Occurrence of QoI Fungicide Resistance in *Cercospora sojina* from Mississippi Soybean

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Abstract

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Frogeye leaf spot, caused by *Cercospora sojina* Hara, is a foliar disease affecting soybean (*Glycine max* (L.) Merr.), often managed by applications of quinone outside inhibitor (QoI) fungicides. In 2013 and 2014, 634 *C. sojina* monoconidial isolates were collected from soybean fields throughout Mississippi. Initially, in vitro bioassays were performed to evaluate the sensitivity of 14 of 634 isolates plus a baseline. Resistant and sensitive isolates were characterized by determining the effective fungicide concentrations at which 50% of conidial germination was inhibited (EC₅₀). The molecular mechanism of resistance was determined for all 634 isolates, using a PCR-RFLP method and comparing

nucleotide sequences of the cytochrome *b* gene. The state of Mississippi was divided into five distinct geographical regions (the Hills, Delta, Pines, Capital, and Coast) based on estimates of total soybean hectares. The greatest proportion (16.7%) of QoI-sensitive isolates was collected in the Hills while the Coast had no QoI-sensitive isolates. QoI-sensitive isolates from the Pines, Capital, and Delta ranged from 1.6 to 7.0%. Results of this study determined that more than 93% of *C. sojina* isolates collected in Mississippi carried the G143A amino acid substitution, indicating a shift to a QoI-resistant population throughout Mississippi soybean fields.

Frogeye leaf spot (FLS) is a disease affecting soybean (*Glycine max* (L.) Merr.) caused by the fungus *Cercospora sojina* Hara. Primarily a disease of foliage, FLS can also affect seed, pods, and stems (Grau et al. 2004). FLS was first reported from Japan in 1915 and in the United States in 1924 (Lehman 1928; Melchers 1925). In 1925, FLS was observed in Mississippi and Louisiana (Haskell 1926; Lehman 1928), and has since been reported from several soybean-producing states and countries worldwide (Akem 1995; Athow and Probst 1952; Phillips 1999; Ploper et al. 2001). In the United States, FLS has predominantly occurred in the southeast, but has more recently been reported affecting soybean in some north-central states (Mengistu et al. 2002; Yang et al. 2001). FLS causes circular to angular lesions that vary in diameter from less than 1 mm to 5 mm. Lesions first appear as dark, water-soaked spots, often with lighter centers, developing into brown spots surrounded by dark, reddish-brown margins (Phillips 1999). Mature lesions may expand and coalesce to form large, irregularly shaped spots. When lesions cover approximately 30% of the leaf surface, the leaves may blight and wither before falling prematurely, resulting in significant yield loss (Phillips 1999). Annual estimates of soybean yield reduction as a result of FLS in the United States has increased from an average of 23,000 metric tons in 1996 to approximately 257,000 metric tons in 2007 (Koenning and Wrather 2010; Wrather and Koenning 2009).

Management of FLS is achieved by planting FLS-resistant soybean cultivars, crop rotation, and the application of foliar fungicides between the full bloom (R2) and beginning seed (R5) growth stages (Fehr and Caviness 1977; Grau et al. 2004; Mian et al. 2008). Fungicides reduced FLS incidence and severity when applied prior to infection in several states, including Indiana, Kentucky, Louisiana,

Mississippi, Ohio, and Virginia (Chappell and Phipps 2006; Hershman et al. 2011; Mills and Dorrance 2008; Price et al. 2014; Sciombato et al. 2006; Shaner and Buechley 2006). As a result, foliar fungicide applications, particularly those belonging to the quinone outside inhibitor (QoI) class (Fungicide Resistance Action Committee [FRAC] Code 11), have become a major tool in FLS management when susceptible cultivars are planted. Several QoI fungicides, most notably azoxystrobin (as Quadris; Syngenta Crop Protection, Greensboro, NC) and pyraclostrobin (as Headline; BASF Corporation, Research Triangle Park, NC), are commercially available and labeled for soybean in the United States (Vincelli 2002).

QoI fungicides are an essential tool in plant disease management programs because of their broad spectrum activity against many phytopathogens (Sauter et al. 1999). However, FRAC classifies the QoIs as “high-risk” for developing resistance in fungal populations due to their single-site mode of action (FRAC 2014). Specifically, the QoI fungicides inhibit mitochondrial respiration by binding to the quinol oxidation (Qo) site of the cytochrome *b* (cyt *b*) gene, blocking electron transfer between cyt *b* and cytochrome *c*₁ (Bartlett et al. 2002). This inhibits respiration and leads to reduced fungal viability (Anke 1995; Balba 2007; Bartlett et al. 2002).

Field resistance to QoIs has been documented in over 30 species representing 20 genera (FRAC 2013) and primarily arises as a result of nucleotide point mutations in the cyt *b* gene (Fernández-Ortuño et al. 2008). These mutations result in specific amino acid substitutions that prevent the fungicide from binding to the Qo site and have been detected in a region of the cyt *b* corresponding to amino acid positions 120 to 155 of the encoded protein. Within this region, three important substitutions have been detected in resistant phytopathogenic fungi and oomycetes. The amino acid substitution from glycine to alanine at position 143 (G143A) is known to confer complete resistance to the QoI fungicides and is associated with a failure to inhibit the pathogen (Fernández-Ortuño et al. 2008). Additional amino acid substitutions from phenylalanine to leucine at position 129 (F129L) or from glycine to arginine at position 137 (G137R) are also associated with reduced sensitivity, with the pathogen typically being managed by either more frequent applications or increased rates of QoI fungicides (Fernández-Ortuño et al. 2008; Gisi

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et al. 2002; Sierotzki et al. 2007). Because they share a common mode of action, cross-resistance to QoIs is frequently observed in fungi carrying the G143A substitution, suggesting that if resistant to one QoI active ingredient, the fungus will be resistant to all QoI active ingredients (Fernández-Ortuño et al. 2008; FRAC 2014; Sierotzki et al. 2000). In *C. sojae*, Zhang et al. (2012a) demonstrated cross-resistance to azoxystrobin, pyraclostrobin, and trifloxystrobin.

Resistance to QoI fungicides in isolates of *C. sojae* was first detected in 2010 from a west Tennessee soybean field where repeated fungicide applications failed to manage FLS (Zhang et al. 2012a). In 2012, amid the documentation of efficacy failures in Tennessee and several adjacent states, *C. sojae* isolates from two Mississippi soybean fields in two separate counties were determined to be resistant to the QoI fungicides based on in vitro bioassays performed by C. Bradley, University of Illinois (Allen 2012). Identification of QoI resistance in *C. sojae* was not unexpected; however, this confirmed the presence of QoI-resistant isolates in Mississippi soybean fields. Fungicide trials conducted in Mississippi between 2003 and 2005 indicated that the QoI fungicides significantly reduced FLS severity and significantly increased yield in the presence of FLS (Spinks 2006). Conversely, field trials conducted in 2013 revealed that the QoI fungicides (e.g., Aproach [picoxystrobin; DuPont Crop Protection, Wilmington, DE], Gem [trifloxystrobin; Bayer CropScience, Research Triangle Park, NC], Headline, and Quadris) had no significant effect on reducing FLS severity nor increasing yield when compared with the nontreated control (Wilkerson et al. 2014a, 2014b, 2014c). These results, along with the identification of QoI-resistant isolates in 2012, provided evidence that the QoI-resistant phenotype was present in Mississippi soybean fields. As such, in 2013 and 2014 Mississippi was divided into five soybean-producing regions to undergo a comprehensive survey of the *C. sojae* population to determine the frequency of QoI-resistant isolates and their pattern within the FLS pathosystem in Mississippi. A study of this magnitude within a soybean-producing state provides new information in terms of defining the prevalence of QoI resistance in this pathosystem as well as determining the need for resistance management in Mississippi soybean production. The main objective of this research was to collect isolates of *C. sojae* from Mississippi soybean fields and determine the prevalence of QoI-resistance in the *C. sojae* population.

Materials and Methods

Sampling pattern, *C. sojae* isolate collection, and preservation of fungal material. To conduct a thorough survey, Mississippi was divided into five geographical regions differing in hectares of soybean production. These regions were called the Hills (16 counties; 125,695 ha), Delta (15 counties; 587,645 ha), Pines (29 counties; 67,046 ha), Capital (16 counties; 32,304 ha), and Coast (6 counties; 728 ha) (NASS 2013a). 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. sojae* (Phillips 1999). When more than one FLS disease focus was present in a field and separated by a minimum of 50 m, samples selected from foci were considered distinct. Sampled leaves were placed in plastic bags and refrigerated (4°C) until pathogen isolation, generally within 5 days. To confirm pathogen identity, leaf lesions were examined using a stereomicroscope to identify *C. sojae* conidia. Species confirmation was initially based on conidia morphology using light microscopy (×100) (Phillips 1999) and subsequently by ITS-based sequencing (White et al. 1990).

Monoconidial isolates were established from individual leaf lesions following a modified protocol outlined by Zhang et al. (2012b). Briefly, when sporulation was observed, 3 µl of a sterile distilled water solution containing 1 drop/liter Tween 20 (SDW+) (Sigma-Aldrich, St. Louis, MO) was placed onto a sporulating lesion using a micropipette (Mettler-Toledo, LLC, Columbus, OH).

The conidial suspension was pipetted from the sporulating lesion onto potato dextrose agar (PDA; 39 g PDA per liter of water) amended with rifampicin (25 mg/liter) in petri plates (15 × 100 mm diameter) (Fisher Scientific, Pittsburgh, PA) and spread across the surface with a sterile glass rod. This process was repeated five times per sample. After 24 h, five germinating conidia per sample were aseptically transferred to five separate petri plates (15 × 60 mm diameter) containing soybean stem lima-bean agar (SSLBA) (Phillips and Boerma 1981) amended with rifampicin (25 mg/liter). Monoconidial isolates were placed in a growth chamber and allowed to incubate under a 12-h day/night cycle with fluorescent and black light sources at 25°C until the colony covered approximately 60% of the plate.

Agar plugs (5 mm diameter) containing mycelia and conidia were removed and placed in sterile 1.5-ml centrifuge tubes (Fisher Scientific, Pittsburgh, PA). The agar plugs were subsequently covered with a 15% glycerol solution and placed in a freezer at -80°C (Zhang et al. 2012b). All isolates collected in this study were prepared for long-term storage using this procedure. 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. sojae* sensitivity to azoxystrobin.** The fungicide azoxystrobin (AZ) was used for the initial screening of *C. sojae* isolates for QoI resistance in 2013. Technical-grade AZ (96% a.i.; Syngenta Crop Protection, Greensboro, NC) was dissolved in 5 ml acetone, and 10-fold serial dilutions were prepared by adding 1 part stock solution to 9 parts acetone. Additional dilutions were performed to reach desired concentrations. The final concentrations in media consisted of 0.0001, 0.001, 0.01, 0.1, 1, 2.25, 3.5, 4.75, 6, and 10 µg/ml, with a non-AZ amended control (0) that contained 1 ml/liter of acetone. Salicylhydroxamic acid (SHAM) was added to prevent conidia from germinating through the alternative respiration pathway (Wood and Hollomon 2003). SHAM was dissolved in a methanol:acetone (1:1; v:v) solution and incorporated into all treatments for a final concentration of 60 µg/ml (Zhang et al. 2012b). Ten-milliliter aliquots of amended PDA were poured into 60-mm petri plates and stored in the dark for use within 24 h.

A *C. sojae* baseline (sensitive) isolate, S86, not previously exposed to AZ, was included for comparison (Zhang et al. 2012b). The isolate's pedigree stated the culture originated from Mississippi (pre-1996), but no county of origin was included. In addition to isolate S86, a subset of 14 *C. sojae* isolates collected in 2013 were included. To generate a sufficient number of conidia for bioassays, hyphal plugs of the isolates retrieved from long-term storage were transferred to SSLBA amended with rifampicin and incubated as previously described. Conidia were harvested using 5 ml of SDW+ solution per colony. The fungal slurry was transferred to multiple plates of SSLBA in aliquots of 700 µl, spread with a sterile glass rod, and returned to the growth chamber to induce conidial production. This process was repeated for each isolate to generate enough conidia to perform bioassays. Ample conidia were produced after 7 to 10 days and harvested by flooding the plates with SDW+. The conidial suspension was adjusted to a final concentration of 1×10^5 conidia/ml using a hemacytometer.

Using a micropipette, 75 µl of previously prepared *C. sojae* 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 $25^\circ\text{C} \pm 2$ for 18 h (Zhang et al. 2012b). 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 (Zhang et al. 2012b).

Statistical analysis. 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 nonamended control. The concentration of AZ that effectively inhibited conidial germination by 50% (EC₅₀) was calculated for each isolate ($n = 15$) using PROC PROBIT in SAS (v. 9.3, SAS Institute, Cary, NC). An analysis of variance was determined for each experiment using PROC GLM and a subsequent analysis was

performed to identify any treatment by experiment interaction before data were pooled. EC₅₀ values were log₁₀-transformed prior to testing for normality using the Kolmogorov-Smirnov test in PROC UNIVARIATE, combined data were analyzed, and Fisher's protected least significant difference test was used to compare the EC₅₀ values of all 15 isolates.

Molecular characterization of *C. sojae* isolates. DNA was extracted from all *C. sojae* isolates collected during 2013 and 2014 ($n = 634$). Isolates were prepared as previously described, mycelia and conidia were collected after 10 to 14 days incubation, by flooding the plate with a 0.9% (w/v) sodium chloride collection solution, and scraping the surface with a sterile cell lifter. The fungal solution was collected in sterile, 1.5-ml centrifuge tubes. Genomic DNA was both extracted and purified using a Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada). Isolates were positively identified based on nucleotide sequences of the internal transcribed spacer (ITS) region determined from two *C. sojae* isolates: 13-2 (Mississippi, 2013); and baseline isolate, S86. Polymerase chain reaction (PCR) amplification was performed using an ITS1/ITS4 primer set (White et al. 1990).

To identify nucleotide point mutations at positions 129 (F129L), 137 (G137R), and 143 (G143A), a set of *cyt b* gene-specific primers were designed. Taking into account that no *cyt b* sequences of the target organism (*C. sojae*) were publically available at the time of research (February 2014), primers were developed based on consensus sequences of this gene present in the GenBank database (NCBI, USA) (Benson et al. 2005), including *C. beticola*, *C. graminicola*, and *C. kikuchii*. Primers CercUN-F (5'-TCTTCTTAGTATACTTACACGTAGG -3') and CercUN-R (5'-AAACCTCTCATAAAACTCAAC -3') were specifically designed to amplify a 238 bp-long portion of *cyt b* containing the three important codons at positions 129, 137, and 143.

PCR was carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) with 1× GoTaq Buffer (Promega, Madison, WI), 2 mM MgCl₂, 0.2 mM dNTPs, 0.1 μM of forward and reverse primer, 1 unit GoTaq polymerase, and 50 ng/μl genomic DNA in a final reaction volume of 50 μl. The cycling conditions were an initial denaturation period at 94°C for 2 min and 20 s; 40 cycles of 94°C for 20 s, 53°C for 30 s, and 72°C for 35 s; with a final extension at 72°C for 10 s.

A PCR-restriction fragment length polymorphism (RFLP) method was used to investigate the potential nucleotide mutation in the codon at position 143 (Sierotzki et al. 2000). Unpurified PCR product was digested with the FastDigest restriction enzyme *AluI* (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's recommendations. 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 (USB Corporation, Cleveland, OH). Amplicons were submitted to Eurofins MWG Operon LLC, Louisville, KY for custom sequencing. Nucleotide sequences were trimmed and contigs were assembled using DNASTAR Lasergene Software (DNASTAR Inc., Madison, WI). Alignment and analysis were performed using ClustalW within the MEGA6 software package (Tamura et al. 2013).

Results

Sampling pattern, *C. sojae* isolate collection, and preservation of fungal material. During 2013, 103 *C. sojae* monoconidial isolates were recovered from 128 samples collected from soybean fields throughout Mississippi. Sampling was intensified in 2014, with 558 samples resulting in 531 monoconidial isolates. These isolates were collected from 89% of the state's counties (Table 1) or approximately 95% of the soybean production area. 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 (Fig. 1). Approximately 87% of

Mississippi's soybean hectares in 2013 were planted in the Delta and the Hills (72 and 15%, respectively) (NASS 2013a).

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. sojae* isolates while no QoI-sensitive isolates were collected from the Coast. The range of QoI-sensitive *C. sojae* 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. QoI-resistant isolates were present in at least one field in all 73 sampled Mississippi counties (Fig. 1). 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 (*data not presented*).

Sensitivity of *C. sojae* isolates to azoxystrobin. The EC₅₀ values determined in this study were not log₁₀-normally distributed ($P < 0.0100$). Nevertheless, the analysis of variance of mean EC₅₀ values following exposure to AZ, indicated EC₅₀ values differed significantly ($P < 0.0001$) (Table 2). Based on EC₅₀ values, isolates were either resistant or sensitive to AZ, compared with baseline isolate S86. On average, the EC₅₀ value of the AZ-resistant *C. sojae* isolates was approximately 42 times greater than that of the sensitive isolates exposed to AZ. Isolates exhibited a range of sensitivity to AZ varying from 192.0 times less sensitive (isolate 13-85) to 1.14×10^4 times less sensitive (isolate 13-11) compared with the baseline isolate. The EC₅₀ of isolates from each region were significantly greater than the baseline isolate (Table 2). Isolate sensitivity to AZ differed among regions, with the Hills isolates being 3.4 times more sensitive to azoxystrobin than isolates originating from the Pines.

Molecular characterization of *C. sojae* isolates. The nucleotide sequences of the ITS region of isolates 13-2 and S86 were mutually identical and shared 100% homology to the corresponding genomic region of *C. sojae* strains CPC 17964 and CBS 132684 from Argentina (GenBank Accession No. JX143662 and JX143660, respectively). The nucleotide sequences for 13-2 and S86 were deposited in the GenBank (Accession Nos. KJ566925 and KJ566926, respectively).

The primers CercUN-F/CercUN-R were initially tested on four selected isolates based upon the results of in vitro sensitivity to AZ (Table 2). Two isolates (S86 and 13-36) had low EC₅₀ values (sensitive), while the other two (13-2 and 13-100) exhibited resistance to the AZ (Table 2). The primers designed in this project proved highly specific as the PCR test yielded a single DNA band of the expected size, 238-bp, in all four isolates. PCR products of the four isolates were custom sequenced and deposited in GenBank (Accession Nos. KJ566927 to KJ566930).

Sequence analysis of PCR products showed the presence of the codons coding for phenylalanine (F) at position 129, and glycine (G) at position 137 of the *cyt b*. Nevertheless, a single nucleotide mutation (G→C) in the codon 143 of *cyt b* (GGT→GCT) was present only in AZ-resistant *C. sojae* isolates 13-2 and 13-100 (Fig. 2a), and not in the two AZ-sensitive isolates.

The difference in nucleotide sequences between resistant and sensitive isolates of *C. sojae* facilitated the design of a simple and fast

Table 1. The origin and number of *Cercospora sojae* 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 of Mississippi were divided into regions based on the average number of hectares in soybean production (NASS 2013a).

^z Isolates carrying the G143A amino acid substitution conferring quinone outside inhibitor resistance as determined using PCR-RFLP and comparing nucleotide sequences.

RFLP test using the restriction enzyme *AluI*. The digestion of PCR products with *AluI* resulted in two distinct profiles. In isolates containing a GCT triplet coding for alanine at position 143 (QoI-resistant), the restriction enzyme *AluI* generated two fragments of approximately 78- and 160-bp (Profile A), while the PCR products from sensitive isolates remained intact upon digestion with the same enzyme (Profile B, Fig. 2b). The analyses of PCR-RFLP profiles from all 634 Mississippi isolates revealed the presence of the GCT codon in a total of 593 *C. sojina* isolates, while the remaining 41 isolates collected from Mississippi soybean fields remained nondigested by *AluI* (Profile B). These results clearly exhibited the presence of molecular indicators for QoI resistance in the majority (93.5%) of *C. sojina* isolates collected in Mississippi over the two years.

In order to better validate the hypothesis of the G143A substitution involvement with the development of QoI resistance in *C. sojina* in Mississippi, and to eliminate the possibility that other point mutations may contribute to this phenomenon, we further sequenced PCR products from 84 isolates: the 41 exhibiting the absence of *AluI* site and a subset of 43 (out of 593) digested during the RFLP. Nucleotide sequence comparisons of these isolates provided confirmation that PCR-RFLP analyses were a robust and reliable method for discrimination between resistant and sensitive *C. sojina* isolates. No differences in nucleotide sequences leading to amino acid substitutions, specifically F129 L and G137R, other than the GCT→GGT mutation responsible for the G143A substitution, were observed between resistant and sensitive isolates of *C. sojina*.

Partial nucleotide sequence data of the *cyt b* gene for an additional 3 QoI-sensitive (13-48, 13-69, and 13-87) and 3 QoI-resistant (13-20, 13-51, and 13-72) *C. sojina* isolates studied in this work were deposited into the GenBank (Accession Nos. KP407548 to KP407553).

Discussion

The results of this study indicate that QoI-resistant *C. sojina* isolates occur throughout the Mississippi soybean production system. Their frequency and distribution are somewhat disconcerting for

the future management of FLS in Mississippi and perhaps the United States. The QoIs are an important FLS management tool that growers need to maintain. The majority of *C. sojina* isolates collected from Mississippi soybean fields in 2013 and 2014 are resistant to the QoIs. The EC₅₀ values observed for the three AZ-sensitive isolates in this study were similar to those reported by Zhang et al. (2012b). The values observed for the AZ-resistant isolates in this study were similar to those found by Zhang et al. (2012a) in that they were >1 µg/ml yet <5 µg/ml. Further support of QoI resistance in Mississippi isolates was documented by the presence of the G143A amino acid substitution in the *cyt b* gene. The G143A amino acid substitution has been identified in many plant pathogens (FRAC 2013), including *Alternaria alternata* (Fr.) Keissl in pistachio and tangerine, *Botrytis cinerea* Pers. in strawberry and grape, *Cercospora beticola* Sacc. in sugar beet, *Colletotrichum cereale* Manns. in creeping bentgrass, and *Venturia inaequalis* (Cooke) G. Winter in apple, among others (Bolton et al. 2013; Fernández-Ortuño et al. 2012; Leroux et al. 2010; Lesniak et al. 2011; Ma et al. 2003; Vega and Dewdney 2014; Young et al. 2010). The F129 L amino acid substitution has been observed in a reduced number of pathogens, some of which are *C. cereale* in creeping bentgrass, *A. solani* Sorauer in potato, and *Pyrenophora teres* Drechsler in barley (Pasche et al. 2005; Semar et al. 2007; Young et al. 2010). The G137R amino acid substitution has only been observed in *P. tritici-repentis* Died in wheat (Sierotzki et al. 2007). Neither the F129 L nor the G137R substitutions were identified in the subset of molecularly characterized *C. sojina* isolates collected in this study, nor in previous studies concerning *C. sojina* (Zeng et al. 2015).

Research conducted on the application of QoI fungicides on various field crops has determined that there may be benefits beyond disease management (Grossmann and Retzlaff 1997; Kyveryga et al. 2013; Wise and Mueller 2011). Fungicide manufacturers have suggested that QoIs can increase yield even in the absence of visible disease symptoms, and as a result, this class of fungicides are often marketed for their potential nonfungicidal physiological effects

Fig. 1. Sensitivity pattern of *Cercospora sojina* to quinone outside inhibitor fungicides in Mississippi. *Cercospora sojina* isolates were collected from 562 soybean fields in 2013 and 2014, totaling 634 *C. sojina* isolates. A = Hills; B = Delta; C = Pines; D = Capital; E = Coast.

(Bradley and Sweets 2008; Dorrance et al. 2010; Kyveryga et al. 2013; Wise and Mueller 2011). However, in some instances, conflicting yield results were reported as a result of fungicide applications in the absence of disease (Swoboda and Pedersen 2009). A study performed by Kyveryga et al. (2013) observed a relationship between yield response following an application of pyraclostrobin

Table 2. The effective concentration of azoxystrobin at which 50% of conidial germination was inhibited for 15 *Cercospora soja* isolates and their in vitro reactions

Isolate ^v	Region ^w	EC ₅₀ µg/ml	Reaction to azoxystrobin	
			Sensitive	Resistant ^x
13-11	Delta	2.2720 a ^y		X
13-2	Capital	2.1548 ab		X
13-58	Pines	2.1047 b		X
13-55	Pines	2.0877 b		X
13-47	Delta	1.8339 c		X
13-61	Pines	1.8153 cd		X
13-43	Hills	1.7009 de		X
13-34	Capital	1.6512 e		X
13-104	Delta	1.4541 f		X
13-37	Capital	1.3204 g		X
13-100	Capital	0.8674 h		X
13-36	Capital	0.0436 i	X	
13-31	Hills	0.0421 i	X	
13-85	Hills	0.0384 j	X	
S86 ^z	-	0.0002 k	X	

^v *Cercospora soja* isolates collected throughout the 2013 growing season in Mississippi.

^w Mississippi region where *C. soja* isolates originated.

^x Resistant isolates exhibited the G143A amino acid substitution conferring resistance to quinone outside inhibitor fungicides.

^y Mean EC₅₀ values (n = 4) within columns followed by the same letter are not significantly different based on Fisher's protected least significant difference test ($P = 0.05$).

^z Isolate S86 originated from a frogeye leaf spot sample collected in MS prior to the release of azoxystrobin (pre-1996) (Zhang et al. 2012b).

Fig. 2. Restriction fragment length polymorphism analysis of the partial sequence of the cytochrome *b* (*cyt b*) gene from *Cercospora soja* isolates sensitive and resistant to QoI fungicides. **A.** Partial nucleotide sequences of the *cyt b* gene. Codon 143 is boxed, the gray shading indicates the mutation responsible for the G143A amino acid substitution. **B.** Restriction enzyme *AluI* cleavage of the 238-bp amplification products at position 143 when GCT was present, resulting in two bands of approximately 78- and 160-bp, no cleavage when GGT was present. Detection of the G143A mutation with restriction enzyme *AluI* was followed by electrophoresis on 2% agarose gels. Isolate names are shown below the respective lanes. M = Norgen 50-bp DNA ladder (Norgen Biotek Corp., Thorold, ON, Canada).

and above-normal spring rainfall. This indicates that there may be economic benefits to making fungicide applications to soybean fields in years where heavy rainfall is observed even when disease symptoms are not present. Annual soybean yields in Mississippi tend to be lower than the national average (NASS 2013b) which may lead to blanket applications of QoIs. This type of management strategy applies additional selection pressure on *C. soja* populations and other nontarget phytopathogens.

In this study, over 98% of *C. soja* isolates collected from Delta fields were QoI-resistant, whereas only 83% of isolates from the Hills region were resistant. The differences between the QoI-sensitivity results from the Delta and Hills regions may be explained by differences in soybean management strategies between these regions. Soybean fields in the Delta are generally irrigated and more frequently planted in high-yielding Maturity Group IV cultivars, which in some cases can be more FLS-susceptible (e.g., Armor DK 4744, Asgrow 4531, Progeny 4510). Also, fields in the Delta typically receive a fungicide application for yield enhancement, rather than for disease management. This practice usually consists of a single QoI fungicide application between R3 (beginning pod) and R4 (full pod) at a reduced rate (292 ml/ha application rather than a 438 ml/ha application; based on azoxystrobin or pyraclostrobin label rates). These fungicide applications are perceived to prevent yield loss as a result of late-season foliar diseases (e.g., anthracnose [*Colletotrichum truncatum*] [Schwein]), *Cercospora* blight [*Cercospora kikuchii*] [Matsumoto & Tomoyasu]), pod and stem blight [*Diaporthe phaseolorum* var. *sojae* [Lehman]], and prevent spread of *C. soja* to noninfected leaf material. Conversely, soybean fields in the Hills have historically been nonirrigated, and are considered to have reduced yield potential. Fungicide applications are far less frequent in the Hills region due to longer crop rotations, fewer fungicide applicators, and the perception that the fungicide applications in the absence of disease will not be as profitable. The reduced frequency of QoI-resistant *C. soja* isolates occurring in the Hills could therefore be due to less reliance on QoI fungicides for general yield benefits or disease management.

The frequency at which *C. soja* QoI-sensitive isolates occurred in the Hills follows the pattern of qualitative sensitivity distribution. Because QoI resistance is monogenic and seemingly stable, the shift to resistant populations is rapid with repeated selection pressure (Latin 2011); this would explain both the differences in these two regions and the high frequency of QoI-resistance identified throughout the state of Mississippi. Alternative disease management practices such as crop rotation, planting FLS-resistant cultivars, and fungicide rotation should be explored for future success in managing FLS in Mississippi soybean. Moreover, resistance monitoring practices for other important foliar soybean pathogens should continue, since the impact of the frequent use of a single fungicide class over several years may subsequently select resistance in additional pathogen populations.

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Literature Cited

- Akem, N. C. 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.
- Allen, T. 2012. Strobilurin-resistant frogeye leaf spot confirmed in Mississippi. Mississippi Crop Situation, Res. and Ext. Serv., Mississippi State Univ. Retrieved from <http://www.mississippi-crops.com/2012/10/05/strobilurin-resistant-frogeye-leaf-spot-confirmed-in-mississippi/>, July 1, 2015.
- Anke, T. 1995. The antifungal strobilurins and their possible ecological role. *Can. J. Bot.* 73:940-945.
- Athow, K. L., and Probst, A. H. 1952. The inheritance of resistance to frogeye leaf spot of soybeans. *Phytopathology* 42:660-662.
- Balba, H. 2007. Review of strobilurin fungicide chemicals. *J. Environ. Sci. Health B* 42:441-451.

- 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.
- Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Wheeler, D. L. 2005. Genbank. *Nucleic Acids Res.* 33:D34-D38.
- Bolton, M. D., Rivera, V., and Secor, G. 2013. Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. *Pest Manag. Sci.* 69:35-39.
- Bradley, K. W., and Sweets, L. E. 2008. Influence of glyphosate and fungicide coapplications on weed control, spray penetration, soybean response, and yield in glyphosate-resistant soybean. *Agron. J.* 100:1360-1365.
- Chappell, G. F., and Phipps, P. M. 2006. A comparison of fungicide spray programs for soybean disease management, 2005. *Fungic. Nematocide Tests.* 1:FC056.
- Dorrance, A. E., Cruz, C., Mills, D., Bender, R., Koenig, M., LaBarge, G., Leeds, R., Mangione, D., McCluer, G., Ruhl, S., Siegrist, H., Sundermeier, A., Sonnenberg, D., Yost, J., Watters, H., Wilson, G., and Hammond, R. B. 2010. Effect of foliar fungicide and insecticide applications on soybeans in Ohio. *Plant Health Progress* 10.1094/PHP-2010-0122-01-RS.
- Fehr, W. R., and Caviness, C. E. 1977. Stages of soybean development. *Spec. Rep.* 80. Iowa Cooperative Ext. Serv., Ames, IA.
- Fernández-Ortuño, D., Chen, F., and Schnabel, G. 2012. Resistance to pyraclostrobin and boscalid in *Botrytis cinerea* isolates from strawberry fields in the Carolinas. *Plant Dis.* 96:1198-1203.
- Fernández-Ortuño, D., Toré, J. A., de Vicente, A., and Pérez-García, A. 2008. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *Int. Microbiol.* 11:1-9.
- Fungicide Resistance Action Committee. 2013. List of plant pathogenic organisms resistant to disease control agents. CropLife Int., Brussels, Belgium. Retrieved from <http://www.frac.info/publications/downloads>, February 2014.
- Fungicide Resistance Action Committee. 2014. FRAC Code List. CropLife Int., Brussels, Belgium. Retrieved from <http://www.frac.info/publications/downloads>, February 2014.
- Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag. Sci.* 58:859-867.
- Grau, C. G., Dorrance, A. E., Bond, J., and Russin, J. S. 2004. Fungal diseases. Pages 679-763. in: *Soybean: Improvement, Production, and Uses*, 3rd ed. H. R. Boerma and J. E. Specht, eds. ASA-CSSA-SSSA, Madison, WI.
- Grossmann, K., and Retzlaff, G. 1997. Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pestic. Sci.* 50: 11-20.
- Haskell, R. J. 1926. Diseases of cereal and forage crops in the United States in 1925. U.S. Dept. Agr. Pl. Dis. Rep. Supl. 48:375-376.
- Hershman, D., Kennedy, B., Yielding, T., and Bachi, P. 2011. Evaluation of selected fungicides for control of foliar diseases of soybean in Kentucky, 2010. *Plant Dis. Manage. Rep.* 5:FC005.
- Koenning, S. R., and Wrather, J. A. 2010. Suppression of soybean yield potential in the continental United States by plant diseases from 2006 to 2009. *Plant Health Progress* 10.1094/PHP-2010-1122-01-RS.
- Kyverya, P. M., Blackmer, T. M., and Mueller, D. S. 2013. When do foliar pyraclostrobin fungicide applications produce profitable soybean yield responses? *Plant Health Progress* 10.1094/PHP-2013-0928-01-RS.
- Latin, R. 2011. Fungicide resistance. Pages 49-77 in: *The Practice Guide to Turfgrass Fungicides*. American Phytopathological Society, St. Paul, MN.
- Lehman, S. G. 1928. Frog-eye leaf spot of soybean caused by *Cercospora diazu* Miura. *J. Agric. Res.* 35:811-833.
- Leroux, P., Gredt, M., Leroux, M., and Walker, A.-S. 2010. Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Appl. Environ. Microbiol.* 76:6615-6630.
- Lesniak, K. E., Proffer, T. J., Beckerman, J. L., and Sundin, G. W. 2011. Occurrence of QoI resistance and detection of the G143A mutation in Michigan populations of *Venturia inaequalis*. *Plant Dis.* 95:927-934.
- Ma, Z., Felts, D., and Michailides, T. J. 2003. Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. *Pestic. Biochem. Physiol.* 77: 66-74.
- Melchers, L. E. 1925. Disease of cereal and forage crops in the United States in 1924. U.S. Dept. Agr. Pl. Dis. Rep. Supl. 40:186.
- Mengistu, A., Kurtzweil, N. C., and Grau, C. R. 2002. First report of frogeye leaf spot (*Cercospora soja*) in Wisconsin. *Plant Dis.* 86:1272.
- 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 soja* 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, 2007. *Plant Dis. Manage. Rep.* 2:FC043.
- NASS. 2013a. Soybeans – All; Crop Acreage, Yield, and Production by County, 2012-2013. USDA-NASS, Washington, DC. Retrieved from http://www.nass.usda.gov/Data_and_Statistics, February 2014.
- NASS. 2013b. 2013 Crop Production. USDA-NASS, Washington, DC. Retrieved from http://www.nass.usda.gov/Data_and_Statistics, February 2014.
- Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Dis.* 89:269-278.
- Phillips, D. V. 1999. Frogeye leaf spot. Pages 20-21 in: *Compendium of Soybean Diseases*, 4th ed. G. L. Hartman, J. B. Sinclair, and J. C. Rupe, eds. American Phytopathological Society, St. Paul, MN.
- Phillips, D. V., and Boerma, H. R. 1981. *Cercospora soja* race 5: A threat to soybeans in the southeastern United States. *Phytopathology* 71:334-336.
- Ploper, L. D., González, V., Gálvez, M. R., Devani, M. R., Ledesma, F., and Zamorano, M. A. 2001. Frogeye leaf spot of soybean caused by *Cercospora soja* in northwestern Argentina. *Plant Dis.* 85:801.
- Price, P., Padgett, G. B., and Purvis, M. A. 2014. The effect of selected fungicides and application timings on frogeye leaf spot, 2013. *Plant Dis. Manage. Rep.* 8:FC223.
- Sauter, H., Steglich, W., and Anke, T. 1999. Strobilurins: Evolution of a new class of active substances. *Angew. Chem. Int. Ed.* 38:1328-1349.
- Sciubato, G. L., Blessitt, J. B., and Poston, D. H. 2006. Fungicides for control of late season foliar diseases, 2005. *F N Tests.* 1:FC066.
- Semar, M., Strobel, D., Koch, A., Klappach, K., and Stammler, G. 2007. Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the cytochrome b gene. *J. Plant Dis. Prot.* 114:117-119.
- Shaner, G., and Buechley, G. 2006. Control of diseases of soybean in central Indiana with foliar fungicides. 2005. *F N Tests.* 1:FC034.
- Sierotzki, H., Frey, R., Wullschlegel, J., Palermo, S., Karlin, S., Godwin, J., and Gisi, U. 2007. Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Manag. Sci.* 63:225-233.
- Sierotzki, H., Parisi, S., Steinfeld, U., Tenzer, I., Poirey, S., and Gisi, U. 2000. Mode of resistance to respiration inhibitors at the cytochrome bc₁ enzyme complex of *Mycosphaerella fijiensis* field isolates. *Pest Manag. Sci.* 56:833-841.
- Spinks, B. L. 2006. Impact of foliar fungicides on yield and net returns in soybean. M.Sc. thesis, Mississippi State University, Mississippi.
- Swoboda, C., and Pedersen, P. 2009. Effect of fungicide on soybean growth and yield. *Agron. J.* 101:352-356.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.
- Vega, B., and Dewdney, M. M. 2014. Distribution of QoI resistance in populations of tangerine-infecting *Alternaria alternata* in Florida. *Plant Dis.* 98:67-76.
- Vincelli, P. 2002. QoI (strobilurin) fungicides: Benefits and Risks. *The Plant Health Instructor* 10.1094/PHI-I-2002-0809-02
- White, T. J., Bruns, 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.
- Wilkerson, T. H., Allen, T. W., and Golden, B. R. 2014a. Evaluation of foliar fungicides to prevent yield loss from frogeye leaf spot in Mississippi full-season soybean, 2013. *Plant Dis. Manage. Rep.* 8:FC243.
- Wilkerson, T. H., Allen, T. W., and Irby, J. T. 2014b. Efficacy of foliar fungicides for management of *Cercospora soja* on soybean in east Mississippi, 2013. *Plant Dis. Manage. Rep.* 8:FC241.
- Wilkerson, T. H., Allen, T. W., and McIntire, P. 2014c. Comparison of BASF protocol to evaluate fungicide efficacy against frogeye leaf spot in Mississippi, 2013. *Plant Dis. Manage. Rep.* 8:FC240.
- Wise, K., and Mueller, D. 2011. Are fungicides no longer just for fungi? An analysis of foliar fungicide use in corn. *APSnet Feature* 10.1094/APSnetFeature-2011-0531.
- 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.
- Wrather, J. A., and Koenning, S. R. 2009. Effects of diseases on soybean yields in the United States 1996 to 2007. Online. *Plant Health Progress* 10.1094/PHP-2009-0401-01-RS.
- Yang, X. B., Uphoff, M. D., and Sanogo, S. 2001. Outbreaks of soybean frogeye leaf spot in Iowa. *Plant Dis.* 85:443.
- Young, J. R., Tomaso-Peterson, M., Tredway, L. P., and de la Cerda, K. 2010. Occurrence and molecular identification of azoxystrobin-resistant *Colletotrichum cereale* isolates from golf course putting greens in the southern United States. *Plant Dis.* 94:751-757.
- Zeng, F., Amaro, E., Zhang, G., Olaya, G., Wullschlegel, 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 soja* and development of diagnostic tools for its identification. *Plant Dis.* 99: 544-550.
- Zhang, G., Pederson, D. K., Phillips, D. V., and Bradley, C. A. 2012b. Sensitivity of *Cercospora soja* isolates to quinone outside inhibitor fungicides. *Crop Prot.* 40:63-68.
- Zhang, G. R., Newman, M. A., and Bradley, C. A. 2012a. First report of soybean frogeye leaf spot fungus (*Cercospora soja*) resistant to quinone outside inhibitor fungicides in North America. *Plant Dis.* 96:767.