MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 61-2015 (FINAL YEAR—NCE)
2016 FINAL REPORT

Title: Investigations into Strobilurin Fungicide Resistance of Soybean Pathogens in Mississippi

Investigator: Maria Tomaso-Peterson – **mariat@pss.msstate.edu**

EXECUTIVE SUMMARY

The objectives of this multi-year project were to:

1. Monitor MS soybean production fields and sentinel plots for QoI resistance within the frogeye leaf spot pathogen (FLS) population (2013–2014).

2. Identify the mechanism(s) of resistance and potential proportion within the prevailing fungal community to better understand the dynamics leading to QoI resistance (2013–2015).

3. Determine potential fitness costs associated with QoI resistance within the soybean pathogen populations present (2015–2017).

Results presented herein demonstrate that fungicide resistance in the FLS pathogen is dominant throughout Mississippi areas of soybean production.

Preliminary results from 2016 suggest the early onset of QoI resistance selection within the target spot pathogen (*Corynospora cassiicola)* population is occurring. The onset of QoI resistance in *C. cassiicola* populations is following the same trend as in *C. sojina* (2010–2017) in Mississippi soybean production.

FLS and target spot were once considered minor foliar diseases, but through repeat applications of QoI fungicides over the last nearly two decades that has resulted in selection pressure for QoI-resistant types, these are now important soybean diseases that growers must manage to avoid yield loss.

The repeated use of/reliance on QoIs may eventually lead to fungicide resistance among many important foliar soybean pathogens.

Resistance management, i.e., preventing complete loss of a highly efficacious class of fungicides, is a recommended practice for Mississippi soybean producers to embrace. This is especially important since results from previous studies show that the QoI-resistant fungal population does not readily revert to a QoI-sensitive population.

The fitness studies conducted in this research project showed the QoI-resistant *C. sojina* isolates causing FLS in Mississippi soybean fields may out-compete the QoI-sensitive, wild-type isolates in terms of virulence.

The QoI-resistant isolates are just as competitive or as fit as the wild-type in terms of vegetative growth and conidia production. Since conidia serve as the primary and secondary inoculum that initiates FLS,

QoI fungicides will not reduce FLS when caused by a QoI-resistant *C. sojina* isolate. Therefore, other fungicide classes, **host resistance**, crop rotation, and tillage management should be considered as a holistic approach to FLS management.

BACKGROUND AND OBJECTIVES

Foliar soybean diseases can result in significant yield loss; therefore cultural and chemical control practices are necessary. The strobilurin or quinone outside inhibitor (QoI) class of fungicides are primarily applied for late-season disease management. The two most commonly used fungicides are azoxystrobin (Quadris) and pyraclostrobin (Headline). The QoI site-specific mode of action puts this particular fungicide class at a high risk for fungicide resistance. Reports in 2010–12 indicated practical resistance to QoI's was identified from a limited number of soybean fields in Mississippi.

This research project was initiated in April of 2013 to focus on screening the frogeye leaf spot (FLS) pathogen, *Cercospora sojina*, collected from Mississippi soybean fields for QoI sensitivity. A gene mutation at the target site (respiration) of the fungicide is responsible for the inability of the QoI fungicide molecule to bind; thus respiration is not inhibited. This results in the fungal pathogen's ability to continue to respire, producing conidia and vegetative mycelium, and initiating polycyclic infections.

A fitness cost may also be associated with this gene mutation. In general, fungal populations that shift from fungicide sensitive to resistant may have a shift in competitiveness, viability (short- and long-term), reproductive capabilities, and most important from the grower's standpoint, virulence, which is the capacity of a pathogen to cause severe disease. This may manifest itself in the reduction of host-plant resistance to FLS.

The objectives of this research project are as follows:

4. **Monitor MS soybean production fields and sentinel plots for QoI resistance within the frogeye leaf spot pathogen population (2013–2014).**

5. **Identify the mechanism(s) of resistance and potential proportion within the prevailing fungal community to better understand the dynamics leading to QoI resistance (2013–2015).**

6. **Determine potential fitness costs associated with QoI resistance within the soybean pathogen populations present (2015–2017).**

RESEARCH ACTIVITY

Objectives 1 and 2 Materials and Methods.

Sampling pattern and *C. sojina* isolate collection. To conduct a thorough survey, Mississippi was divided into five geographical regions which differed in acres of soybean production. These regions were called the Hills (16 counties; 310,600 acres), Delta (15 counties; 1,452,102 acres), Pines (29 counties; 165,674 acres), Capital (16 counties; 79,824 acres), and Coast (6 counties; 1,798 acres). Sampling was carried out during the soybean production months of 2013 and 2014. Sampled fields were initially chosen in 2013 when growers or consultants reported QoI control failures; however, as

sampling intensified in 2014, many fields were sampled with no prior knowledge of production practices or control failures.

A sample consisted of 10 to 20 fresh leaves with FLS symptoms colonized by sporulating *C. sojina*. Mono-conidial isolates were established from individual leaf lesions and placed in a growth chamber and allowed to incubate under 12 h day/night with fluorescent and black light sources at 77ºF until the colony covered approximately 60% of the plate. All *C. sojina* isolates collected in this study were prepared for long-term storage. Additionally, infected leaf samples were pressed and added to the Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology herbarium at Mississippi State University.

***C. sojina* sensitivity to azoxystrobin.** The fungicide azoxystrobin (AZ) was used for the initial screening of *C. sojina* isolates for QoI resistance in 2013 using dilutions of 0.0001, 0.001, 0.01, 0.1, 1, 2.25, 3.5, 4.75, 6, and 10 ppm within a potato dextrose agar medium. A *C. sojina* baseline (sensitive) isolate, not previously exposed to AZ, was included for comparison. Using a micropipette, 75 µl of previously prepared *C. sojina* conidial suspensions were added to AZ-amended PDA plates. Conidia were distributed over the surface of the medium using a sterile glass rod. The infested plates were incubated in the dark at 77ºF for 18 h.

A compound microscope (×100) was used to determine germination of 50 conidia per petri plate. A conidium was considered germinated if the germ tube was at least as long as the length of the conidium itself. AZ-sensitivity assays were arranged in a completely randomized design and performed by running two separate experiments including two replicates for each isolate per fungicide concentration and non-amended control. The concentration of AZ that effectively inhibited conidial germination by 50% (EC_{50}) was calculated for each isolate (n=15) using PROC PROBIT in SAS.

An analysis of variance was conducted for each experiment using PROC GLM, and a subsequent analysis was performed to identify any treatment by experiment interaction before data were pooled. EC_{50} values were $\log_{10}$-transformed prior to testing for normality using the Kolmogorov-Smirnov test in PROC UNIVARIATE. Combined data were then analyzed, and Fisher's protected least significant difference test was used to compare the EC_{50} values of all 15 isolates.

Molecular characterization of *C. sojina* isolates. DNA was extracted from all *C. sojina* isolates collected during 2013 and 2014 (n=634). To identify nucleotide point mutations at positions 129 (F129L), 137 (G137R), and 143 (G143A) responsible for QoI resistance, a set of cyt *b* gene specific primers was designed in our lab. The cyt *b* gene fragments were amplified using PCR. A PCR-restriction fragment length polymorphism (RFLP) method was used to investigate the potential nucleotide mutation in the codon at position 143 which confers complete QoI resistance in a fungus. Unpurified PCR product was digested with the FastDigest restriction enzyme *AluI*. PCR products and enzyme-digested fragments were visualized by electrophoresis in 1.5% and 2% agarose gels, respectively, using UV light. To confirm the accuracy of the PCR-RFLP method, PCR products from all sensitive and randomly selected QoI-resistant isolates were purified using ExoSAP-IT. Amplicons were submitted for custom sequencing. Nucleotide sequences were trimmed and contigs were assembled using DNASTAR Lasergene Software. Alignment and analysis were performed using ClustalW within the MEGA6 software package.

Objectives 1 and 2 Results.

During 2013, 103 *C. sojina* mono-conidial isolates were recovered from 128 samples collected from soybean fields throughout Mississippi. Sampling was intensified in 2014, with 558 samples resulting in 531 mono-conidial isolates. These isolates were collected from 89% of the state's counties or approximately 95% of the soybean production area (Table 1). Over the two-year period, the Delta accounted for the greatest number of isolates, 40%, resulting in more isolates collected than in the Capital (16%), Coast (1%) and Pines (20%) regions combined. The Hills region accounted for the remaining 23%.

In 2013 and 2014, soybean was produced in 78 out of 82 Mississippi counties. Approximately 87% of Mississippi's soybean acres in 2013 were planted in the Delta and the Hills (72 and 15%, respectively). The percentage of resistant isolates differed between the five geographical regions established in this study. The Hills had the greatest proportion of QoI-sensitive (16.7%) *C. sojina* isolates while no QoI-sensitive isolates were collected from the Coast. The range of QoI-sensitive *C. sojina* isolates from the Pines, Capital, and Delta was 1.6 to 7.0%. Of the 634 total isolates evaluated, 93.5% carried the G143A amino acid substitution conferring QoI resistance. QoI-resistant isolates were present in at least one field in all 73 sampled Mississippi counties. In 2013, 11 QoI-sensitive isolates were recovered, while 30 QoI-sensitive isolates were recovered in 2014, representing 6.5% of all isolates collected from Mississippi.

Table 1. The origin and number of *Cercospora sojina* isolates collected and carrying the G143A amino acid substitution that confers resistance to the quinone outside inhibitor fungicides from Mississippi soybean fields in 2013 and 2014.

Region[y]	Total counties	Counties sampled	Total isolates	# carrying G143A[z]
Capital	16	16	102	98
Coast	6	3	8	8
Delta	15	15	252	248
Hills	16	16	144	120
Pines	29	23	128	119
TOTAL	82	73	634	593

[y] Counties within Mississippi were split into region based on the average number of acres in soybean production.
[z] Isolates carrying the G143A amino acid substitution conferring quinone outside inhibitor resistance as determined using PCR-RFLP and comparing nucleotide sequences.

Objective 3 Materials and Methods.

Selection of *Cercospora sojina* isolates and fitness measurements. Twenty-four *C. sojina* isolates, 13 QoI-resistant and 11 QoI-sensitive, were selected from a repository of isolates collected in 2013 and 2014 from FLS soybean in Mississippi (Table 2). The selected isolates were derived from mono-conidial isolates originating from five geographic regions of Mississippi (Table 1).

Independent measurements of fitness included measurements or counts for AZ EC_{50} determination and stability, colony growth, conidia production, conidia germination, and virulence. Measurements of

fitness were taken from the mother plate (initial culture) and the tenth subculture (obtained by consecutively transferring a hyphal plug from a 14-day-old culture to a fresh plate of soybean stem lima bean agar 10 times beginning with the mother plate) for all isolates selected for the current studies. Each *C. sojina* isolate was replicated four times within a fitness measurement study, and all studies were repeated twice.

An in vitro bioassay was conducted on the selected *C. sojina* isolates using AZ to determine the EC_{50}. Following a 14-day incubation, relative growth of an isolate was determined as a percentage of colony growth compared to the non-AZ-amended control for each *C. sojina* isolate. Colony growth was determined by taking two colony diameter measurements at perpendicular points and then averaged following a 14-day incubation. Conidia production was determined using the 14-day-old colonies previously measured for colony growth.

The conidia collected for the previous production measurements were then incubated to 18 hours at 77ºF to determine percentage germination. *C. sojina* virulence (degree in which a pathogen causes disease) was determined by inoculating Dyna-Gro 37RY47 soybean seedlings with a conidial suspension generated from each isolate. Inoculated seedlings were placed into a humid chamber in the greenhouse at > 90% relative humidity for two days, then reduced to > 80% for an additional 19 days. Virulence was determined by taking photograph images of the first three trifoliates of each inoculated seedling using an Epson Perfection V700 photo scanner. Percentage leaf area exhibiting necrosis due to FLS lesions was analyzed using Assess 2.0 Image Analysis Software.

Measurements of fitness were conducted from the initial mother plate of each isolate and the tenth subculture of each isolate. Conidia obtained from the tenth subculture were the source for the post-subculture AZ EC_{50}, colony growth, conidia production, and conidia germination for each isolate as previously described. Six selected isolates (3 each of QoI-resistant and -sensitive isolates) were used to conduct a second virulence study using conidia derived from the tenth subculture; this study also included conidia from the mother plate of the same six isolates to serve as a positive control. The study was conducted in the same manner as previously described.

Table 2. *Cercospora sojina* isolates collected from soybean fields in Mississippi in 2013 and 2014 and used in the current study.

Isolate Name	MS Geographic Region	MS County	Year Collected	Azoxystrobin Sensitivity[z]
MS Cs 31	Hills	Desoto	2013	S
MS Cs 36	Capital/River	Hinds	2013	S
MS Cs 48	Delta	Leflore	2013	S
MS Cs 13	Delta	Holmes	2014	S
MS Cs 53	Hills	Prentiss	2014	S
MS Cs 94	Capital/River	Amite	2014	S
MS Cs 109	Pines	Kemper	2014	S
MS Cs 213	Pines	Lamar	2014	S
MS Cs 390	Pines	Lowndes	2014	S
MS Cs 418	Hills	Tippah	2014	S
MS Cs 500	Pines	Wayne	2014	S
MS Cs 11	Delta	Bolivar	2013	R
MS Cs 34	Capital/River	Hinds	2013	R
MS Cs 61	Pines	Oktibbeha	2013	R
MS Cs 73	Delta	Quitman	2013	R
MS Cs 102	Hills	Yalobusha	2013	R
MS Cs 104	Delta	Yazoo	2013	R
MS Cs 100	Capital/River	Rankin	2014	R
MS Cs 200	Hills	Benton	2014	R
MS Cs 260	Pines	Jefferson Davis	2014	R
MS Cs 310	Hills	Prentiss	2014	R
MS Cs 396	Pines	Monroe	2014	R
MS Cs 401	Pines	Leake	2014	R
MS Cs 505	Coast	Jackson	2014	R

[z] S = sensitive and R = resistant. Sensitive and resistant phenotypes were determined by using a polymerase chain reaction-restriction fragment length polymorphism and comparing nucleotide sequences of the cytochrome *b* gene.

Phylogenetic analysis. Genomic DNA was extracted from the 24 selected Mississippi *C. sojina* isolates. PCR was performed to obtain partial sequence fragments of five loci, the internal transcribed spacer (ITS) region of the ribosomal ribonucleic acid (rRNA) gene, actin (*ACT*) gene, translation elongation factor 1-alpha gene (*TEF*), calmodulin (*CAL*) gene, and histone H3 (*HIS*) gene. Sequences of Mississippi *C. sojina* isolates were compared to reference sequences of *C. sojina* and other species of *Cercospora* isolated from soybean located in Argentina, Japan, Mexico, and South Korea.

Objective 3 Results.

All QoI-resistant *C. sojina* isolates collected from Mississippi soybean production maintained EC_{50} values > 10 ppm following 10 subcultures while the QoI-sensitive isolates maintained EC_{50} values < 0.1 ppm. These results indicate genetic stability at the cyt *b* binding site of QoI fungicides. No differences were noted between QoI-resistant and -sensitive isolates when colony growth was determined at

temperatures of 63, 77, or 90ºF. Nor were there differences when fitness measurements were compared from the mother plate and tenth subculture within a *C. sojina* isolate. Only when virulence for *C. sojina* isolates was evaluated did we see differences between the QoI-resistant and -sensitive isolates at $P \leq 0.10$.

Based on greenhouse studies, we measured an 86% increase in percentage leaf necrosis when soybean seedlings were infected by QoI-resistant isolates compared to -sensitive isolates (Fig. 1). No differences in virulence, based on percentage necrotic leaf area, were noted when conidia derived from the mother plate nor tenth subculture were used to inoculate soybean seedlings. The phylogenetic tree derived from the combined five loci showed all the Mississippi isolates reside in subclade, regardless of QoI sensitivity.

As a group, the Mississippi isolates were divergent from the Argentina and South Korea *C. sojina* isolates. The fitness results of Objective 3 suggest that QoI-resistant *C. sojina* isolates from soybean in Mississippi are as equally fit as the QoI-sensitive *C. sojina* isolates in terms of colony growth, conidia production and germination, and virulence. The results also suggest, based on the phylogenetic analysis, that the QoI-resistant *C. sojina* isolates are genetically similar to the QoI-sensitive *C. sojina* isolates from Mississippi. When considering virulence, the results indicate that there may be a fitness benefit associated with QoI resistance in *C. sojina* from Mississippi soybean.

Our research has documented a population shift from QoI-sensitive *C. sojina* isolates to a dominate presence of QoI-resistant *C. sojina* isolates which cause FLS in soybean. The QoI-resistant isolates are genetically stable and more virulent than QoI-resistant isolates. These results support a serious need to manage FLS in terms of host resistance, crop rotation, tillage practices, and fungicide rotation to include different modes of action.

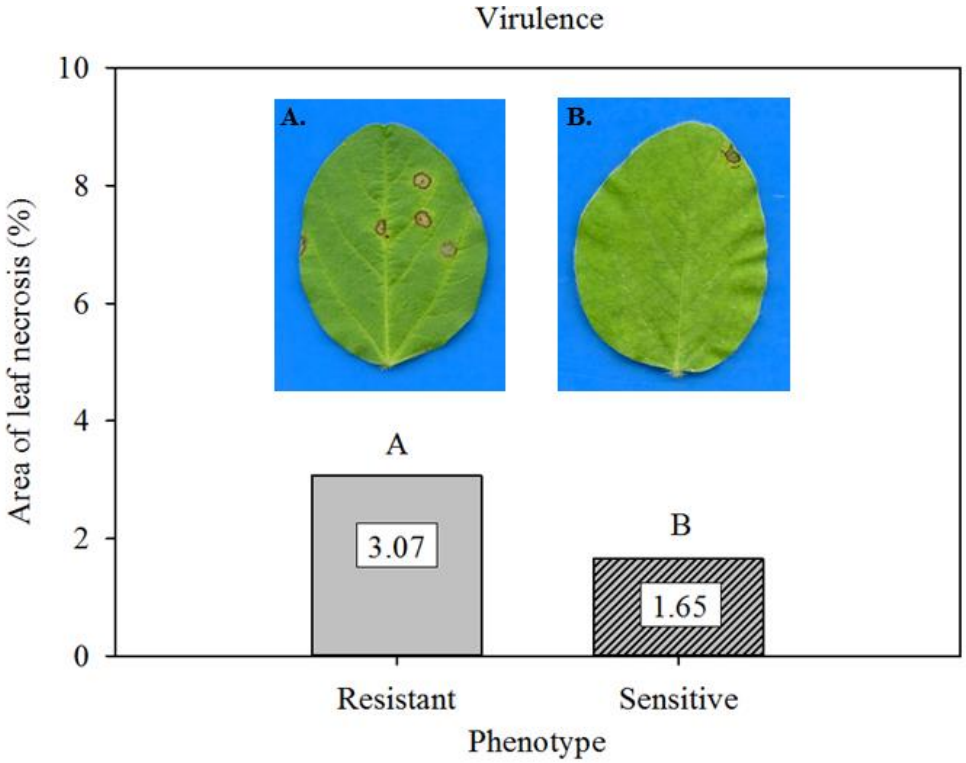

Figure 1. Percent area of leaf necrosis of the resistant and sensitive phenotypes of the *Cercospora sojina* isolates from Mississippi. Means are significantly different at $P \leq 0.1$. **A**) leaflet inoculated with a QoI-resistant isolate showing an area of leaf necrosis rating of 3.07%. **B**) leaflet inoculated with a QoI-sensitive isolate showing an area of leaf necrosis rating of 1.65%.

Target spot of soybean

While addressing soybean disease monitoring throughout Mississippi, Dr. Tom Allen has observed an increase in target spot incidence, caused by *Corynospora cassiicola*, specifically in fields where QoI fungicides have been applied. This may be a result of non-target effects of widespread QoI use.

Drs. Tom Allen and Trent Irby collected target spot samples throughout the state during the 2016 growing season. Dr. Allen recovered 87 *C. cassiicola* isolates from infected foliar samples. We received the isolates in pure culture in our laboratory at MSU to conduct QoI sensitivity evaluations using the same protocols as previously describe with *C. sojina*. A subset of 20 *C. cassiicola* isolates were evaluated for QoI sensitivity *in vitro*. No inhibition of mycelial growth was observed when *C. cassiicola* isolates were exposed to 10 ppm AZ, indicating a lack of QoI sensitivity. Genomic DNA was isolated from eight of the *C. cassiicola* isolates to amplify *cyt b* using PCR. The sequenced *cyt b* gene fragments showed three of the isolates carried the G143A substitution which confers complete resistance to QoI's. Given these results, a limited level of QoI resistance has occurred and continued use of non-target fungicides, such as QoIs, especially in the absence of disease, means target spot management will be difficult to achieve.

IMPACTS AND BENEFITS TO MISSISSIPPI SOYBEAN PRODUCERS

This four-year project demonstrated that fungicide resistance in the FLS pathogen is dominant throughout Mississippi soybean production. A preliminary study also suggests the early onset of QoI resistance selection within the target spot pathogen (*Corynospora cassiicola)* population.

FLS and target spot were once considered minor foliar diseases, but because of repeat applications of QoIs, they are now important soybean diseases that growers must manage to avoid yield loss.

The onset of QoI resistance in *C. cassiicola* populations is following the same trend as *C. sojina* (2010–2017) in Mississippi soybean production. The repeated use and reliance of QoIs may eventually lead to fungicide resistance among many important foliar soybean pathogens.

Resistance management, i.e., preventing complete loss of a highly efficacious class of fungicides, is a recommended practice for Mississippi soybean producers to embrace. Based on studies of QoI resistance in other crops, the QoI-resistant fungal population does not readily revert to a QoI-sensitive population.

The fitness studies demonstrated in this research project showed the QoI-resistant *C. sojina* isolates causing FLS in Mississippi soybean fields may out-compete the QoI-sensitive, wild-type isolates in terms of virulence. Also, the QoI-resistant isolates are just as competitive or as fit as the wild-type in terms of vegetative growth and conidia production. Conidia serve as the primary and secondary inoculum that initiates FLS. QoI fungicides will not reduce FLS when caused by a QoI-resistant *C. sojina* isolate; therefore, other fungicide classes, host resistance, crop rotation, and tillage management should be considered as a holistic approach to FLS management.

END PRODUCTS– THE NUMBER OF PROJECT ACTIVITIES

	Year				
Publications	**2013**	**2014**	**2015**	**2016**	**2017***
Refereed			1		
MSc Thesis			1		1
Abstracts	1	3	3	1	
Presentations--Oral					
National		1		1	
Regional		2	2	2	1
State	1	3	3	2	
Posters--National	1	1	1		
Total output	**3**	**10**	**11**	**6**	**2**

* A manuscript, based on fitness (obj. 3) is currently being prepared for publication in a refereed journal. Anticipated date of publication is late 2017 or early 2018.