRACT

Fungicides specifically within the class of quinone outside inhibitors (QoIs) have been widely used in the United States for the management of frogeye leaf spot (FLS), caused by the fungal pathogen *Cercospora sojina* K., in soybean (*Glycine max* L. (Merr.). Continued use has resulted in the development of resistance in many states. In 2018 and 2019, 80 isolates of *C. sojina* were recovered from six counties in Georgia and were screened for fungicide resistance using a PCR-RFLP method with resistant isolates recovered in three of the six counties. Additionally, 50 isolates, including a "baseline isolate" with no prior fungicide exposure, were used to determine the percent reduction of mycelial growth to two fungicides, azoxystrobin and pyraclostrobin, at six concentrations: 0.0001, 0.001, 0.01, 0.1, 1, and 10 μg ml^{-1}. Mycelial growth observed for resistant isolates varied significantly from both the sensitive isolates and the baseline isolate for azoxystrobin concentrations of 10, 1, 0.1, and 0.01 μg ml^{-1}, but did not differ for concentrations of 0.001 and 0.0001 μg/ml. Moreover, for pyraclostrobin, resistant isolates differed significantly at concentrations of 10, 1, 0.1, 0.01 and 0.001 μg ml^{-1} to those of the baseline and sensitive isolates, but not at the concentration of 0.0001 μg ml^{-1}. The results of this study indicate QoI resistance is present among the Georgia population of *C. sojina*, suggesting the management of this disease moving forward should rely more on the use of host resistance and fungicides with two or more modes of action.

INTRODUCTION

Soybean, *Glycine max* L. (Merr.), is grown throughout the state of Georgia, yet is more widely cultivated in the northwestern parts of the state, with counties within the region historically having the highest production each year. Yet, depending on year and soybean prices,

other regions of Georgia, such as central and southwestern Georgia, can also see their production increase and planted acres reach or surpass acres planted in the northwestern region of the state (NASS 2017a). In 2016, soybean was planted on over 320,000 acres in Georgia with production reaching over $72 million. In subsequent years, soybean acreage decreased to 250,000 in 2017, 170,000 in 2018, and 130,000 acres in 2019 (NASS 2016, NASS 2017, NASS 2018, NASS 2019).

Due to the high relative humidity and warm temperatures present in Georgia, the most common pests and diseases of soybeans there differ from other states in the soybean producing region of the U.S. where soybean cyst nematode is more prevalent. Historically, soybean cyst nematode (*Heterodera glycines*) has been the most yield limiting pest in the U.S., yet in Georgia, soil conditions, low in organic matter and fertility, do not lead to significant yield reductions from *H. glycines* (Freeman et al. 2019). In Georgia, other species of nematodes are more problematic, with southern root-knot nematode (*Meloidogyne incognita*) being the most problematic and widespread, but depending on field and prior crop, peanut root-knot nematode (*M. arenaria*) and javanese root-knot nematode (*M. javanica*) can also be problematic (Freeman et al. 2019). In 2019, the top three yield-limiting pest and diseases in Georgia were root-knot nematodes (*Meloidogyne* spp.), southern blight (*Sclerotium rolfsii*), and cercospora leaf blight (*Cercospora kikuchii*) and frogeye leaf spot (*C. sojina*), respectively (Little 2019).

Frogeye leaf spot (FLS), caused by the fungal pathogen *Cercospora sojina* K. Hara, is a foliar disease of soybeans responsible for yield reductions throughout the major soybean producing regions in the world (Hartman et al. 2015; Mian et al. 2008). Historically in the U.S., FLS has been present in the southeastern U.S. due to warm temperatures and high relative humidity. However, since the early 2000's, FLS has been reported affecting soybeans in more

north-central states (Cruz and Dorrance 2009; Mengistu et al. 2002; Yang et al. 2001) due to planting of more susceptible varieties, increased no-till production, and warming climatic conditions (Hartman et al. 2015).

The pathogen, *C. sojina*, produces asexual conidia that act as both primary and secondary sources of inoculum. Conidia primarily infect soybean foliage, although pods, seeds, and stems can also be infected (Grau et al. 2004; Hartman et al. 2015). Soybean foliage displaying symptoms of FLS start out as brown to red water-soaked lesions with a circular to angular appearance and as lesions mature, the dark centers turn to light brown-white centers with dark brown to reddish colored margins (Hartman et al. 2015). If foliar infection increases, lesions may expand and coalesce, resulting in blighting of foliage if lesions cover approximately 30% of the leaf surface area (Hartman et. al. 2015). Soybean pods, seeds, and stems infected with FLS are less common than foliage and often occur late in the growing season.

C. sojina predominately survives and overwinters in infected soybean debris left in fields as mycelium or in infected seeds (Hartman et al. 2015; Mian et al. 2008). Soybean seeds infected with *C. sojina* tend to germinate to produce weak seedlings with lesions present on cotyledons. Wind and water splashing from rain and over-head irrigation aid in the dispersal of *C. sojina*, with sporulating lesions on cotyledons and infected soybean residue serving as the primary source of inoculum to infect neighboring soybean seedlings (Hartman et al. 2015). *C. sojina* infection is dependent on environmental conditions, and when present can infect soybean throughout the majority of its life cycle (Grau et al. 2004; Phillips 1999).

Soybean growth stage and the presence or absence of pathogen inoculum are significant factors in the development and severity of FLS. In Georgia, the optimum planting period is from May 10 to June 10, or when soil temperatures are greater than 21°C (70°F), with the growing

season extending to the end of October or early November (Freeman et al. 2019). Soybean growth and development is dependent on photoperiod and temperature (Major et al. 1975), with the majority of soybeans in Georgia reaching reproductive growth stages during end of June to July (Freeman et al. 2019). During these months, high temperatures and adequate rain fall can lead to conducive environments that can promote and manifest FLS symptoms. If these conditions arise and infection occurs prior to flowering yield reductions can range from 10-60% (Akem and Dashiell 1994; Dashiell and Akem 1991; Mian et al. 1998).

Prevention of FLS is dependent on cultural practices. Planting of high-quality pathogen-free seed, two-year rotation away from a host crop, and tillage practices are recommended to reduce pathogen inoculum levels (Dorrance and Mills 2011; Grau et al. 2004; Mian et al. 2008). Resistant cultivars containing the *Rcs3* gene or a combination of the *Rcs1* and *Rcs2* genes are also recommended in locations where varieties are adapted and have high yield potential (Hartman et al. 2015). After the furrow has been closed and the choice to use or not to use a resistant cultivar has been made, applications of fungicides can also provide adequate control of FLS when applied between growth stages R2 and R5, or full bloom and beginning pod, respectively (Hartman et al. 2015; Mian et al. 2008a). Of the foliar fungicides labeled for use in soybeans, products in the fungicide class quinone outside inhibitor (QoI) (Fungicide Resistance Action Committee [FRAC] Code 11) have been a widely used method of control of FLS in the U.S. (Hartman et al. 2015).

Quinone outside inhibitors (QoIs), also referred to as strobilurins, were first discovered from naturally occurring anti-fungal compounds and have broad-spectrum of activity on all four major groups of plant-pathogenic fungi with a single-site mode of action (MOA) (Bartlett et al. 2002). The fungicidal activity of QoI products results from their ability to bind to the outer

quinol oxidation (Q_o) site in complex III of the mitochondria, preventing the shuttling of electrons down the electron transport chain (ETC), and thus ceasing the production of ATP (Bartlett et al. 2002; Becker at al. 1981; Vincelli 2012). Due to their wide-spread use in many pathosystems and single-site MOA, resistance to these products has developed in over 40 different fungal species (FRAC 2018). Resistance to QoIs has been linked to the presence of three point mutations within the mitochondrial cytochrome *b* gene. These mutations result in amino acid substitutions at position 129, 137 and 143; yet, only the mutation present at position 143 displays complete resistance, which if present results in fungicide failure (Fernández-Ortuño et al. 2008).

The first report of QoI resistant *C. sojina* containing the G143A mutation and displaying complete resistance occurred in Tennessee in 2010. Isolates recovered from the field also displayed cross-resistance to three products within the strobilurin class: azoxystrobin, pyraclostrobin, and trifloxystrobin (Zhang et al. 2012a). Since 2010, QoI resistance has been documented in many of the major soybean producing states in the U.S. including Alabama, Arkansas, Delaware, Illinois, Indiana, Iowa, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Ohio, Tennessee, and Virginia (Standish et al. 2015; Zhang et al. 2012a; Zhang et al. 2018). Between 2017 and 2018, commercial fields in northwest Georgia showed increased FLS severity after applications of fungicides within the QoI class leading to the suspected development of QoI resistance in the Georgia population of *C. sojina* (R. Kemerait, *personal communication*). The purpose of this study was to conduct a survey of commercial and state variety testing locations in Georgia with soybean displaying symptoms of FLS to collect isolates of *C. sojina* to determine sensitivity to QoI fungicides through molecular and in vitro assays.

MATERIALS AND METHODS

Frogeye Leaf Spot Sampling

In 2018 and 2019 in the months of August through October, commercial and state variety testing locations throughout the state of Georgia were sampled for soybean foliage displaying symptoms of frogeye leaf spot. Commercial soybean fields were chosen for sampling with the help of University of Georgia Extension agents who knew of soybean displaying FLS symptoms or of soybean fields were a fungicide had been applied. A sample consisted of 20 to 50 leaves exhibiting foliar symptoms resembling those of FLS with sampling being conducted by members of the Buck Lab or University of Georgia Extension agents. Sampled leaves were placed in plastic bags and kept on ice until refrigerated at (4°C). Samples submitted to the Buck Lab by county extension agents were placed in plastic bags and over-night shipped and then refrigerated upon arrival. Samples containing leaves displaying FLS symptoms were refrigerated until pathogen isolation, generally within 5 days of sampling.

Isolation of *Cercospora sojina*

Leaves displaying FLS symptoms were first rinsed with water to remove any soil and debris left from the field and blotted dry. Next, foliage was examined under the dissecting microscope (20x) for the presence of sporulating lesions, depicted by tuffs of conidiophores bearing conidia. If lesions exhibited sporulation, a sterile hypodermic needle was used to gently collect conidia and transfer them to V8 agar plates amended with chloramphenicol (1 ml L^{-1}). If lesions were not producing conidia, approximately 15 to 20 leaves were placed in 150 mm petri dishes (Fisher Scientific, Pittsburgh, PA) containing damp filter paper, sealed with Parafilm®

(Sigma-Aldrich, St. Louis, MO), and placed in plastic bags with a damp paper towel for 24 h to induce sporulation. After 24 h, leaves were reexamined for sporulation under the dissecting microscope and if found to have sporulating lesions, conidia were collected using a sterile hypodermic needle and transferred to V8 agar plates amended with chloramphenicol as described above.

After 24 h, plates were examined for germinating conidia. If plates displayed germinating conidia, hyphal tips were aseptically transferred to fresh V8 agar plates amended with chloramphenicol. Plates were then kept at ambient temperature for approximately 21 days to allow for hyphal colonization of the plates. After 21 days, 5 mm hyphal plugs were removed and placed on 10 ml V8 agar slants amended with chloramphenicol and were stored at 4°C for future use. Glycerol stocks of each mono-conidial isolate were also created by adding 3 ml of sterile tween solution (1 drop/liter Tween 20 in 1 L sterile distilled water) (Sigma-Aldrich, St. Louis, MO) to each plate and gently scraping with a sterile scalpel to remove mycelia and spores. Next, 750 µl of each mycelia/conidia suspension was added to 750 µl of 30% glycerol solution in a sterile 1.8 ml cryotube (Fisher Scientific, Pittsburgh, PA), and placed in the freezer at -80°C for long-term storage. All isolates collected from 2018 and 2019 were prepared for future use and long-term storage using these procedures.

DNA Extraction

Plugs from actively growing colonies were transferred to fresh V8 agar amended with chloramphenicol and allowed to grow for approximately 14 days, or until sufficient mycelial growth was present for extraction, at ambient temperature with an alternating 12 h photoperiod. Next, mycelia and conidia were harvested by adding 3 ml of 0.9% (w/v) sodium chloride

collection solution and gently scrapping and removing mycelia and conidia using a sterile scalpel. A volume of 1 ml of the mycelia/spore suspension was collected and placed in 1.5 ml centrifuge tubes.

DNA extraction for all isolates recovered from 2018 and 2019 was performed using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, ON, Canada). Centrifuge tubes containing 1 ml of mycelia/conidia suspension were centrifuged at 14,000 RPM for 1 min and the resulting supernatant discarded. The cell pellet was resuspended in 500 µl of lysis buffer L and then transferred to a provided bead tube. Bead tubes containing lysis buffer and mycelia/conidia suspension were vortexed for 2 mins and 30 sec using a Geno-Grinder® (SPEX, Metuchen, NJ), removed and inverted 3 to 4 times, and then returned and vortexed for an additional 2 mins and 30 sec. After vortexing, tubes were incubated at 65°C for 10 min, removed and inverted 3 to 4 times, and then returned and incubated for an additional 10 min. The rest of the steps were conducted as outlined in the Norgen Fungi/Yeast Genomic DNA Isolation Kit product insert. Samples were then stored at -8°C until used for polymerase chain reaction.

Polymerase Chain Reaction

Isolates collected in 2018 and 2019 and two confirmed *C. sojina* isolates (CS1036 and 18CS741) from Dr. Carl Bradley at the University of Kentucky were positively identified as *C. sojina* using polymerase chain reaction (PCR) amplification of the internal transcribed spacer region using an ITS1/ITS4 primer set (White et al. 1990). PCR was conducted using a Bio-Rad T100™ Thermal Cyler (Bio-Rad Laboratories, Inc., Hercules, CA) with the cycling conditions set at an initial denaturation period for 3 min at 90°C; and then 34 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and elongation at 72°C for 2 min; followed by a

final extension step at 72℃ for 10 min. PCR products were cast on 2% agarose gels containing 3 µl of GelRed® (Biotium, Inc., Fremont, CA) and run in 1X Tris-Acetate-EDTA (TAE) buffer and then viewed under UV light.

In order to detect the presence or absence of point mutations within the cytochrome *b* gene that give rise to QoI resistance, primers CercUN-F (5' – TCTTCTTAGTATACTTACACGTAG – 3') and CercUN-R (5' – AAACTCCTCATAAAAACTCAAC – 3') created by Standish et al. were used (2015). PCR amplification was again conducted using a Bio-Rad T100™ Thermal Cyler, cycling conditions set at an initial denaturation period at 94℃ for 2 min and 20 sec; then 40 cycles of denaturation at 94℃ for 20 sec, primer annealing at 53℃ for 30 sec, and elongation at 72℃ for 35 sec; followed by a final extension set at 72℃ for 10 min (Standish et al. 2015). PCR products were cast on 2% agarose gels containing 3 µl of GelRed® and run in 1X TAE buffer and then viewed under UV light.

Restriction Fragment Length Polymorphism

To determine the presence or absence of point-mutations in the mitochondrial cytochrome *b* gene, a restriction fragment length polymorphism (RFLP) assay was conducted on all *C. sojina* isolates collected in 2018 and 2019. In the presence of a point-mutation at amino acid codon position 143, the nucleotide base change from a guanine to a cytosine creates a restriction site to which the restriction enzyme *AluI* is able to bind and cut the DNA resulting in two distinct products in resistant isolates, compared to one product in sensitive isolates when cast on an agarose gel (Sierotzki et al. 2000; Standish et al. 2015).

To perform RFLP, all steps were followed using a modified protocol supplied with the Thermo Fisher FastDigest kit (Thermo Fisher Scientific Inc., Waltham, MA). Briefly, 10 µl of unpurified cytochrome *b* PCR product obtained above, 17 µl of nuclease-free water, and 2 µl of 10x FastDigest Buffer was added to 200 µl microcentrifuge tube. Next, the tube was vortex briefly and then 1 µl of FastDigest restriction enzyme *AluI* was added and the tube incubated at 37℃ for 45 mins. After incubation, RFLP products were cast on 2% agarose gels containing 3 µl of GelRed® and run in 1X TAE buffer and then viewed under UV light.

To confirm the accuracy of the RFLP assay, cytochrome *b* PCR products from 10 sensitive and 5 resistant isolates were submitted to Eurofins Genomics (Louisville, Kentucky) for sequencing. Geneious Prime 2020.2.2 (Biomatters Ltd., Auckland, New Zealand) was used to trim, edit, and assemble consensus sequences for each isolate using both the forward and reverse sequences. Consensus sequences were used to BLAST selected isolates to sequences of known resistant and sensitive isolates that are deposited in GenBank, accession numbers: KJ566927 to KJ566930 (Standish et al. 2015).

Cercospora sojina Fungicide Sensitivity

To determine the fungicide sensitivity profile to two QoI fungicides, azoxystrobin and pyraclostrobin, 49 isolates, making up a representative sample of the overall *C. sojina* collection from Georgia, were used to conduct an invitro mycelial growth fungicide sensitivity study. Also included was one isolate (baseline; FELS-23) collected in 1993, which predated the production of these fungicides, and to the best of our knowledge has had no prior fungicide exposure. Technical grade azoxystrobin and pyraclostrobin (Sigma-Aldrich, St. Louis, MO) were each dissolved in 10 ml of acetone creating 100 µg ml^{-1} stock solutions of each and then ten-fold

serial dilutions were prepared by adding 1-part stock solution to 9 parts acetone. In total, 6 different concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 10 μg ml^{-1}) of each fungicide were created by adding 1 ml of each final fungicide concentration to 1 liter of PDA after it had cooled to approximately 55℃. A non-fungicide amended control was also included that contained 1 ml of acetone per liter. To prevent conidia from germinating through the alternative respiration pathway, salicylhydroxamic acid (SHAM) (Wood and Holloman 2013) dissolved in methanol was added to all fungicide amended and non-fungicide amended agar at a final concentration of 60 μg ml^{-1} after agar had cooled to approximately 55℃ (Zhang et al. 2012b).

To prepare for the in vitro assay, *C. sojina* isolates were grown on V8 agar amended with chloramphenicol for 21 days at ambient temperature in the dark. After 21days, mycelial disks were removed from actively growing margins with a sterile 5 mm cork-borer and transferred to fresh PDA amended with each fungicide concentration and non-amended control. Plates were stored at 25℃ in the dark with average colony diameter being recorded after 7, 14, and 21 days after plating (DAP).

Statistical Analysis

In vitro assays were performed in a completely randomized design (CRD) with two replicates and the experiment repeated. The percent growth reduction was calculated for each isolate on azoxystrobin amended agar using the formula: ([colony diameter of isolate on non-fungicide amended agar – 5 mm] – [colony diameter on azoxystrobin-amended agar – 5 mm]) / (colony diameter of isolate on non-fungicide amended agar – 5 mm) x 100 and each isolate on pyraclostrobin amended agar using the formula: ([colony diameter of isolate on non-fungicide amended agar – 5 mm] – [colony diameter on pyraclostrobin-amended agar – 5 mm]) / (colony

diameter of isolate on non-fungicide amended agar – 5 mm) x 100 (Ishii et al. 2007; Mengistu et al. 2020). Analysis of variance was determined for each isolate, fungicide, and concentration and their interactions utilizing PROC GLIMMIX in SAS (Version 9.4; SAS Institute, Cary, NC), and means were compared using Tukey's test ($\alpha = 0.05$). The percent reduction for each fungicide and concentration was tested for normality using Kolmogorov-Smirnov test (PROC CAPABILITY) in SAS.

RESULTS

Frogeye Leaf Spot Sampling

During 2018 and 2019, commercial and state variety testing locations known to have planted soybean were sampled during the months of August through October for soybean foliage displaying symptoms associated with FLS. In 2018, 71 isolates of *C. sojina* were recovered from four counties in Georgia: Gordon, Oconee, Pike, and Sumter (Figure 2.1). Weather conditions in 2018 were more favorable during the months of July through September, with more rainfall, and thus FLS was more widely observed. In 2019, sampling was increased to include more commercial locations with 22 counties in Georgia sampled for FLS, but low soybean acreage and dry climatic conditions in the months of July through September resulted in very little FLS symptomatic soybean. However, isolates of *C. sojina* were recovered from two additional counties, Jefferson and Walker, in 2019.

Restriction Fragment Length Polymorphism

The results from the PCR-RFLP assay conducted on the 80 *C. sojina* isolates recovered from soybean fields in Georgia to determine the presence of QoI resistance showed 5 of the 80

isolates tested (Table 2.1) displayed the necessary "two band" fragments (Figure 2.2) associated with the presence of the mutation at position 143. Sequencing results of 10 sensitive isolates: BL 18-8, BL 18-17, BL 18-25, CAL 18-5, CAL 18-19, IH 18-21, PL 18-1, WK 19-3, WK 19-4, and WK 19-5, along with all 5 resistant isolates: IH 18-7, JF19-1, WK 19-6, WK 19-7, and WK 19-8, determined by PCR-RFLP, confirmed these results. Resistant isolates showed the presence of the nucleotide mutation of a guanine to a cytosine (G to C) at position 143 resulting in the codon GCT coding for alanine in all 5 isolates. All 10 sensitive isolates did not show the presence of the mutation at position 143, thus contained the codon GGT coding for glycine.

Cercospora sojina Fungicide Sensitivity

For each isolate, the data from the two trials were compared statistically and found to be homogeneous and lacked significant interactions and thus were combined for analyses. Isolates were grouped based on their PCR-RFLP results as either sensitive (S), 44 isolates; resistant (R), 5 isolates; or baseline (B), 1 isolate, for analysis of variance, with percent reduction being normally distributed. Results from analysis of variance showed all main effects and the interaction of isolates*fungicide*concentration to be significant ($P = <.0001$) (Table 2.2). Percent reduction of radial growth for azoxystrobin ranged from -81.8 to 100.0, with a mean of 43.7, and a median of 21.1. Percent reduction of radial growth for pyraclostrobin ranged from -93.3 to 100.0, with a mean of 58.2, and a median of 76.2.

The results of this study for the fungicide azoxystrobin (Figure 2.6) showed significant differences among the resistant and sensitive groups of isolates at concentrations of 10, 1, 0.1, and 0.01 μg ml^{-1}. At 10 μg ml^{-1} sensitive isolates had a 99.5% reduction of mycelia growth compared to 21.7% reduction of growth for resistant isolates. At 1 μg ml^{-1}, sensitive isolates had

a 97.2% reduction compared to -1.8% for resistant isolates. At 0.1 μg ml^{-1} sensitive isolates had an 83.9% reduction compared to -3.7% for resistant isolates. At 0.01 μg ml^{-1} sensitive isolates had a 10.9% reduction compared to -4.3% for resistant isolates.

The results of this study for the fungicide pyraclostrobin (Figure 2.7) showed significant differences among the resistant and sensitive groups of isolates at concentrations of 10, 1, 0.1, 0.01, and 0.001 μg ml^{-1}. At 10 μg ml^{-1}, sensitive isolates had a 99.6% reduction of mycelia growth compared to 72.2% reduction of growth for resistant isolates. At 1 μg ml^{-1}, sensitive isolates had a 95.0% reduction compared to 24.5% for resistant isolates. At 0.1 μg ml^{-1}, sensitive isolates had an 86.0% reduction compared to -0.5% for resistant isolates. At 0.01 μg ml^{-1}, sensitive isolates had a 73.6% reduction compared to -4.5% for resistant isolates. At 0.001 μg ml^{-1}, sensitive isolates had a 17.6% reduction compared to -6.8% for resistant isolates.

DISCUSSION

The use of fungicides has been an integral part of the management of FLS for in-season control of the disease in the U.S. However, overuse, or the continuous use, of products within the class of QoI fungicides without a rotation of chemistry, along with their broad spectrum of activity and single site mode of action, has seen resistance develop in *C. sojina* in many of the major soybean producing states (Hartman et al. 2015; Mian et al. 2008; Zhang et al. 2018). In this study, sampling of commercial and state variety testing locations containing soybean was used to evaluate the population of *C. sojina* in Georgia to QoIs through molecular and in vitro fungicide assays. The results of this study indicate resistance to QoI fungicides is present within the population of *C. sojina* from Georgia. The resistance found in this study does not seem widespread, when compared to other states (Standish et al. 2015), but lack of additional sampling

in 2019, due to dry weather conditions and the decrease in soybean acreage, allows for speculation that resistance could be present in more counties in Georgia and perhaps at a larger frequency.

Of the counties sampled found to have resistant isolates, two counties: Oconee and Walker, in fields where isolates were recovered, had no prior fungicide history. The field from Oconee County was a state variety testing location used for testing numerous soybean varieties for performance to various conditions within the location and thus received no fungicide applications. In addition, the grower of the commercial field location where resistant isolates were recovered in Walker County confirmed that no fungicide had been applied to the field in which the soybeans were grown. The lack of fungicide applications, specifically QoI fungicides, in these two locations suggest that resistant isolates are moving into these locations from other locations where applications of QoIs has been frequent. The movement of the pathogen via wind and rain splash is possible for local dissemination, however, the infection of soybean seeds with *C. sojina* could result in more long-distance dispersal of the pathogen. The movement of QoI resistant *C. sojina* on infected seeds could account for presence of resistant isolates within the two locations with no known fungicide application, but also creates cause for concern for the use of QoI products going forward as tool for the management of FLS even in locations without the prior use of the products.

The results from the in vitro fungicide assay of 50 *C. sojina* isolates, including one baseline isolate with no prior fungicide history, showed significant differences in percent reduction among the resistant isolates compared to the baseline and the group of sensitive isolates for both azoxystrobin and pyraclostrobin. There were no significant effects between the baseline and the group of sensitive isolates for either fungicide or any of the fungicides at the 6

concentrations, so in terms of the analysis, the baseline was included in the group with the sensitive isolates. The results with azoxystrobin indicated there was a signification difference in control of sensitive isolates observed between 0.1 and 0.01 μg ml^{-1} of azoxystrobin, 83.9% to 10.9%, respectively, which implies that at concentrations below 0.1 μg ml^{-1} of azoxystrobin are not effective in the control of *C. sojina* in sensitive populations. At concentrations of 0.001 and 0.0001 μg ml^{-1} the differences among sensitive and resistant isolates were not significant, with both sensitive and resistant isolates increasing in growth compared to their non-treated control. As for pyraclostrobin, there was a significant difference in control of sensitive isolates observed between 0.01 and 0.001 μg ml^{-1} of pyraclostrobin, 73.6% to 17.6%, respectively, which implies that at concentrations below 0.01 μg ml^{-1} of pyraclostrobin are not effective in the control of *C. sojina* in sensitive populations. At the concentration of 0.0001 μg ml^{-1} the differences among sensitive and resistant isolates were not significant.

Differences in the intrinsic activity between azoxystrobin and pyraclostrobin were observed in this study. Azoxystrobin was less effective in controlling sensitive isolates at lower concentrations such as 0.01 and 0.001 μg ml^{-1} when compared to pyraclostrobin. Pyraclostrobin also displayed more activity in controlling resistant isolates with positive reductions in mycelia growth observed at 10 and 1 μg ml^{-1}, 72.2% and 24.6% reduction, respectively. Whereas, azoxystrobin displayed minimal activity on resistant isolates with a positive reduction only observed at 10 μg ml^{-1}, 21.7%. These findings are similar to those reported for *C. sojina* to QoI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin when evaluated for their activity on conidial germination, with pyraclostrobin being more effective than azoxystrobin and trifloxystrobin (Zhang et al. 2012b).

The presence of QoI resistant *C. sojina* in Georgia could have significant impact on the management of soybean diseases with the use of fungicides going forward. Over the last several years, growers have been more widely planting indeterminate soybean varieties (maturity group (MG) 000 – IV) over determinate varieties (MG V – X) in Georgia, which could require a longer in-season widow for disease management due to the continued production of vegetive growth even after flowering (R. Kemerait, *personal communication*). The switch from indeterminate to determinate varieties in turn could require more applications of fungicides in order to protect new vegetative growth emerging after flowering, whereas with determinate varieties, timely applications at or between R2 and R5, or full bloom and beginning pod, once vegetative growth has ceased could provide a protective barrier on foliage to last through R6, where yields have typically been made and any infection occurring after this stage does not result in yield reductions. The requirement of more in season fungicide applications could enhance the population of QoI resistant *C. sojina* in Georgia.

The question of QoI resistant *C. sojina* moving via seed also raises concern for the management of FLS moving forward. The use of seed treatments, often containing a fungicide, to improve soybean seedling vigor and to help fight seedling diseases is common in early planted soybean production systems in which conditions are often cool and wet (Giesler and Miller 2017). If soybean seed lots are infected with *C. sojina* and a seed treatment is applied containing a QoI, a selection pressure has already been placed on the pathogen even before a foliar application. Furthermore, if seeds lots are infected with QoI resistant *C. sojina*, control failures with seed treatments containing QoI products will be observed, resulting in local or field level dissemination of resistance without a foliar application of a QoI fungicide. The movement of QoI resistant *C. sojina* via seed could explain the presence of resistant *C. sojina* isolates from Oconee

and Walker counties where no prior fungicide had been applied. The potential development of QoI resistant *C. sojina* moving via seed places an increased importance on fungicide resistance management, with management of FLS going forward relying on host resistance and when needed fungicide applications containing mixed modes of action.

LITERATURE CITED

Akem, C. N., and Dashiell, K. E. 1994. Effect of planting date on severity of frogeye leaf spot and grain yield of soybeans. Crop Prot. 13:607–610.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. Pest Manag. Sci. 58:649-662.

Becker, W. F., von Jagow, G., Anke, T., and Steglich, W. 1981. Oudemansin, strobilurin A, strobilurin B and myxothizaol: new inhibitors of the cytochrome bc1 segment of the respiratory chain with an E-ß-methoxyacrylate system as a common structural element. FEBS Letters 132:329-333.

Cruz, C. D., and Dorrance, A. E. 2009. Characterization and survival of *Cercospora sojina* in Ohio. Plant Health Prog. doi: 10.1094/php-2009-0512-03-rs

Dashiell, K. E., and Akem, C. N. 1991. Yield losses in soybeans from frogeye leaf spot caused by *Cercospora sojina*. Crop Prot. 10:465-468.

Dorrance, A. E., and Mills, D. 2010. Frogeye leaf spot of soybean. Ohio State University Extension Fact Sheet AC 53-10. Ohio State University Extension, Columbus, OH.

Fernández-Ortuño, D., Torés, J. A., De Vicente, A., and Pérez-García, A. 2010. The QoI Fungicides: the rise and fall of a successful class of agricultural fungicides. Pages 203-220 in: Fungicides. Odille Carisse, ed.

Freeman, M., Harris, G., Kemerait, R., Prostko, E., Roberts, P., Smith, A., Rabinowitz, A., and Porter, W. 2019. 2019 Georgia Soybean Production Guide, GA: University of Georgia Cooperative Extension Service Publ., Athens, GA.

Fungicide Resistance Action Committee. 2018. List of Plant Pathogenic organisms resistant to disease control agents. Online. http://www.frac.info/frac/index.htm.

Giesler, L. J. and Miller, J. J. 2017. Seed treatment fungicides for soybeans. University of Nebraska-Lincoln Extension Service Publ, Lincoln, NE.

Gisi, U., and Sierotzki, H. 2008. Fungicide modes of action and resistance in downy mildews. Eur. J. Plant Pathology 122:157-167.

Grau, C. G., Dorrance, A. E., Bond, J., and Russin, J. S. 2004. Fungal diseases. p. 679-763, *In*: H. R. Boerma, J. E. Specht (Eds.), Soybean: Improvement, production, and uses, third edition. Am Soc. Agron., Crop Sci. Soc. Amer., and Soil Sci. Soc. Amer. Madison, WI.

Hartman, G. L., Rupe, J. C., Sikora, E. J., Domier, L. L., Davis, J. A., and Steffey, K. L. 2015. Compendium of soybean diseases. Fifth Edition. American Phytopathological Society, St. Paul, MN.

Ishii, H., Yano, K., Date, H., Furuta, A., Sagehashi, Y., and Yamaguchi, T. 2007. Molecular characterization and diagnosis of QoI resistance in cucumber and eggplant fungal pathogens. Phytopathology 97:1458-1466.

Lehman, S.G. 1928. Frog-eye leaf spot of soybean caused by *Cercospora diazu* Miura. J. Agric. Res. 35:811–833.

Little, E. 2019. 2017 Georgia Plant Disease Loss Estimates, GA: University of Georgia Cooperative Extension Service Publ., Athens, GA.

Major, D. J., Johnson, D. R., Tanner, J. W., and Anderson, I. C. 1975. Effects of daylength and temperature on soybean development. Crop Sci. 15:174-179.

Mengistu, A., Kurtzweil, N. C., and Grau, C. R. 2002. First report of frogeye leaf spot (*Cercospora sojina*) in Wisconsin. Plant Dis. 86:1272.

Mengistu, A., Ray, J. D., Kelly, H. M., Lin, B., Yu, H., Smith, J. R., Arelli, P. R., and Bellaloui, N. 2020. Pathotype grouping of *Cercospora sojina* isolates on soybean and sensitivity to QoI fungicides. Plant Dis. 104:373-380.

Mian, M. A. R., Boerma, H. R., Phillips, D. V., Kenty, M. M., Shannon, G., Shipe, E. R., Soffes Blount, A. R., and Weaver, D. B. 1998. Performance of frogeye leaf spot resistant and susceptible near isolines of soybean. Plant Dis. 82:1017-1021.

Mian, M. A. R., Missaoui, A. M., Walker, D. R., Phillips, D. V., and Boerma, H. R. 2008. Frogeye leaf spot of soybean: a review and proposed race designations for isolates of *Cercospora sojina* Hara. Crop Sci. 48:14-24.

Mian, R., Bond, J., Joobeur, T., Mengistu, A., Weibold, W., Shannon, G., and Wrather, A. 2008a. Identification of soybean genotypes resistant to *Cercospora sojina* by field screening and molecular makers. Plant Dis. 93:408-411.

NASS. 2016. United States Department of Agriculture National Agricultural Statistics Service. 2016 Georgia Soybean Production. Online. http://www.nass.usda.gov/

NASS. 2017. United States Department of Agriculture National Agricultural Statistics Service. 2017 Georgia Soybean Production. Online. http://www.nass.usda.gov/

NASS. 2017a. United States Department of Agriculture National Agricultural Statistics Service. Georgia County Estimates Soybean 2016-2017. Online. http://www.nass.usda.gov/

NASS. 2018. United States Department of Agriculture National Agricultural Statistics Service. 2018 Georgia Soybean Production. Online. http://www.nass.usda.gov/

NASS. 2019. United States Department of Agriculture National Agricultural Statistics Service. 2019 Georgia Soybean Production. Online. http://www.nass.usda.gov/

Sierotzki, H., Parisi, S., Steinfeld, U., Tenzer, I., Poirey, S., and Gisi, U. 2000. Mode of resistance to respiration inhibitors at the cytochrome bc_1 enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest Manag. Sci. 56:833-841.

Standish, J. R., Tomaso-Peterson, M., Allen, T. W., Sabanadzovic, S., and Aboughanem-Sabanadzovic, N. 2015. Occurrence of QoI fungicide resistance in *Cercospora sojina* from Mississippi soybean. Plant Dis. 99:1347-1352.

Vincelli, P. 2012. QoI (Strobilurin) Fungicides: Benefits and Risks. APSnet Features. doi:10.1094/PHI-I-2002-0809-02.

White, T. J., Burns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Application. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.

Wood, P. M., and Hollomon, D. W. 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.

Yang, X.B., Uphoff, M. D., and Sanogo, S. 2001. Outbreaks of soybean frogeye leaf spot in Iowa. Plant Dis. 85:443.

Zhang, G., Allen, T. W., Bond, J. P., Fakhoury, A. M., Dorrance, A. E., Weber, L., Faske, T. R., Giesler, L. J., Hershman, D. E., Kennedy, B. S., Neves, D. L., Hollier, C. A., Kelly, H. M., Newman, M. A., Kleczewski, N. M., Koenning, S. R., Thiessen, L. D., Mehl, H. L., Zhou, T., Meyer, M. D., Mueller, D. S., Kandel, Y. R., Price III, P. P., Rupe, J. C., Sikora, E. J., Standish, J. R., Tomaso-Peterson, M., Wise, K. A., and Bradley, C. A. 2018. Widespread occurrence of quinone outside inhibitor fungicide-resistant isolates of *Cercospora sojina*, causal agent of frogeye leaf spot of soybean, in the United States. Plant Health Prog. 19:295-302.

Zhang, G. R., Newman M. A., and Bradley, C. A. 2012a. First Report of the soybean frogeye leaf spot fungus (*Cercospora sojina*) resistant to quinone outside inhibitor fungicides in North America. Plant Dis. 96:767-767.

Zhang, G., Pederson, D, K., Phillips, D. V., and Bradley, C. A. 2012b. Sensitivity of *Cercospora sojina* isolates to quinone outside inhibitor fungicides. Crop Prot. 40:63-68.

Table 2.1. Location, year, and number of QoI resistant *Cercospora sojina* isolates recovered from Georgia soybean in 2018 and 2019.

County	Number of isolates / Year		Total	Total QoI resistant[a]
	2018	2019		
Gordon	17	-	17	0
Jefferson	-	1	1	1
Oconee	27	-	27	1
Pike	26	-	26	0
Sumter	1	-	1	0
Walker	-	8	8	3
TOTAL	71	9	80	5

[a]Resistant isolates determined to have the G143A mutation conferring resistance to QoI fungicides by PCR-RFLP assay and confirmed by sequencing of the cytochrome *b* gene.

Table 2.2. Analysis of variance of percent reduction of radial growth of *Cercospora sojina* isolates to two fungicides, azoxystrobin and pyraclostrobin, at six concentrations.

Effect	Degrees of freedom	*F*-value	*P>F*
Isolate[a]	2	6288.73	<.0001
Fungicide	1	397.86	<.0001
Concentration[b]	5	1754.66	<.0001
Isolate*Fungicide*Concentration	27	458.87	<.0001
DAP[c]	2	198.58	<.0001

[a]Isolates grouped based on QoI sensitivity or resistance determined by PCR-RFLP results, with 44 isolates grouped as sensitive (S), 5 isolates grouped as resistant (R), and 1 baseline isolate (B) with no prior fungicide exposure.

[b]Isolates were grown on amended agar of each fungicide at 6 concentrations: 0.0001, 0.001, 0.01, 0.1, 1, and 10 μg ml^{-1}.

[c]Average colony diameter was recorded at 7, 14, and 21 days after plating (DAP).

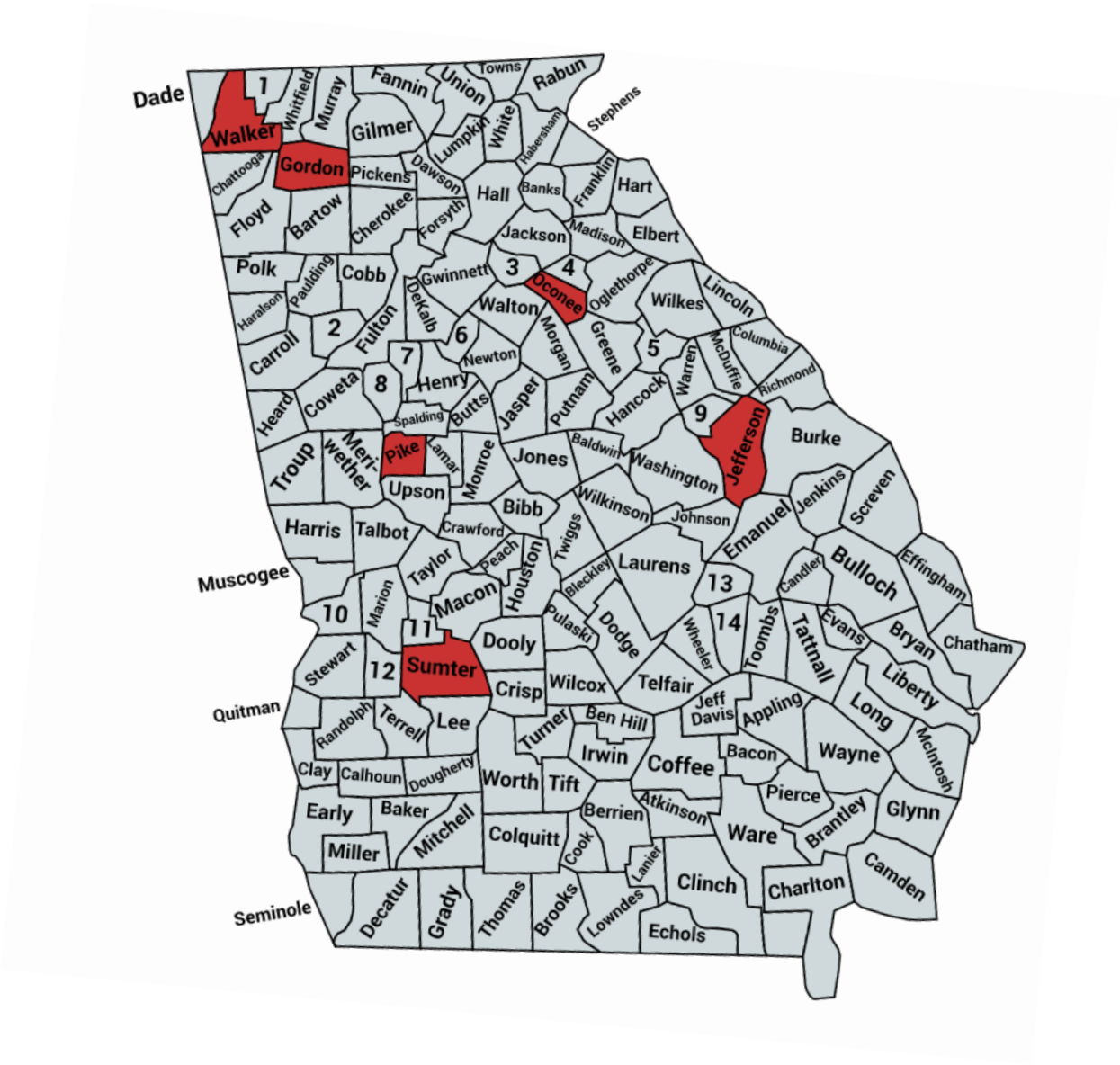

Figure 2.1. County map of Georgia, with *Cercospora sojina* isolates recovered from counties highlighted in red.

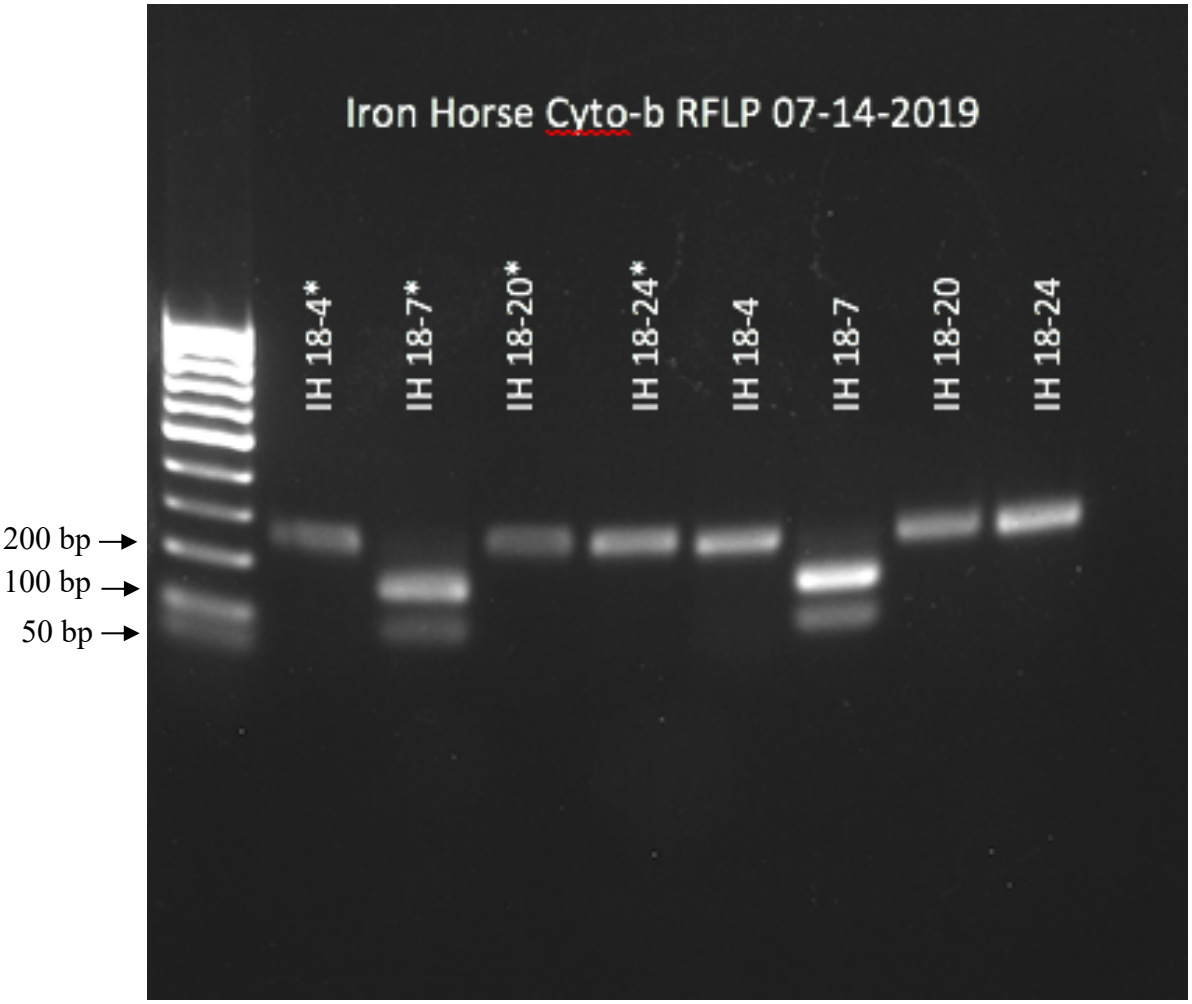

Figure 2.2. Results from PCR-RFLP of the 238-bp product of the cytochrome *b* region using restriction enzyme *AluI*. Cleavage of the band fragment, indicating the presence of the guanine to cytosine nucleotide base change resulting in QoI resistance, is shown in two band fragment sizes approximately 78- and 160-bp, respectively. Sensitive isolates, not containing the mutation responsible for the G143A mutation, display a single band fragment approximately 238-bp in length.

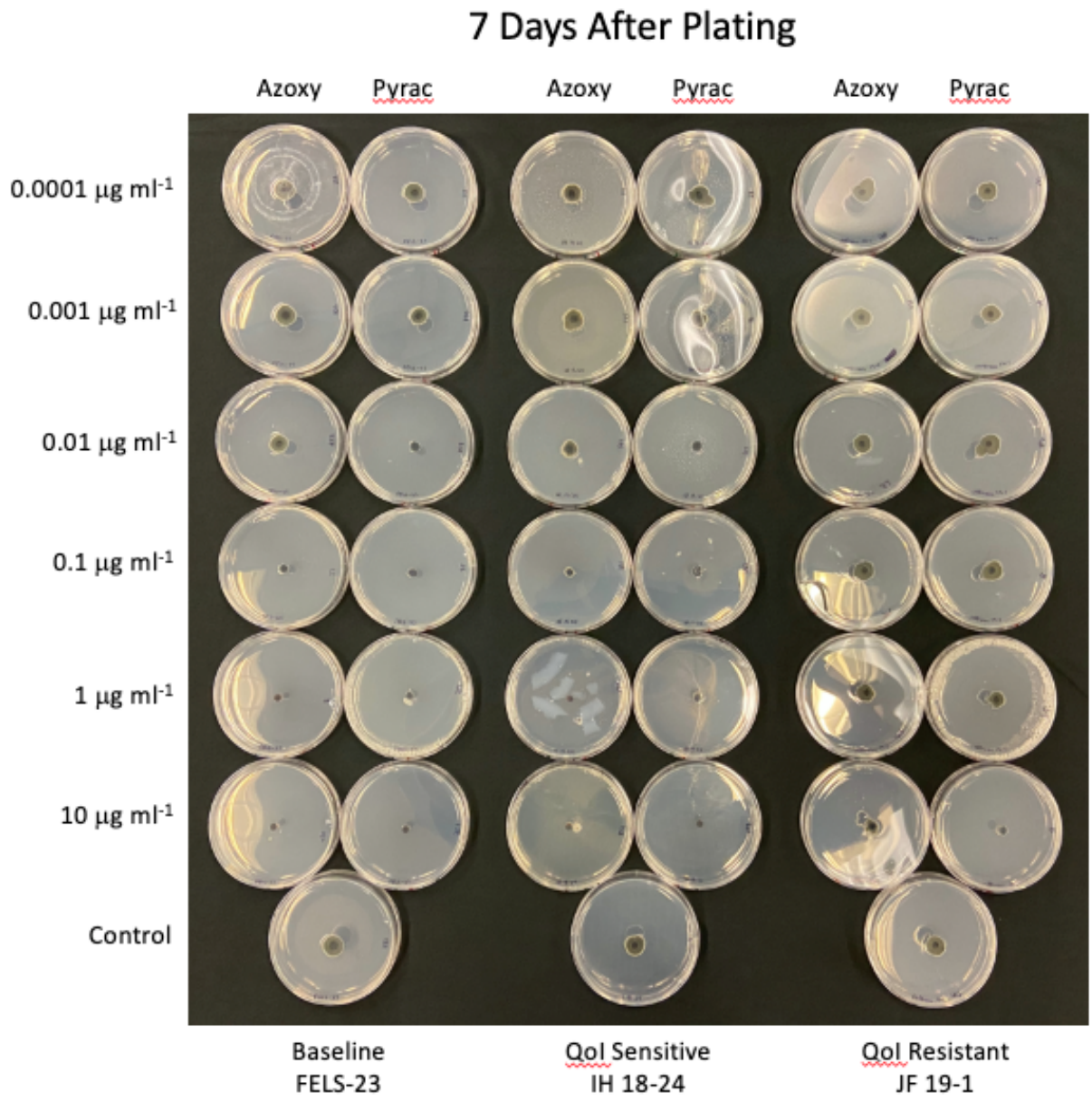

Figure 2.3. Sensitivity of three *Cercospora sojina* isolates: one QoI sensitive (middle), one QoI resistant (right), and one baseline isolate with no prior exposure to QoI fungicides (left) to 6 concentrations of both azoxystrobin (left column) and pyraclostrobin (right column) 7 days after plating.

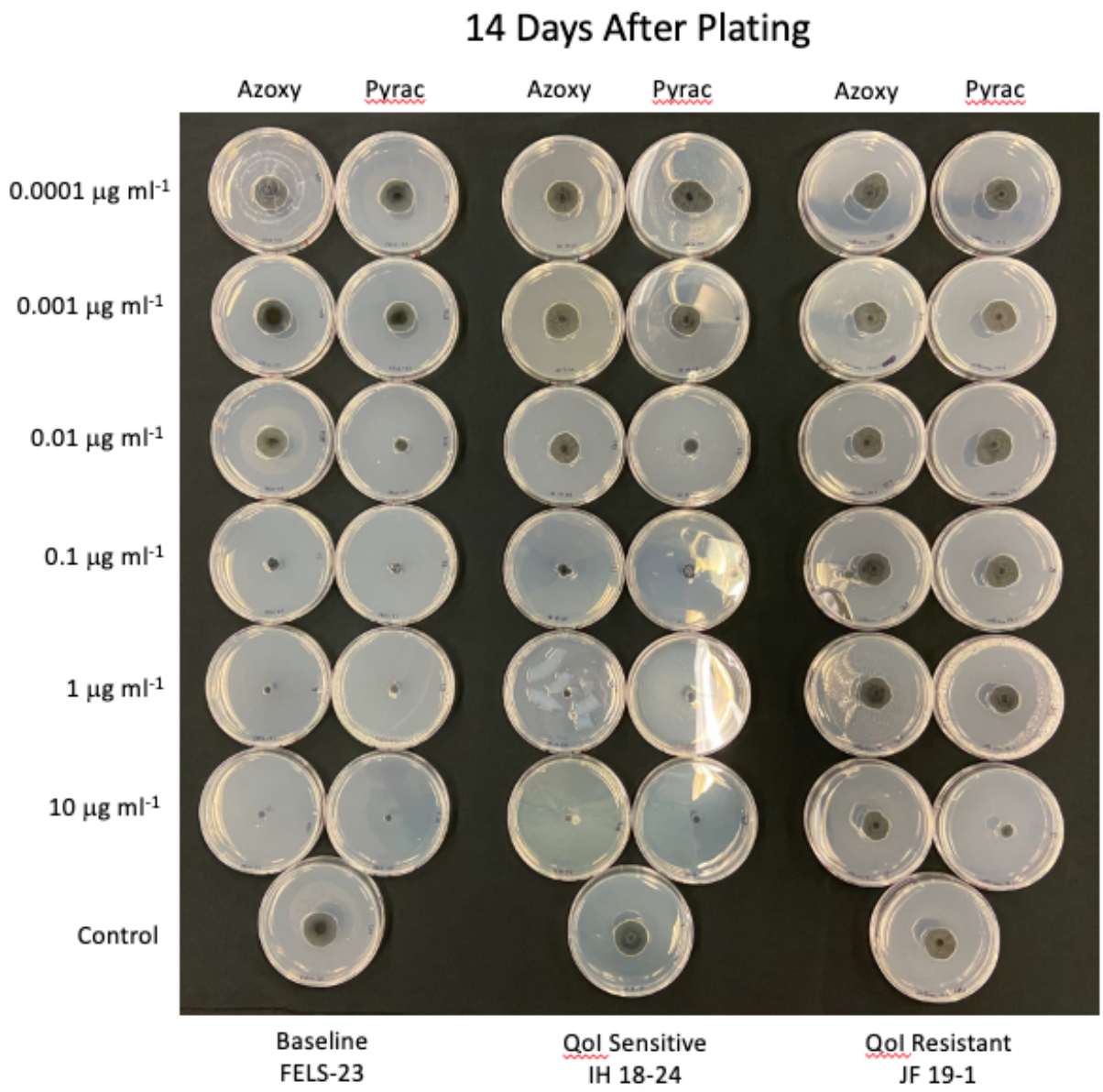

Figure 2.4. Sensitivity of three *Cercospora sojina* isolates: one QoI sensitive (middle), one QoI resistant (right), and one baseline isolate with no prior exposure to QoI fungicides (left) to 6 concentrations of both azoxystrobin (left column) and pyraclostrobin (right column) 14 days after plating.

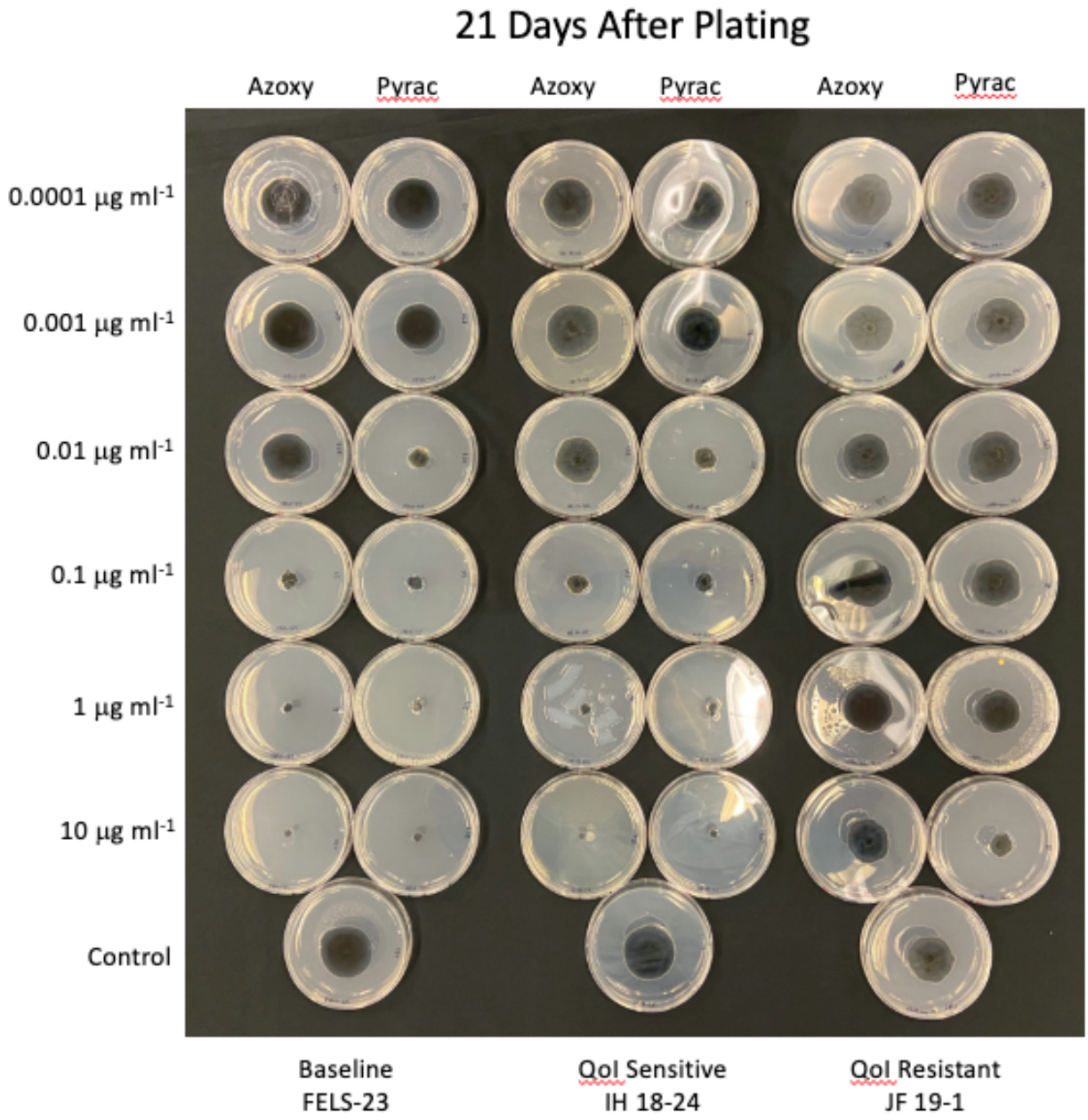

Figure 2.5. Sensitivity of three *Cercospora sojina* isolates: one QoI sensitive (middle), one QoI resistant (right), and one baseline isolate with no prior exposure to QoI fungicides (left) to 6 concentrations of both azoxystrobin (left column) and pyraclostrobin (right column) 21 days after plating.

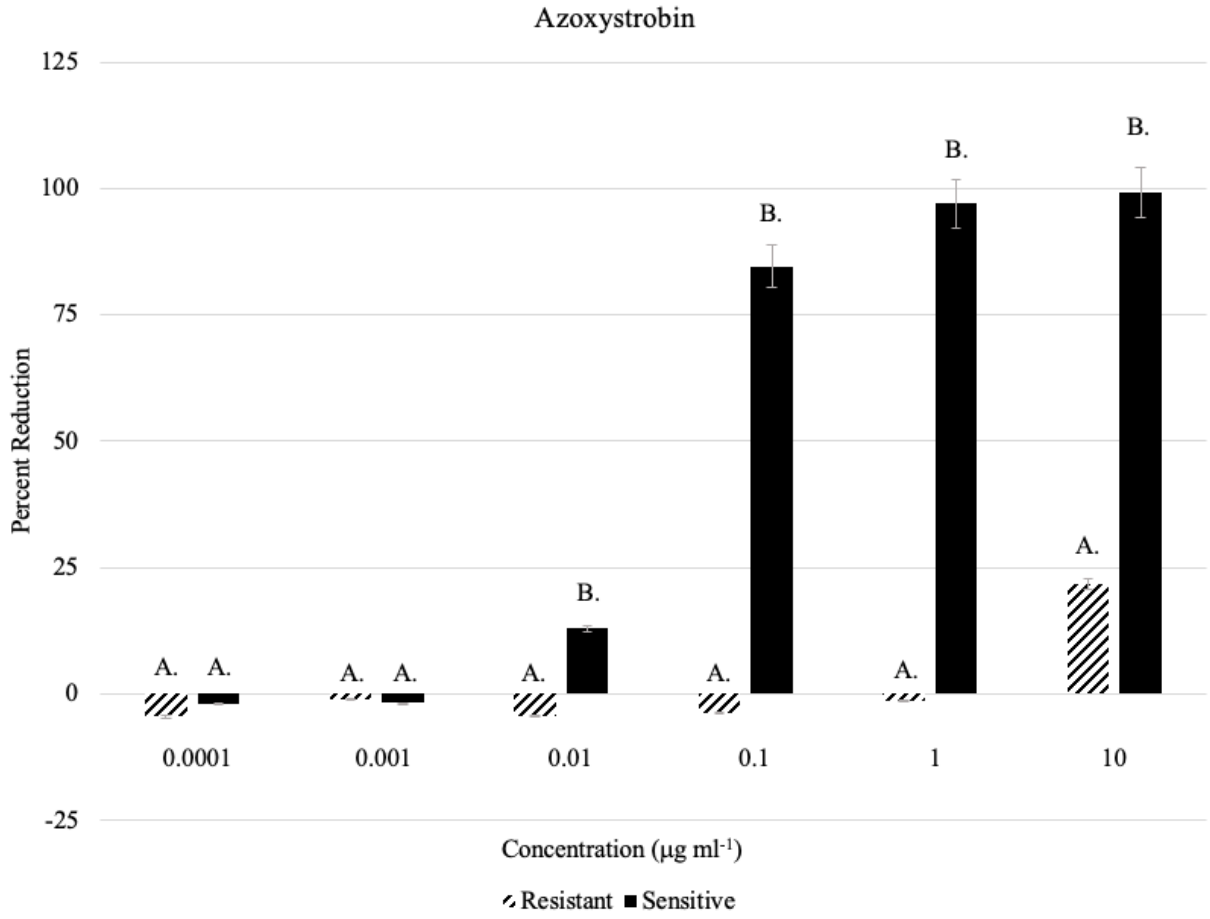

Figure 2.6. Percent reduction of mycelia growth, compared to nonamended control, of quinone outside inhibitor resistant and sensitive isolates of *Cercospora sojina* recovered from soybean in Georgia in 2018 and 2019 to azoxystrobin at 0.0001, 0.001, 0.01, 0.1, 1 and 10 µg ml^{-1}.

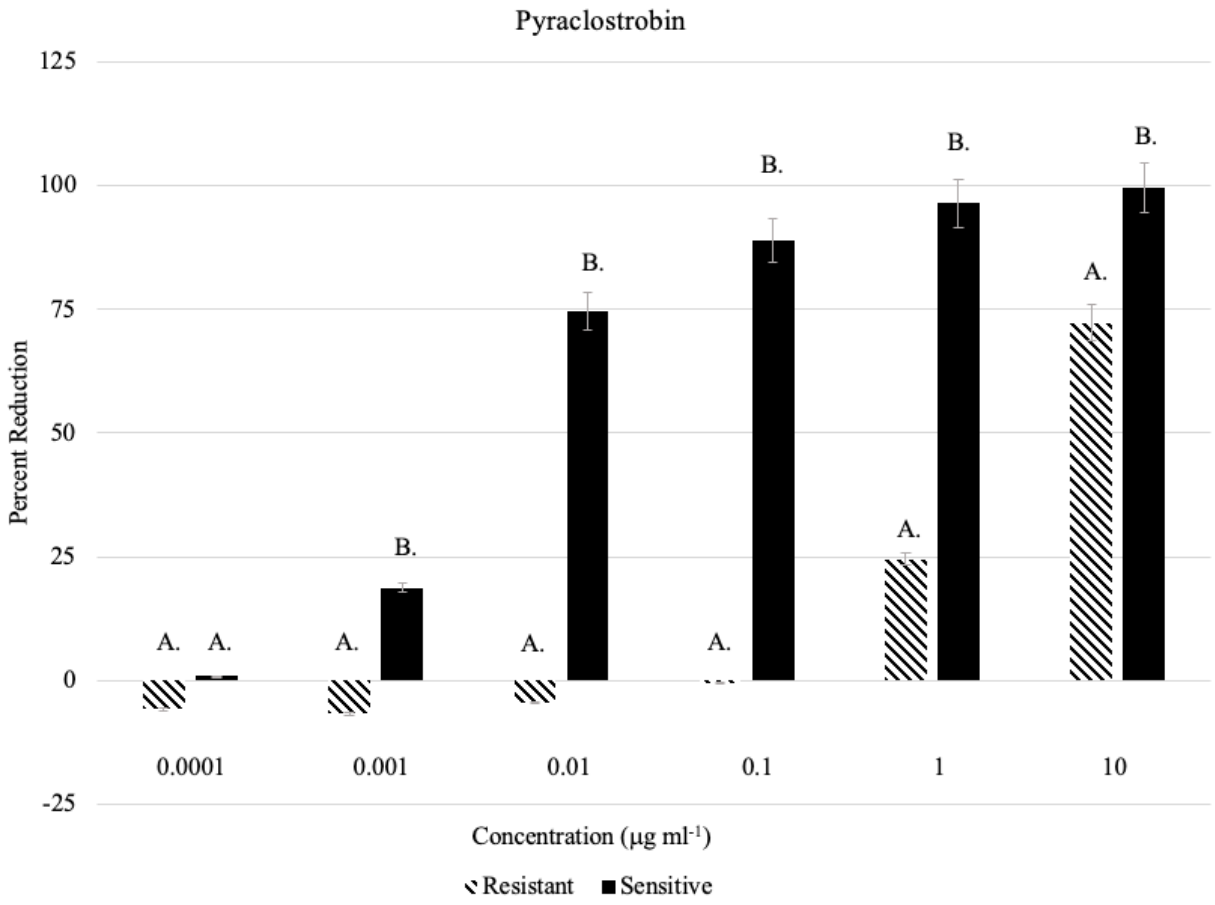

Figure 2.7. Percent reduction of mycelia growth, compared to nonamended control, of quinone outside inhibitor resistant and sensitive isolates of *Cercospora sojina* recovered from soybean in Georgia in 2018 and 2019 to pyraclostrobin at 0.0001, 0.001, 0.01, 0.1, 1 and 10 μg ml^{-1}.

CHAPTER III

EVALUATION OF PATHOGEN RACE OF *CERCOSPORA SOJINA* RECOVERED FROM GEORGIA SOYBEAN[2]

[2] Harrelson, B. C., Culbreath, A. K., Kemerait, Jr., R. C., and Buck, J. W. To be submitted to *Plant Disease*.

ABSTRACT

Frogeye leaf spot (FLS), caused by the fungal plant pathogen *Cercospora sojina* K. Hara, is a common foliar disease of soybean (*Glycine max* L. (Merr.)) known to cause yield reductions wherever soybean is grown, but is more problematic in regions with high temperatures and humidity. In the United States, resistant cultivars have been relied on heavily for the management of this disease, with commercially available varieties containing a combination of single resistance genes *Rcs1* and *Rcs2* or *Rcs3* alone. Yet, with numerous races of *C. sojina* reported around the world and the deployment of single genes for resistance, there is concern for new races of the pathogen overcoming host resistance. In 2018 and 2019, 80 isolates of *C. sojina* recovered from soybean fields in Georgia, were used to inoculate 6 soybean differential cultivars, 'Davis', 'Hood', 'Lee', 'Lincoln', 'Tracy' and 'Blackhawk' to determine pathogenic races. Soybean differentials were grown on average for 14-days and then inoculated with conidial suspensions adjusted to approximately 6×10^4 spores/ml and then re-inoculated after 24-hours. After 14-days, soybeans were assessed as either susceptible or resistant. Isolate reactions on each differential cultivar suggested 12 different races of *C. sojina* present in Georgia, four of which have not been previously described. However, no isolates were pathogenic on differential cultivar 'Davis', containing the *Rcs3* gene, suggesting the gene is still an effective source of resistance in Georgia.

INTRODUCTION

Frogeye leaf spot (FLS), caused by the fungal pathogen *Cercospora sojina* K. Hara, is a foliar disease of soybean (*Glycine max* L. (Merr.), and is known to cause yield reductions throughout the world wherever soybean is grown (Hartman et al. 2015). Symptoms associated

with FLS occur primarily on soybean foliage, yet pods, seeds and stems can also become infected (Grau et al. 2004; Mian et al. 2008). Foliar symptoms start out as water-soaked spots and as they mature result in circular to angular lesions with dark brown to reddish colored margins and gray to white colored centers (Phillips 1999). If foliar infection progresses, lesions can expand and coalesce resulting in premature blighting of foliage if approximately 30% of the leaf surface area is infected (Dashiell and Akem 1991). Yield reductions resulting from FLS is dependent on loss of photosynethic leaf area and can range from 10 to 60% depending on environment and cultivar susceptibility (Akem and Dashiell 1994; Dashiell and Akem 1991; Mian et al. 1998).

An integrated disease management approach incorporating cultural practices, use of resistant cultivars, and preventative fungicide applications is the most effective strategy for managing FLS. The use of pathogen free seed, a two-year rotation to a non-host crop, and tillage can be effective in limiting pathogen inoculum, as *C. sojina* overwinters as infested soybean residue and infected soybean seeds, which act as primary inoculum into to the next growing season (Grau et al. 2004; Hartman et al. 2015; Heatherly and Hodges 1998). After planting, preventative foliar fungicide applications can be an effective tool in controlling FLS when applied between growth stages R2 and R5, full bloom and beginning pod, respectively (Akem 1995; Mills and Dorrance 2008; Grau et al. 2004; Price et al. 2014). However, the use of resistant cultivars provides the most economical and effective means of control and is recommended with commercially available seed containing the *Rcs3* gene or a combination of the *Rcs1* and *Rcs2* genes, with the *Rcs3* gene providing control to previously reported races of FLS in the U.S. (Hartman et al. 2015; Mian et al. 2008).

Until the 2000s, the Soybean Genetics Committee only recognized three single genes, *Rcs1*, *Rcs2*, and *Rcs3* that conferred resistance to FLS. *Rcs1*, discovered in the differential cultivar 'Lincoln', was the first gene found to display resistance to *C. sojina* race 1 in the early 1950s (Athow and Probst 1952). *Rcs2*, discovered in 'Kent', was identified to confer resistance to race 2 (Athow et al. 1962), followed by *Rcs3*, discovered in 'Davis', which is known to convey resistance to all previously described races of *C. sojina* in the U.S. (Boerma and Phillips 1983; Phillips and Boerma 1982). Yet in 2012, the Soybean Genetics Committee approved two additional genes found to confer a high level of resistance to *C. sojina* in PI 594891 and PI 594774 from China and were designated *Rcs*(PI 594891) and *Rcs*(PI 594774) (Hoskins 2011; Pham et al. 2015). Several other genes have been proposed to confer resistance to *C. sojina*, *Rcs4* and *Rcs7* (Buzzell 1988; Zou et al. 1999), but were never proven to be non-allelic to the *Rcs* genes, thus never approved (Baker et al. 1999). Soybean cultivars containing *Rcs* genes can however still become infected with FLS, yet lesions are often small and non-sporulating (Athow and Probst 1952).

The use of resistant cultivars was the predominate means of control for FLS in the U.S. in the early 1900s, providing adequate control of the disease until the late 1950s when race 2 of *C. sojina* appeared (Athow et al. 1962). After the discovery of race 2 in the 1950s, races 3 and 4 were discovered in the 1960s (Ross 1968). In the late 1970s, race 5 was discovered in Georgia and was thought to pose a serious threat to soybean production in the southeastern U.S. due to a large number of widely planted soybean cultivars being susceptible to the newly identified race (Phillips and Boerma 1981). In the early 2000s, 12 physiological races of *C. sojina* were thought to be present in the U.S. (Grau et al. 2004), however, there are discrepancies when comparing

these races to other described races in other countries due to varying sets of soybean differential cultivars used to screen these races (Mian et al. 2008).

In 2008, Mian et al. proposed a set of 12 soybean differential cultivars ('Davis', 'Peking', 'Kent', 'CNS', 'Palmetto', 'Tracy', 'Hood', 'Lincoln', 'Lee', 'Richland', 'S 100', and 'Blackhawk') to serve as a universally accepted set of differential cultivars for the identification and characterization of *C. sojina* isolates, establishing 11 new races designated 5 through 15. Since their establishment, a study conducted in Ohio revealed 20 races of *C. sojina* present from within the state, 9 of which had not been previously described (Cruz and Dorrance 2009). However, issues arise when screening isolates on the 12 differentials purposed by Mian et al. (2008) due to the lack of 12 known genes conferring resistance to result in 12 different possible reactions among the given cultivars. Some of the cultivars proposed, contain genes that are non-allelic but very tightly linked to the *Rcs3* locus and thus produce the same reactions as other cultivars (Mian et al. 2008). To account for this, a study conducted in Arkansas used 6 of the 12 differentials, 'Davis', 'Tracy', 'Hood', 'Lincoln', 'Lee' and 'Blackhawk', and determined 6 races to be present in Arkansas (Fagundes et al. 2018).

With the confirmation of QoI fungicide resistance in Georgia, as determined in this study and in many of the major soybean producing states in the U.S. (Standish et al. 2015; Zhang 2012; Zhang et al. 2018), and with the discovery of previously undescribed races of *C. sojina* in the U. S. (Cruz and Dorrance 2009), an increased importance has been placed on host resistance for the management of FLS. With that, the purpose of this study was to collect and identify *C. sojina* isolates from Georgia to assess pathogen race by screening these isolates on given soybean differential cultivars to determine if the *Rcs* genes still serve as an effective source of resistance to FLS in Georgia.

MATERIALS AND METHODS

Differential Cultivars

Six soybean differentials were used to determine pathogenic race of *C. sojina* in the present study (Fagundes et al. 2018). Cultivars 'Davis', 'Hood', 'Lee', 'Lincoln', 'Tracy', and 'Blackhawk' were obtained from seed stocks from both the University of Georgia Soybean Breeding Program, as well as Dr. Burt Bluhm's lab at the University of Arkansas. Differential cultivar 'Davis', containing the *Rcs3* gene, which confers resistance to all previously reported races of *C. sojina* in the U. S. (Mian et al. 2008), was included as a resistant check to screen *C. sojina* isolates. Differentials 'Hood' and 'Lee' were included due to their respective low and high frequency of susceptible reactions when screened on isolates from Brazil, China, and the U.S. (Cruz and Dorrance 2009; Mian et al. 2008). 'Lincoln' contains the *Rcs1* gene and 'Tracy' is an ancestral line developed for the southern U.S. (Mian et al. 2008). Also included in each experiment was the cultivar 'Blackhawk', presumed to have no resistance to *C. sojina* (Mian et al. 2008) to serve as a susceptible check. Differential 'Kent', thought to contain the *Rcs2* gene, was not included in this study due to new findings determining the resistance present within the soybean cultivar is actually linked to both *Rcs1* and *Rcs3* rather than *Rcs2* (McDonald et al. 2020). Soybean seeds were stored in a cold room with controlled humidity approximately 45 %RH and 4°C until used in greenhouse studies.

Isolates of *C. sojina*

In 2018 and 2019, 80 isolates of *C. sojina* were collected from commercial fields and state variety test locations in Georgia, where soybean foliage was displaying symptoms of FLS.

Symptomatic soybean foliage was observed under the dissecting microscope (20x) for the presence of sporulating lesions, and if present, conidia were aseptically transferred using a sterile hypodermic needle from lesions to V8 agar plates amended with 50 µg ml^{-1} chloramphenicol. If lesions were not sporulating, symptomatic leaves were placed in 150 mm petri dishes (Fisher Scientific, Pittsburgh, PA) containing damp filter paper, sealed with Parafilm® (Sigma-Aldrich, St. Louis, MO), and placed inside plastic bags to induce sporulation and reexamined after 24 h. If found to be sporulating, lesions were isolated using the method mentioned above. Twenty-four hours after transferring germinated conidia, if present, were used to make mono-conidial cultures for each isolate by aseptically isolating actively growing hyphal tips and transferring each isolate to fresh V8 agar plates amended with chloramphenicol. Mono-conidial isolates were allowed to grow for approximately 21 days, after which 5 mm hyphal plugs of each isolate were transferred to 10 ml V8 slants containing chloramphenicol and were stored at 4°C until use in greenhouse studies.

Molecular Identification of *C. sojina* isolates

Plugs from actively growing colonies were transferred to fresh V8 agar with chloramphenicol and allowed to grow for approximately 14 days, or until sufficient mycelial growth was present for extraction, at ambient temperature with an alternating 12 h photoperiod. Next, mycelia and spores were harvested by adding 3 ml of 0.9% (w/v) sodium chloride collection solution and gently scrapping using a sterile scalpel. A volume of 1 ml of the mycelia/spore suspension were collected and placed in 1.5 ml centrifuge tubes.

DNA extraction for all isolates recovered from 2018 and 2019 was performed using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, ON, Canada). Centrifuge

tubes containing 1 ml of mycelia/spore suspension were centrifuged at 14,000 RPM for 1 min and the resulting supernatant discarded. Next, 500 µl of lysis buffer L was used to resuspend the cell pellet and this was then transferred to a provided bead tube. Bead tubes containing lysis buffer and mycelia/spore solution were vortexed for 2 min and 30 sec using a Geno-Grinder® (SPEX, Metuchen, NJ), tubes were then inverted 3 to 4 times and vortexed for an additional 2 mins and 30 sec. After vortexing, tubes were incubated at 65°C for 10 min, then removed and inverted 3 to 4 times, and then returned and incubated for an additional 10 min. The rest of the steps were conducted as outlined in the Norgen Fungi/Yeast Genomic DNA Isolation Kit product insert. Samples were stored at -8°C until used for polymerase chain reaction (PCR).

C. sojina isolates collected in 2018 and 2019 from locations in Georgia were positively identified compared to two confirmed *C. sojina* isolates (CS1036 and 18CS741) from Dr. Carl Bradley at the University of Kentucky using PCR amplification of the internal transcribed spacer (ITS) region using an ITS1/ITS4 primer set (White et al. 1990). PCR was conducted using a Bio-Rad T100™ Thermal Cyler (Bio-Rad Laboratories, Inc., Hercules, CA) with the cycling conditions set at an initial denaturation period for 3 min at 90°C; and then 34 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and elongation at 72°C for 2 min; followed by a final extension step at 72°C for 10 min. PCR products were cast on 2% agarose gels containing 3 µl of GelRed® (Biotium, Inc., Fremont, CA) and run in 1X Tris-Acetate-EDTA (TAE) buffer and then viewed under UV light.

To confirm isolate identity, ITS1/ITS4 PCR products for five randomly selected *C. sojina* isolates were submitted to Eurofins Genomics (Louisville, Kentucky) for sequencing. Geneious Prime 2020.2.2 (Biomatters Ltd., Auckland, New Zealand) was used to trim, edit, and assemble consensus sequences for each isolate using both the forward and reverse sequences. Consensus

sequences were used to BLAST selected isolates to sequences of known *C. sojina* isolates deposited in GenBank, accession number: NR_147265.

Inoculum Preparation

C. sojina isolates were transferred to fresh V8 agar containing chloramphenicol and allowed to grow for approximately 14 days at ambient temperature. After 14 days, large agar discs of mycelia, approximately 10 mm x 10 mm in size, containing spores, were removed from actively growing margins of each plate. These were then inverted onto a 300 µl droplet of sterile tween solution (1 drop/L Tween 20 in 1 L sterile distilled water) (Sigma-Aldrich, St. Louis, MO) and spread across the surface of fresh V8 agar plates amended containing chloramphenicol to transfer conidia. To ensure enough conidia were produced for plant inoculations, this process was repeated for 10 to 15 plates for each isolate. After allowing plates to dry in the laminar flow hood overnight, plates were sealed in plastic bags and allowed to grow for approximately 14 days at ambient temperature. After 14 days, conidial suspensions of each isolate were created by flooding petri plates containing the colonies of *C. sojina* with approximately 3 ml of tween solution and lightly scraping the agar surface with a sterile scalpel to dislodge conidia. The mycelial/conidial suspension was then passed through two layers of cheesecloth to remove any large mycelia fragments. Lastly, conidial suspensions for each isolate were adjusted to an approximate final concentration of 6×10^4 spores ml^{-1} using a hemocytometer and placed in 15 ml Falcon tube (Thermo Fisher Scientific Inc., Waltham, MA).

Greenhouse Inoculations

Soybean differential cultivars 'Davis', 'Hood', 'Lee', 'Lincoln', 'Tracy', and 'Blackhawk' were grown in 10 x 10 cm square pots containing a soilless peat-based potting mix (Fafard® 4P Mix, Sun Gro® Horticulture, Agawam, MA) in greenhouses (average daytime and nighttime temperature of 26°C and 22°C, respectively) at the University of Georgia – Griffin campus, Griffin, GA. Plants were fertilized weekly with Peters Professional 20-20-20 water-soluble fertilizer (Everris NA, Inc., The Neatherlands) and watered as needed. Each pot contained three seeds of a given cultivar and were arranged in flats containing 12 pots and allowed to grow for approximately 7 days. After 7 days, seedlings were thinned in each pot from three to two and seedlings were allowed to grow for an additional 7 days or until their first trifoliate had expanded (V1 to V2).

After soybean seedlings had reached growth stages V1 to V2, fully expanded trifoliates were spray-inoculated with a *C. sojina* conidial suspension adjusted to a concentration of 6×10^4 conidia ml^{-1}, using a Paasche H Series airbrush (Paasche Airbrush, Kenosha, WI), modified to attach to a 15 ml Falcon tube, at approximately 30 PSI, with each trifoliate receiving approximately 0.3 ml to insure complete coverage. Inoculated plants were placed in 100% relative humidity chambers for 24 h. in the dark. Plants were then removed from the chambers, re-inoculated following the aforementioned methods, and then returned to humidity chambers for an additional 24 h. After the additional 24 h, plants were then returned to greenhouse benches and placed in a randomized complete block design (RCBD) with 3 replicates with each replicate containing two plants of each isolate by cultivar interaction, total 6 plants per experiment. Soybean differential cultivars were rated 14-days post inoculation for disease reaction, susceptible (producing lesions characteristic to *C. sojina* infection) or resistant (no lesions

present). Data were inputted into the 'hagis' package (McCoy et al. 2019) in R (R Core Team 2020, Vienna, Austria) to determine a pathotype code for each differential based on reaction with each isolate. Due to the large number of isolates screened in this study and greenhouse space limitations, 8 isolates were screened at a time over 10 independent experiments between 2018 and 2019.

RESULTS

In total, 12 different races of *C. sojina* were identified (Table 3.1) among the 80 isolates recovered from Georgia soybean in 2018 and 2019. Of these 12 races, 8 races had previously been described (Mian et al. 2008) including: race 5, 6, 8, 9, 10, 11, 12, and 14. In addition to the previously described races, 4 previously undescribed pathotypes were also identified and termed 'NA' or not applicable followed by the numerical order in which they were found: NA-1, NA-2, NA-3, and NA-4 The 4 unidentified pathotypes, produced susceptible reactions on differentials 'Lincoln', 'Tracy', and 'Blackhawk', denoted NA-1; on 'Hood', 'Lincoln', 'Tracy', and 'Blackhawk', NA-2; on 'Lee' and 'Blackhawk', NA-3; and on 'Lee', 'Tracy', and 'Blackhawk', NA-4. To our knowledge, this is the first report of pathotypes of *C. sojina* producing reactions on these given soybean differential cultivars. Race 12 was the most common race observed (Figure 3.1), present in 21.3% of the total isolates tested; then race 14, 15%; followed by race 11, 13.8%. Of the previously undescribed races, NA-1 was the most common, 11.3% of tested isolates.

Of the 6 differential cultivars used to screen *C. sojina* isolates, 5 of the 6 (Figure 3.2) produced compatible, or susceptible reactions, resulting in lesions consistent with *C. sojina* infection (Figure 3.3). Universal susceptible 'Blackhawk' produced susceptible reactions on 100% of the isolates tested. Differential cultivar 'Lincoln', containing the *Rcs1* gene, produced

susceptible reactions on 68.8%; followed by ‘Lee’, 67.5%; then ‘Tracy’, 57.5%; and then ‘Hood’, 22.5%. ‘Davis’, containing the *Rcs3* gene, conferred resistance to all of the Georgia isolates of *C. sojina*.

DISCUSSION

The identification of *C. sojina* isolates resistant to quinone outside inhibitor fungicides in Georgia and many other states, along with the previous emergence of new races of *C. sojina* in the U.S., (Athow et al. 1962; Cruz and Dorrance 2009; Mian et al. 2008; Phillips and Boerma 1981; Ross 1968) which have overcome host resistance, has been placed an increased importance on the development of soybean cultivars with more durable sources of resistance. Historically, the *Rcs3* gene has provided resistance to all known races of *C. sojina* in the U.S. (Mian et al. 2008; Missaoui et al. 2007), but with increasing selection pressure being placed on a single gene for resistance, the emergence of a new race could be detrimental.

Due to the low acreage of soybean planted in Georgia in 2019 and dry climatic conditions during the months of July through September, very little FLS was observed and recovered from commercial and state variety testing locations. Thus, the majority of the isolates used in this study originate from 2018 where FLS was more widely observed. Results from this study identified 12 races of *C. sojina* present among the 80 isolates recovered from Georgia soybean fields in 2018 and 2019. Of the 12 races, 4 novel pathotypes were discovered and were denoted NA-1, NA-2, NA-3, and NA-4. Race 12 was the most common of the pathotypes, which was consistent with what was reported from Ohio (Cruz and Dorrance 2009), accounting for 21.3% of the isolates collected and was found in 4 of the 6 counties sampled. As a whole, the 4 novel pathotypes identified accounted 26.3% of the isolates recovered. NA-1 was the abundant of the

previously undescribed pathotypes, making up 11.3% of the total isolates and was recovered in 3 of the 6 counties sampled. Despite race 12 being the most abundant race overall, within a given soybean field, multiple races were found (Table 3.2), with the average number of races identified within a location being 5.2. In fact, fields sampled in Pike, Gordon, and Oconee counties had 9, 8, and 7 of the 12 identified races, respectively.

The large number of races identified within each location indicates a high volume of genetic diversity within the Georgia population of *C. sojina* not just at a regional level, but a field level. Although a sexual stage of *C. sojina* has not been observed in nature, a study conducted in Arkansas showed that populations of the pathogen within Arkansas had both mating types, were genetically diverse, and are most likely undergoing sexual reproduction (Kim et al. 2013). The results from this study conducted in Georgia, along with the findings from Ohio also reporting previously undescribed pathotypes of the pathogen (Cruz and Dorrance 2009), further validate the findings found in Arkansas and suggest that likely more races of the *C. sojina* are present within Georgia. Furthermore, since such a large number of races were identified within locations, this suggests that the pathogen population does not appear to be shifting towards a single or more virulent race of *C. sojina*. Therefore, any recent outbreaks of the pathogen are more likely due to conducive environmental conditions and the increased planting of susceptible soybean cultivars rather than the emergence of a new pathogen race which has overcome host resistance.

With the increase in QoI resistant *C. sojina* being reported in the major soybean producing regions of the U.S., and now in Georgia, the question of resistance being linked to the emergence of a new race or a previously identified race was asked. However, *C. sojina* isolates recovered from soybean fields in Georgia that displayed QoI resistance when screened with the

RFLP assay and confirmed by the presence of the G143A mutation when sequenced, did not fall within one race or pathotype, but were found within 4 different pathotype groups, races 11, 12, and 14, along with novel pathotype NA-2. The presence of QoI resistance in different pathotype groups seems to indicate that resistance developed independently within each pathotype. Although one single race or pathotype does not appear to be responsible for QoI resistance, high genetic variability and the possibility of sexual recombination occurring in nature could result in the transfer of resistance between races of *C. sojina* even without the exposure of a QoI fungicide application.

Greenhouse inoculation studies indicated varying levels of resistance within the 6 soybean differential cultivars. Differential cultivar 'Lincoln' had a resistance index of 31.3% when tested by the 80 isolates of *C. sojina* from Georgia, second lowest to universally susceptible 'Blackhawk'. While these results indicate that the *Rcs1* gene cannot be an effective source of resistance alone, it may still be effective if added in combination with other resistance genes with higher resistance indexes, such as those found in differential 'Pecking' with a resistance index 95.7% (Mian et al. 2008), with cultivars containing combinations of the *Rcs1* gene with other resistance genes still commercially available (Hartman et al. 2015). Differential cultivar 'Hood', when screened with the *C. sojina* isolates produced a resistance index of 77.5% second highest to 'Davis'. These findings indicate some level of genetic resistance, either a single gene or multiple genes displaying partial resistance, are present within 'Hood'. Future studies will be needed to identify the gene or genes involved. Differential 'Davis', containing the *Rcs3* gene, produced a 100% resistance index on the isolates recovered from soybean in Georgia. Even with the identification of 4 new pathotypes, the *Rcs3* gene still seems to be an effective source of resistance to *C. sojina*.

Universally susceptible cultivar ‘Blackhawk’ produced susceptible reactions on all 80 *C. sojina* isolates tested. However, in two independent trials, trials 3 and 4, 7 isolates (CAL 18-4, CAL 18-6, CAL 18-7, CAL 18-10, IH 18-5, IH 18-10, and IH 18-12) did not produce susceptible reactions on differential cultivar ‘Blackhawk’. Many factors could have contributed the lack of susceptible reactions of each isolate on the given cultivar, including spore viability, greenhouse temperatures, etc. Over the course of the 10 experiments conducted with the 80 isolates, numerous ‘Blackhawk’ plants were found to have incompatible or resistant reactions with certain isolates, but over the course of 3 replications compatible or susceptible reactions could be found. Nevertheless, an additional trial was conducted for the 7 isolates organized in a randomized complete block design with 3 replicates in a growth chamber with controlled conditions of: 18 h photoperiod and a day/night temperature of 28 and 21°C, respectively. All 7 isolates were found to have compatible reactions on ‘Blackhawk’.

In order to improve soybean breeding efforts going forward, molecular studies looking into the pathogen structure of *C. sojina* are needed. With the identification of new pathotypes in Georgia and in other parts of the U.S., molecular differentiation of these pathotypes are needed to discern the relationship between pathotypes and how they arose. Additionally, pathotype virulence needs to be addressed moving forward. Studies conducted in Tennessee with *C. sojina* isolates from Brazil, China, and the U.S. found distinct differences in virulence among isolates and pathotypes and proposed grouping isolates based on virulence in pathogenicity groups (Mengistu et al. 2020). Although virulence was not measured in this study, visual observations of isolates on given differential cultivars showed clear differences in virulence among isolates with some isolates producing many lesions (>3 lesions), while others produced few lesions (1-3 lesions). Differences in virulence within individual isolates was also observed between

differential cultivars, where a given isolate would produce numerous lesions on one differential, but only few on another. These observations indicate there could be variability in virulence not only among pathotypes, but also among isolates within each pathotype. Being able to decipher these differences could be important for the screening of resistant cultivars in the future as well as to improve management recommendations for FLS moving forward.

LITERATURE CITED

Akem, C. N. 1995. The effect of timing of fungicide applications on control of frogeye leaf spot and grain yield of soybeans. Eur. J. Plant Pathol. 101:183-187.

Akem, C. N., and Dashiell, K. E. 1994. Effect of planting date on severity of frogeye leaf spot and grain yield of soybeans. Crop Prot. 13:607–610.

Athow, K. L., and Probst, A. H. 1952. The inheritance of resistance to frogeye leaf spot of soybeans. Phytopathology 42:660-662.

Athow, K. L., Probst, A. H., Kartzman, C. P., and Laviolette, F. A. 1962. A newly identified physiological race of *Cercospora sojina* on soybean. Phytopathology 52:712-714.

Baker, W. A., Weaver, D. B., Qiu, J., and Pace, P. F. 1999. Genetic analysis of frogeye leaf spot resistance in PI54610 and Pecking soybean. Crop Sci. 39:1021-1025.

Boerma, H. R., and Phillips, D. V. 1983. Genetic implications of the susceptibility of Kent soybeans to *Cercospora sojina*. Phytopathology 74:1666-1668.

Buzzell, R. I. 1988. Soybean genetics committee report. Soybean Genet. Newsl. 15:2-8.

Cruz, C. D., and Dorrance, A. E. 2009. Characterization and survival of *Cercospora sojina* in Ohio. Plant Health Prog. doi: 10.1094/php-2009-0512-03-rs

Dashiell, K. E., and Akem, C. N. 1991. Yield losses in soybeans from frogeye leaf spot caused by *Cercospora sojina*. Crop Prot. 10:465-468.

Fagundes, W., Zaccaron, M., Zaccaron, A., and Bluhm, B. H. 2018. Elucidating the race population structure of *Cercospora sojina* through genotypic patters. Southern Soybean Disease Workers Conference. March 7-8, 2018. Pensacola Beach, Florida.

Grau, C. G., Dorrance, A. E., Bond, J., and Russin, J. S. 2004. Fungal diseases. p. 679-763, *In*: H. R. Boerma, J. E. Specht (Eds.), Soybean: Improvement, production, and uses, third edition. Am. Soc. Agron., Crop Sci. Soc. Amer., and Soil Sci. Soc. Amer. Madison, WI.

Hartman, G. L., Rupe, J. C., Sikora, E. J., Domier, L. L., Davis, J. A., and Steffey, K. L. 2015. Compendium of soybean diseases. Fifth Edition. American Phytopathological Society, St. Paul, MN.

Heatherly, L. G., and Hodges, H. F. 1998. Soybean Production in the Midsouth. CRC Press. Boca Raton, FL.

Hoskin, A. 2011. Genetic mapping of soybean resistance genes to frogeye leaf spot in five Chinese Plant Introductions and efficiency of early generation selection for low phytate soybean lines. Institute of Plant Breeding, Genetics, and Genomics. PhD Thesis. University of Georgia.

Kim, H., Newell, A. D., Cota-Sieckmeyer, R. G., Rupe, J. C., Fakhoury, A. M., and Bluhm, B. H. 2013. Mating-type distribution and genetic diversity of *Cercospora sojina* populations on soybean from Arkansas: evidence for potential sexual reproduction. Phytopathology 103:1045-1051.

McCoy, A. G., Noel, Z., Sparks, A. H., and Chilvers, M. 2019. 'hagis', an R package resource for pathotype analysis of *Phytophthora sojae* causing stem and root rot of soybean. MPMI 32:1574-1576.

McDonald, S., Buck, J., Song, Q., and Li, Z. 2020. Dissecting the genetics of soybean resistance to frogeye leaf spot using a genomewide scan of diverse accessions and a biparental population. Soybean Breeder's Workshop. St. Louis, MO.

Mengistu, A., Kelly, H. M., Bellaloui, N., Arelli, P. R., Reddy, K. N., and Wrather, A. J. 2014. Tillage, fungicide, and cultivar effects on frogeye leaf spot severity and yield in soybean. Plant Dis. 98:1476-1484.

Mengistu, A., Ray, J. D., Kelly, H. M., Lin, B., Yu, H., Smith, J. R., Arelli, P. R., and Bellaloui, N. 2020. Pathotype grouping of *Cercospora sojina* isolates on soybean and sensitivity to QoI fungicides. Plant Dis. 104:373-380.

Mian, M. A. R., Boerma, H. R., Phillips, D. V., Kenty, M. M., Shannon, G., Shipe, E. R., Soffes Blount, A. R., and Weaver, D. B. 1998. Performance of frogeye leaf spot resistant and susceptible near isolines of soybean. Plant Dis. 82:1017-1021.

Mian, M. A. R., Missaoui, A. M., Walker, D. R., Phillips, D. V., and Boerma, H. R. 2008. Frogeye leaf spot of soybean: a review and proposed race designations for isolates of *Cercospora sojina* Hara. Crop Sci. 48:14-24.

Mills, D. R., and Dorrance, A. E. 2008. Evaluation of soybean fungicides for yield response and frogeye leaf spot control in Ohio. Plant Dis. Manag. Rep. 2:FC043.

Missaoui, A. M., Phillips, D. V., and Boerma, H. R. 2007. DNA marker analysis of 'Davis' soybean and its descendants for the *Rcs3* gene conferring resistance to *Cercospora sojina*. Crop Sci. 47:1263-1270.

Pham, A-T., Harris, D. K., Buck, J., Hoskins, A., Serrano, J., Abdel-Haleem, H., Cregan, P., Song, Q., Boerma, H. R., and Li, Z. 2015. Fine mapping and characterization of candidate genes that control resistance to *Cercospora sojina* K. Hara in two soybean germplasm accessions. PLoS ONE 10(5): e0126753. doi:10.137/journal.pone.0126753

Phillips, D. V. 1999. Frogeye leaf spot. p. 20-21. *In* G. L. Hartman, J. B. Sinclair, and J.C. Rupe

(ed). Compendium of soybean diseases. American Phytopathological Society

Phillips, D. V., and Boerma, H. R. 1981. *Cercospora sojina* race 5: A threat to soybean in the southeastern United States. Phytopathology 71:334-336.

Phillips, D. V., and Boerma, H. R. 1982. Two genes for resistance to race 5 of *Cercospora sojina* in soybeans. Phytopathology 72:764-766.

Price, P., Padgett, G. B., and Purvis, M. A. 2014. The effect of selected fungicides and application timings on frogeye leaf spot. Plant Dis. Manag. Rep. 8:FC223.

Ross, J. T. 1968. Additional physiologic races of *Cercospora sojina* on soybean in North Carolina. Phytopathology 58:708-709.

Standish, J. R., Tomaso-Peterson, M., Allen, T. W., Sabanadzovic, S., and Aboughanem-Sabanadzovic, N. 2015. Occurrence of QoI fungicide resistance in *Cercospora sojina* from Mississippi soybean. Plant Dis. 99:1347-1352.

Zhang, G. 2012. *Cercospora sojina*: Overwinter survival and fungicide resistance. Department of Crop Science. PhD Thesis. University of Illinois at Urbana-Champaign. Urbana, Illinois.

Zhang, G., Allen, T. W., Bond, J. P., Fakhoury, A. M., Dorrance, A. E., Weber, L., Faske, T. R., Giesler, L. J., Hershman, D. E., Kennedy, B. S., Neves, D. L., Hollier, C. A., Kelly, H. M., Newman, M. A., Kleczewski, N. M., Koenning, S. R., Thiessen, L. D., Mehl, H. L., Zhou, T., Meyer, M. D., Mueller, D. S., Kandel, Y. R., Price III, P. P., Rupe, J. C., Sikora, E. J., Standish, J. R., Tomaso-Peterson, M., Wise, K. A., and Bradley, C. A. 2018. Widespread occurrence of Quinone outside inhibitor fungicide-resistant isolates of *Cercospora sojina*, causal agent of frogeye leaf spot of soybean, in the United States. Plant Health Prog. 19:295-302.

Zou, J., Dong, W., Yang, Q., Cao, Y., and Chen, S. 1999. Inheritance of resistance to race 7 of *Cercospora sojina* in soybeans and RAPD tagging of the resistance gene. Chin Sci. Bull. 44:452-455.

Table 3.1. Proposed races of *Cercospora sojina* recovered from Georgia soybean based on their reaction on 6 soybean differential cultivars.

Differential cultivar	***C. sojina* designated races**[a]											
	Race 5	**Race 6**	**Race 8**	**Race 9**	**Race 10**	**Race 11**	**Race 12**	**Race 14**	**NA-1**[b]	**NA-2**	**NA-3**	**NA-4**
Davis	-	-	-	-	-	-	-	-	-	-	-	-
Hood	+	-	-	-	+	-	-	+	-	+	-	-
Tracy	-	-	+	-	-	-	+	+	+	+	-	+
Lincoln	-	-	-	+	-	+	+	+	+	+	-	-
Lee	-	-	-	-	+	+	+	+	-	-	+	+
Blackhawk	+	+	+	+	+	+	+	+	+	+	+	+

[a]*C. sojina* designated races were determined 14-days post-inoculation based on isolate reactions on each differential cultivar (N=6). Compatible reactions (susceptible), denoted as a plus sign, produced lesions consistent with those of *C. sojina* infection. Incompatible reactions (resistant), denoted as a negative sign, produced no lesions.

[b]Novel pathotypes were designated NA, not applicable, followed by the numerical order in which they were discovered.

Table 3.2. Location, year, and number of *Cercospora sojina* races identified within each location.

County	Number of isolates / Year		Number of races identified[a]
	2018	2019	
Gordon	16	-	8
Jefferson	-	1	1
Oconee	27	-	7
Pike	26	-	9
Sumter	1	-	1
Walker	-	8	5

[a]Races of *C. sojina* were identified by screening recovered isolates from 2018 and 2019 on 6 soybean differential cultivars and determining disease reaction as susceptible or resistant.

Table 3.3. Resistance index of 6 soybean differential cultivars to 80 isolates of *Cercospora sojina* from Georgia.

Differential cultivar	Selection criteria[a]	Compatible reaction[b]		Resistance index (%)[c]
		Positive	Negative	
Davis	*Rcs3* gene	0	80	100.0
Hood	Additional cultivar	18	62	77.5
Tracy	Ancestor	46	34	42.5
Lee	Additional cultivar	54	26	32.5
Lincoln	*Rcs1* gene	55	25	31.3
Blackhawk	Susceptible	80	0	0.0

[a]Selection criteria as determined by Mian et al. 2008.

[b]Compatible reaction either being positive / susceptible (lesions present), or negative / resistant (no lesions) on a given differential cultivar.

[c]Percentage of resistance = number of isolates to which the differential is resistant / 80 (number of isolates tested) x 100.

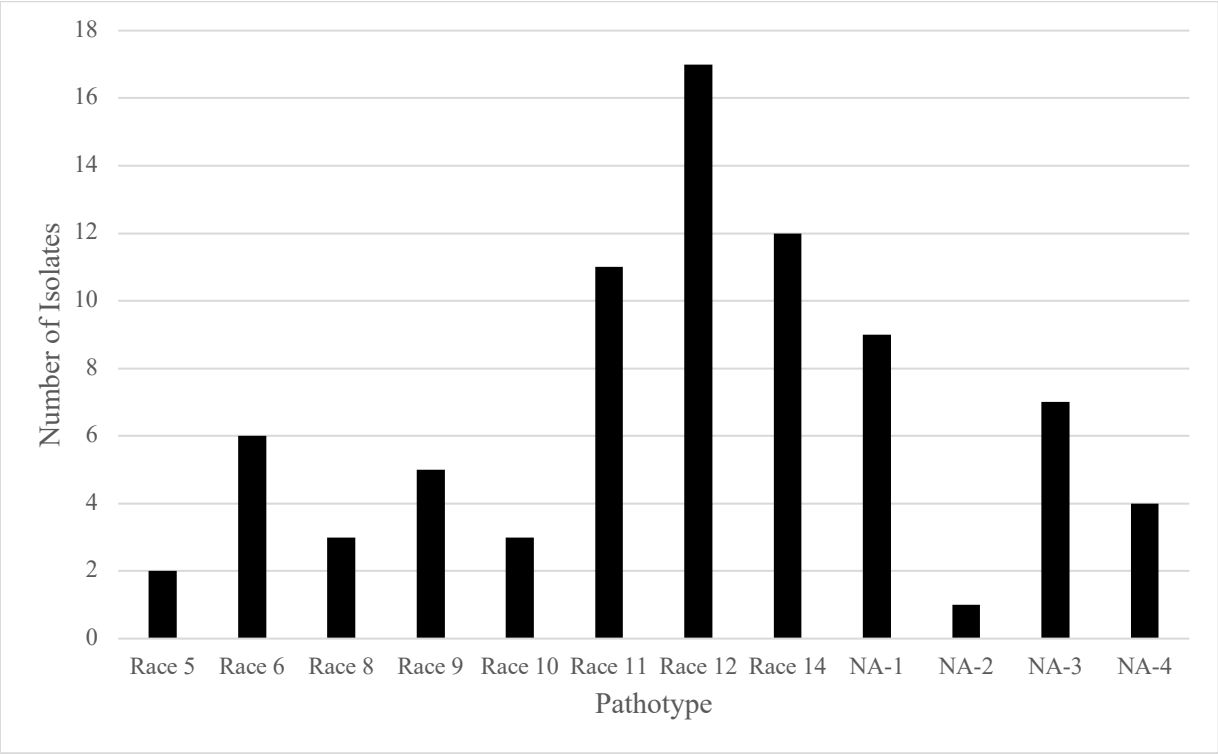

Figure 3.1. Number of Georgia isolates of *Cercospora sojina* within each pathotype designation.

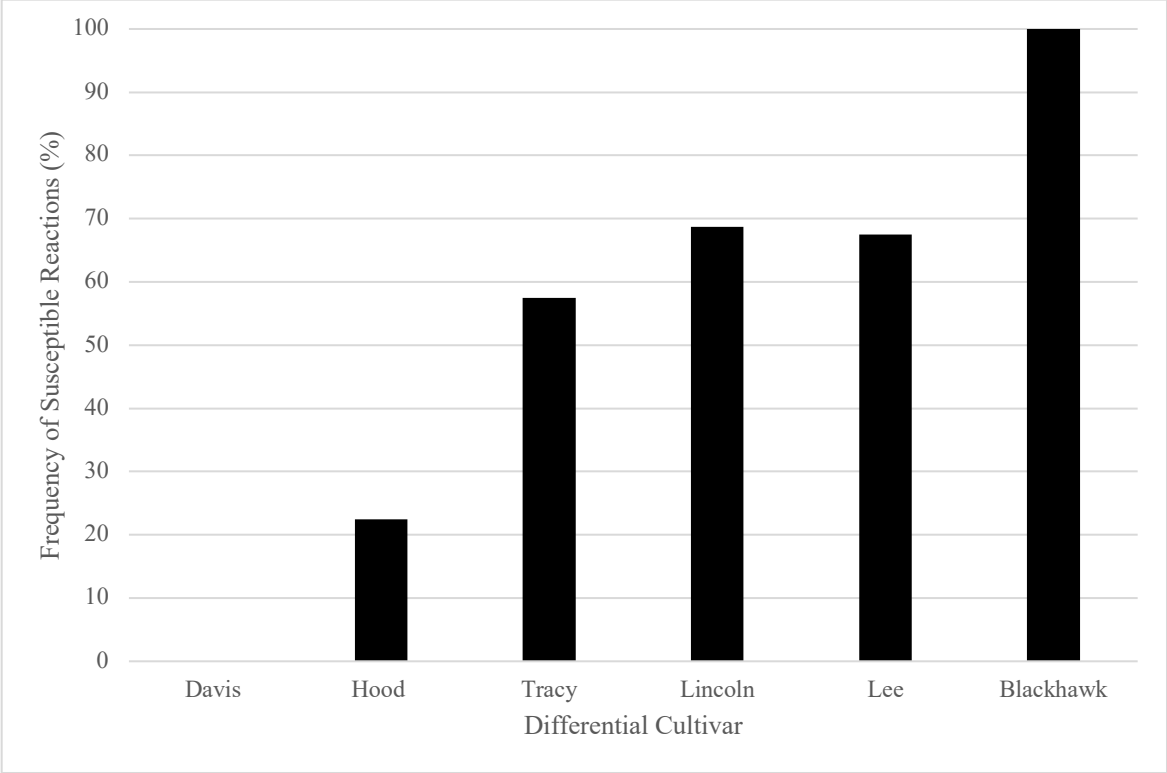

Figure 3.2. Frequency of susceptible reactions of each differential cultivar to 80 isolates of *Cercospora sojina* recovered from Georgia.

Figure 3.3. Soybean differential cultivars inoculated with *Cercospora sojina* displaying different reaction phenotypes: **A.** incompatible or resistant reaction on 'Davis' resulting in no lesions, or **B.** compatible or susceptible reactions on 'Blackhawk' resulting in symptoms associated with *C. sojina* infection.