# *CERCOSPORA SOJINA*: OVER-WINTER SURVIVAL AND FUNGICIDE RESISTANCE

BY

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DISSERTATION

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## ABSTRACT

Historically, frogeye leaf spot (FLS; caused by *Cercospora sojina*) of soybean has been observed more frequently in the southern U.S. than the north central U.S. However, in recent years, FLS field observations have been on the increase in the north central U.S., including Illinois. To better understand the survival rate of *C. sojina* in Illinois, a field study was conducted at three locations: Monmouth (west-central Illinois), Urbana (east-central Illinois), and Dixon Springs (southeastern Illinois). At each location, soybean leaves affected by FLS were placed at depths of 0, 10, and 20 cm and retrieved after 12, 19, and 24 months. To determine the viability of *C. sojina* in the collected leaves, a greenhouse bioassay was developed. Survival of *C. sojina* declined with time equally at all three locations through 19 months. After 24 months, *C. sojina* from leaves collected from Monmouth and Urbana was no longer viable, but the fungus was still active in leaves collected from Dixon Springs. Depth of leaf placement had no effect on survival of *C. sojina*. These results suggest that planting a non-host crop for two years in central Illinois will reduce the level of *C. sojina* inoculum to a negligible amount; however, soybean farmers in southern Illinois may need a longer rotation for FLS management.

Another topic addressed in this dissertation was the monitoring of Quinone outside inhibitor (QoI) fungicide resistance in *C. sojina*. QoI fungicides have been effective in managing frogeye leaf spot, but the risk of selecting *C. sojina* strains with resistance to this class of fungicides is considered high. A QoI fungicide resistance monitoring program was initiated, in which sensitivities to azoxystrobin, pyraclostrobin, and trifloxystrobin were determined in *C. sojina* isolates collected prior to QoI fungicide use on soybean (baseline population) and *C. sojina* isolates collected from soybean fields in 2007, 2008, and 2009. For the baseline

population, the mean effective fungicide concentration at which 50% of the conidial germination was inhibited ($EC_{50}$) was determined to be 0.01287, 0.00028, and 0.00116 µg/ml for azoxystrobin, pyraclostrobin, and trifloxystrobin, respectively. When mean $EC_{50}$ levels of 2007, 2008, and 2009 *C. sojina* isolates were compared to baseline *C. sojina* $EC_{50}$ levels, a small but statistically significant ($P \leq 0.05$) shift towards less sensitivity was observed for trifloxystrobin in 2009. In 2010, QoI fungicide resistant isolates were found at two locations in Illinois, one location in Kentucky, and two locations in Tennessee. QoI fungicide sensitivity levels of the resistant isolates were over 200-fold higher than baseline isolates using petri dish assays. A greenhouse trial was conducted with a QoI-resistant *C. sojina* isolate from Tennessee and a QoI-sensitive baseline isolate. FLS caused by the QoI-resistant isolate was not significantly ($P \leq 0.05$) reduced with QoI fungicides compared to a water control, but FLS caused by the QoI-sensitive isolate was significantly reduced with QoI fungicides compared to a water control. Several fungicides in the demethylation inhibitor (DMI) group and the methyl benzimidazole carbamate (MBC) fungicide, thiophanate methyl significantly reduced FLS caused by the QoI-resistant or QoI-sensitive isolate compared to their respective water controls. These results indicate that *C. sojina* isolates resistant to QoI fungicides are present in Illinois, Kentucky, and Tennessee, and that FLS caused by QoI-resistant isolates may be managed with DMI or MBC fungicides. To develop the best management tactics for control of FLS caused by QoI resistant *C. sojina* and the best fungicide resistance management tactics, a better understanding of how QoI resistant *C. sojina* isolates compare to QoI sensitive isolates in their biology and their aggressiveness in causing FLS on different soybean cultivars is needed. Results from a laboratory study indicated that no differences in mycelial morphology, number of spores produced after 5 days, and radial growth after 6 or 12 days were observed between QoI resistant

and sensitive *C. sojina* isolates. Results from a greenhouse study indicated that on a FLS susceptible cultivar ('Blackhawk'), QoI resistant *C. sojina* isolates caused significantly ($P \leq 0.05$) greater disease severity than QoI sensitive isolates 7 to 8 days after inoculation, but no differences in severity were observed after 9 days. On a FLS resistant cultivar with the *Rcs3* gene for resistance ('Davis'), QoI resistant *C. sojina* isolates caused significantly greater disease severity than QoI sensitive isolates 8 to 14 days after inoculation. In general, these comparisons between QoI resistant and sensitive *C. sojina* isolates indicate that they are similar in growth and sporulation, but the QoI resistant isolates were slightly more aggressive in causing greater FLS severity on soybean.

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# CHAPTER ONE: INTRODUCTION AND OVERVIEW

## *Cercospora sojina*

Frogeye leaf spot (FLS), caused by *Cercospora sojina* Hara, was first reported in Japan in 1915 (Hara, 1915). The first occurrence of *C. sojina* causing FLS on soybean in the United States was in South Carolina in 1924 (Melchers, 1925). After 1924, FLS was also found in Mississippi and Louisiana (Haskell, 1926; Lehman, 1928). Since 1929, FLS has been observed in several soybean–producing states at various times (Athow and Probst, 1952). FLS also has been reported from Australia, Canada, China, Germany, Japan, Manchuria, and Russia (Athow and Probst, 1952). In countries with a tropical climate, such as Brazil, Argentina, South Africa, Nigeria and Zimbabwe, FLS can cause severe yield losses. In the United States, FLS traditionally has occurred mostly in the southern parts of the United States, but recently it has caused soybean yield reductions in Iowa (Yang et al., 2001) and Wisconsin (Mengistu, et al., 2002). The disease is favored by a combination of warm winter temperatures and the cultivation of susceptible soybean cultivars in the northern states. The practice of conservation tillage that leaves pathogen-infested plant debris on the soil surface may also be a factor in the increased occurrence of this disease. The estimate of soybean yield suppression due to FLS in the USA from 1996 (about 23,1000 tons) to 2007 (about 270,1000 tons) was about 11 times greater than the level observed in the other main soybean-producing countries in 2006 (Wrather and Koenning, 2009). Estimated reduction of soybean yields (thousand metric tons) due to FLS was about 10 times (350 to 35) higher than that observed in other countries in 2008 (Wrather et al., 2010). The recent increase in the range and severity of FLS has caused concern and interest among the soybean research community throughout the United States (Mian et al., 2008).

*C. sojina* overwinters in infected seeds and infested soybean residue. Seeds initially are infected from pod lesions, but healthy seeds may become contaminated with conidia or mycelia during harvest. Heavily infected seeds generally have poor germination and lead to weak and stunted seedlings with lesions on cotyledons. Spores from cotyledons may continue to infect leaves. Young leaves that are not fully expanded are highly susceptible, while fully expanded leaves are more resistant to invasion (Phillips, 1999). Conidia on lesions normally appear 8-12 days following inoculation. However, under continuous moist, warm conditions, conidia may appear as early as 48 hrs after inoculation. Conidia are carried short distances by air currents and splashing rain, and they cause secondary infections throughout the season under favorable conditions. Zhong et al. (1991) found there were no differences in disease severity among the treatments in which seeds infected with different lesion rates were planted into separate plots. This indicates that the infested seeds are not a major source of inoculum. The parts (leaf, pod and stem) of the soybean were buried in different environments and at different depths, in the following year, the observations of different plant parts at different times showed that conidia were produced on both leaves and pods, which indicated that infected residue is the major source of inoculum for FLS (Ma and Li, 1987).

In the field, symptoms most often develop after flowering. The early common symptoms are small circular dark-brownish spots that appear on the leaves. These spots finally enlarge to a diameter of 1-5 mm, and the brown spots are surrounded by narrow, dark reddish brown margins. Older spots are light to dark brown, translucent, and have white centers. Several spots may merge into larger, irregular spots. When spots cover about 50% of the leaf area, leaves blight, wither, and then fall prematurely. The number of lesions on the plant will continue to increase as

long as the weather is favorable for infection. If favorable conditions for infection continue late into the season, the fungus will infect pods and seeds.

Three major dominant genes in soybean confering reistance to *C. sojina* are *Rcs1, Rcs2,* and *Rcs3* (Athow and Probst, 1952; Athow, et al., 1962; Phillips and Boerma, 1982). Physiological races of *C. sojina* with virulence to *Rcs1* and *Rcs2* have been reported (Athow and Probst, 1952; Athow et al., 1962). Cultivars with *Rcs* genes may have only occasional spots, which are often small and nonsporulating (Athow and Probst, 1952). The *Rcs3* gene, which was found in the soybean cultivar 'Davis', has been able to confer resistance to all known races of *C. sojina* that occurred in the United States (Phillips and Boerma, 1982; Pace et al., 1993). Seed-applied fungicides can reduce the risk of seed borne *C. sojina* inoculum. Foliar-applied fungicides sprayed at late flowering and early seed (R2-R5) growth stages (Fehr and Caviness, 1977) may provide some protection against *C. sojina* infection (Akem, 1995). Crop rotation and burying affected crop residues will reduce disease incidence (Phillips, 1999).

**QoI fungicide and fungicide resistance**

The quinone outside inhibitor (QoI) class of fungicides is one of the most important for plant disease management in agricultural systems. QoI fungicides represent a relatively new class of compounds developed from the natural fungicidal derivation strobilurin A, which is a secondary metabolite produced by the basidiomycota wood-rotting fungus, *Strobilurus tenacellus* (Clough et al., 1996). These fungicides have the ability to inhibit mitochondrial respiration by binding at the Qo site of cytochrome *b* (Gerth et al., 1980; Zhang et al., 1998). This blocks electron transfer between cytochrome *b* and cytochrome $c_1$, which, in turn, disrupts the energy cycle within the fungus by blocking the production of ATP (Becker et al., 1981; Anke, 1995; Bartlett et al., 2002). Many studies have shown that alternative oxidase (AOX) plays an

important role in the branched respiratory chain. AOX is a strobilurin–insensitive terminal oxidase that allows electrons to bypass Complex III from ubiquinol. Salicylhydroxamic acid (SHAM) is one of the characteristic inhibitors of AOX (Wood and Hollomon, 2003).

The QoI fungicides currently are used for the management of several agronomic and horticultural crop diseases. These fungicides provide control of fungal pathogens from major divisions of fungi, such as Ascomycota and Basidiomycota, and provide control of some pathogens in Oomycota (Ammermann et al., 1992; Jordan et al., 1999). Studies with azoxystrobin (Godwin, 1994, 1997), trifloxystrobin (Margot et al., 1998), and pyraclostrobin (Ammermann et al., 2000, Stierl et al., 2000) have demonstrated that the spore germination and the zoospore development are particularly sensitive to QoI fungicide. Because of this, QoI fungicides have a high level of preventative activity. Curative activity and antisporulation activity also have been reported (Bartlett et al., 2002; Wong and Wilcox, 2001; Anesiadis et al., 2003). Some diseases are not controlled at all by QoI fungicides, and several pathogens have quickly developed resistance.

Resistance to QoI fungicides has been reported in over twenty fungal species (Fungicide resistance action committee, 2011), and the Fungicide Resistance Action Committee (FRAC) has determined that QoI fungicides have a high risk of selecting for fungicide resistant strains. The target site cytochrome bc-1 protein is encoded by a mitochondrial gene for which DNA repair mechanisms are less effective than for nuclear DNA genes. Consequently, genes encoded in the mitochondrial DNA are more likely to mutate (Zheng and Kokller, 1997). According to recent FRAC reports, three mutations are responsible for QoI fungicide resistance (Fungicide resistance action committee, 2006). These mutations occur in the cytochrome b gene, and are single amino acid shifts. When glycine is replaced with alanine at position 143, it is known as the G143A

mutation; this is the most common of all the QoI fungicide resistance mutations. When phenyalanine is replaced with leucine at position 129, it is known as the F129L mutation, and when glycine is replaced with arginine at position 137, it is known as the G137A mutation. Of these mutations, the G143A is the most severe, because the resistance factor (sensitivity of resistant strain / sensitivity of sensitive strain) generally is greater than 100.

**Fitness, aggressiveness, and adaptation of fungicide resistant isolates**

Fitness of fungicide resistant isolates is an important parameter affecting the risk of developing practical resistance. Understanding the fitness of QoI-resistant strains would have significant benefits for fungicide resistance management. Studies on a QoI resistant mutant of *Saccharomyces cerevisiae* revealed that mitochondria of most mutations were impaired, and electron flow through the cytochrome *bc*1 complex was reduced (Köller et al., 2001). Further evidence of fitness penalties were observed for several QoI resistant isolates of fungi such as *Ustilago maydis* (Ziogas et al., 2002), *Cercospora beticola* (Malandraki et al., 2006), and *Botrytis cinerea* (Markoglou et al., 2006). However, in other studies, no fitness penalties were found in some of QoI resistant isolates, such as a G143A mutant of *Blumeria graminis* (Chin et al., 2001), and laboratory – selected G143A mutants of *Magnaporthe grisea* (Avila-Adame and Köller, 2003). In many studies with other fungicides, fitness was estimated by measuring spore formation, mycelium growth rate, pathogenicity, or survival, but it is unclear whether these parameters correlate with the fitness of QoI resistance. Van der Plank (1963) developed the concept that the cost of qualitative virulence was the reduction in pathogen fitness induced by a mutation from avirulence to qualitative virulence. Recent progress in plant pathogen genomics has shown that mutation from avirulence to qualitative virulence gene modification was caused by single–base mutation or a large chromosome deletion (Gout et al., 2007). Therefore, genetic

correlation between traits, either positive or negative (trade–off) may constrain quantitative trait evolution, such as aggressiveness (disease severity, infection efficiency, latent period, spore production rate, infectious period and lesion size) and fitness. The study of the relationship between aggressiveness and fitness showed that *Cochliobolus carbonum* presented a low aggressiveness level on maize, but greater survival rate; whereas *Cochliobolus beterostrophus* presented greater aggressiveness level, but had a low survival rate (Leonard et al., 1988). Carson (1998) also found a negative correlation between the survival of *C. beterostrophus* on the soil surface and their aggressiveness. Another study of *Phytophthora infestans* on potato tubers found no correlations between aggressiveness mearsured as lesion size, sporulation and latent period and overwinter survival (Montarry et al., 2007). These results indicate that pathogen aggressiveness played an important role for the population studies on pathogen adaptation. Research on latent period and sporulation per lesion of *Pyrenophora teres* fungicide resistant isolates showed that pathogen aggressiveness was not associated with resistance to triadimenol or propiconazol (Peever and Milgroom, 1994). Potato leaf lesions caused by metalaxyl-resistant isolates of *P. infestans* were larger than those lesions caused by metalaxyl-sensitives isolates of *P. infestans* in the absence of selection pressure. However, there was no significant difference in sporulation capacity (Kadish and Cohen, 1998a; Kadish and Cohen, 1998b). Additionally, no significant difference in aggressiveness was observed between mefenoxam-resistant and -sensitive *P. erythroseptica* isolates in the absence or presence of mefenoxamselection pressure (Taylor et al., 2006). Another interesting report was that isolates of *M. graminicola* from wheat plots receiving fungicide applications were more aggressive than isolates from wheat plots unprotected by fungicide (Cowger and Mundt, 2002). However, it is important to note that very

few reports on the aggressiveness and adaptation of QoI resistant isolates of fungal phytopathogens are currently available.

**Fungicide resistance management**

Fungicide usage to control of Cercospora leaf spot caused by *C. beticola* in North Dakota and Minnesota is a very well documented example of fungicide resistance management. *C. beticola* has a history of developing resistance to different fungicides (Weiland et al., 2001). Resistance to the methyl benzimidazole carbamate (MBC) fungicide thiophanate methyl became widespread in the 1980s. Resistance to triphenyltin hydroxide was first reported in 1994, and resistance to demthylation inhibitor (DMI) fungicides began in 2000. However, in the last 10 years, fungicide resistance is being successfully managed in the sugar beet production areas in North Dakota and Minnesota; resistance to thiophanate-methyl is declining because of less thiophanate-methyl usage in 2006 and 2008. No triphenyltin hydroxide resistant isolates were found because of an absence of selection pressure; and isolates with reduced-sensitivity to DMI fungicides became less prevelant because of alternative fungicide usage (Secor, et al., 2010). Tank-mixing and rotating different fungicide classes were also used to manage fungicide resistance on *C. beticola*. The above strategies may be used for management of QoI fungicide resistance in other crops.

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# CHAPTER TWO: SURVIVAL OF *CERCOSPORA SOJINA* ON SOYBEAN DEBRIS IN ILLINOIS


## ABSTRACT

Historically, frogeye leaf spot (FLS); caused by *Cercospora sojina*) of soybean has been observed more frequently in the southern U.S. than in the North Central U.S. However, in recent years, FLS field observations have been on the increase in the North Central U.S., including Illinois. To better understand the survival rate of *C. sojina* in Illinois, a field study was conducted at three locations: Monmouth (west-central Illinois), Urbana (east-central Illinois), and Dixon Springs (southeastern Illinois). In fields at each location, soybean leaves affected by FLS were placed at depths of 0, 10, and 20 cm and retrieved after 12, 19, and 24 months. To determine the viability of *C. sojina* in the collected leaves, a greenhouse bioassay was developed. Survival of *C. sojina* declined with time equally at all three locations over 19 months. After 24 months, *C. sojina* from leaves collected from Monmouth and Urbana was no longer viable, but the fungus was still active in leaves collected from Dixon Springs. Depth of leaf placement had no effect on the survival of *C. sojina*. These results suggest that planting a non-host crop for two years in central Illinois will reduce the level of *C. sojina* inoculum to a negligible amount; however, soybean farmers in southern Illinois may need a longer rotation for FLS management.


## INTRODUCTION

Frogeye leaf spots (FLS), caused by *Cercospora sojina* Hara, is one of the most important diseases of soybean (*Glycine max* (L.) Merr.). This disease is especially severe in the warm humid areas of the world (Akem, 1992; Ploper, 2001; Phillips and Boerma, 1981; Phillips, 1999). In the U.S., it has recently been identified as far north as Iowa, Wisconsin and Ohio

(Yang et al., 2001; Mengistu et al., 2002; Cruz and Dorrance, 2009). Soybean yield losses due to FLS primarily occur because of a reduction in photosynthetic area and premature defoliation (Akem, 1994). The estimated annual soybean yield reductions caused by frogeye leaf spot in the United States ranged between 183,868 and 345,148 metric tons from 2006 to 2009 (Koenning and Wrather, 2010). In U.S. experimental field research plots, the greatest soybean yield reductions caused by FLS were about 21% and 31% (Laviolette et al., 1970; Mian et al., 1998).

*Cercospora sojina* survival in infected seeds and residue of soybean are the major inoculum sources of FLS (Ma et al., 1987; Grau et al., 2004). *C. sojina* in soybean residue in different environments buried in 0 to 20 cm soil survived after one year in the northeast China (Ma et al., 1987). It was generally thought that *C. sojina* could not survive in northern areas of the U.S. (Grau et al, 2004), but it was reported that the pathogen survived successfully after one winter in infested soybean leaves on the surface of fields in Ohio (Cruz and Dorrance, 2009). FLS is a polycyclic disease, and warm and humid weather is necessary for infection and multiple disease cycles. In practice, planting pathogen-free seed, burying infected soybean residue, rotating with non-host crops, planting resistant cultivars, and applying foliar fungicides are used to manage FLS (Phillips, 1999).

Research on the biology of *C. sojina* has provided evidence that suitable temperatures for conidia germination are from 24-26°C, that conidia were viable for 7 days at 20-22°C, and that the optimal temperature for mycelium growth is between 21 and 26°C (Zhong et al., 1989; Cruz and Dorrance, 2009). However, the viability and longevity of this pathogen in soybean residue in northern U.S. regions have not been determined.

The objective of this study was to evaluate the survival rate of *C. sojina* in affected soybean leaf debris at different regions of in Illinois when buried at three depths over a two-year period.

## MATERIALS AND METHODS

### Sample collection and treatment in the field

Soybean leaves severely infected with *C. sojina* (>70% disease severity) were collected from fields at Dixon Springs, IL in September 2008. Ten leaflets were placed in nylon bags and sealed in protective fiberglass mesh sacks. The sacks were placed on the soil surface or buried in the soil at depths of 10 or 20 cm in fields at University of Illinois research farms located near Urbana, Monmouth and Dixon Springs, IL (Fig. 2.1). Soil characters, latitude and longitude of the three locations are listed in Table 2.1. Temperature and relative humidity (RH) sensors (WatchDog, Spectrum$^{@}$ technology, Inc. Plainfield, IL) were placed at three depths to record temperature and RH hourly. Precipitation data were provided by research farm staff. Treatments at the three depths were replicated four times at each site. Sacks were placed in the field in November 2008. Twelve sample sacks (four from each depth) from each location were retrieved at each sample time, and samples from Monmouth and Dixon Springs were collected at 7, 12, 19 and 24 months after placement (June and November in two years). Due to a collection mistake, samples at Urbana were collected at 12, 19, and 24 months, but not at 7 months. The retrieved leaves were removed from the sacks, air dried at room temperature for at least 5 days, after which they were ground to a fine powder by hand and placed in a sterile plastic vial to be used to inoculate soybean leaves in the greenhouse.

**Inoculation of *C. sojina in* the greenhouse**

The viability of *C. sojina* was tested on the leaves of the susceptible soybean cultivar 'Asgrow 3101' in an air conditioned room in the greenhouse. Soybean plants were grown in trays (26.46 x 52.96 x 5.84 cm) with 24 individual pots (7.88 x 5.72 x 5.84 cm) that contained a potting mix (Sunshine Mix 1, Sun Gro Horticulture Inc., Bellevue, WA). Pots were placed in a larger tray (53.66 x 26.67 x 6.35 cm) so that plants could be watered from the bottom. Before inoculation, soybean plants were watered until the potting mix was saturated, and wet paper towels were placed on the soil surface between plants to increase the relative humidity (RH). Ten day old soybean seedlings (VC stage) were inoculated with the previously prepared diseased leaf powder. The abaxial surface of unifoliolate leaves (50-75% unrolled) was sprayed with a solution of 0.005 % Tween 20 in $dH_2O$ until leaves were wet , then 0.05 g of powder inoculum was placed on one abaxial leaf surface for each treatment. The control treatments included inoculation with sterilized soybean leaf powder and a non-inoculated control. All of the treatments were arranged in one tray using a completely randomized design. All samples were replicated 4 times in separate trays. The inoculated plants were then covered with a transparent plastic dome (15.24 x 53.34 x 27.94 cm) and incubated for 6 days to maintain high RH (≥90%). After 6 days, plastic domes were removed, and plants were maintained for 15 days, after which time the number of lesions per leaf was counted. The temperature of the potting mixture surface was 25 ± 2 °C without plastic domes and 23 ± 2 °C with plastic domes. Supplemental lighting was used (high pressure sodium 1000 watt) with a photoperiod of 14 hours. Plants were watered daily as needed. Because of limited space, inoculations of all samples from different locations collected at different times were assayed over multiple trials. Therefore, inoculation with $1 \times 10^4$

conidia suspension of *C. sojina* on the leaves at every trial was used as an internal control to check optimal conditions for *C. sojina* infection.

**Data analysis**

Because data from the 7 month collection time were missing from the Urbana location, only data from the 12, 19, and 24 month collection times were used for data analyses to compare locations, collection times, and burial depths. Levene's test for variance homogeneity of conidia –inoculated control trials indicated that trial variances were not statistically different from each other; therefore data from all trials were combined together for further analysis. Analysis of variance (ANOVA) was conducted using the general linear model procedure (PROC GLM) in SAS (version 9.2, SAS Inc., Cary, NC) to determine the effects of the fixed nested three-factors (time, location and depth) on the viability of *C. sojina*. All factors were fixed and depth nested within locations. Least significant different (LSD) at $\alpha=0.05$ was calculated to compare differences in average number of lesions per leaf produced from inocula buried at different depths over time at different locations. Simple linear regression analysis was performed to predict inoculum viability and combined the three depths with time using the mathematical equation $y= aX+b$, in which Y= predicted log transformation lesion number per leaf, b=number of lesions produced at the beginning of the experiment (or viability calculated at time zero), and a=rate of viability decline at time X. For regression analysis, the data transformed for normality and the data from the 7 month collection time were included for Dixon Springs and Monmouth.

**RESULTS**

The main factors of location and sampling time had a significant ($P < 0.0001$) effect on survival of *C. sojina*, but the main factor of depth did not (Table 2.2). No interactive effects

were significant. The greatest survival of *C. sojina* was observed in leaf debris collected at Dixon Springs (southeastern Illinois), which was significantly better than survival in leaf debris collected at Urbana (east-central Illinois) or Monmouth (west-central Illinois) (Fig. 2.2). No differences in *C. sojina* survival were observed between leaf debris collected at Urbana and Monmouth. Survival of *C. sojina* was greatest in leaf debris collected after 12 or 19 months, which was significantly better than the survival in debris collected after 24 months (Fig. 2.3).

Combined across the different burial depths, regression analysis results indicate that the rate of survival was decreased at similar rates (rate = 0.03 to 0.04) for all three locations (Fig. 2.4). The coefficients of determinations were relatively high for each location, indicating that the linear regression models fit the data relatively well. The models that fit the data the best were from Dixon Springs and Monmouth ($R^2$ = 0.72 and 0.70, respectively), while the model for Urbana had a slightly lower coefficient of determination ($R^2$ = 0.52).

The three locations represented three different kinds of weather in Illinois. The mean daily temperature of Dixons Spring was about 2°C higher than Urbana over the entire year. The spring, summer, and fall mean daily temperatures were similar at Urbana and Monmouth, but in winter, Monmouth was about 1 to 2°C colder than Urbana. The lowest mean daily temperature of all three locations was from December to February: -12°C at Dixon Springs, -21°C at Urbana and -25°C at Monmouth. Highest daily temperatures (about 30°C) were similar from the middle of June to August at all three locations. There was a great deal of fluctuation in the mean daily temperature at the soil surface, and it was sometimes warmer than the temperature beneath the surface, but the sub-surface temperatures were generally 1 to 2°C warmer than the soil surface temperatures. During the winter months, temperatures at the 20 cm soil depth were 1 to 2°C

warmer than those at the 10 cm soil depth, but soil at the 10 cm depth was warmer than soil at the 20 cm depth during the other seasons. Soil temperatures are summarized in Fig. 2.5.

The mean daily relative humidity of the three locations ranged from 45% to 95% (Fig. 2.6). The mean daily precipitation at Dixon Springs and Monmouth was similar in two different years, but the precipitation at Urbana was somewhat less (Fig. 2.7).

## DISCUSSION

In this study, *C. sojina* inoculum in soybean leaf debris was viable after overwintering successfully at three different locations in Illinois for up to 24 months, which is the longest field survival period reported for this pathogen. Cruz and Dorrance (2009) reported that *C. sojina* survived in leaf debris for 7 months at two locations in Ohio, and Ma and Li (1987) reported that *C. sojina* could survive for up to 12 months in China. In a similarly-designed study to the one conducted in Illinois, Khan et al. (2008) evaluated the survival of a similar fungus, *C. beticola*, in North Dakota, and they reported that *C. beticola* survived up to 22 months. In light of the results from the Illinois study, soybean growers in Illinois should consider rotating to a non-host crop for two seasons after a severe outbreak of frogeye leaf spot in a soybean field. In the Illinois study, the greatest reduction in *C. sojina* viability was found between the 19 month and 24 month collection periods. The 19 month collection period would approximately represent June in the second season following the soybean crop initially affected by frogeye leaf spot. This 19 month collection period would be the approximate time that *C. sojina* conidia would be present, splashing onto leaves, and causing infections in that second season.

Survival of *C. sojina* was the highest at the Dixon Springs location in southeastern Illinois. The Dixon Springs location had the warmest winter temperatures compared to the other locations. Of two counties in Ohio, more *C. sojina* conidia were obtained from the leaf debris

that had overwintered in the southern most county (Clark) compared to a county that was more northern (Wayne) after 7 months (Cruz and Dorrance, 2009). Historically, frogeye leaf spot has caused more severe losses to soybean in the southern U.S. compared to the north central U.S. (Wrather and Koenning, 2009). Warmer temperatures in the southern U.S. and southern areas of Illinois likely provide a better climate for *C. sojina* survival and increase the risk of frogeye leaf spot damage in those areas. It is not known why warmer soil temperatures benefited *C. sojina* survival, other than the simple conclusion that the cold temperatures were detrimental to the survival of *C.sojina*. Differences in soil temperature could have resulted in differences in microbial activity at the different locations, which could have led to different decomposition rates of leaf debris. Other factors may have had an effect on the survival of *C. sojina*, such as soil type, soil pH, and soil moisture content. Based on these findings, soybean growers in southern Illinois should be more aware of the threat of frogeye leaf spot and should use preventative tactics to manage frogeye leaf spot because of the increased risk due to better survival of *C. sojina* and potentially greater inoculum levels each season.

In this study, burial depth of leaf debris had no effect on *C. sojina* survival. Burying infested soybean debris using tillage is recommended as a potential frogeye leaf spot management tactic (Phillips, 1999). In light of the Illinois findings, burying the soybean debris will not affect survival of *C. sojina*; however, it may reduce the impact of *C. sojina* inoculum from infested debris, since conidia that are buried would not be able to splash onto soybean leaves and cause infections. The results of the Illinois trial are different from those reported by Khan et al. (2008), who, working with a similar pathogen, reported that *C. beticola* survival was greatest in sugarbeet leaf debris left on the soil surface compared to debris that was buried at 10

or 20 cm, and they attributed the differences to a greater debris decomposition rate from greater microorganism activity within the soil.

In this study, soybean leaves infected by *C. sojina* were able to support *C. sojina* survival for at least 12 months at different locations in Illinois with the greatest survival occurring in southeastern Illinois (Dixon Springs). Based on these results, soybean farmers in southern Illinois may need to rotate to a non-host crop for two years, while farmers in central Illinois may only require rotating to a non-host crop for one year to manage the risk of losses due to frogeye leaf spot. Because no differences in *C. sojina* survival were observed among leaves at different soil depths, tillage may not be needed as a frogeye leaf spot management practice in Illinois.

## TABLES

Table 2.1. Soil parameters and locations of field research sites.

| Location | Soil type | pH | Soil Drainage | Latitude | Longitude |
|---|---|---|---|---|---|
| Dixon Springs | Grantsburg silt | 6.0 | Moderately well drained | 37° 26'05.31"N | 88°40'07.46"W |
| Monmouth | Osca Silt Loam | 6.6 | Very good | 40° 56'13.14" N | 90°43'23.93"W |
| Urbana | Drummer | 6.5 | Average | 40° 04'13.72"N | 88°13'08.06"W |

Table 2.2. Analysis of variance of *Cercospora sojna* survival at various soil depths over time at three locations in Illinois.

| **Source** | ***F* value** | ***P* > *F*** |
|---|---|---|
| Location | 21.83 | <0.0001 |
| Month | 113.22 | <0.0001 |
| Location*Month | 0.78 | 0.5415 |
| Depth (Location) | 1.62 | 0.1502 |

**FIGURES**

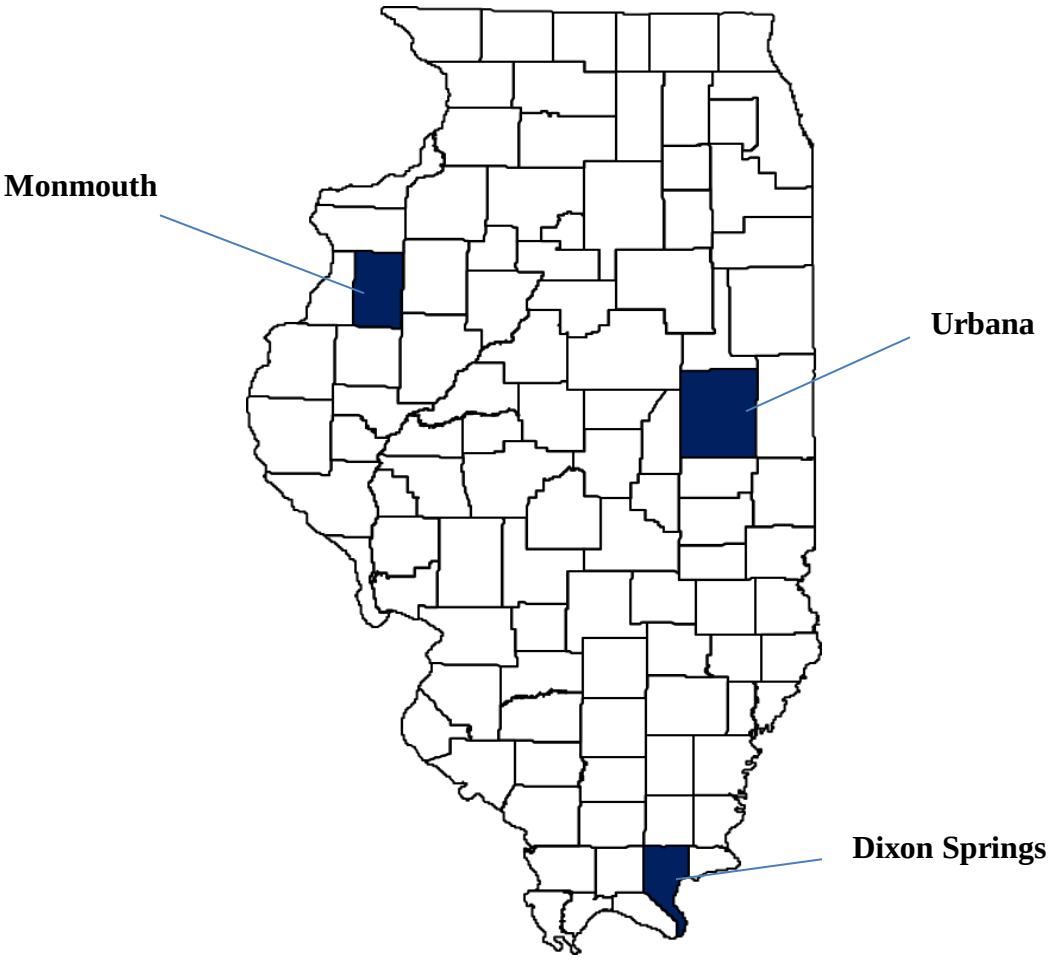



Figure 2.1. Locations of soybean leaf burial sites at Dixon Springs, Urbana and Monmouth, IL.

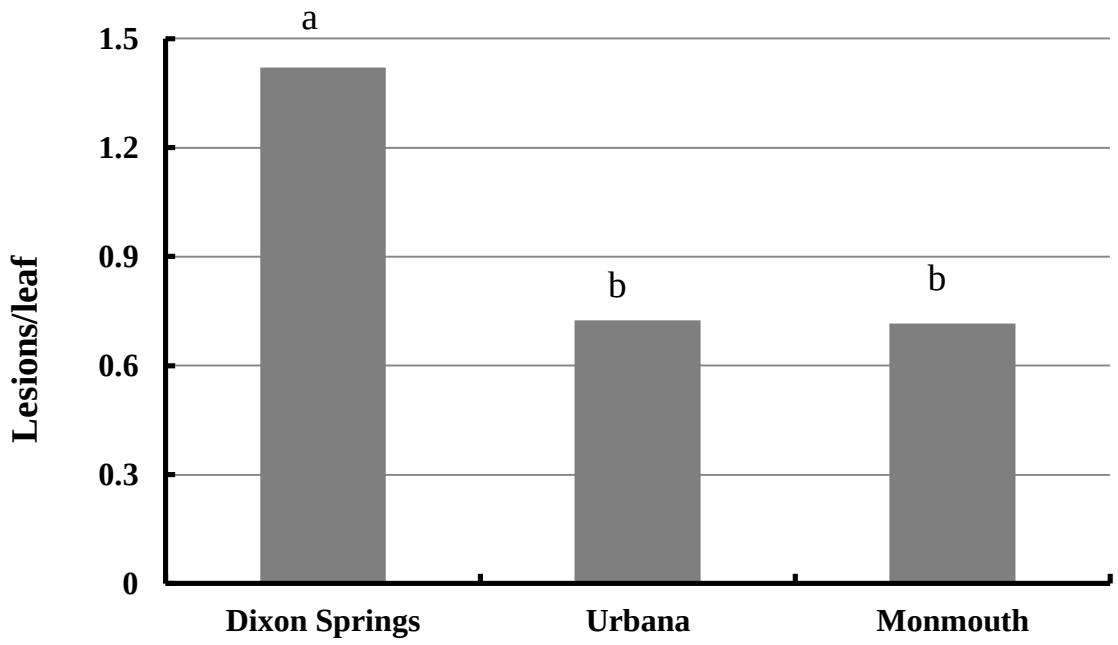



Figure 2.2. Frogeye leaf spot lesions on soybean leaves inoculated with *Cercospora sojina* infected leaf residue that was buried in Dixon Springs, Urbana and Monmouth, IL. Values presented are combined over times of collection (12, 19, and 24 months) and depths of leaf burial (0, 10, and 20 cm) ($\alpha$=0.05).

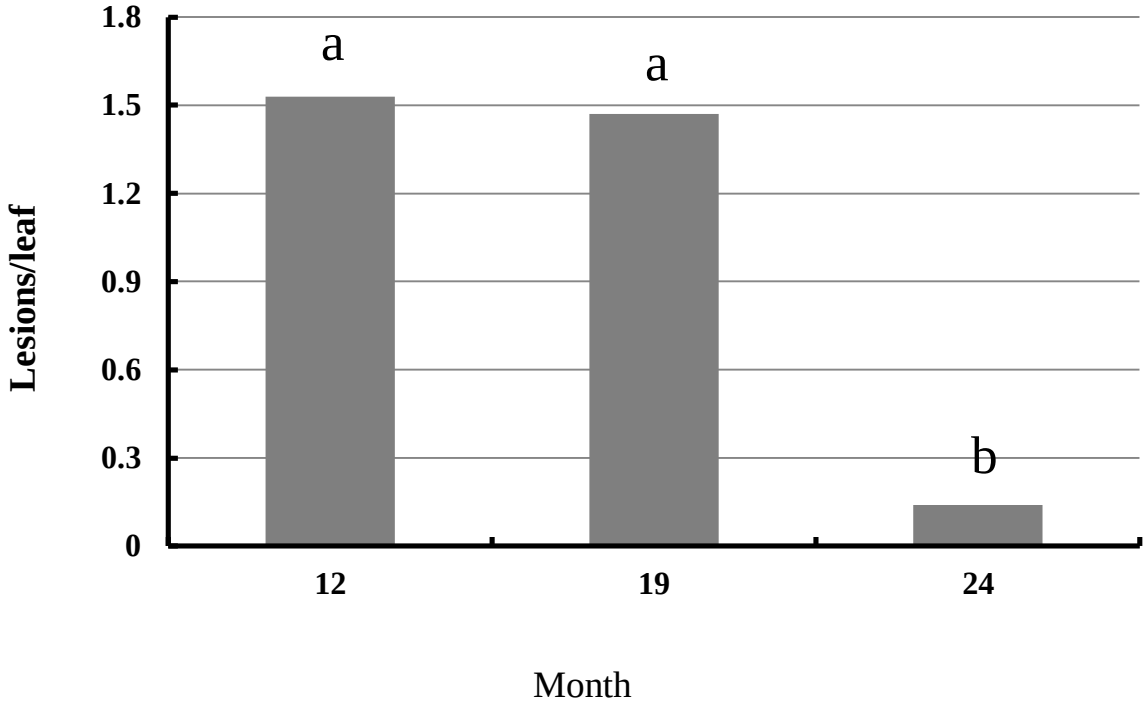



Figure 2.3. Frogeye leaf spot lesions on soybean leaves inoculated with *Cercospora sojina* infected leaf residue that was buried in Dixon Springs, Urbana and Monmouth, IL at 0, 10 and 20 cm depths. Values presented represent means over location and depth of leaf burial ($\alpha$=0.05).

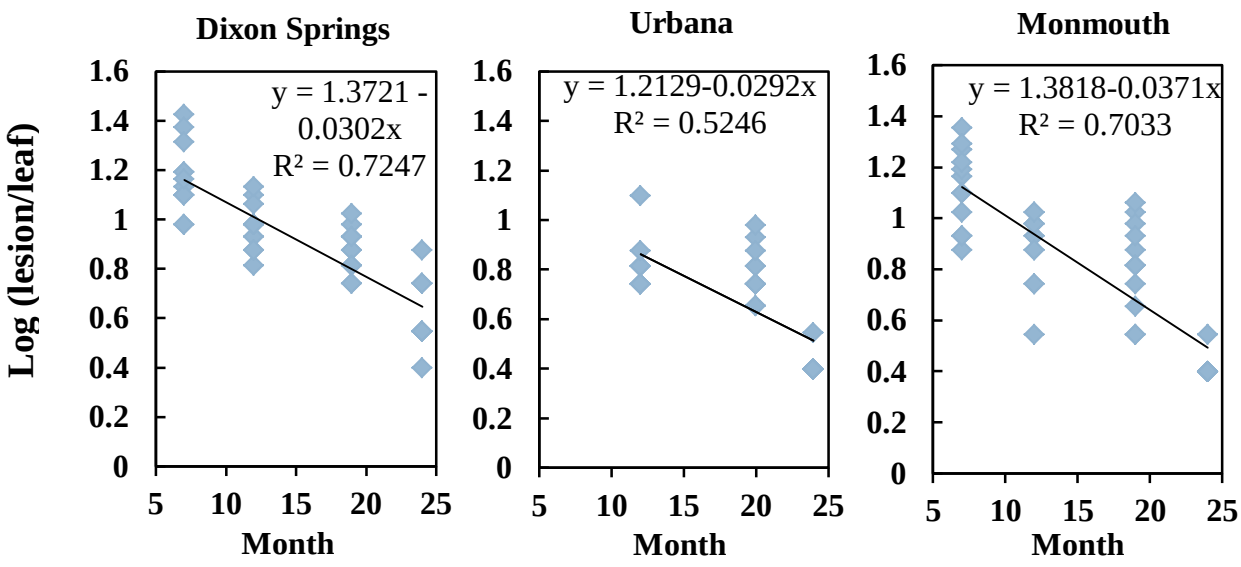



Figure 2.4. Linear regression of log(lesion/leaf) values combined over three leaf burial depths (0, 10, and 20 cm) at Dixon Springs, Urbana and Monmouth, IL over four sampling times.

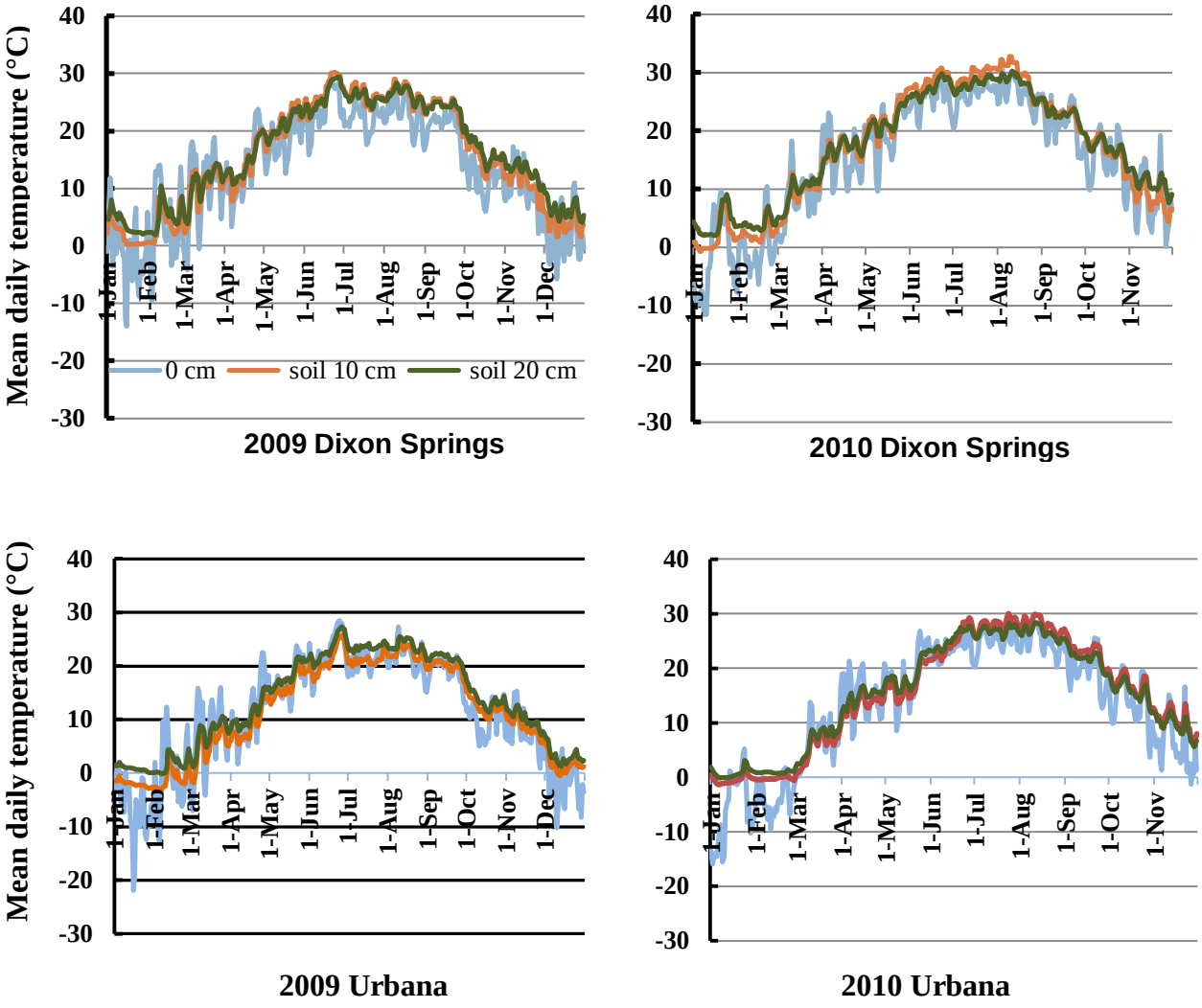
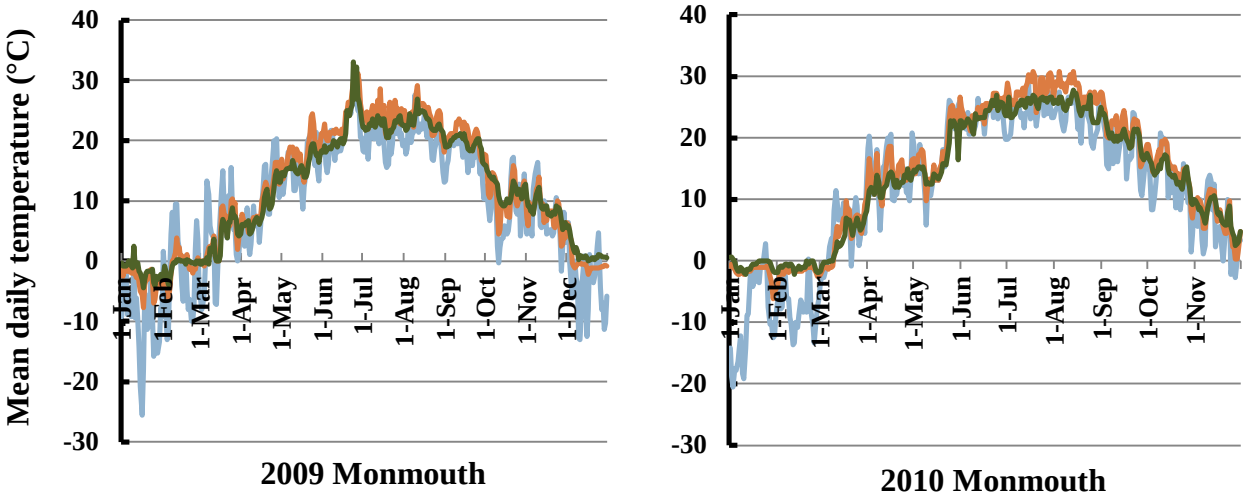



Figure 2.5. Mean daily temperatures at three locations over two years.

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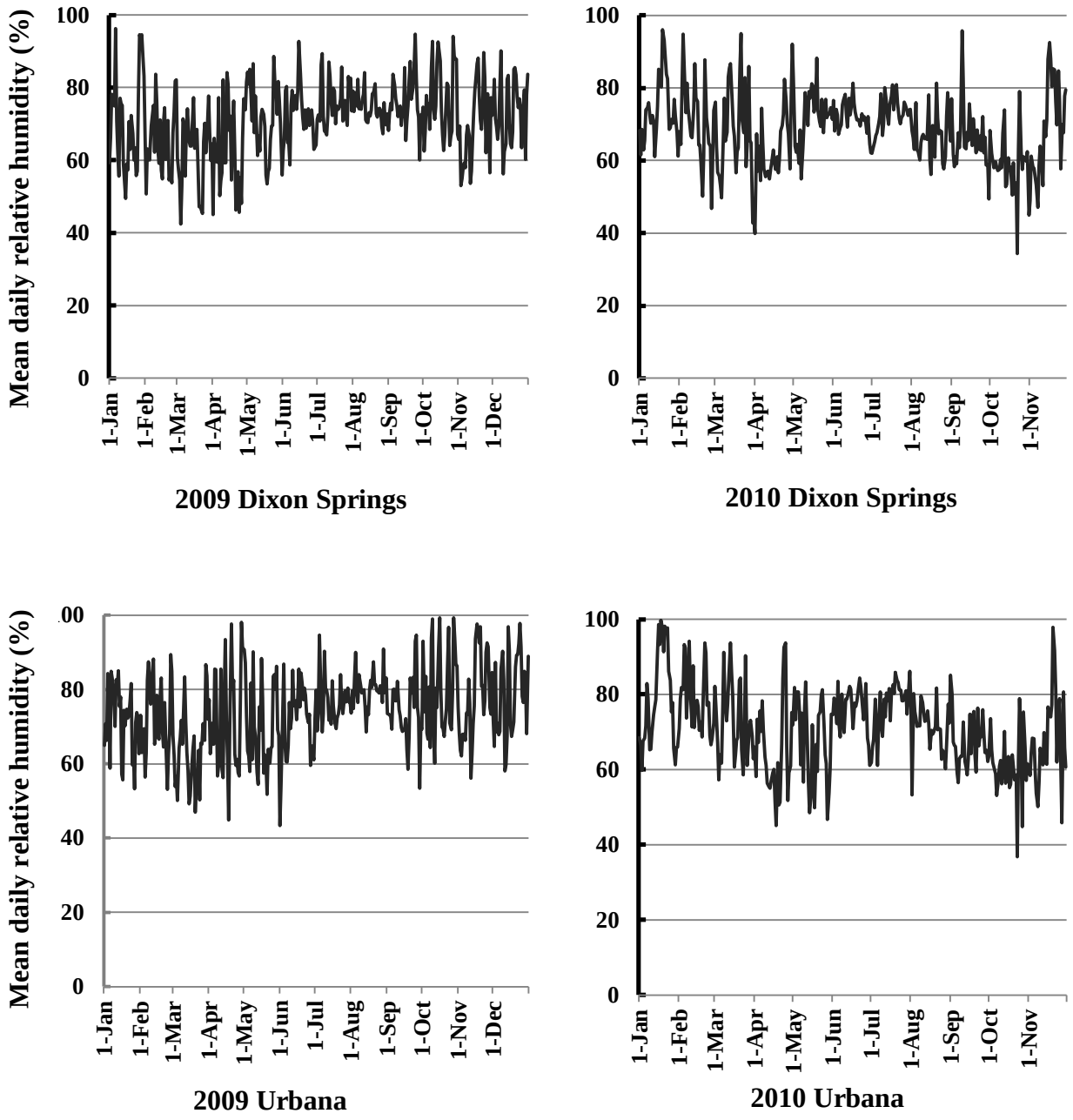


Figure 2.6. Mean daily relative humidity at three locations over two years.

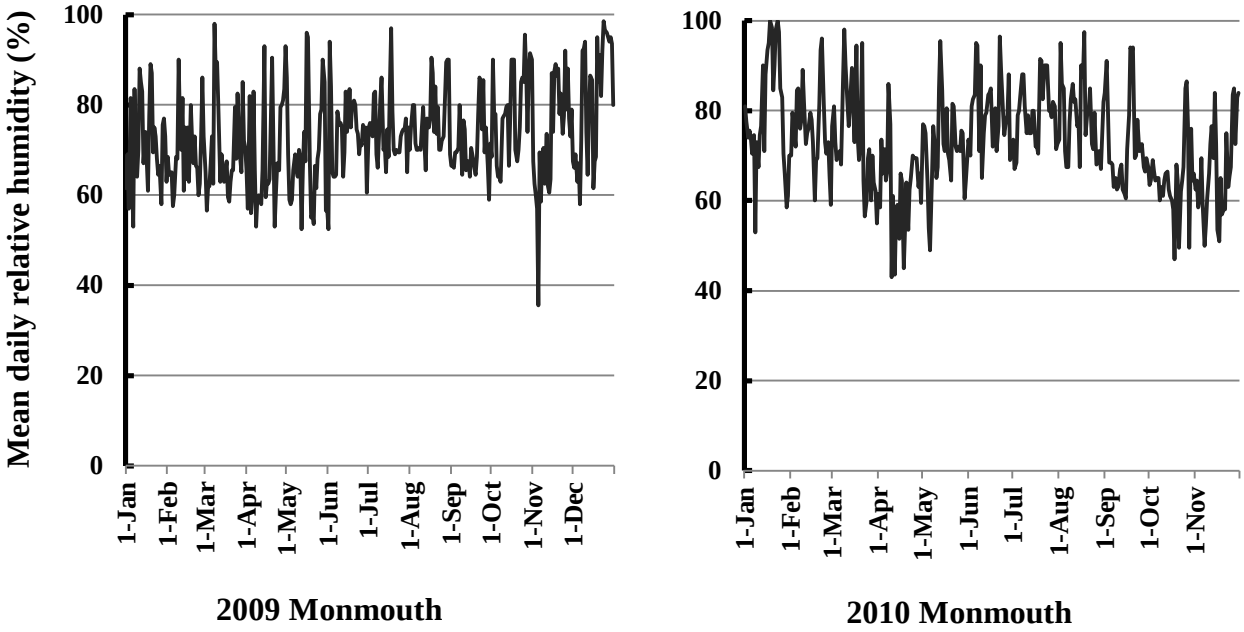


Figure 2.6. Continued.

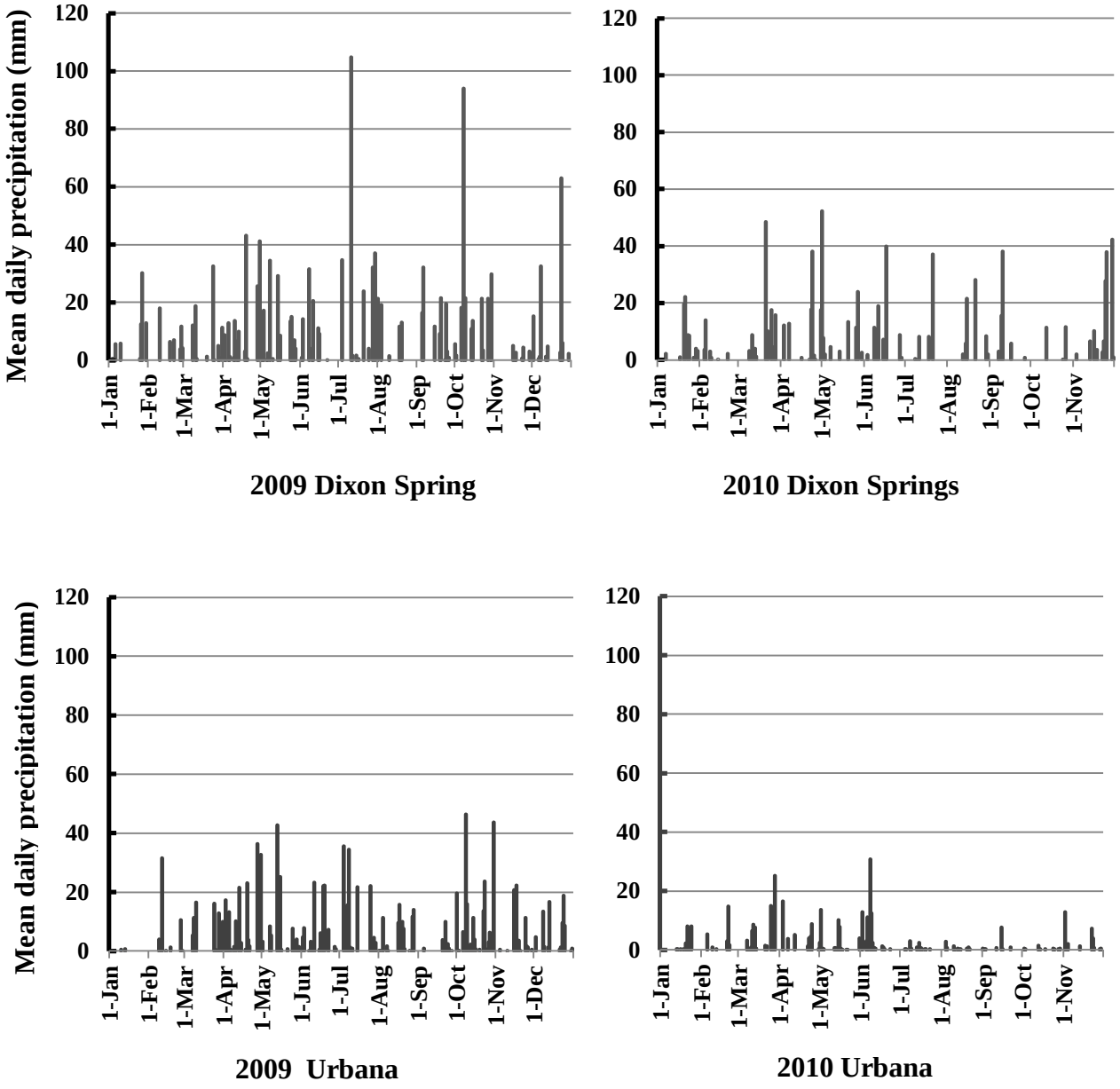


Figure 2.7. Mean daily precipitation levels at three locations over two years.

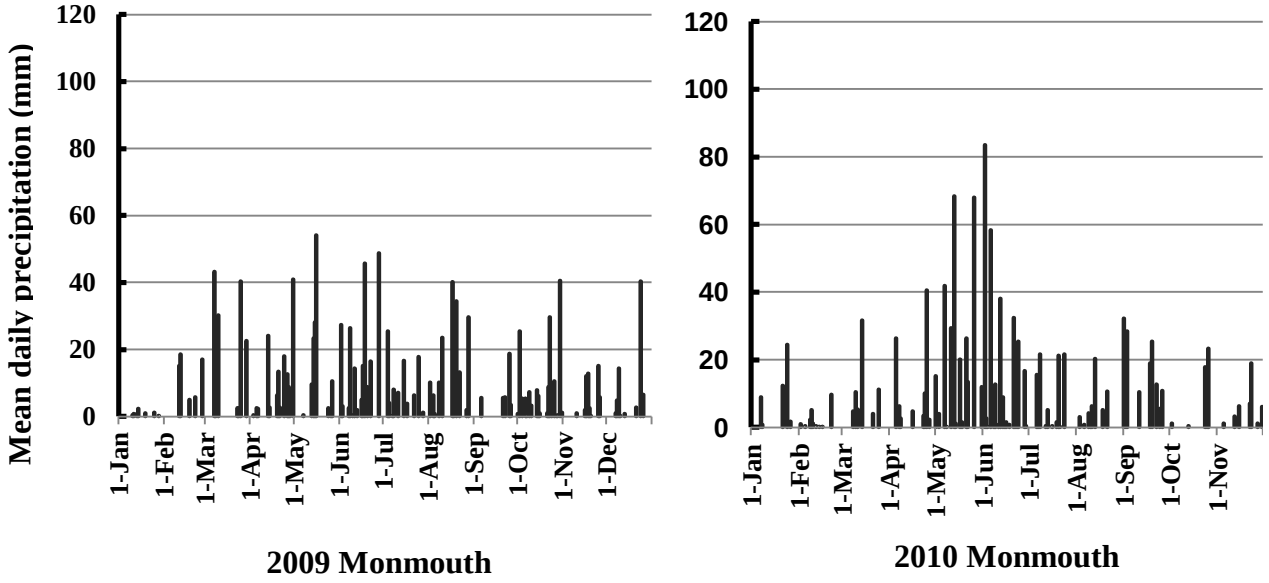


Figure 2.7. Continued.

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# CHAPTER THREE: SENSITIVITY OF *CERCOSPORA SOJINA* POPULATION TO QUINONE OUTSIDE INHIBITOR FUNGICIDES


## ABSTRACT

Frogeye leaf spot, caused by *Cercospora sojina*, causes yield reductions to soybean (*Glycine max* (L) Merr.) grown worldwide. Quinone outside inhibitor (QoI) fungicides have been effective in managing frogeye leaf spot, but the risk of selecting *C. sojina* strains with resistance to this class of fungicides is considered to be high. A QoI fungicide resistance monitoring program was initiated, in which levels of sensitiviy to azoxystrobin, pyraclostrobin, and trifloxystrobin were determined in *C. sojina* isolates collected prior to QoI fungicide use on soybean (baseline population) and in *C. sojina* isolates collected from soybean fields in 2007, 2008, and 2009. For the baseline population, the mean effective fungicide concentration at which 50% of the conidial germination was inhibited ($EC_{50}$) was determined to be 0.01287, 0.00028, and 0.00116 µg/ml for azoxystrobin, pyraclostrobin, and trifloxystrobin, respectively. When mean $EC_{50}$ levels of 2007, 2008, and 2009 collected *C. sojina* isolates were compared to baseline *C. sojina* $EC_{50}$ levels, a small but statistically significant ($P \leq 0.05$) shift towards less sensitivity was observed for trifloxystrobin in 2009. Although small (<1.5-fold), this shift in sensitivity indicates a risk of selecting for *C. sojina* strains with reduced sensitivity to QoI fungicides, and fungicide sensitivities should continue to be monitored in the future.

## INTRODUCTION

Frogeye leaf spot, caused by the fungus *Cercospora sojina* Hara, causes yield reductions to soybean (Glycine max (L.) Merr.) worldwide (Wrather et al., 2010). In the United States, estimated annual soybean yield reductions caused by frogeye leaf spot ranged between 183,868 and 345,148 metric tons from 2006 to 2009 (Koenning and Wrather, 2010). In experimental field research plots in the United States, the greatest soybean yield reductions caused by frogeye leaf spot were reported to be 21% and 31% (Laviolette et al., 1970; Mian et al., 1998). The use of host resistance has been an economical and effective way to manage frogeye leaf spot, but the development of new virulent races of *C. sojina* has been a historical and current threat (Athow et al., 1962; Mian et al., 2008; Phillips and Boerma, 1980; Ross, 1968).

Foliar fungicides have been evaluated for their effectiveness in controlling frogeye leaf spot. In past studies, benomyl, a methyl benzimidazole carbamate (MBC) fungicide, was shown to significantly reduce frogeye leaf spot severity in treated research plots (Akem, 1995; Akem and Dashiell, 1994; Backman et al., 1979; Dashiell and Akem, 1991). More recently, quinones outside inhibitor (QoI) and demethylation inhibitor (DMI) fungicides have proven to be efficacious in controlling frogeye leaf spot (Dorrance et al., 2010; Galloway, 2008; Nelson et al., 2010). Although effective in managing frogeye leaf spot, QoI fungicides have been determined to have a high risk of target fungi developing resistance to them and over 30 fungal pathogen species across 20 genera have been reported to show field resistance toward QoI fungicides (Fungicide Resistance Action Committee, 2011).

Because of the high risk of resistance developing to QoI fungicides, it is important that baseline sensitivities of isolates of *C. sojina* to QoI fungicides be established and a QoI fungicide resistance monitoring program be established that will be able to detect shifts in sensitivity. The

objectives of this research were to (i) establish baseline sensitivities of *C. sojina* to the QoI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin and (ii) compare QoI fungicide sensitivities of *C. sojina* isolates collected from fields applied with QoI fungicides in 2007, 2008, and 2009 with the baseline sensitivities.

## MATERIALS AND METHODS

### Collection, isolation, maintenance, and preparation of *C. sojina* isolates

For determining baseline sensitivity levels, 55 *C. sojina* isolates were obtained from 7 different states (Table 3.1). All baseline isolates were collected from soybean fields prior to QoI fungicide registration on soybean in the United States. In 2007, 2008, and 2009, *C. sojina* isolates were collected from soybean fields (Table 3.2). In the majority of the fields in which isolates were collected in 2007-2009, either a QoI fungicide or a QoI + DMI fungicide mixture had been applied. The soybean fields in which *C. sojina* isolates were collected from 2007 to 2009 included both university research and commercial production fields.

To obtain pure *C. sojina* cultures from soybean leaflets, frogeye leaf spot lesions were evaluated for *C. sojina* sporulation using a dissecting microscope. If the lesions were not sporulating, leaflets were placed in a humidity chamber (a sealed clear plastic box with sterile distilled water-dampened filter paper at the bottom) for 12-24 hours to encourage sporulation. Sterile distilled water (2 µl) was placed onto sporulating lesions with a micropipette, and conidia and water were then drawn back up and deposited onto soybean stem lima bean agar (SSLBA) (Phillips and Boerma, 1980) amended with rifampicin (25 mg/L) in petri dishes (100 mm diameter) and spread with a sterile bent glass rod. After 18 hours, a germinating conidium from each isolate was selected and aseptically transferred to a separate petri dish containing SSLBA,

incubated for 7 days under 12 hours fluorescent and black lights / 12 hours darkness at 25°C. Agar plugs (0.6 $cm^2$) with mycelia and conidia were stored in a 15% glycerol solution in sterile cryogenic tubes at -80°C.

For all QoI fungicide sensitivity experiments, isolates were prepared using methods adapted from Bradley and Pedersen (2011). Thawed fungal plugs were placed onto SSLB media amended with rifampicin and were incubated for 7 days at 25°C under 12 hours fluorescent and black lights / 12 hours darkness to produce conidia. These isolates were sub-cultured several times to produce enough conidia to run the assays. Conidial suspensions were prepared by placing 4 or 5 plugs (1 $cm^2$) containing conidia, mycelia, and SSLB media into 20 ml sterile glass tubes, adding 4 ml sterile distilled water, and vortexing for 30 seconds. The conidial suspension was filtered through 4 layers of cheese cloth into a sterile glass tube.The concentration of the conidial suspension was adjusted to $1 \times 10^5$ conidia/ml after initial concentration estimates were determined using a hemacytometer.

**Determination of alternative respiration in *C. sojina***

*C. sojina* when exposed to a QoI fungicide (azoxystrobin). Previous research has shown that some fungi possess an alternative respiration pathway which allows them to bypass complex III and IV in the The objective of this experiment was to determine whether alternative respiration is induced in mitochondrial respiration chain, which is accounted for by the presence of the respiratory enzyme alternative oxidase (Kubicek *et al*, 1980; Minagawa and Yoshimoto, 1987). The alternative respiration pathway has allowed some fungal spores to germinate in the presence of high doses of QoI fungicides in vitro (Olaya and Koller, 1999; Olaya et al., 1998; Vincelli and Dixon, 2002; Ziogas et al., 1997). The addition of salicylhydroxamic acid (SHAM;

Sigma-Aldrich, St. Louis, MO) has been used effectively in QoI fungicide sensitivity assays to prevent fungi from using an alternative respiration pathway (Olaya and Koller, 1999).

Five *C. sojina* baseline isolates (S5, S9, S10, S13, and S22) were randomly selected to test the effect of azoxystrobin fungicide on conidial germination with and without the addition of SHAM in the media. A stock solution of technical-grade azoxystrobin (96% a.i.; Syngenta Crop Protection) was prepared at a concentration of 100 mg/ml in acetone. Serial dilutions of the azoxystrobin stock solution were prepared in acetone and amended to potato dextrose agar (PDA; Becton, Dickinson, and Company, Franklin Lakes, NJ) at 0, 0.0001, 0.001, 0.01, and 0.1µg/ml. In addition, SHAM at 60 µg/ml dissolved in methanol was either added or not added to the PDA. All amendments were added to the autoclaved PDA after it had cooled to 55°C. *C. sojina* conidial suspensions were prepared for each isolate as previously described above, and 75 µl of the conidial suspensions were pipetted onto the amended PDA surface, spread with a sterile bent glass rod, and incubated in the dark for 15-18 hours at 26°C. Two replicate petri dishes (60 mm diameter) were prepared for each azoxystrobin-SHAM × *C. sojina* isolate combination. Using a compound microscope, germination was determined for 50 conidia per petri dish. A conidium was considered to be germinated if the germ tube was at least as long as the length of the conidium.

For each of the replicate plates, conidial germination was converted to percent inhibition compared with the no-azoxystrobin control plates by 100 – ([percent germination of azoxystrobin-amended]/[mean percent germination of control]). The azoxystrobin fungicide concentration that effectively inhibited conidial germination by 50% of the azoxystrobin control ($EC_{50}$) was determined for each *C. sojina* isolate by linear interpolation using the two concentrations that bracketed 50% (Wise et al., 2008). The experiment was arranged in using a

completely randomized design (CRD) and was repeated once. Data from each experiment were first analyzed separately using the general linear model procedure (PROC GLM) in SAS (Version 9.2; SAS Institute, Inc., Cary, NC) to compute variances; then, a two-tailed F test for equality of variances was conducted to determine whether data from trials could be combined. Combined data were analyzed using PROC GLM in SAS, and least-square means t tests (PDIFF option in SAS) were used to compare $EC_{50}$ values of the eight *C. sojina* isolates with SHAM versus without SHAM.

**Fungicide sensitivity testing of *C. sojina* populations**

Technical grade formulations of azoxystrobin (96% a.i.; Syngenta Crop Protection), pyraclostrobin (98% a.i.; BASF Corporation), and trifloxystrobin (98% a.i.; Bayer CropScience) were used to prepare stock solutions at concentrations of 100 mg/ml in acetone. Serial dilutions in acetone were prepared for each fungicide. *C. sojina* fungicide sensitivity was assessed by determining conidial germination on PDA amended with different concentrations of each fungicide. Azoxystrobin was added to PDA at 0.0001, 0.001, 0.01, 0.1, and 1 µg/ml, and pyraclostrobin and trifloxystrobin were each added to PDA at 0.00001, 0.0001, 0.001, 0.01, and 0.1µg/ml after PDA had cooled to 55°C after autoclaving. Non-fungicide-amended PDA also was included. SHAM dissolved in methanol was added to all media at 60µg/ml when the media had cooled to 55°C after autoclaving. Azoxystrobin, pyraclostrobin, and trifloxystrobin $EC_{50}$ values were determined for the *C. sojina* baseline isolates (Table 2.1), and isolates collected in 2007, 2008, and 2009 (Table 3.2) using the methods described above.

Because of limited space, fungicide sensitivities of all *C. sojina* isolates (baseline, 2007, 2008, and 2009) were assayed over multiple trials. A reproducibility test described by Wong and Wilcox (2000) was used to validate each trial. An internal control *C. sojina* baseline isolate (S9)

was randomly selected, and $EC_{50}$ values were determined in eight separate trials for azoxystrobin, pyraclostrobin, and trifloxystrobin using methods described in section of determination of alternative respiration. The mean, standard error, and 95% confidence interval were calculated for this isolate as described by Wong and Wilcox (2000). If the $EC_{50}$ value of the internal control isolate did not fall within the 95% confidence interval in the trials designed to establish baseline sensitivities for all of the isolates, those trials were dropped and repeated until the $EC_{50}$ of the internal control isolate was within the 95% confidence interval. For each trial, isolates were assayed on two replicate plates. Each isolate was assayed in at least two separate trials.

For each fungicide – *C. sojina* population (baseline, 2007, 2008, and 2009) combination, mean and median EC50 values were calculated. Within a fungicide, the mean $EC_{50}$ values for each *C. sojina* population were compared using Tukey's honestly significant difference (HSD) test (alpha = 0.05) in SAS.

## RESULTS

### Determination of alternative respiration in *C. sojina*

The analysis of variance of $EC_{50}$ values of the eight *C. sojina* isolates exposed to azoxystrobin with and without SHAM indicated that the main effects of isolate and SHAM were significant (P = 0.0119 and 0.0001, respectively), but the interaction of isolate × SHAM was not significant (P = 0.1271). In three of the five isolates tested, $EC_{50}$ values were significantly ($P \leq 0.05$) greater when SHAM was not included in the azoxystrobin-amended media (Table 3.3). These results indicated that alternative respiration may exist in *C. sojina*; therefore, SHAM was used to inhibit alternative respiration in subsequent *C. sojina* QoI fungicide sensitivity assays.

**Fungicide sensitivity testing of *C. sojina* populations**

The range of $EC_{50}$ values determined for *C. sojina* baseline isolates exposed to azoxystrobin was 0.00297-0.03241 µg/ml, with a mean and median of 0.01287 and 0.01252 µg/ml, respectively. The azoxystrobin baseline $EC_{50}$ values were not normally distributed (P = 0.0023) (Figure 3.1). The ranges of azoxystrobin $EC_{50}$ values determined for *C. sojina* isolates collected in 2007, 2008, and 2009 were 0.00297-0.02574, 0.00171-0.03088, and 0.00249-0.03162 µg/ml, respectively. The mean and median azoxystrobin $EC_{50}$ values for *C. sojina* isolates collected in 2007, 2008, and 2009 were 0.00966 and 0.00903µg/ml, 0.01506 and 0.01499µg/ml, and 0.01627 and 0.01614 µg/ml, respectively.

The range of $EC_{50}$ values determined for *C. sojina* baseline isolates exposed to pyraclostrobin was 0.00014-0.00076 µg/ml, with a mean and median of 0.00028 and 0.00025 µg/ml, respectively. The pyraclostrobin baseline $EC_{50}$ values were not distributed normally (P = 0.0001) (Figure 3.2). The ranges of pyraclostrobin $EC_{50}$ values determined for *C. sojina* isolates collected in 2007, 2008, and 2009 were 0.00007-0.00057, 0.00004-0.00052, and 0.00015-0.00074 µg/ml, respectively. The mean and median pyraclostrobin $EC_{50}$ values for *C. sojina* isolates collected in 2007, 2008, and 2009 were 0.00026 and 0.00026µg/ml, 0.00027 and 0.00029µg/ml, and 0.00031 and 0.00029 µg/ml, respectively.

The range of $EC_{50}$ values determined for *C. sojina* baseline isolates exposed to trifloxystrobin was 0.00018-0.00311 µg/ml, with a mean and median of 0.00116 and 0.00107 µg/ml, respectively. The trifloxystrobin baseline $EC_{50}$ values were not distributed normally (P = 0.0006) (Figure 3.3). The ranges of trifloxystrobin $EC_{50}$ values determined for *C. sojina* isolates collected in 2007, 2008, and 2009 were 0.00015-0.00293, 0.00005-0.00309, and 0.00013-0.00277 µg/ml, respectively. The mean and median trifloxystrobin $EC_{50}$ values for *C. sojina*

isolates collected in 2007, 2008, and 2009 were 0.00116 and 0.00114µg/ml, 0.00153 and 0.00149µg/ml, and 0.00163 and 0.00158 µg/ml, respectively.

Compared to the mean azoxystrobin and pyraclostrobin $EC_{50}$ values of the baseline isolates, mean azoxystrobin and pyraclostrobin $EC_{50}$ values of isolates collected in 2007, 2008, or 2009 did not significantly differ from their respective mean baseline $EC_{50}$ value (Table 3.4). Compared to the mean trifloxystrobin $EC_{50}$ value of the baseline isolates, isolates collected in 2009 had a significantly greater mean trifloxystrobin $EC_{50}$ value.

**DISCUSSION**

Results of our research indicate that some *C. sojina* isolates had significantly greater azoxystrobin $EC_{50}$ values when SHAM was not added to the media. This is an indication that *C. sojina* may be able to use the alternative respiration pathway to overcome the inhibitory action of QoI fungicides in vitro. This has been reported in a number of different fungi (Olaya and Koller, 1999; Vincelli and Dixon, 2002; Wise et al., 2008; Ziogas et al., 1997), including *C. zeae-maydis* (Bradley and Pedersen, 2011), which is in the same genus as *C. sojina*. Reportedly, the alternative respiration pathway is not believed to occur in nature because plant-produced flavones prevent the induction of alternative oxidase (Mizutani et al., 1996; Olaya and Koller, 1999; Olaya et al., 1998). In light of our results, SHAM should be added to media to prevent alternative oxidase when conducting in vitro QoI fungicide sensitivity assays with *C. sojina*.

Differences in the intrinsic activity on *C. sojina* conidial germination were observed among the QoI fungicides evaluated in this study. Based on mean $EC_{50}$ levels, intrinsic activity was greatest with pyraclostrobin, followed by trifloxystrobin, followed by azoxystrobin. This is similar to what was reported with *C. zeae-maydis* when the same QoI fungicides were evaluated for their activity on conidial germination (Bradley and Pedersen, 2011). In *C. beticola*, the

intrinsic activity of pyraclostrobin on conidial germination was greater than that of trifloxystrobin (Secor et al., 2010). Although these differences in in vitro activity on *C. sojina* conidial germination were observed in our laboratory setting, differences in efficacy for frogeye leaf spot control are not always observed among fungicide products containing these QoI active ingredients when evaluating them for control of frogeye leaf spot in the field (Grybauskas and Reed, 2006; Shaner and Buechley, 2007).

Shifts toward reduced fungicide sensitivity historically have been common in plant pathogenic species of *Cercospora*. Populations of *C. arachidicola* and *C. beticola* with resistance to methyl benzimidazole carbamate (MBC) fungicides were first reported several years ago (Bugbee, 1996; Campbell et al., 1998; Clark et al., 1974; Georgopoulos and Dovas, 1973; Littrell, 1974; Rupel and Scott, 1974; Smith and Littrell, 1980; Weiland and Halloin, 2001), and populations of *C. kikuchii*, another soybean pathogen, have been reported to have resistance to thiophanate methyl, an MBC fungicide (Imazaki et al., 2006). In addition, populations of *C. beticola* with reduced sensitivity to triphenyltin hydroxide (Bugbee, 1995; Bugbee, 1996; Campbell et al., 1998; Giannopolitis, 1978) and DMI fungicides (Karaoglanidis et al., 2000; Karaoglanidis et al., 2002; Secor et al., 2010) have been reported. Field resistance to QoI fungicides in speices of *Cercospora* has not been reported, but Malandrakis et al. (2006) obtained pyraclostrobin fungicide-resistant strains of *C. beticola* in the laboratory through ultraviolet mutagenesis. In addition, Secor et al. (2010) reported that some individual isolates of *C. beticola* had a 400-fold shift towards reduced sensitivity to pyraclostrobin. In light of the history of other *Cercospora* species shifting towards less sensitivity and resistance to some fungicides, in addition to our results showing a statistically significant shift toward less sensitivity to the QoI fungicide trifloxystrobin, it is important that *C. sojina* populations continue

to be monitored for their sensitivities to QoI fungicides. In addition, baseline sensitivities and resistance monitoring of *C. sojina* to other fungicide classes (such as DMI and MBC fungicides) should be initiated.

## TABLES

Table 3.1. Isolates of *Cercospora sojina* from soybean collected prior to 2001 used to determine baseline sensitivities to quinone outside inhibitor fungicides.

| State | Isolates |
|---|---|
| Alabama | S28, S29, S31, S32, S33, S35, S37, S39 |
| Arkansas | S96, S97, S99, S100 |
| Georgia | S1, S3, S5, S6, S7, S9, S10, S11, S12, S13, S14, S16, S18, S19, S40, S134 |
| Illinois | S127 |
| Iowa | S123, S124, S125 |
| Louisiana | S82, S83, S111, S114 |
| Mississippi | S85, S86, S90, S92, S95, S101, S102, S105, S106, S107, S108, S115, S116, S119, S121, S122 |
| South Carolina | S128, S130 |
| Wisconsin | S126 |

Table 3.2. Isolates of *Cercospora sojina* collected from soybean in 2007, 2008, and 2009.

| Year | State | County | No. of isolates |
|---|---|---|---|
| 2007 | Illinois | Gallatin | 4 |
| | | Henry | 4 |
| | | Logan | 1 |
| | | Pope | 5 |
| | | Tazewell | 1 |
| | | Vermillion | 3 |
| | | Warren | 4 |
| 2008 | Illinois | Alexander | 42 |
| | | Warren | 2 |
| | Missouri | Sainte Genevieve | 2 |
| 2009 | Illinois | Alexander | 16 |
| | | Gallatin | 2 |
| | | Piatt | 11 |
| | | Pope | 3 |
| | | White | 1 |
| | Missouri | Sainte Genevieve | 7 |

Table 3.3. Sensetivity of *Cercospora sojina* baseline isolates to azoxystrobin measured as effective concentration at which 50% of conidial germination was inhibited ($EC_{50}$) in nonamended potato dextrose agar and salicylhydroxamic acid (SHAM)-amended potato dextrose agar.

| | **$EC_{50}$ (µg/ml)** | | |
|---|---|---|---|
| **Isolate** | **Nonamended** | **SHAM-amended** | ***P* value**[a] |
| S5 | 0.0237 | 0.0200 | 0.4381 |
| S9 | 0.0208 | 0.0182 | 0.0173 |
| S10 | 0.0274 | 0.0185 | 0.0182 |
| S13 | 0.0228 | 0.0205 | 0.3166 |
| S22 | 0.0274 | 0.0226 | 0.0074 |
| Mean | 0.0243 | 0.0200 | 0.0001 |

[a] *P* value for individual isolates were determined using least-square means *t* tests; *P* value for comparison of overall isolate means of nonamended and SHAM-amended was determined from an *F* test.

Table 3.4. Comparison of mean effective fungicide concentrations that inhibited conidial germination by 50% ($EC_{50}$) for baseline *Cercospora sojina* isolates and isolates collected in 2007, 2008, and 2009.

| **Population** | **Azoxystrobin $EC_{50}$ (µg/ml)[a]** | **Pyraclostrobin $EC_{50}$ (µg/ml)[a]** | **Trifloxystrobin $EC_{50}$ (µg/ml)[a]** |
|---|---|---|---|
| Baseline | 0.01287 ab | 0.00028 a | 0.00116 b |
| 2007 | 0.00966 b | 0.00026 a | 0.00116 b |
| 2008 | 0.01506 a | 0.00027 a | 0.00153 ab |
| 2009 | 0.01627 a | 0.00031 a | 0.00163 a |

[a] Means within a column followed by the same letter are not significantly different according to Tukey's honestly significant difference test (alpha = 0.05).

**FIGURES**

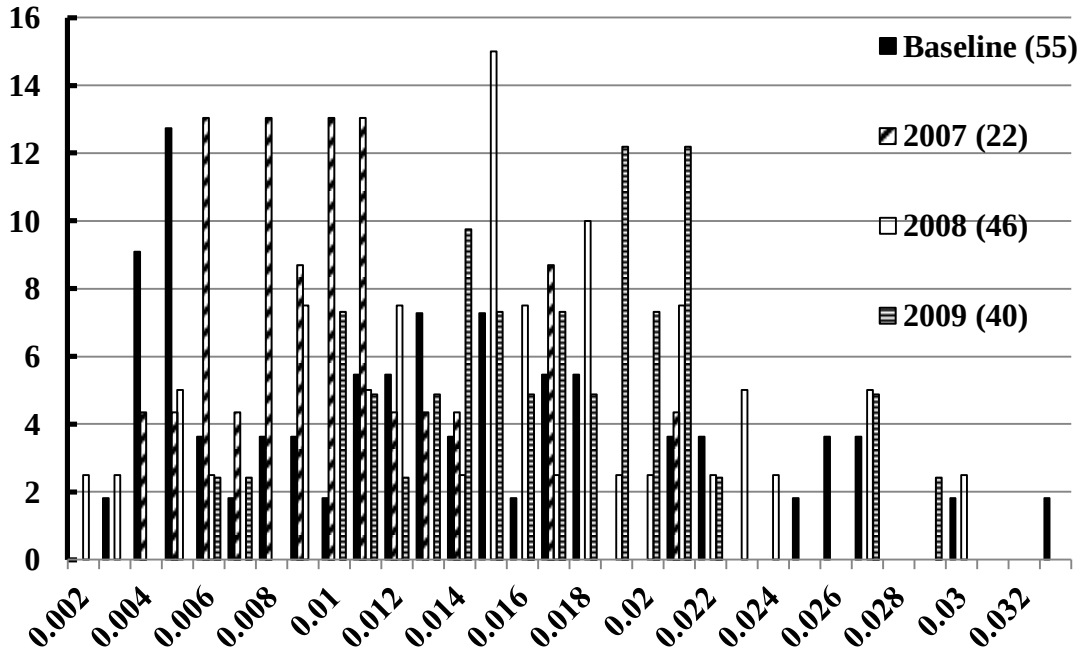


Figure 3.1. Frequency distribution of effective azoxystrobin concentrations that inhibited conidial germination by 50% ($EC_{50}$) for baseline *Cercospora sojina* isolates and isolates collected in 2007, 2008, and 2009.

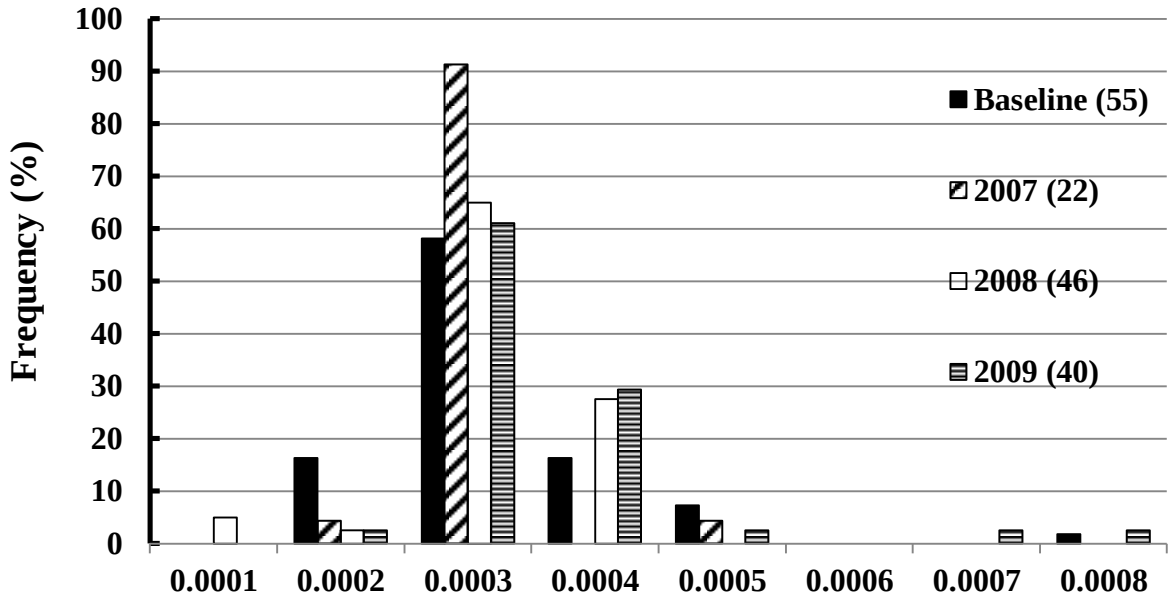


Figure 3.2. Frequency distribution of effective pyraclostrobin concentrations that inhibited conidial germination by 50% ($EC_{50}$) for baseline *Cercospora sojina* isolates and isolates collected in 2007, 2008, and 2009.

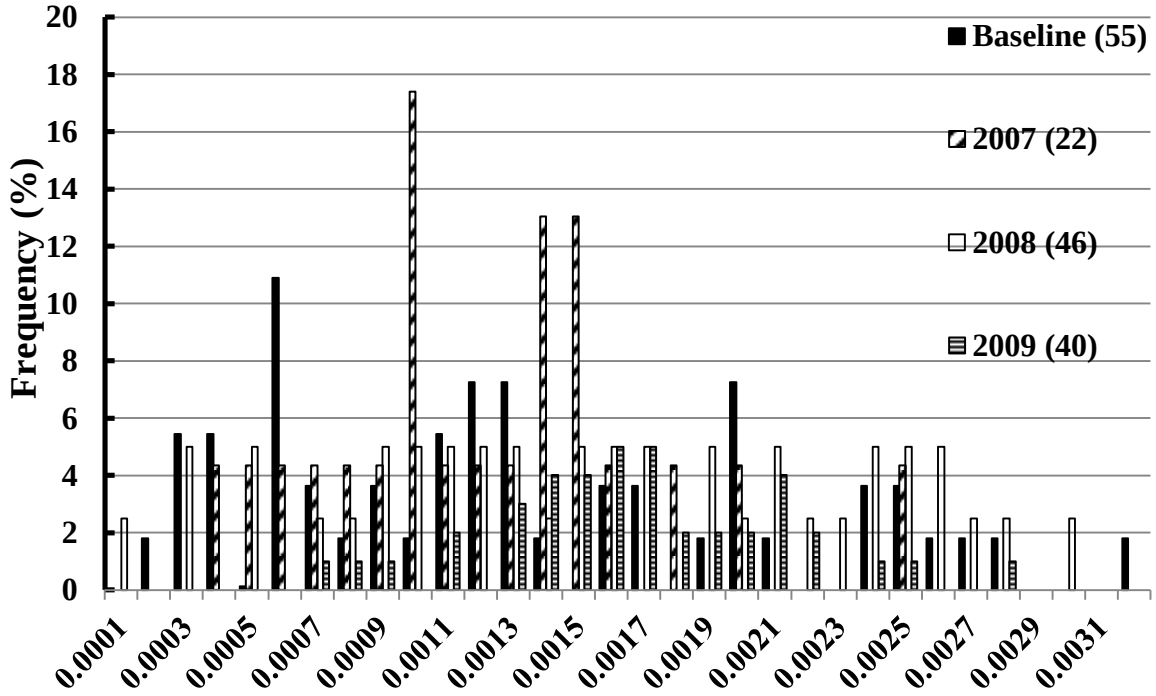


Figure 3.3. Frequency distribution of effective trifloxystrobin concentrations that inhibited conidial germination by 50% ($EC_{50}$) for baseline *Cercospora sojina* isolates and isolates collected in 2007, 2008, and 2009.

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# CHAPTER FOUR: QOI FUNGICIDE RESISTANT *CERCOSPORA SOJINA* AND MANAGEMENT WITH ALTERNATIVE FUNGICIDE CHEMISTRIES

## ABSTRACT

Frogeye leaf spot (FLS), caused by *Cercospora sojina*, causes yield reductions of soybean (*Glycine max* (L) Merr.) grown in the United States and other countries. A primary method of managing FLS is the use of foliar fungicides in the quinone outside inhibitor (QoI) class. Because QoI fungicides have a high risk of fungal pathogens developing resistance to them, a fungicide resistance monitoring program was established for *C. sojina* which included the determination of baseline sensitivities to QoI fungicides. QoI fungicide resistant isolates were found at two locations in Illinois, one location in Kentucky, and two locations in Tennessee. QoI fungicide sensitivity levels of the resistant isolates were over 200-fold higher than baseline isolates using petri dish assays. A greenhouse trial was conducted with a QoI-resistant *C. sojina* isolate from Tennessee and a QoI-sensitive baseline isolate to: i) confirm that the putative QoI-resistant isolates could cause FLS on leaves treated with QoI fungicides; and ii) determine if fungicides with other modes of action could control FLS caused by the QoI-resistant isolates. In greenhouse trials, FLS caused by the QoI-resistant isolate was not significantly ($P \leq 0.05$) reduced with QoI fungicides compared to a water control, but FLS caused by the QoI-sensitive isolate was significantly reduced with QoI fungicides compared to a water control. Several fungicides in the demethylation inhibitor (DMI) group and the methyl benzimidazole carbamate (MBC) fungicide, thiophanate methyl, significantly reduced FLS caused by the QoI-resistant and QoI-sensitive isolates compared to their respective water controls. These results indicate that *C. sojina* isolates resistant to QoI fungicides are present in Illinois, Kentucky, and Tennessee, and that FLS caused by QoI-resistant isolates may be managed with DMI or MBC fungicides.

## INTRODUCTION

Frogeye leaf spot (FLS) of soybean is caused by the fungal pathogen, *Cercospora sojina* Hara, and is an economically important disease in hot humid soybean-producing regions such as Brazil, China, Nigeria, and the southern U.S. (Phillips, 1999). In the U.S., it has recently been identified in areas as far north as Iowa, Wisconsin and Ohio (Yang et al., 2001, Mengistu et al., 2002). The estimate of soybean yield suppression due to FLS in the U.S. increased from approximately 23,000 to 270,000 metric tons from 1996 to 2007 (Wrather and Koenning, 2009). The recent increase in FLS range and severity has caused concern and interest among the soybean disease research groups throughout the U. S. (Mian, 2008). The use of resistant soybean cultivars is one of the best ways to manage frogeye leaf spot. Regardless of the availability of resistant soybean cultivars, foliar fungicides are still used as a method to manage FLS and other foliar diseases of soybean.

Quinone outside inhibitor (QoI) fungicides are one of the most recent groups of chemicals that play an important role in plant protection against many phytopathogenic fungi including (Anke et al 1997; Ammermann et al., 1992). QoI fungicides specifically inhibit cell respiration by binding at the ubiquinol oxidation center (Qo site) of the mitochondrial cytochrome bc1 complex II (Sauter et al., 1999; Bartlett et al., 2002). QoI fungicides have been classified as having a high risk of fungi developing resistance to them because of their specific, single–site mode of action (Brent, 2007). The first QoI fungicide resistant phytopathogenic fungus observed was *Blumeria graminis* on wheat, which was reported in Germany in 1998 (Sierotzki et al., 2000b; Chin et al., 2001). About 27 QoI fungicide resistant fungal species have been documented in the world and are listed by the Fungicide Resistance Action Committee (2012) such as *Blumeria graminis* (Sierotzki et al., 2000a), *Mycosphaerella fijiensis* (Sierotzki et al.,

2000a), *Venturia inaequalis* (Steinfeld et al., 2001), *Mycosphaerella graminicola* (Fraaije et al., 2005), *Colletotrichum graminicola* (Avila-Cruz et al., 2003), *Plasmopara viticola* (Chen et al., 2007), and *Alternaria spp.* (Ma et al., 2003). In most phytopathogenic fungi, resistance was conferred by the single point mutation known as G143A, a glycine-to-alanine substitution at amino acid codon 143. This G143A mutation expresses high (complete) resistance, and has no significantly negative effect on enzyme activity (Brasseur et al., 1996). Other mutations, such as the F129L mutation (phenylalanine to leucine substitution at codon 129) and the G137R mutation (glycine to arginine substitution at codon 137), have been shown to cause moderate (partial) resistance (Sierotzki et al., 2000a, 2000b). Because of the single-site mode of action, intensive use of QoI fungicides can increase selection pressure to favor resistant strains in pathogen populations (Bartlett et al., 2002; Gisi et al., 2002).

Fungicide applications to soybean have significantly increased over the past five years due to claims of both a reduction in foliar disease and an increase in "plant health"(Wise and Mueller, 2011). QoI fungicide active ingredients currently registered on soybean include azoxystrobin, fluoxastrobin, pyraclostrobin, and trifloxystrobin. The most important effect of QoI fungicides is their high efficacy in controlling infections at the first developmental stages (spore germination, zoospore release and motility) of the pathogen (Bartlett et al., 2002). Two families of fungicides are primarily used to effectively manage soybean foliar disease in practice. QoI fungicides are most effective when they are applied before infection takes place or at the very early stages of disease development (Bartlett et al., 2002). Demethylation inhibitor (DMI) fungicides can be applied after infection takes place but are most effective when applied at early stages of disease development to inhibit germ tube elongation, fungal penetration and mycelial growth (Mercer, 1991; Burden, et al., 1989). Some fungicide products registered for use on soybean contain both

a QoI and a DMI active ingredient, such as Quilt or Quilt Xcel (azoxystrobin + propiconazole; Syngenta Crop Protection, Greensboro, NC), Stratego (trifloxystrobin + propiconazole; Bayer CropScience, Research Triangle Park, NC), and Stratego YLD (trifloxystrobin + prothioconazole; Bayer CropScience). These products are useful for broad spectrum disease control and may help slow down the selection of isolates that are resistant to a particular class of fungicides (Giesler and Gustafson, 2008). Thiophanate methyl, a methyl benzimidazole carbamate (MBC) fungicide, sometimes is used to manage soybean diseases too. All three of the fungicide classes mentioned (QoI, DMI, and MBC) face resistance problems because of their specific sites of action. In Europe, some of the DMI fungicides have disappeared from the marketplace as resistance to them developed, and they no longer provided any benefit or advantage in plant disease control programs (Mueller and Bradley, 2008). Control failure due to resistance development to MBC was found in brown rot (*M. fructicola*) within a few years of MBC introduction in the mid-1960s (Anonymous, 1980). In the 1970s, benomyl, an MBC fungicide, was used extensively to control eyespot on wheat until resistance developed. After resistance developed to benomyl, none of the MBC fungicides could be used for managing this disease because the resistant strains of eyespot were as fit as susceptible strains (King and Griffin, 1985; Murray et al., 1990; Murray, 1996). Another well-documented example is the sustained resistance of *C. beticola* on sugar beet to MBC fungicides in Greece (Dovas et al., 1976).

Based on the results of QoI fungicide sensitivity assays on the *C. sojina* isolates collected in 2007, 2008 and 2009, the sensitivity distributions of *C. sojina* to QoI fungicides had slightly shifted over the years (see Chapter 3). The objectives of this study were to: (i) monitor for QoI fungicide resistance in *C. sojina* isolates collected from different states in 2010, and (ii)

determine if FLS caused by *C. sojina* isolates with resistance to QoI fungicides *in vitro* could be controlled by QoI fungicides and other fungicide classes under greenhouse conditions.

## MATERIALS AND METHODS

### QoI fungicide resistance monitoring

In total, 179 isolates of *C. sojina* were collected in 2010 from soybean production fields receiving QoI fungicide applications. Of the 179 isolates, 54 were collected from two Tennessee counties, 13 from one Kentucky County, 88 from six Illinois counties, 9 from two Mississippi counties, and 15 from two Indiana counties (Table 4.1). Drs. Melvin Newman (University of Tennessee), Don Hershman (University of Kentucky), Tom Allen (Mississippi State University), and Kiersten Wise (Purdue University) collected and sent the FLS-affected soybean leaf samples to the University of Illinois from Tennessee, Kentucky, Mississippi, and Indiana, respectively. Leaf samples from Illinois were collected by personnel in Dr. Carl Bradley's research program at the University of Illinois.

*C. sojina* isolates were obtained via single spore isolation directly from diseased tissue. Single conidia were transferred to petri dishes (100 mm diameter) containing soybean stem and lime bean agar (SSLB) with rifampicin (25 mg/liter) (Phillips and Boerma, 1980). These isolates were then incubated about one week under alternate light (fluorescent with black light 12 hours) and darkness (12 hours) in a growth chamber (25 ± 2°C) until the agar surface was covered with mycelia and conidia. Plugs with mycelia and conidia were stored in 1.5 ml micro centrifuge tubes containing 15% glycerin and frozen at -80°C for later use.

Technical grade formulations of azoxystrobin, trifloxystrobin, and pyraclostrobin were used to prepare stock solutions at a concentration of 100µg/ml in acetone. Serial dilutions in

acetone were prepared for each fungicide. Azoxystrobin was amended to potato dextrose agar (PDA) at 0.0001, 0.001, 0.01, 0.1, and 1 µg/ml. Trifloxystrobin or pyraclostrobin were amended to PDA at 0.00001, 0.0001, 0.001, 0.01 and 0.1µg/ml after PDA had been cooled to about 60°C after autoclaving. A stock solution of salicylhydroxamic acid (SHAM) was prepared at a concentration of 100 mg/ml in methanol. It was then added to all fungicide-amended media at a concentration of 60 µg/ml to inhibit the effects of the alternative oxidative pathways that some fungi use to overcome QoI fungicide toxicity in fungicide sensitivity assays *in vitro* (Bartlett et al., 2002; Wise et al., 2008).

*C. sojina* isolate preparation and conidia harvesting methods were adapted from Bradley and Pedersen (2011) and Wise et al. (2008). Thawed fungal plugs were placed onto SSLB media amended with rifampicin and were incubated in a growth chamber about one week to produce conidia. These isolates were subcultured on new SSLB agar several times to produce enough conidia to run the assays. Conidial suspensions were prepared by placing 4 or 5 plugs ($1cm^2$) containing conidia, mycelia, and SSLB media into 20 ml sterile glass tubes, adding 4ml sterile distilled water, and vortexing for 30 seconds. The conidial suspension was filtered through four layers of cheesecloth a sterile tube to remove any hyphal filaments. The concentration of the conidial suspension was adjusted to approximately $1 \times 10^5$ conidia per ml using a hemacytometer, and 75 µl of the conidial suspension was pipetted onto the fungicide-amended media surface, spread with a sterile bent glass rod, and incubated for 15-18 hours at 26°C in the dark. Using a compound microscope (10× magnification), fifty conidia were evaluated per petri dish by visually assessing conidia germination. Conidia with germination tubes at least as long as the conidia were considered germinated. For each of the two replicate dishes, conidial germination was converted to percent inhibition compared with the 0 µg/ml fungicide control

treatment by: 100 ([percent germination of fungicide-amended/[mean percent germination of 0µg/ml]). The concentration of fungicide that effectively reduced conidial germination by 50% relative to the untreated control ($EC_{50}$) was determined for each *C. sojina* isolate by linear interpolation using the two concentrations (Wise et al., 2008).

Because of limited space and time, fungicide sensitivities of *C. sojina* isolates were assayed over multiple trials. The reproducibility test described by Wong and Wilcox (2000) was used to validate each trial. *C. sojina* isolate S9 was used as an internal control for its sensitivity to azoxystrobin, trifloxystrobin and pyraclostrobin. If the $EC_{50}$ value of the internal control isolate did not fall within the 95% confidence interval in the test trial, the trial was dropped and repeated until the $EC_{50}$ of the internal control isolate was within its 95% confidence interval.

Initially, all 179 *C. sojina* isolates collected in 2010 were going to be assayed, and their $EC_{50}$ values of QoI fungicides were going to be calculated using the methods above. However, when the *C. sojina* isolates sent from Lauderdale County, TN were assayed, their $EC_{50}$ values were >100 fold higher than mean $EC_{50}$ values of baseline *C. sojina* isolates (see Chapter 3 for mean $EC_{50}$ values of baseline isolates). To increase the efficiency of monitoring for QoI resistant isolates, it was decided that discriminatory doses of azoxystrobin, pyraclostrobin, and trifloxystrobin would be established that would distinguish between QoI sensitive and resistant *C. sojina* isolates. Once established, the discriminatory doses would then be used to evaluate the rest of the *C. sojina* isolates. If additional QoI resistant *C. sojina* isolates were identified using the discriminatory doses, those isolates would then be assayed on all of the fungicide concentrations and their $EC_{50}$ values would be calculated using the methods above.

To establish discriminatory doses of QoI fungicides that would distinguish QoI resistant and sensitive *C. sojina* isolates, twenty-six *C. sojina* isolates from Lauderdale County, TN that

had been found to have very high $EC_{50}$ values of QoI fungicides and 26 *C. sojina* baseline isolates were used for this study. Isolates were tested on PDA amended with azoxystrobin or trifloxystrobin at 0, 0.01, 0.1, 1, 10, or 100µg/ml or PDA amended with pyraclostrobin at 0, 0.0001, 0.01, 0.1, 1, or 10µg/ml. SHAM was added to all of the fungicide concentration treatments at 60µg/ml.

The method to determination of discriminatory dose was the same as the fungicide assay used to determine for baseline sensitivities (see Chapter 3). Seventy five µl of the conidial suspension (1 x $10^5$ conidia per ml) were pipetted onto the fungicide-amended media surface spread with a sterile bent glass rod and incubated for 15-18 hours at 26°C in the dark. Using a compound microscope, fifty conidia were counted per treatment replication. Conidia with germination tubes at least as long as the length of conidia was considered germinated. Two replicate petri dishes (60 mm diameter) were prepared for each fungicide × *C. sojina* isolate combination for each fungicide concentration test. The petri dishes were arranged using a completely randomized design. Isolates were assayed on two replicate plates. Each isolate was assayed in at least two separate trials. QoI resistant isolate CS1036 and sensitive baseline isolate S9 were used as internal controls.

**Greenhouse fungicide study**

Seeds of a soybean cultivar susceptible to FLS (Asgrow 3101) were planted in 5 x 5 cm pots with Sunshine Mix 1 (Sun Gro Horticulture Inc., Bellevue, WA), placed in 20 x 30 cm trays, and grown under 1000 watt high pressure sodium bulbs set for a 12-h photoperiod, at 23 ± 1°C. After emergence, plants were thinned to 3 seedlings per pot; the plants in two pots were used as the experiment unit. Fungicide treatments and a water control were applied to 10 day old soybean plants inside a mechanized spray chamber. Fungicide treatments included the QoIs

azoxystrobin at 0.28 kg a.i./ha (Quadris, Syngenta Crop Protection), pyraclostrobin at 0.22 kg a.i./ha (Headline, BASF Corp. Research Triangle Park, NC), fluoxastrobin at 0.20 kg a.i./ha (Evito, Arysta LifeScience, Cary, NC), and trifloxystrobin at 0.13 kg a.i./ha (Gem, Bayer CropScience). DMI fungicide treatments included prothioconazole at 0.10 kg a.i./ha (Proline, Bayer CropScience), propiconazole at 0.19 kg a.i./ha (Tilt, Syngenta Crop Protection), tebuconazole at 0.13 kg a.i./ha (Bayer CropScience), tetraconazole at 0.08 kg a.i./ha (Domark, Valent BioSciences, Walnut Creek, CA), and flutriafol at 0.13 kg a.i./ha (Cheminova Inc., Research Triangle Park, NC). In addition, the MBC fungicide thiophanate methyl (Topsin M, United Phosphorus Inc., King of Prussia, PA) was evaluated at 0.59 kg a.i./ha. One day after fungicide treatments were applied, the unifoliolate leaves of the soybean plants were inoculated with either a QoI-resistant (isolate CS1036) or a sensitive (isolate S9) *C. sojina* isolate with a hand mist sprayer containing a conidial suppension ($6 \times 10^4$ condia per ml). The inoculated plants were then covered with a transparent plastic dome for 4 days to maintain high relative humidity (RH) (≥90%). Disease severity was rated 10 days after inoculation by visually estimating the percent area of the unifoliate leaves covered with FLS lesions. The experiment was designed as a split-plot arrangement in a randomized complete block (RCB). Isolates were considered as the whole plot factors and fungicide treatment as the subplot with four replicates. All main effects were considered fixed factors. The average disease severity was calculated for 6 plants from each experimental unit. The trial was repeated once over time.

**Data analysis for greenhouse study**

Data from each experiment were analyzed first separately using the general linear model procedure (PROC GLM) in SAS (version 9.2; SAS Institute, Inc., Cary, NC) to compute the variances. If the Brown and Forsythe's F test indicated homogenous variance, data from trials

were combined for further analysis. Disease severity data of greenhouse fungicide treatment were transformed to percent disease control through (1-(severity of treatment /severity of water))*100. The disease control data were transformed using logArcsin prior to analysis. PROC MIXED in SAS (version 9.2; SAS Institute, Inc., Cary, NC) was used to evaluate analysis of variance. Multiple comparisons (LSD) of fungicide interactions with pathogen strain were calculated using pdmix800 ($\alpha = 0.05$) in SAS (A. M. Saxton, University of Tennessee, Knoxville, TN).

## RESULTS

### QoI fungicide resistance monitoring

The analysis of variance from in vitro fungicide sensitivity assays showed that error variance of mean $EC_{50}$ values were homogenous ($P \geq 0.05$). Thus, the four trials of different fungicide assays for *C. sojina* isolates collected in 2010 and baseline isolates were combined for further analysis. For the establishment of discriminatory doses of fungicides, the concentrations that completely inhibited conidial germination of the QoI sensitive isolates but allowed at least 50% of the conidia of the QoI resistant isolates to germinate were 1 µg/ml of azoxystrobin, 1 µg/ml of trifloxystrobin, and 0.1 µg/ml of pyraclostrobin. These fungicide concentrations were determined to be the discriminatory doses that can be used to distinguish between QoI resistant and QoI sensitive *C. sojina* isolates.

Twenty-seven *C. sojina* isolates collected from Tennessee, 3 isolates from Kentucky, and 3 isolates from Illinois had $EC_{50}$ values at least 100-fold greater than the mean $EC_{50}$ values from *C. sojina* baseline isolates for azoxystrobin, pyraclostrobin, and trifloxystrobin and were considered to be resistant to QoI fungicides (Tables 4.1 and 4.2). *C. sojina* isolates with these

high $EC_{50}$ values were found in two different locations in Tennessee (Gibson and Lauderdale Counties), one location in Kentucky (Caldwell County), and two different locations in Illinois (Gallatin and Pope Counties). All of the isolates (26 out of 26) from the Lauderdale County, TN location had very high $EC_{50}$ levels, but the frequency of isolates with high $EC_{50}$ values were lower at the other locations, ranging from 3.5% (Gibson County, TN; one out of 28 isolates) to 23.1% (Caldwell County, KY; 3 out of 13 isolates).

**Greenhouse fungicide study**

The data from the two greenhouse experiments had homogeneous variances according to the Brown and Forsythe's Test; therefore, the data from the two experiments were combined together for further analysis. Significant interactions were observed between *C. sojina* isolates and fungicide treatments ($P < 0.0001$). Significant main effects of the fungicides and the *C. sojina* isolates also were observed ($P < 0.0001$) (Table 4.3). All fungicides tested provided moderate to high levels of control of FLS caused by the QoI sensitive *C. sojina* isolate (at least 60% control; Fig. 4.1). The QoI fungicides tested (azoxystrobin, pyraclostrobin, fluoxastrobin, and trifloxystrobin) provided little to no control of FLS caused by the QoI resistant *C. sojina* isolate (≤25% control); however, all of the DMI fungicides and thiophanate methyl provided moderate to high levels of control of FLS caused by the QoI resistant *C. sojina* isolates (approximately ≥75% control).

**DISCUSSION**

Isolates of *C. sojina* with high $EC_{50}$ values of the QoI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin were identified at two locations in Tennessee, two locations in Illinois, and one location in Kentucky. FLS caused by one of these isolates was not controlled

with QoI fungicides in the greenhouse, indicating that these isolates are resistant to QoI fungicides. The fields in which the QoI resistant isolates were collected differed in their histories of management practices. The field located in Lauderdale County, TN had been cropped to soybean since at least 2007 using no-tillage practices. QoI fungicides had been applied to the crops since at least 2009, and the field had a history of FLS (M. Newman, personal communication). The farmer of this field had reported a lack of FLS control in 2010 after two applications of fungicides containing QoI active ingredients. The Gibson County, TN field had been cropped to soybean since at least 2007 and had been used as a University of Tennessee research site since then to test foliar fungicides from different classes on soybean (M. Newman, personal communication). The two fields in Illinois and the field in Kentucky were most recently planted to soybean in 2008, and FLS had been observed in the past but was not a frequent problem (C. Bradley and D. Hershman, personal communications). The Illinois and Kentucky fields were being used as research sites to test foliar fungicides from different classes on soybean by the University of Illinois and University of Kentucky in 2010. Foliar fungicides had been applied to the Illinois and Kentucky fields or in adjacent fields in the past, but on a very infrequent basis.

All of the *C. sojina* isolates recovered from the Lauderdale County, TN field were resistant to QoI fungicides, while less than 25% of the *C. sojina* isolates recovered from the Illinois and Kentucky fields were resistant to QoI fungicides. Considering that the Lauderdale County, TN field had been continuously cropped to soybean without tillage for several years, the level of *C. sojina* inoculum likely was high. In addition, QoI fungicides (and no other classes) had been applied to the same field for several years. Considering all of this, the selection pressure being applied to the *C. sojina* population was greatest in the Lauderdale County, TN

field, which likely was the reason that all recovered *C. sojina* isolates were resistant to QoI fungicides. Selection pressure was lowest at the Illinois and Kentucky fields, where the fields had been rotated to other crops and fungicide use was infrequent, which is why QoI resistant *C. sojina* isolates were recovered less frequently than QoI sensitive isolates. Although the Gibson County, TN field had a history of fungicide use, fungicides from other classes in addition to QoI fungicides had been applied. The fact that other classes of fungicides were applied in this field may have slowed down the selection for *C. sojina* isolates with QoI resistance, which may be why QoI resistant isolates made up less than 4% of the isolates recovered. The differences in frequencies of recovered QoI resistant *C. sojina* isolates indicate that in situations where the only FLS management practice used was the application of QoI fungicides (i.e. Lauderdale County, TN field), the risk of fungicide resistance and the frequency of fungicide resistant isolates may be greatest. In addition, in fields where additional practices are used to manage FLS, such as crop rotation and use of different fungicide classes (i.e. Illinois and Kentucky fields), the risk of fungicide resistance and the frequency of fungicide resistant isolates may be lower. In light of these findings, it is apparent that a risk of selecting QoI resistant isolates, even in areas where fungicides are not frequently used, is still present. The high genetic variability within *C. sojina*, as observed by Bradley et al. (unpublished), may be a component of this risk, and only one QoI fungicide application may result in the selection of QoI resistant isolates in such a highly variable fungus.

The magnitude of the shift in QoI fungicide sensitivity from the *C. sojina* baseline isolates compared to the QoI resistant isolates is very high (>100 fold). In general, isolates that are at least 100 fold less sensitive to QoI fungicides have the G143A mutation (Fungicide

Resistance Action Committee, 2004). Additional research is needed to verify if resistance to QoI fungicides in *C. sojina* is due to the G143A mutation.

In light of the confirmation of the presence of QoI resistant *C. sojina* isolates in several states, additional surveys should be conducted to determine if resitant isolates are present in other counties and states where soybean is grown. The development of the discriminatory doses will help speed up the diagnosis of QoI resistant isolates, since isolates can be tested on media amended with only one concentration of fungicide rather than several. If the specific mutation responsible for QoI resistance in *C. sojina* is determined, PCR assays may be developed that can identify QoI resistant *C. sojina* isolates directly from *C. sojina* – infected soybean leaf DNA. This would significantly increase the efficiency of detecting QoI resistant isolates, as *C. sojina* would not have to be isolated and cultured from the plants. Such PCR assays have been successfully utilized in other fungicide resistance monitoring programs (Ma and Michailides, 2005; Avenot and Michailides, 2010).

In areas with high risk of FLS causing yield reductions to soybean, fungicide resistance management tactics should be implemented. According to Hewitt (1998), important anti-resistance strategies include: using fungicides only when necessary; applying fungicides at the manufacturer's recommended rate; avoiding multiple applications of fungicides with the same mode of action; applying combinations of fungicides with different modes of action; and avoiding treatments of large areas. Results of the greenhouse fungicide study indicated that fungicides in the DMI and MBC classes could be used to control FLS caused by a QoI resistant *C. sojina* isolate. When foliar fungicides are needed to control FLS, farmers should consider applying fungicides from the DMI or MBC classes, perhaps in combination with a QoI

fungicide. This practice should provide adequate control of FLS, while implementing a recommended anti-resistance strategy.

One of the fungicide resistance management strategies reported by Peever and Milgroom (1995) is to delay the evolution of resistance by using cultural methods, such as host plant resistance and crop rotation, to reduce the growth rates of both resistant and sensitive isolates. Soybean cultivars with high levels of resistance to *C. sojina* are available for farmers to plant, especially those in the southern U.S. In fields with histories of FLS, farmers should first use cultural practices, such as crop rotation and resistant cultivars, to manage FLS and not rely solely on fungicides. Cultivars that contain the *Rcs3* gene have been shown to confer resistance to all known races of *C. sojina* that occur in the U.S. (Cruz and Dorrance, 2009; Mian et al., 1998; Phillips, 1999; Phillips and Boerma, 1982).

As recommended by Hewitt (1998), using fungicides only when necessary is an important anti-resistance strategy. The use of QoI foliar fungicides on field crops for reasons other than disease control (i.e. physiological benefits) is being promoted (Wise and Mueller, 2011). In controlled studies, physiological effects, such as delaying senescence, altering amounts of plant hormones, increasing activity of antioxidative enzymes, and increasing nitrate reductase, have been reported (Glaab and Kaiser, 1999; Grossman et al., 1999; Grossman and Retzlaff, 1997; Ruske et al., 2003; Wu and von Tiedemann, 2001; Wu and von Tiedemann, 2002; Ypema and Gold, 1999). The increased use of QoI fungicides on field crops for reasons other than disease control could increase the selection of QoI resistant *C. sojina* isolates and isolates of other fungal pathogens of field crops; thus, it is important that farmers should apply fungicides only for purposes of disease management.

## TABLES

Table 4.1. Collection locations, total number of isolates, and number of QoI resistant isolates of *C. sojina* collected in 2010.

| State | County | Total no. of isolates | No. of QoI resistant isolates |
|---|---|---|---|
| Tennessee | Gibson | 28 | 1 |
| Tennessee | Lauderdale | 26 | 26 |
| Kentucky | Caldwell | 13 | 3 |
| Illinois | Gallatin | 19 | 2 |
| Illinois | Pope | 5 | 1 |
| Illinois | Warren | 19 | 0 |
| Illinois | DeKalb | 13 | 0 |
| Illinois | St. Clair | 20 | 0 |
| Illinois | Alexander | 12 | 0 |
| Mississippi | Rankin | 5 | 0 |
| Mississippi | Washington | 4 | 0 |
| Indiana | Tippecanoe | 15 | 0 |

Table 4.2. Effective concentrations of azoxystrobin, pyraclostrobin, and trifloxystrobin fungicides in which 50% conidial germination was inhibited ($EC_{50}$) for QoI fungicide resistant *Cercospora sojina* isolates collected in 2010 and the mean $EC_{50}$ values for baseline *C. sojina* isolates.

| **State** | **County** | **Isolate** | **Azoxystrobin** | **Pyraclostrobin** | **Trifloxystrobin** |
|---|---|---|---|---|---|
| | | | | **$EC_{50}$ (µg/ml)** | |
| Tennessee | Gibson | CS10117 | 2.9951 | 0.3964 | 1.1309 |
| Tennesee | Lauderdale | CS1023 | 3.0129 | 0.3318 | 1.0489 |
| | | CS1024 | 3.5403 | 0.4461 | 1.3954 |
| | | CS1025 | 3.1068 | 0.3182 | 0.9028 |
| | | CS1026 | 3.0041 | 0.2980 | 1.0111 |
| | | CS1027 | 3.0460 | 0.3486 | 1.2362 |
| | | CS1028 | 3.6752 | 0.3047 | 1.1454 |
| | | CS1029 | 3.9555 | 0.2975 | 0.4140 |
| | | CS1030 | 3.1448 | 0.3068 | 0.8148 |
| | | CS1031 | 3.0515 | 0.3051 | 0.7086 |
| | | CS1032 | 3.0684 | 0.2994 | 0.8070 |
| | | CS1033 | 3.1623 | 0.2879 | 0.8150 |
| | | CS1034 | 2.9762 | 0.2995 | 0.4381 |
| | | CS1035 | 3.0124 | 0.3214 | 0.8034 |
| | | CS1036 | 3.0515 | 0.2971 | 0.6904 |
| | | CS1037 | 3.2581 | 0.2998 | 0.7031 |
| | | CS1038 | 3.1376 | 0.3256 | 1.1222 |
| | | CS1039 | 3.0390 | 0.3051 | 1.3777 |
| | | CS1040 | 3.0295 | 0.3453 | 0.6035 |
| | | CS1041 | 3.2037 | 0.3088 | 0.6133 |
| | | CS1042 | 3.1240 | 0.3068 | 0.6771 |
| | | CS1043 | 3.4387 | 0.3321 | 0.9843 |
| | | CS1044 | 3.3651 | 0.3452 | 0.7172 |
| | | CS1045 | 3.2639 | 0.3113 | 1.1045 |
| | | CS1046 | 3.2042 | 0.3415 | 0.6834 |
| | | CS1047 | 3.1662 | 0.3475 | 1.2108 |
| Kentucky | Caldwell | CS1084 | 3.2750 | 0.3813 | 2.2565 |
| | | CS1090 | 3.1255 | 0.3162 | 0.5733 |
| | | CS1093 | 3.1623 | 0.3648 | 1.7004 |
| Illinois | Gallatin | CS1065 | 3.1623 | 0.3854 | 1.9012 |
| | | CS1076 | 3.0804 | 0.3045 | 0.8216 |
| Illinois | Pope | CS10187 | 3.5664 | 0.4204 | 1.8248 |
| | | Baseline Isolates | 0.01287 | 0.00028 | 0.00116 |

Table 4.3. Analysis of variance of frogeye leaf spot control with fungicides when soybean plants were inoculated with quinone outside inhibitor (QoI) fungicide resistant and QoI sensitive *Cercospora sojina* isolates in a greenhouse trial.

| **Effect** | **Degrees of freedom** | ***F* value** | ***P* > *F*** |
|---|---|---|---|
| Pathogen | 1 | 387.70 | <0.0001 |
| Fungicides | 12 | 49.85 | <0.0001 |
| Pathogen × fungicides | 12 | 13.21 | <0.0001 |

**FIGURES**

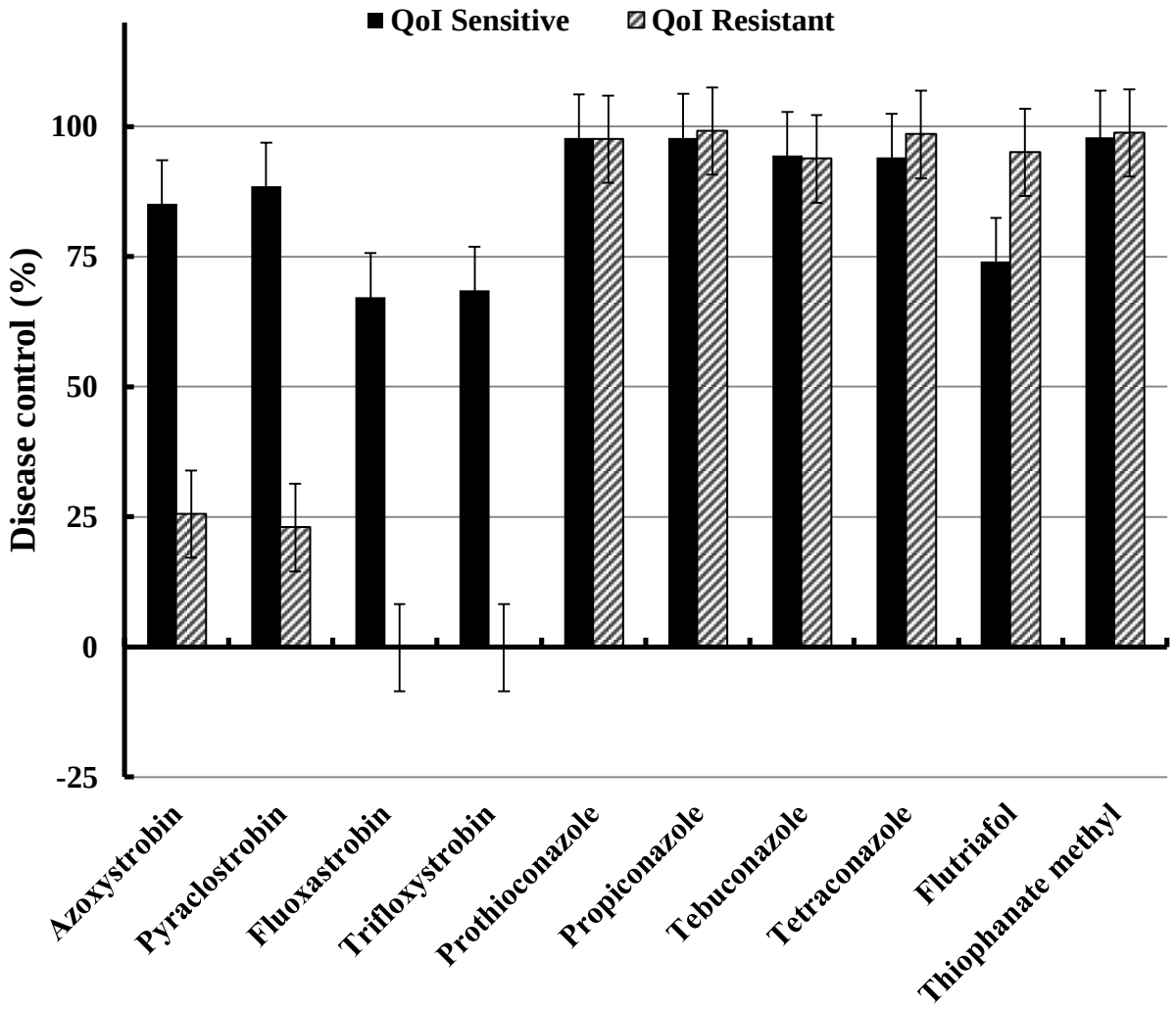


Figure 4.1. Percent disease control ( (1-(severity of treatment /severity of water))*100) of frog eye leafspot resulting from infection by QoI-sensitive and QoI-resistant *Cercospora sojina* isolates of soybean plants following the application of foliar fungicides in a greenhouse trial.

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# CHAPTER FIVE: COMPARISON OF QUINONE OUTSIDE INHIBITOR FUNGICIDE RESISTANT AND SENSITIVE ISOLATES OF *CERCOSPORA SOJINA*


## ABSTRACT

Quinones outside inhibitor (QoI) fungicide resistant isolates of *Cercospora sojina*, causal agent of frogeye leaf spot (FLS) of soybean, have been reported in Illinois, Tennessee and Kentucky in the United States. To develop the best management tactics for control of FLS caused by QoI resistant *C. sojina* and the best fungicide resistance management tactics, a better understanding of how QoI resistant *C. sojina* isolates compare to QoI sensitive isolates in their biology and their aggressiveness in causing FLS on different soybean cultivars is needed. Results from a laboratory study indicated that no differences in mycelial morphology, number of spores produced after 5 days, and radial growth after 6 or 12 days were observed between QoI resistant and sensitive *C. sojina* isolates. Results from a greenhouse study indicated that on a FLS susceptible cultivar ('Blackhawk'), QoI resistant *C. sojina* isolates caused significantly ($P \leq 0.05$) greater disease severity than QoI sensitive isolates 7 to 8 days after inoculation, but no differences in severity were observed after 9 days. On an FLS resistant cultivar with the *Rcs3* gene for resistance ('Davis'), QoI resistant *C. sojina* isolates caused significantly greater disease severity than QoI sensitive isolates 8 to 14 days after inoculation. In general, these comparisons between QoI resistant and sensitive *C. sojina* isolates indicate that they were similar in growth and sporulation, but the QoI resistant isolates were slightly more aggressive in causing greater FLS severity on soybean.

## INTRODUCTION

Frogeye leaf spot (FLS), caused by *Cercospora sojina* Hara causes yield reductions to soybean in the southern and north central United States. FLS can be managed by applying foliar fungicides, planting resistant soybean cultivars, rotating to non-host crops, and tilling to speed up decomposition of infested soybean debris (Phillips, 1999). Despite the multiple tactics available to manage FLS, many farmers have relied almost exclusively on the application of quinone outside inhibitor (QoI) fungicides for control of this disease. Because of this heavy reliance on QoI fungicides, *C. sojina* isolates resistant to QoI fungicides began to be detected in 2010 (see Chapter 4). These QoI resistant *C. sojina* isolates were collected from soybean fields in Tennessee, Kentucky, and Illinois in 2010. In addition to the fact that these *C. sojina* isolates are >100 fold less sensitive to QoI fungicides than baseline isolates ( isolates collected before fungicide exposuring to them), preliminary results from characterization of the cytochrome b gene of these isolates indicate that the G143A mutation is responsible for QoI resistance in these isolates (Bradley et al., unpublished). The G143A mutation occurs when glycine is substituted for alanine at codon 143 (Fungicide Resistance Action Committee, 2006). Results of studies on QoI fungicide resistant G143A mutations in *Sacchariomyces cerevisiae, Venturia inaequalis, Ustilago maydis,* and *Plasmopara viticola* indicate that a fitness penalty occurred with these isolates because functionally impaired mitochondria had reduced electron flow through the cytochrome bc1 complex (Köller et al., 2001; Zheng et al., 2000; Ziogas et al., 2002; Heaney et al., 2000). However, no fitness penalty was found with some G143A mutations such as those in *Blumeria graminis* (Heaney et al., 2000), *Magnaporthe grisea* (Avila-Adame and Köller, 2003) and *Magnaporthe oryzae* (Ma and Uddin, 2009). Fungicide resistant mutants of *M. grisea* were significantly less virulent than the sensitive strains, based on disease severity assessments. No

differences in QoI resistant and sensitive *M.grisea* isolates were observed for colony size and conidia formation. (Avila-Adame and Köller, 2003).

Pathogen fitness has sometimes been linked to isolates with greater aggressiveness. In a study with *Tapesia yallundae* and *T. acuformis* (causal agents of eyespot on wheat), isolates resistant to the methyl benzimidazole carbamate (MBC) fungicide carbendazim were evaluated (Bierman et al., 2000). In this study, a 50% stable coexistence of carbendazim resistant and sensitive strains was observed in carbendazim treated plots from 1987 to 2000. However, dicarboximide fungicide resistant *Botrytis cinerea* on geranium completely replaced the sensitive strain after two applications of vincozolin, while the resistant isolate remained at the initial level of 0.02% of the population in absence of vinclozolin (Vali and Moorman, 1992). A QoI-resistant population of *Erysiphe graminis* f.sp. *tritici* on wheat increased slowly and remained steady on untreated host tissue over three generations (Chin et al., 2001). However QoI resistant *Plasmorphara viticola* failed to increase in the pathogen population because of being less fit than sensitive wild types (Heaney et al., 2000). In another report, an *M. graminicola* isolate from chlorothalonil fungicide sprayed plots was more aggressive than isolates from plots that did not receive fungicide applications, and more frequenty fungicide applications and higher dosages were associated more strongly with increased pathogen aggressiveness (Kema et al., 1996).

*C. sojina* is a dynamic pathogen with extensive virulence or race diversity. Mian et al. (2008) proposed 12 soybean differentials and 11 *C. sojina* races, representing the major diversity among the population of isolates in the U.S. Races 2, 3, 4 and 5 of *C. sojina* have been identified as the major sources of FLS in the U.S. (Athow et al., 1962; Ross, 1968; Phillips and Boerma, 1981). Three resistant genes have been shown to condition resistance to these races; the *Rcs1* gene in `Lincoln' confers resistance to race 1 of *C. sojina* (Athow and Probst, 1952), the *Rcs2*

gene in `Kent' confers resistance to race 2 (Athow et al., 1962), and the *Rcs3* gene from 'Davis' confers resistance to race 5 and to all other known races of *C. sojina* in the U.S. (Boerma and Phillips, 1984; Phillips and Boerma, 1982) and to all isolates from Brazil (Yorinori, 1992). Some other dominant genes for resistance to race 5 were found in 'Ransom', 'Stonewall', and 'Lee' (Pace et al., 1993), and another single dominant gene that conditioned resistance to many isolates of *C. sojina* was found in 'Peking' (Baker et al., 1999). However, the aggressiveness and host adaptation of QoI fungicide resistant *C. sojina* isolates are unknown.

The objective of our study was to compare QoI resistant and sensitive *C. sojina* isolates for: (i) number of spores produced in culture; (ii) radial growth in culture; and (iii) aggressiveness in causing FLS on a susceptible and a resistant soybean cultivars in the greenhouse based assays.

## MATERIALS AND METHODS

### Sporulation and radial growth studies

A total of 24 (11 QoI resistant isolates and 13 QoI sensitive isolates) *C. sojina* isolates were used in sporulation and radial growth studies (Table 5.1). Each isolate was cultured from a single spore, obtained from a frogeye leaf spot lesion on a soybean leaf, and maintained on soybean stem and lima bean agar (SSLB) with rifampicin (25 mg/L) (Phillips and Boerma, 1980). The cultures were incubated for 5 days under alternating light (fluorescent with black light 12 hours) and darkness (12 hours) at 25±2°C until the agar surface was covered with mycelia and conidia. Conidia were washed with 5 µl of sterilized water with a pipette and transferred onto SSLB agar in petri dishes, then spread across the agar surface using a sterile bent glass rod. After 16 hours of incubation, six germinated spores were individually transferred onto SSLB agar. All of the plates were arranged randomly into two stacks at the same place on the laboratory bench at

room temperature. After five days, three well-sporulated colonies were cut and transferred separately into 1 ml microcentrifuge tubes containing 500 µl sterilized water for making suspension to calculate spores produced per colony. The colonies with spores and media were homogenized using toothpicks and mixed well by agitating with a vortex mixer. 10 µl of the resulting suspensions were then used to determine the concentration of spores with the aid of a hemacytometer. Another three colonies were left for measurement of radial growth. After 6 and 12 days, colonies growing in petri dishes were scanned using a flatbed scanner (Expression 1000XL; Epson America Inc., Long Beach, CA) and the mycelium growth area per colony was calculated using Assess 2.0 software (American Phytopathological Society, St. Paul, MN). Data were converted from area to radius values. Each isolate was assayed with two replications with 6 subsamples for sporulation and radial growth. The experiment for sporulation and mycelium growth was arranged using a completely randomized design (CRD) and was repeated once.

**Comparison of isolates for aggressiveness in the greenhouse**

A total of 10 *C. sojina* isolates were used to inoculate soybean plants in the greenhouse. Five isolates were resistant to QoI fungicides and five isolates were sensitive to QoI fungicides (Table 5.2). Cultures of each *C. sojina* isolate were maintained and conidia for inoculation were produced on SSLB agar. Conidia were collected from 5 day old colonies by placing all mycelia plugs with conidia into 150 ml sterilized water and agitating with the use of a vortex mixer for 2 minutes. The suspensions were passed through 4 layers of cheesecloth to remove large mycelia fragments. Conidial suspensions were adjusted to approximately $6 \times 10^4$ conidia per ml prior to use for inoculating plants in the greenhouse.

Seeds of soybean cultivars ‘Blackhawk’ (susceptible to FLS) and ‘Davis’ (resistant to FLS with the *Rcs3* gene) were planted in 5 x 5 cm pots containing Sun Shine Mix 1 planting

medium (Sun Gro Horticulture Inc., Bellevue, WA). Plants in the pots were placed in a 20 x 30 cm tray and grown under 1000 watt high pressure sodium bulbs set for a 12-h photoperiod, at 23±1°C on benches in an air-conditioned greenhouse. In one tray, 12 pots were planted with 'Davis', and another 12 pots were planted with 'Blackhawk'. After emergence, seedlings were thinned to one per pot. Ten days after emergence, the unifoliolate leaves were inoculated with one of the *C. sojina* isolates. Two leaves per plant were sprayed with a *C. sojina* conidial suspension. The inoculated plants were then covered with a transparent plastic dome and incubated for 4 days to maintain high relative humidity (RH) levels (≥90%). Wet paper towels were placed on the soil surface to enhace humidity levels. Each cultivar × isolate treatment had three replicates, and the experiment was repeated twice over time.

In trial 1, percent diseased leaf area per leaf (disease severity) was visually estimated and recorded at 11 and 14 days after inoculation. In trial 2, disease severity was recorded at 7, 8, 9, 10, 11, and 14 days after inoculation.

**Statistical analysis**

The experiment for sporulation and mycelium growth was analyzed as a completely randomized design (CRD) and was repeated once. Data from each experiment were first analyzed separately using the general linear model procedure (PROC GLM) in SAS (Version 9.3; SAS Institute, Inc., Cary, NC) to compute variances; then, a two-tailed F test for equality of variances was conducted to determine whether data from trials could be combined. Combined data were analyzed using PROC GLM in SAS, and least significant difference (LSD; $\alpha = 0.05$) analysis was used to compare sporulation and mycelial growth of different *C. sojina* isolates. Contrasts were used to compare QoI resistant and sensitive isolates of *C. sojina.*

In the greenhouse study, the variance of the two trials was not homogeneous. Therefore, the data of two trials were analyzed separately. The data were transformed using log-transformations inorder to normalize distributions. Data were analyzed as a factorial design with repeated measures. The mixed model procedure (PROC MIXED) (SAS 9.3) was used to calculate the analysis of variance, and multiple comparisons of soybean cultivars, isolates, and dates, as well as their interactions, were conducted using pdmix800 ($\alpha = 0.05$) in SAS (Arnold M. Saxton, University of Tennessee, Knoxville, TN). Contrast statements in SAS were used to compare groups of QoI resistant and sensitive isolates.

## RESULTS

### Sporulation and radial growth studies

Sporulation on SSLB agar varied among the 11 QoI resistants and 13 sensitive isolates (Table 5.1). The highest sporulation level observed was 11,083 conidia per area of colony with isolate CS1036, and the lowest level observed was 2,654 conidia per area of colony with isolate CS1033. Among all isolates, 8% had sporulation higher than 7,000 conidia per area of colony, and 17% were lower than 4,000 conidia per area of colony. Sporulation levels of isolates from Tennessee and Kentucky were more variable than were the sporulation levels of isolates from Illinois. Significant differences in sporulation were found among isolates within locations and within fungicide resistant and sensitive groups. However, the overall mean sporulation levels of QoI resistant isolates and sensitive isolates did not significantly differ from each other (Table 5. 1).

Hyphal morphology and average radial growth of QoI resistant isolates were the same as the sensitive isolates after 6 and 12 days (Table 5.3). Analysis of growth of all isolates at 6 and

12 days showed that growth of isolates collected from the same locations were similar. Mycelium growth of all isolates was less variable than sporulation.

**Comparison of disease severity on soyeans among isolates in greenhouse based assays**

Analysis of disease severity from two separate experiments showed similar results for 11 and 14 repeated measurement (experiment 1 recorded disease data only at 11 and 14 days). Therefore, only the data from experiment 2 were used for analyzing repeated measures data at 7, 8, 9, 10, 11, and 14 days.

Results of the analysis of variance showed significant differences in disease severity for main effects (soybean, isolate, and day), two way interaction effects (soybean × isolate and soybean × day) and three way interaction effect (soybean× isolate × day) (Table 5.4).

Frogeye leaf spot severity caused by the 10 *C. sojina* isolates significantly varied (Table 5. 5). On the FLS susceptible soybean cultivar 'Blackhawk', FLS severity caused by the QoI resistant isolates was significantly greater than that caused by the QoI sensitive isolates at 7 and 8 days after inoculation (Table 5.6; Fig. 5.1). However, at 9 to 14 days after inoculation, no significant differences between QoI resistant and sensitive isolates were observed for FLS severity. On the FLS resistant soybean cultivar 'Davis', FLS severity levels resulting from infection by the QoI resistant isolates did not significantly differ from those resulting from infection by the QoI sensitive isolates at 7 days after inoculation; however, from 8 to 14 days after inoculation, the QoI resistant isolates caused greater FLS severity than the QoI sensitive isolates. Overall, FLS severity levels on 'Blackhawk' were greater than those that developed on 'Davis', no matter which isolates were used (Figs. 5.2 and 5.3).

## DISCUSSION

### Sporulation and radial growth

A previous study of fitness-determining characteristics of pyraclostrobin (QoI fungicide) resistant mutants of *Cercospora beticola* showed that the mutations were pleiotropic, and had adverse effects on most of the mutant strains, such as reduction in sporulation (Malandrakis et al., 2006). In another study, the laboratory mutation(s) of *Botrytis cinerea* for resistance to pyraclostrobin also had adverse effects on the ability of mutants to compete with the wild-type strain due to their reduced sporulation and/or spore germination, but had no effect on the mycelial growth of mutant isolates (Markoglou et al., 2006). However, the G143A mutant of *Magnaporthe grisea* was not different from the wild-type strain when sporulation and mycelium growth were compared (Avila-Adame and Koller, 2003). In our study of *C. sogina* sporulation on SSLB agar, mean sporulation levels of QoI resistant isolates were not different from sporulation levels of sensitive *C. sojina* isolates. However, sporulation levels of both QoI resistant and sensitive isolates varied significantly. For example, mutant isolate CS1036, produced 2 to 5 times more conidia than most other isolates. Moreover, radial growth of CS1036 was greater than most other isolates at 6 and 12 days, which indicates that it might compete successfully with QoI sensitive isolates and become a practical problem in soybean fields. Even though mean sporulation and radial growth of QoI resistant isolates were not different on average, there were significant differences in sporulation and radial growth among individual isolates. It is interesting that some of the QoI resistant isolates sporulated more rapdily than did the sensitive isolates; consequently, these resistant isolates produced more conidia than the sensitive isolates after only three days (data not shown). This may be related with why lesion development was one day earlier on ‘Blackhawk’ when inoculated with a QoI resistant *C. sojina* isolate as

compared to inoculation with a sensitive isolate. Further studies are needed to determine whether mutations in QoI resistance genes affect other fitness components (spore viability and susceptibility to changes in environment) and aggressiveness (lesion size, sporulation capacities on host leaf, and latent period) that might influence the competitive ability of QoI resistant isolates under field conditions.

**Comparison of isolates for aggressiveness**

In the current experiment, aggressiveness was characterized/quantified in terms of the level of disease severity resulting from infection. Regardless of QoI sensitivity, isolates varied greatly in their aggressiveness levels. A high level of variability among *C. sojina* isolates also was observed in an AFLP genetic diversity study (Bradley et al., unpublished). The interesting result was that most of the QoI resistant isolates were more aggressive and showed greater levels of variation than did the sensitive isolates. Even though the sensitive isolates were collected from different geographical locations, they demonstrated similar levels of aggressiveness. These results indicate that aggressiveness of QoI resistance may be linked with fungicide resistant trait. Previous reports also showed that isolates of *M. graminicola* from fungicide treated fields were significantly more aggressive than isolates from the same cultivars that had not been treated with fungicides (Kema et al., 1996; Cowger and Mundt, 2002), which may indicate some reduction in fitness in the absence of fungicide. Fitness costs associated with resistance genes are important from an evolutionary perspective because they allow selection against resistance in absence of the fungicide, leading to a decrease in the frequency of resistant genes in the pathogen population (Brent, 1995; Fry and Milgroom, 1990). Examples of this are the decline in the frequency of the resistance characteristic in populations of *Magnaporthe oryzae* to azoxystrobin (QoI),

*Cercospora beticola* to demethylation inhibitors (DMI), and *Phytophthora capsici* to metalaxyl (Ma and Uddin, 2009; Staub,1991; Bruin and Edgington,1981).

Interactions of fungicide sensitive and insensitive isolates on cultivars may vary. Metalaxyl sensitive phenotypes of *Phytophthora infestans* were more aggressive than insensitive phenotypes on potato cultivars Cara and Stirling but not on Maris Piper (Day and Shattock, 1997). Although some differences were observed between QoI resistant and sensitive isolates in their aggressiveness on the soybean cultivars Blackhawk and Davis, the differences were sometimes minor and were not always observed throughout the course of the experiment. Other reports have shown that resistant hosts select for more aggressive pathogens than do susceptible hosts (Schouten and Beniers, 1997; Pink et al., 1992; Cowger and Mundt, 2002). It is not surprising that, the model of Gandon and Michalakis (2000) predicts that increasing levels of quantitative host resistance will select for increasing levels of damage caused by a parasite to the host. Our results may further confirm previous findings that isolates collected from fungicide treated cultivars were more aggressive than those from non-treated cultivars (Kema et al., 1996; Cowger and Mundt, 2002). The multisite fungicide and partial host resistance resulted in qualitatively similar selective pressures on the fungal populations exposed to them. Even QoI resistant isolates were more aggressive on 'Davis' than sensitive isolates, and the disease severity caused by QoI resistant isolates on 'Davis' still was much lower than that which developed on 'Blackhawk' (Table 5.4; Figs. 5.2 and 5.3). This indicates that the *Rcs3* allele present in 'Davis" conditioned resistance to both QoI resistant and sensitive *C. sojina* isolates, and that farmers should consider growing resistant cultivars with the *Rcs3* allele as part of FLS management and fungicide resistance management programs.

Overall, the genetic diversity and phenotypic differentiation of QoI resistant and sensitive isolates could have important implications for disease management. QoI resistant isolates of *C. sojina* might compete successfully with QoI sensitive isolates because of their aggressive potential to cause severe disease.

**TABLES**

Table 5.1. Comparison of QoI fungicide resistant and sensitive *Cercospora sojina* isolates for sporulation (number of conidia) 5 days after single conidia were placed on soybean stem lime bean agar.

| Isolate | QoI sensitivity | Geographic origin | Number of conidia[a] |
|---|---|---|---|
| CS1036 | Resistant | Lauderdale Co., TN | 11083 a |
| CS1091 | Sensitive | Caldwell Co., KY | 7421 b |
| CS10127 | Sensitive | Gibson Co., TN | 6854 bc |
| CS1065 | Resistant | Gallatin Co., IL | 6583 bcd |
| CS1090 | Resistant | Caldwell Co., KY | 6554 bcd |
| CS10110 | Sensitive | Gibson Co., TN | 6533 bcd |
| CS10190 | Sensitive | Pope Co., IL | 6196 bcde |
| CS1084 | Resistant | Caldwell Co., KY | 6088 bcde |
| CS1054 | Sensitive | DeKalb Co., IL | 5779 cdef |
| CS1076 | Resistant | Gallatin Co., IL | 5442 cdefg |
| CS10117 | Resistant | Gibson Co., TN | 5146 defgh |
| CS10187 | Resistant | Pope Co., IL | 4954 efghi |
| CS1068 | Sensitive | Gallatin Co., IL | 4925 efghi |
| CS1093 | Resistant | Caldwell Co., KY | 4788 efghi |
| CS10186 | Sensitive | Pope Co., IL | 4688 fghi |
| CS10116 | Sensitive | Gibson Co., TN | 4613 fghi |
| CS1049 | Sensitive | DeKalb Co., IL | 4271 ghij |
| CS1082 | Sensitive | Gallatin Co., IL | 4258 ghij |
| CS1031 | Resistant | Lauderdale Co., TN | 4038 ghijk |
| CS107 | Sensitive | Warren Co., IL | 3792 hijk |
| CS1013 | Sensitive | Warren Co., IL | 3604 ijk |
| CS1044 | Resistant | Lauderdale Co., TN | 3504 ijk |
| CS1097 | Sensitive | Caldwell Co., KY | 2788 jk |
| CS1033 | Resistant | Lauderdale Co., TN | 2654 k |
| **Mean of QoI resistant isolates** | | | **5530 a** |
| **Mean of QoI sensitive isolates** | | | **5055 a** |

[a]Means followed by the same letter are not significantly different from each other ($\alpha = 0.05$).

Table 5.2. Comparison of QoI fungicide resistant and sensitive *Cercospora sojina* isolates for radial growth 6 days and 12 days after single conidia were placed on soybean stem lime bean agar.

| Isolate | Radius of mycelial growth after 6 days (mm)[a] | Isolate | Radius of mycelial growth after 12 days (mm)[a] |
|---|---|---|---|
| CS1036* | 4.49 a | CS1036* | 9.36 a |
| CS1033* | 4.21 ab | CS10190 | 8.66 ab |
| CS10190 | 4.21 ab | CS1033* | 8.50 abc |
| CS1031* | 4.18 abc | CS1031* | 8.40 abcd |
| CS10186 | 4.14 abcd | CS1065* | 8.30 bcde |
| CS1068 | 4.08 abcde | CS1054 | 7.73 bcde |
| CS1054 | 3.99 abcdef | CS1097 | 7.70 bcde |
| CS1093* | 3.97 abcdefg | CS10186 | 7.62 cdef |
| CS107 | 3.96 abcdefg | CS10187* | 7.58 cdef |
| CS10187* | 3.91 abcdefg | CS1068 | 7.47 defg |
| CS1090* | 3.88 abcdefgh | CS1076* | 7.42 defgh |
| CS1097 | 3.79 bcdegh | CS1091 | 7.34 efgh |
| CS1049 | 3.77 bcdefghi | CS1049 | 7.26 fgh |
| CS1065* | 3.74 bcdefghi | CS1090* | 7.24 fgh |
| CS1084* | 3.71 bcdefghi | CS107 | 7.24 fgh |
| CS1044* | 3.68 bcdefghi | CS1044* | 7.20 fgh |
| CS1091 | 3.66 cdefghi | CS10110 | 7.17 fgh |
| CS10110 | 3.63 defghi | CS1084* | 7.13 fgh |
| CS1082 | 3.59 efghi | CS10116 | 7.08 fgh |
| CS1076* | 3.54 efghi | CS1093* | 7.05 fgh |
| CS1013 | 3.52 fghi | CS1082 | 6.91 fgh |
| CS10117* | 3.45 ghi | CS10127 | 6.84 fgh |
| CS10127 | 3.43 ghi | CS10117* | 6.53 gh |
| CS10116 | 3.36 hi | CS1013 | 6.43 gh |
| **Resistant isolates mean** | **3.88 a** | **Resistant isolates mean** | **7.70 a** |
| **Sensitive isolates mean** | **3.78 a** | **Sensitive isolates mean** | **7.34 a** |

*Isolate is resistant to QoI fungicides.

[a]Means followed by the same letter are not significantly different from each other ($\alpha = 0.05$).

Table 5.3. *Cercospora sojina* isolates used to inoculate soybean cultivars 'Blackhawk' and 'Davis' in a greenhouse study where isolates were compared for their aggressiveness in causing frogeye leaf spot.

| **Isolate** | **QoI fungicide sensitivity** | **Geographic origin** |
|---|---|---|
| CS10187 | Resistant | Pope Co., IL |
| CS10117 | Resistant | Gibson Co., TN |
| CS1036 | Resistant | Lauderdale Co., TN |
| CS1065 | Resistant | Gallatin Co., IL |
| CS1093 | Resistant | Caldwell Co., KY |
| CS10190 | Sensitive | Pope Co., IL |
| CS10116 | Sensitive | Gibson Co., TN |
| CS10654 | Sensitive | DeKalb Co., IL |
| CS1082 | Sensitive | Gallatin Co., IL |
| CS1091 | Sensitive | Caldwell Co., KY |

Table 5.4. Analysis of variance of frogeye leaf spot severity with the main and interactive effects of soybean cultivar, QoI sensitivity of *Cercospora sojina* isolates, and days after inoculation in a greenhouse study.

| Effect | Degrees of freedom | *F*-value | *P* > *F* |
|---|---|---|---|
| Cultivar | 1 | 1302.0 | 0.0001 |
| QoI sensitivity | 1 | 54.1 | 0.0001 |
| Cultivar × QoI sensitivity | 1 | 13.4 | 0.0003 |
| Day | 5 | 93.4 | 0.0001 |
| QoI sensitivity × day | 5 | 26.1 | 0.0001 |
| Cultivar × day | 5 | 0.7 | 0.6426 |
| Cultivar × day × QoI sensitivity | 5 | 4.9 | 0.0003 |

Table 5.5. Effect of *Cercospora sojina* isolates resistant and sensitive to quinone outside inhibitor (QoI) fungicides on frogeye leaf spot severity. Mean results presented are averaged over two soybean cultivars (Davis and Blackhawk) and averaged over different rating dates (7, 8, 9, 10, 11, and 14 days after inoculation) in the greenhouse.

| **Isolates** | **Severity (%)[a]** |
|---|---|
| CS10117* | 12.30 a |
| CS1065* | 8.43 b |
| CS1036* | 8.25 b |
| CS1093* | 6.36 c |
| CS1054 | 5.72 cd |
| CS10116 | 5.46 cd |
| CS10187* | 4.69 de |
| CS10190 | 4.68 de |
| CS1082 | 4.52 de |
| CS1091 | 4.16 e |
| **Resistant isolates mean** | **7.61 a** |
| **Sensitive isolates mean** | **4.87 b** |

[a]Means followed by the same letter are not significantly different from each other ($\alpha = 0.05$).

*Resistant to QoI fungicides

Table 5.6. Results of a greenhouse study comparing the effect of quinone outside inhibitor (QoI) fungicide resistant and sensitive *C. sojina* isolates' aggressiveness in causing frogeye leaf spot severity on a susceptible ('Blackhawk') and resistant ('Davis') soybean cultivar at 7, 8, 9, 10, 11, and 14 days after inoculation.

| **Cultivar** | **QoI sensitivity** | **Days after inoculation** | **Disease severity (%)[a]** |
|---|---|---|---|
| Blackhawk | Resistant | 7 | 4.63 gh |
| Blackhawk | Sensitive | 7 | 1.73 kl |
| Blackhawk | Resistant | 8 | 12.15 e |
| Blackhawk | Sensitive | 8 | 7.81 f |
| Blackhawk | Resistant | 9 | 21.04 cd |
| Blackhawk | Sensitive | 9 | 19.01 d |
| Blackhawk | Resistant | 10 | 31.24 bc |
| Blackhawk | Sensitive | 10 | 31.59 bc |
| Blackhawk | Resistant | 11 | 38.59 ab |
| Blackhawk | Sensitive | 11 | 43.89 ab |
| Blackhawk | Resistant | 14 | 49.38 a |
| Blackhawk | Sensitive | 14 | 51.59 a |
| Davis | Resistant | 7 | 1.21 lm |
| Davis | Sensitive | 7 | 1.19 m |
| Davis | Resistant | 8 | 2.67 ij |
| Davis | Sensitive | 8 | 1.36 lm |
| Davis | Resistant | 9 | 2.83 ij |
| Davis | Sensitive | 9 | 1.40 lm |
| Davis | Resistant | 10 | 2.97 ij |
| Davis | Sensitive | 10 | 1.49 klm |
| Davis | Resistant | 11 | 3.36 hi |
| Davis | Sensitive | 11 | 1.58 kl |
| Davis | Resistant | 14 | 5.83 fg |
| Davis | Sensitive | 14 | 2.19 jk |

[a]Means followed by the same letter are not significantly different from each other ($\alpha = 0.05$).

**FIGURES**

Figure 5.1. Symptoms of frogeye leaf spot caused by a quinone outside inhibitor (QoI) fungicide sensitive *Cercospora sojina* isolate (CS1036) (left) and a QoI resistant *C. sojina* isolate (CS1091) (right) on the susceptible soybean cultivar 'Blackhawk' 7 days after inoculation in the greenhouse.

Figure 5.2. Symptoms of frogeye leaf spot caused by a quinone outside inhibitor (QoI) fungicide resistant *Cercospora sojina* isolate (CS10117) on the resistant soybean cultivar 'Davis' ( left ) and susceptible cultivar 'Blackhawk' (right) at 14 days after inoculation in the greenhouse.

Figure 5.3. Symptoms of frogeye leaf spot caused by a quinone outside inhibitor (QoI) fungicide sensitive *Cercospora sojina* isolate (CS1054) on the resistant soybean cultivar 'Davis' ( left ) and susceptible cultivar 'Blackhawk' (right) at 14 days after inoculation in the greenhouse

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