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Evaluation of fungicide efficacy against *Cercospora sojina* and selection for QoI-fungicide resistance in soybean

Alicia Mercedes Cochran
University of Tennessee - Knoxville, acochr10@vols.utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Alicia Mercedes Cochran entitled "Evaluation of fungicide efficacy against *Cercospora sojina* and selection for QoI-fungicide resistance in soybean." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Heather M. Kelly, Major Professor

We have read this thesis and recommend its acceptance:

Alemu Mengistu, Angela McClure, Kurt Lamour

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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DEDICATION

I dedicate this work to my Dad.

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ABSTRACT

Frogeye leaf spot (FLS) of soybean is caused by *Cercospora sojina*. In 2010, resistance to the quinone outside inhibitor (QoI) fungicides was reported. Since then, evaluating FLS for QoI-resistance has been of particular interest in Tennessee and other soybean-producing states. In order to determine alternative fungicide options, fungicides with solo and combination mode of action were tested in 2013-2014. The objectives were,1) to evaluate fungicide efficacy for disease control and yield protection, and 2) evaluate selection pressure for QoI-resistance. Treatments included the following fungicide groups: QoI, DMI, MBC, Chlorothalonil, QoI+DMI, SDHI+QoI, SDHI+QoI+DMI, and DMI+MBC. QoI and Chlorothalonil treatments failed to control FLS. Any product with a QoI-component listed as an active ingredient exerted greater selection for QoI-resistance than products lacking QoI-components. Combination-QoI treatments provide better disease control than solo strobilurin treatments, but still exhibited selection pressure for resistance. Chlorothalonil and SDHI+QoI treatments were not as effective as alternate modes of action at controlling FLS when there was a high proportion of resistance in a field. The four fungicide groups with the greatest efficacy were the solo-DMI, solo-MBC, combination DMI+MBC, and combination SDHI+QoI+DMI treatments. The 3rd objective was to assess *C.sojina* epidemiology in Tennessee and whether or not it varied across the state. This was accomplished by sampling non-treated portions of farmer's fields in 2014 and 2015 across west and middle Tennessee, referred to as sentinel plots. The sentinel plot samples revealed that selection for QoI resistance and progression of FLS disease was less favorable in middle Tennessee compared to west

Tennessee. Furthermore, upper canopies of soybeans were more likely to harbor greater levels of QoI-resistant *C.sojina* than lower canopies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Soybean History and Production

Modern soybean (*Glycine max* Merrill) is the product of domestication of *Glycine soja* in China more than 5,000 years ago (Li et al., 2013). Pre-domesticated soybean would produce black seeds and display weed-like growth; however, modern breeding selected for smaller, erect plants, with less branching, and greater yields (Li et al., 2013). In order to germinate, soybeans require 50% moisture per dry weight and soil temperatures of at least 10°C; soybean growth is favored by soil pH between 5.8-7.0, as well as ambient temperatures between 25-30°C (Compendium of Soybean Diseases, 1999). Soybean production is common in temperate and subtropical climates. Soybean cultivars are assigned to one of 13 maturity groups (000-X) on the basis of their response to day length because they vary in latitude adaptations. Soybeans in the southern United States typically display determinate growth, but indeterminate growth is more common in the northern states (McWilliams & Berglund, 1999). Soybeans grow in a series of vegetative stages described according to the number of trifoliate leaves produced, but production of the first flower signals the beginning of the reproductive growth stage (R1) and the plants reach maturity at R8 when 95% of the pods turn brown (McWilliams & Berglund, 1999).

Although soybeans were initially introduced in 1765, prior to the 20th century large-scale production of soybean was uncommon in the United States (USDA-ERS, 2012). Soybean crops became favorable due to improved yields, and reduced production costs primarily as a result of the introduction of herbicide-tolerant varieties

(USDA-ERS, 2012). Soybeans are used as a protein source for human and animal consumption, in vegetable oil, and in industrial processes (Compendium of Soybean Diseases, 1999).

In the U.S., soybeans follow corn as the most frequently planted crop (77.5 million acres in 2009), and account for 90% of oilseed production (USDA-ERS, 2012). More than two-thirds of U.S. soybeans are grown in the upper Midwest, but production also occurs down along the Mississippi river through to Louisiana, as well as in the southeastern states (USDA-NASS, 2010). In 1994, the top ten soybean producing countries were: U.S., Brazil, China, Argentina, India, Canada, Paraguay, Indonesia, Italy, and Bolivia. That year, in these countries, an estimated $3.31 billion in revenue were lost due to reduced soybean yield (~15 million metric tons) as a result of soybean diseases, particularly soybean cyst nematode (*Heterodera glycines*), stem canker (*Diaporthe phaeseolorum* var. *caulivora*), brown spot (*Septoria glycines*), and charcoal rot (*Macrophomina phaseolina*) (Wrather et al., 1997). Prior to 2005, seed treatments were the common fungicide application soybeans received; however, the detection of Asian soybean rust (*Phakopsora pachyrhizi*) in North and South America beginning in the early 2000s resulted in increased application of foliar fungicide applications in many soybean production systems (Dorrance, et al., 2009).

In 1994, *Cercospora sojina*, causal agent of frogeye leaf spot (FLS) disease, was responsible for an estimated 506,800 metric ton reduction in yield within the top 10 soybean producing countries (Wrather et al., 1997). Previous research has indicated that there is a negative correlation between FLS severity and soybean yield in terms of

diminished seed weight, particularly if infection occurs prior to flowering (Dashiell & Akem, 1991). The decrease in plant photosynthesis, as a result of FLS infection, will negatively impact pod-fill and could potentially cause up to 66% yield loss in a susceptible cultivar (Dashiell & Akem, 1991). In 2009, FLS was responsible for the loss of an estimated 7.5 million soybean bushels amongst 28 U.S. states (Koenning & Wrather, 2010). Dr. Melvin Newman from the University of Tennessee estimated that, within the span of 5 years (2009-2013), FLS was responsible for a 16% yield loss in Tennessee soybean production, translating to a loss of 8.2 million bushels which, given an average market price of $11.45, would total over $ 90 million in lost revenue (Kelly, 2013).

Frogeye leaf spot

Pathogen History

The genus *Cercospora* was first established by Fresenius in 1863 (Groenewald et al., 2013). *Cercospora sojina* Hara (syn. *Cercospora diazu* Miura) was originally identified in Japan in 1914 (Chupp, 1954), followed by a secondary identification in Manchuria (present day China) in 1918 on soybean leaves (Lehman, 1928). Although there is speculation that the first incidence of frogeye leaf spot (FLS) disease on soybean in the United States occurred in South Carolina in 1924, specimens were neither stored nor evaluated; therefore, the first confident reports of *C.sojina* on soybean originated from Louisiana and North Carolina in 1925 (Lehman, 1928). Frogeye leaf spot lesions primarily affect the soybean foliage which, depending on the percent leaf area affected, can significantly impact the photosynthetic capacity of the

plant and lead to premature defoliation, posing a serious threat to soybean yield (Lehman, 1928).

Pathogen Description and Disease Development

FLS is a globally-distributed soybean disease with reported yield losses as high as 60% (Mian, et al., 2008). While common in the southern United States due to the warm and humid conditions, FLS has been reported in northern states such as Wisconsin and Ohio (Cruz & Dorrance, 2009; Mengistu, Kurtzweil, & Grau, 2002). Fungal mycelium will overwinter and sporulate from infested soybean debris (Cruz & Dorrance, 2009). Viable *C.sojina* specimens have been recovered from soybean leaf debris after 2 years in an Illinois field (Zhang, 2012). The pathogen can infect soybean seeds by entering through pores and cracks in the seed coat and then spreading to neighboring tissues (Singh & Sinclair, 1985). Within the seed, hyphae are rarely found on the cotyledons or the seed embryo; however, seedlings germinated from infected seed may be stunted and display lesions on the cotyledons (Mian et al., 2008; Singh & Sinclair, 1985). Gray to brown discolorations are typical on infected seed (Singh & Sinclair, 1985).

FLS lesions may be observed on soybean leaves, stems, and pods (Lehman, 1934). Stem lesions are reddish-brown in color, twice as long as they are wide and often observed late in the season, if at all; pod lesions are light gray-black and will depress the pod tissues (Mian et al., 2008). Foliar lesions are the most common and initially appear as small, red-brown spots which widen in diameter as the disease progresses, but eventually appear as circular or irregularly-shaped spots with pale gray-

brown centers around which a red-brown border is maintained (Lehman, 1928). Lesions are usually 1-3 mm in diameter but may reach 10 mm across, and become more irregular, once coalesced (Lehman, 1928). In older lesions, the brown band will appear raised above neighboring healthy leaf tissues but, unlike leaf spots caused by other soybean pathogens, no chlorosis is observed beyond the confines of the lesion (Lehman, 1928). Plant cells within the confines of the lesion demonstrate “complete collapse”, but are not colonized with mycelium except at the margin, which suggests that the fungus produces a compound to degrade the host tissues (Lehman, 1928).

C.sojina has been reported to produce the cercosporin toxin common to the genus; however, alternative studies indicate that *C.sojina* may have lost the ability to produce this toxin (Agarwal & Sinclair, 1996; Goodwin, Dunkle, & Zismann, 2001). Cercosporin is a light- activated toxin which generates reactive oxygen species that can disrupt plant cell membranes and cause leakage of cellular contents; thus, providing the fungus with access to nutrients (Daub & Chung, 2007). In the area bordering the necrotic FLS lesion, plant cells will appear jumbled, and display an accumulation of chlorophyll and greater levels of starch than healthy tissues (Benedict & Fucikovsky, 1966). Clusters of darkly-pigmented conidiophores (52-120µ x 4-4.5µ) can be seen emerging from the middle of the lesions on either side of the leaf, but tend to be produced with greater prevalence on the abaxial surface (Lehman, 1928).

Conidia are generated at the tips of the conidiophores and curved scars can be detected microscopically on the conidiophores at the sites of spore production; a single conidiophore may produce 1-11 asexual conidia (Lehman, 1928). Conidia (5-7µm x 39-

70µm) may be produced on infested debris and seeds, and can germinate in the presence of adequate moisture one hour after coming in contact with susceptible tissue (Mian et al., 2008). These asexual conidia will be spread by rain and wind, and may cause secondary infections within a season, potentially as soon as four weeks after the initial infection (Mengistu et al., 2011; Mian et al., 2008). The conidia produced by *C.sojina* are typically wider in the middle and taper slightly at one or both ends; the conidia are colorless and display multiple septa but specific dimensions depend significantly on environmental conditions, for example conidia tend to be more slender under abundant moisture conditions (Lehman, 1928). Conidia will germinate and produce hyphae within 18 hours in tap water at 25°C, and may retain viability even after 3 months on dry leaf tissue; however, the neighboring cells within a conidium may not display the same level of viability and the non-viable compartments appear less turgid than adjacent sections (Lehman, 1928). No sexual cycle has been confirmed for *C.sojina*, but various *Cercospora* species have been associated with teleomorphs in the *Mycosphaerella* genus and the relatively equal distribution of mating type loci in *C.sojina* field specimens suggests that sexual reproduction is occurring (Bradley et al., 2012; Goodwin et al., 2001; Kim et al,, 2013).

Frogeye leaf spot disease on soybean is favored by warm (25-30°C) and humid (>90%) conditions, but the pathogen can withstand below zero overwintering conditions (Cruz & Dorrance, 2009; UT Crops, 2013). *C.sojina* conidia can be isolated from FLS lesions and transferred to a variety of different agar media to induce germination and/or sporulation (Gomez & Reis, 2013). Under artificial light conditions (12 hrs) at 25±2 °C

and in the presence of certain substrates (potato dextrose agar, soybean extract agar, soybean seed extract agar, tomato extract agar, oat meal agar, and V8 agar), there is some indication that the use of filter paper to separate the conidia from the agar may result in greater sporulation as compared to unfilter-papered cultures under the same conditions; however, the absence of light and/or filter paper does not have a significant negative impact on spore production (Gomez & Reis, 2013). *C.sojina* has been observed to sporulate on plain agar media (Lehman, 1928). Fungal colonies take on a velvety, gray-brown appearance and concentric growths with dark gray to olive-brown interiors are often observed on potato dextrose agar (Lehman, 1928). The fungus will grow normally from pH 3.6-9.6, but displays abnormal growth at pH 2.6 (Lehman, 1928). The optimal temperature for hyphal growth of *C.sojina* on V8 agar is 25°C (Cruz & Dorrance, 2009). Greenhouse conditions with high humidity and temperature averaging 22°C are conducive for FLS disease development 2 weeks post-inoculation with conidial suspensions, and young soybean leaves are more susceptible to infection since they will display greater numbers and larger lesions than older leaves (Lehman, 1928). Under favorable greenhouse conditions artificially-inoculated, fully-expanded leaves display few lesions (Mian et al., 2008).

Disease Control

Crop rotation out of soybean for two years, tillage, planting resistant cultivars and pathogen-free seed are management strategies to reduce selection for fungicide resistant strains of the fungus (Mian et al., 2008; Zhang et al., 2012). Plowing may reduce spread of inoculum, but will not necessarily impact viability of *C.sojina* within a

field (Zhang, 2012). Additionally, a recent study (Mengistu et al., 2014) indicated that tilled and no-till fields display no significant difference, in FLS disease severity, in the absence of fungicide application; however, when a fungicide was applied tilled fields had less FLS disease than no-till fields. When a fungicide treatment is necessary, an application between the late-flowering (R1) and beginning-pod (R5) stage of soybean development is typically recommended (Mian et al., 2008). Some studies suggest that a fungicide application, at both R1 and R3 soybean growth stages, may offer better FLS control and higher soybean yields than applications made at alternative times (Akem, 1995).

Resistant soybean cultivars managed to keep FLS under control in the U.S. until multiple novel races of *C.sojina* were reported beginning in the 1950s (Mian et al., 2008). There are three soybean genes acknowledged as conferring resistance to FLS (*Rcs1*, *Rcs2*, and *Rcs3*), of which only *Rcs3* has demonstrated resistance to all *C.sojina* isolates within the U.S. (Mengistu et al., 2011). Field screening experiments indicate that soybean in moist environments are more likely to acquire FLS resistance than those in drier regions (Mengistu et al., 2011). Discrepancies exist with regards to the specific number of *C.sojina* races, and 22 races have been identified in Brazil (Yorinori, 1992), 14 races in China (Ma and Li, 1997), and 12 races in the U.S. (Grau et al, 2004). Mian et al (2008) evaluated 93 *C.sojina* isolates, predominantly from the U.S. but also some from Brazil and China, and used 10 soybean differential cultivars to identify 11 *C.sojina* races 5-15.

While fungicides and resistance genes are common disease management strategies in soybean, alternative management practices are being evaluated. Silicon (Si) has been reported to diminish the severity of many soilborne and foliar plant diseases (Datnoff, 2007). In soybean, a foliar application of potassium silicate was associated with fewer *Phakopsora pachyrhizi* pustules, and plants grown in soil amended with calcium silicate demonstrated increased chitinase and β-1,3-glucanase activity (Cruz et al., 2013; Rodrigues et al., 2009). To evaluate the influence of silicon on frogeye leaf spot disease, soybean seedlings were supplied with a nutrient solution containing 0 or 2 mM Si and, subsequently, inoculated with a conidial suspension of *C.sojina*; the plants supplemented with silicon experienced greater FLS disease severity than plants lacking the silicon amendment, suggesting that Si does not improve soybean resistance to this fungus (Nascimento et al., 2014).

The use of microbial agents to control phytopathogens provides an alternative to chemical management strategies. Simonetti et al., isolated and characterized *Psuedomonas fluorescens* and *Bacillus amyloliquefaciens* bacteria from the soybean rhizosphere in Argentina. The researchers assessed the efficacy of these bacteria as biological controls against the foliar fungus *C.sojina*, and observed inhibition of fungal growth; however, the in vitro and in vivo test results were not and additional studies remain to be conducted, particularly in field-applied situations (Simonetti et al., 2012). A similar study reported an increase in expression of the defense-related gene GmAOS in soybean plants inoculated with native *Bacillus* sp. CHEP5, which reduced FLS disease

severity and, thus, displays potential as a biocontrol agent for *C.sojina* by inducing systemic resistance in the plant (Tonelli & Fabra, 2014).

Strobilurin Fungicides

Prior to the 1940s, the availability of chemical controls for phytopathogens was limited and the use of inorganic chemicals such as salt, copper sulfate, and lime was common (Staub & Morton, 2008). Inorganic compounds were not necessarily effective at controlling plant disease, and sometimes damaged the plant due to the high application rates (Staub & Morton, 2008). Thiram, (fungicide class: dithiocarbamate) was first introduced in 1942 and demonstrated greater efficacy and reduced phytotoxicity than the inorganic fungicides; since that time, organic chemical controls (seed treatments and fungicides) are some of the most popular plant disease control methods (Staub & Morton, 2008). While there are potential risks to consumers and the environment, as far as the use of these chemical control measures are concerned, the benefits are believed to exceed the risks and in the U.S. fungicide use is estimated to increase farm income by $13 billion annually (Staub & Morton, 2008).

The strobilurin products marketed in the U.S. are the result of laboratory modifications to reduce photosensitivity, and are considered by the Environmental Protection Agency (EPA) to be “reduced-risk” (Vincelli, 2012). They are effective on many plants against various diseases (Vincelli, 2012). Quinone outside inhibitors (QoIs) exhibit translaminar activity which means they can diffuse from the top of the leaf surface to the underside, so both sides of the leaf are protected (Vincelli, 2012). Depending on the specific active ingredient, some strobilurins may also move within the

plant's vascular system (Vincelli, 2012). QoIs display preventative action and are known to inhibit spore germination, but their curative action is not very effective since the fungicide prefers to bind with the waxy leaf cuticle, such that the chemical does not reside within the leaf in large quantities (Vincelli, 2012). Certain plants (grapes, apples, cherries) display phytotoxicity in response to certain strobilurin active ingredients (Vincelli, 2012).

Strobilurins are a class of broad spectrum fungicides which were first introduced in 1996, and as of 2005 were second only to demethylation inhibitor (DMI) fungicides in terms of importance within the fungicide market (Staub & Morton, 2008). Strobilurins are named after the wood-rotting fungi, such as *Strobilurus tenacellus*, from which the first derivatives were isolated, but are also referred to as quinone outside inhibitor (QoI) fungicides because they bind to the outer quinol binding site of the cytochrome bc_1 complex (aka complex III) which is located in the interior membrane of the mitochondria (Vincelli, 2012). The catalytic core of this complex is composed of an iron-sulphur protein, a cytochrome b subunit, and a cytochrome c_1 subunit (Fisher & Meunier, 2008). Cytochrome b has two quinol binding sites (Q_o and Q_i) and, as part of the mitochondrial respiration process, two quinol molecules will be oxidized at the outer binding site (Q_o) and one quinone molecule will be reduced at the inner binding site (Q_i), in addition to the transfer of two protons across the inner mitochondrial membrane (Fisher & Meunier, 2008). The complex is used during mitochondrial respiration to shuttle electrons down the electron transport chain and aides in generation of the proton gradient that will ultimately facilitate ATP production (Fisher & Meunier, 2008). Because the cytochrome

b gene is encoded by the mitochondrial genome, it is more susceptible to mutation than nuclear-encoded genes (Fisher & Meunier, 2008). Strobilurin fungicides are subject to being overcome due to their single site specificity; thus, a point mutation at the target site, which alters the expected amino acid (F129L, G137R, G143A, Y279), may confer fungicide resistance (Fisher & Meunier, 2008). Resistance does not necessarily indicate increased virulence by the pathogen, because some QoI resistant phytopathogens demonstrate diminished fitness (Fisher & Meunier, 2008).

The first report of QoI-fungicide resistant *C.sojina* in North America originated in 2010 from a Tennessee soybean field being treated with a strobilurin fungicide (Zhang et al., 2012). Pathogens may acquire resistance to QoI fungicides due to target site mutations, but may also overcome the action of the fungicide, at least in vitro, by the production of the alternative oxidase enzyme (Avila-Adame & Koller, 2003; Bartlett et al., 2002). There are no visually detectable differences between QoI resistant and QoI sensitive isolates of *C.sojina*; and while greenhouse inoculations suggest that resistant isolates demonstrate greater initial virulence, given 1-2 weeks the resulting disease severity did not differ according to the QoI sensitivity of the isolate (Zhang, 2012).

Fungicide Resistance

It is common knowledge that the use of chemicals, such as antibiotics and herbicides, to manage pest problems often leads to selection within the pest population for resistance to the applied chemical(s). Fungicide resistance is defined by the Fungicide Resistance Action Committee (FRAC) as “an acquired, heritable reduction in

sensitivity of a fungus to a specific anti-fungal agent" (http://www.frac.info/resistance-overview). The primary purpose of FRAC is to prolong the life of fungicides in order to minimize crop loss associated with fungicide resistance. FRAC is responsible for assigning the number and/or letter combinations present on fungicide labels which distinguish fungicide groups and indicate fungicide mode of action. Because compounds with similar chemical structure can often be expected to behave in a typical way, fungicidal compounds are often classified based on structural similarity. Once resistance occurs against one specific fungicide, there is a possibility for cross-resistance, or resistance to all members of that fungicide group. Alternatively, members of the same chemical class may not exhibit the same disposition for resistance acquisition (Brent & Hollowman, 2007). Some fungicides (MBCs, QoIs) are simply more prone to resistance acquisition than others (phthalamides) (Brent & Hollowman, 2007).

Fungicide resistance tends to occur within pathogen populations, oftentimes as a result of random genetic mutations, or after exposure to mutagens. Fungicide resistant strains may remain in the population at low levels due to reduced fitness or die-out as a result of random events (Hobbelen, et al., 2014). Application of certain fungicides may increase selection for resistant mutants by eliminating the sensitive isolates, such that the resistant isolates have less competition and may repopulate the field (Hobbelen, et al., 2014). Fungicides with single target sites are particularly susceptible to fungicide resistance, because something as simple as a single nucleotide point mutation may alter the active site in the organism enough to confer resistance; however, multiple mutations would be required for resistance to occur against fungicides with multiple

target sites, which is a less common occurrence (Brent & Hollowman, 2007). QoI-resistance, for example, has been reported in to emerge in multiple pathogen systems (*Mycosphaerella graminicola* and *Plasmopora viticola*) as a result of independent mutations (Chen et al., 2007; Torriani et al., 2009).

Multiple studies have been conducted in an effort to identify methods of reducing selection for resistance. A recent study generated a population dynamics model to evaluate how mixtures of low-risk and high-risk fungicides might influence selection for resistance, and determined that establishing resistance in the population is largely dependent on the fitness costs associated with the acquisition of resistance (Mikaberidze et al., 2014). A different modeling study indicated that a mixture consisting of a high-risk and a low-risk fungicide could delay the development of resistance, when compared to solo applications of the high-risk fungicide (Hobbelen et al., 2014). When the assumption is that resistance is not associated with any fitness costs to the organism, studies indicate that fungicide mixtures composed of a high-risk and low-risk fungicide will still select for fungicide resistance in the population; however, when fungicide-resistance is associated with high fitness costs, it is possible to find a ratio of low-risk to high-risk fungicides which can be applied to preferentially select for the sensitive strains to outcompete resistant strains (Hobbelen et al., 2011; Mikaberidze, et al., 2014)

References

Agarwal, V. K., & Sinclair, J. B. (1996). *Principles of Seed Pathology* (second ed.): CRC Press, Inc.

Akem, C. (1995). The effect of timing of fungicide applications on control of frogeye leaf spot and grain yield of soybeans. *European Journal of Plant Pathology, 101*(2), 183-187. doi: 10.1007/BF01874764

Avila-Adame, C., & Koller, W. (2003). Impact of alternative respiration and target-site mutations on responses of germinating conidia of Magnaporthe grisea to Qo-inhibiting fungicides. *Pest Management Science, 59*(3), 303-309. doi: 10.1002/ps.638

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Management Science, 58*(7), 649-662. doi: 10.1002/ps.520

Benedict, W. G., & Fucikovsky, L. (1966). CERCOSPORA SOJINA LESIONS ON SOYBEAN. *Canadian Journal of Plant Science, 46*(5), 567-568. doi: 10.4141/cjps66-094

Bradley, C. A., Wood, A., Zhang, G. R., Murray, J. E., Phillips, D. V., & Ming, R. (2012). Genetic diversity ofCercospora sojinarevealed by amplified fragment length polymorphism markers. *Canadian Journal of Plant Pathology, 34*(3), 410-416. doi: 10.1080/07060661.2012.711775

Brent, K. J., & Hollomon, D. W. (2007). *Fungicide Resistance: The Assessment of Risk , FRAC Monograph No. 2 second (revised) edition*: Fungicide Resistance Action Committee.

Chen, W.-J., Delmotte, F., Cervera, S. R., Douence, L., Greif, C., & Corio-Costet, M.-F. (2007). At Least Two Origins of Fungicide Resistance in Grapevine Downy Mildew Populations. Applied and Environmental Microbiology, 73(16), 5162-5172. doi:10.1128/AEM.00507-07

Chupp, C. (1954). *A monograph of the fungus genus Cercospora*. Ithaca, New York: Chupp, Charles.

Compendium of Soybean Diseases. (1999). (G. L. Hartman, J. B. Sinclair & J. C. Rupe Eds. fourth edition ed.): The American Phytopathological Society.

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

Cruz, M. F. A., Rodrigues, F. A., Rodriguez, L. P., Curvelo, C. R. S.,, & Nascimento, K. J. T., Moreira, M. A., and Barros, E. G. (2013). Inducers of resistance and silicon on the activity of defense enzymes in the soybean– Phakopsora pachyrhizi interaction. *Bragantia, 72*, 162-172.

Datnoff, L. E., Rodrigues, F. A., and Seebold, K. (2007). *Silicon and plant disease* (W. E. E. Datnoff, and D. M. Huber, eds. Ed.). St. Paul, MN: The American Phytopathological Society.

Dashiell, K., & Akem, C. (1991). Yield losses in soybeans from frogeye leaf spot caused by Cercospora sojina. *Crop Protection, 10*(6), 465-468. doi: 10.1016/S0261-2194(91)80134-2

Daub, M., & Chung, K. (2007). Cercosporin: A Phytoactivated Toxin in Plant Disease. *APS net Features*. doi:doi: 10.1094/APSnetFeature/2007-0207

Dorrance, A., Hoeft, R., Onstad, D., Orf, J., Porter, P., Spurlock, S., and Young, B. (2009). Sustainability of U.S. Soybean Production: Conventional, Transgenic, and Organic Production Systems. Council for Agricultural Science and Technology, Ames, IA. Spec. Publ. 30. www.cast-science.org

Fisher, N., & Meunier, B. (2008). Molecular basis of resistance to cytochrome bc1 inhibitors. *FEMS Yeast Research, 8*(2), 183-192. doi: 10.1111/j.1567-1364.2007.00328.

Gomez, D., & Reis, E. (2013). Influence of substrates, light, filter paper and pH on the sporulation of Cercospora sojina. *Summa Phytopathologica, 39*(2), 126-129.

Goodwin, S. B., Dunkle, L. D., & Zismann, V. L. (2001). Phylogenetic Analysis of Cercospora and Mycosphaerella Based on the Internal Transcribed Spacer Region of Ribosomal DNA. *Phytopathology*, 11.

Grau, C.R., A.E. Dorrance, J. Bond, and J.S. Russin. (2004). *Fungal diseases*. 679–763. In H.R. Boerma and J.E. Specht (ed.) Soybeans: Improvement, production, and uses. 3rd ed. Agron. Monogr. 16. ASA, CSSA, and SSSA, Madison, WI.

Groenewald, J. Z., Nakashima, C., Nishikawa, J., Shin, H. D., Park, J. H., Jama, A. N., Crous, P. W. (2013). Species concepts in Cercospora: spotting the weeds among the roses. *Stud Mycol, 75*(1), 115-170. doi: 10.3114/sim0012

Hobbelen, P. H. F., Paveley, N. D., & van den Bosch, F. (2011). Delaying Selection for Fungicide Insensitivity by Mixing Fungicides at a Low and High Risk of Resistance Development: A Modeling Analysis. *Phytopathology, 101*(10), 1224-1233. doi: 10.1094/PHYTO-10-10-0290

Hobbelen, P. H. F., Paveley, N. D., & van den Bosch, F. (2014). The Emergence of Resistance to Fungicides. *PLoS ONE*, *9*(3), e91910. http://doi.org/10.1371/journal.pone.0091910

Kelly, H. (2013). Frogeye Leaf Spot Information. UT Crops.

Kim, H., Newell, A., Cota-Sieckmeyer, R., Rupe, J., Fakhoury, A., & Bluhm, B. (2013). Mating-Type Distribution and Genetic Diversity of Cercospora sojina Populations on Soybean from Arkansas: Evidence for Potential Sexual Reproduction. *Phytopathology, 103*(10), 1045-1051. doi: 10.1094/PHYTO-09-12-0229-R

Koenning, S., & Wrather, J. (2010). Suppression of soybean yield potential in the continental United States by plant diseases from 2006 to 2009. *Plant Health Progress*. doi:10.1094/PHP-2010-1122-01-RS

Lehman, S. G. (1928). Frogeye Leaf Spot of Soy Bean caused by Cercospora Diazu Miura. *Journal of Agricultural Research, 36*(9), 811-833

Lehman, S. G. (1934). Frog-eye (Cercospora diazu Miura) on stems, pods, and seeds of Soybean, and the relation of these infections to recurrence of the disease. *Journal of Agricultural Research, 48*(2), 131-147 pp.

Li, Y., Shan-cen Zhao, J.-x. M., Dong Li, Long Yan, Jun Li, Xiao-tian Qi, Xiao-sen Guo,, Le Zhang, W.-m. H., Ru-zhen Chang, Qin-si Liang, Yong Guo1, Chen Ye2, Xiao-bo Wang1, Yong Tao2,5,, Rong-xia Guan1, J.-y. W., 6, Yu-lin Liu1, Long-guo Jin1, Xiu-qing Zhang2, Zhang-xiong Liu1, Li-juan Zhang1,, Jie Chen2, K.-j. W., Rasmus Nielsen2,5,7, Rui-qiang Li2, Peng-yin Chen8, Wen-bin Li9, Jochen C Reif10,, & Michael Purugganan11, J. W., Meng-chen Zhang4, Jun Wang2,5* and

Li-juan Qiu1*. (2013). Molecular footprints of domestication andimprovement in soybean revealed by whole genome re-sequencing. *Biomedical Central, 14*, 579-591.

Ma, S.M., and B.Y. Li. (1997). Primary report on the identifi cation for physiological races of Cercospora sojina Hara in northeast China. Acta Phytopathol. Sin. 27(2):180

McWilliams, D., & Berglund, D. (1999). Soybean Growth and Management Quickguide. In N. D. S. University (Ed.), (pp. 1-8): NDSU Extension Service.

Mengistu, A., Bond, J., Mian, R., Nelson, R., Shannon, G., & Wrather, A. (2011). Identification of Soybean Accessions Resistant to by Field Screening, Molecular Markers, and Phenotyping. *Crop Science, 51*(3), 1101. doi: 10.2135/cropsci2010.09.0529

Mengistu, A., Kelly, H. M., Bellaloui, N., Arelli, P. R., Reddy, K. N., & Wrather, A. J. (2014). Tillage, Fungicide, and Cultivar Effects on Frogeye Leaf Spot Severity and Yield in Soybean. *Plant Disease, 98*(11), 1476-1484. doi: 10.1094/PDIS-12-13-1268-RE

Mengistu, A., Kurtzweil, N. C., & Grau, C. R. (2002). First Report of Frogeye Leaf Spot (Cercospora sojina) in Wisconsin. *Plant Disease, 86*(11), 1272-1272. doi: 10.1094/PDIS.2002.86.11.1272B

Mian, M. A. R., Missaoui, A. M., Walker, D. R., Phillips, D. V., & Boerma, H. R. (2008). Frogeye Leaf Spot of Soybean: A Review and Proposed Race Designations for Isolates of Hara. *Crop Science, 48*(1), 14. doi: 10.2135/cropsci2007.08.0432

Mikaberidze, A., McDonald, B. A., & Bonhoeffer, S. (2014). Can High-Risk Fungicides be Used in Mixtures Without Selecting for Fungicide Resistance? *Phytopathology, 104*(4), 324-331. doi: 10.1094/PHYTO-07-13-0204-R

Nascimento, K. J., Debona, D., Franca, S. K., Goncalves, M. G., DaMatta, F. M., & Rodrigues, F. A. (2014). Soybean Resistance to Cercospora sojina Infection Is Reduced by Silicon. *Phytopathology, 104*(11), 1183-1191. doi: 10.1094/PHYTO-02-14-0047-R

Rodrigues, F. A., Duarte, H. S. S., Domiciano, G. P., Souza, C. A., Korndörfer, G. H., & and Zambolim, L. (2009). Foliar application of potassium silicate reduces the intensity of soybean rust. *Australasian Plant Pathol., 38*, 15-21.

Simonetti E, Carmona MA, Scandiani MM, García AF, Luque AG, et al. 2012. Evaluation of indigenous bacterial strains for biocontrol of the frogeye leaf spot of soya bean caused by Cercospora sojina. *Letters in Applied Microbiology* 55:170-173.

Singh, T., & Sinclair, J. B. (1985). Histopathology of Cercospora sojina in Soybean Seeds. *Phytopathology, 75*(2), 185-189.

Staub, T., & Morton, V. (2008). A short history of fungicides. *APS net Features*. doi:10.1094/APSnetFeature-2008-0308

Tonelli, M. L., & Fabra, A. (2014). The biocontrol agent Bacillus sp. CHEP5 primes the defense response against Cercospora sojina. *World Journal of Microbiology and Biotechnology, 30*(9), 2503-2509. doi: 10.1007/s11274-014-1675-3

Torriani, S. F., Brunner, P. C., McDonald, B. A., & Sierotzki, H. (2009). QoI resistance emerged independently at least 4 times in European populations of Mycosphaerella graminicola. Pest Manag Sci, 65(2), 155-162. doi:10.1002/ps.1662

USDA-ERS. (2012). Soybeans & Oil Crops:Background. 1-3.

USDA-NASS. (2010). Field crops usual planting and harvesting dates (pp. 1-51).

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

Wrather, J., Anderson, T., Gai, J., Arysad, D., Pota-Puglia, A., Ram, H., & Yorinori, J. (1997). Soybean Disease Loss Estimates for the Top 10 Soybean Producing Countries in 1994. *Plant Disease, 81*, 107-110.

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

Zeng, F., Arnao, E., Zhang, G., Olaya, G., Wullschleger, J., Sierotzki, H., Bradley, C. A. (2014). Characterization of quinone outside inhibitor fungicide resistance in Cercospora sojina and development of diagnostic tools for its identification. *Plant Disease*, 141029130041000. doi: 10.1094/pdis-05-14-0460-re

Zhang, G. (2012). Cercospora sojina:Overwinter survival and fungicide resistance. (Doctor of Philosophy in Crop Sciences Dissertation), University of Illinois.

Zhang, G. R., Newman, M. A., & Bradley, C. A. (2012). First Report of the Soybean Frogeye Leaf Spot Fungus (Cercospora sojina) Resistant to Quinone Outside

Inhibitor Fungicides in North America. *Plant Disease, 96*(5), 767-767. doi: 10.1094/PDIS-10-11-0915-PDN.

CHAPTER I
EPIDEMIOLOGY OF FROGYE LEAF SPOT OF SOYBEAN

Abstract

It is important to understand variation in fungicide sensitivity of a pathogen population in order to design optimal management strategies. Understanding the potential variation in QoI- fungicide sensitivity on a small field scale, as well as on a broader state-wide scale could allow for the design of more efficacious management schemes. QoI-fungicide resistance in frogeye leaf spot disease on soybean was evaluated in 2014 and 2015 in multiple counties across Tennessee. The area under disease progress curve (AUDPC) for both incidence and severity of FLS was greater in counties in climate division 3 and 4 than in climate division 2. A conidial germination assay and a TaqMan qPCR assay were used to evaluate QoI- fungicide sensitivity. The level of QoI-resistance in West Tennessee was greater than counties in the central part of the state. A directional study evaluated variation in QoI-fungicide sensitivity at various depths in the canopy within a 5 foot area of a field and it was determined that while sensitivity could vary a lot within a small area, QoI-resistance tended to be greater in the upper canopy of soybeans.

Keywords: strobilurin, QoI-resistance, *C.sojina*, frogeye leaf spot, epidemiology

Introduction

Frogeye leaf spot (FLS) is a disease caused by the fungus *Cercospora sojina* Hara which affects soybean (Chupp, 1954). FLS was first reported in Japan in 1914, but it was not until the early 1920s that the pathogen was reported in the southern United States, where it remains prevalent; however, it has since spread to more northern states, such as Wisconsin and Ohio (Chupp, 1954; Cruz & Dorrance, 2009; Lehman, 1928; Mengistu et al., 2002). Although the pathogen can withstand below-zero overwintering conditions, FLS disease progression is favored by warm and humid conditions which enable spore production to occur as early as 48 hours after symptoms manifest (Cruz & Dorrance, 2009; Mian et al., 2008). The fungus will overwinter and sporulate on infested soybean debris, and remains viable on debris even after two years in the field (Cruz & Dorrance, 2009; G. Zhang, 2012). A study evaluating *C.sojina* populations across multiple fields in Arkansas determined that mating type loci (*MAT1-1* and *MAT1-2*) are found at relatively equal proportions in the population which, combined with the high degree of genetic diversity between *C.sojina* isolates, indicates that sexual reproduction is occurring (Kim et al., 2013). The majority of *Cercospora* teleomorphs belong to the *Mycosphaerella* genus (Goodwin, Dunkle, & Zismann, 2001).

FLS disease is characterized by the angular to circular lesions which typically appear on soybean leaves. Initially, lesions appear as small, dark brown spots but eventually develop into larger spots with discolored gray or beige centers, surrounded by a dark red or brown border. Lesions may coalesce as the disease progresses and contribute to premature defoliation. While foliar symptoms are the most commonly

observed, the leaves, stems, and pods may also be affected (Lehman, 1934). Stem lesions are typically red-brown in color, pod lesions are typically dark and sunken (Mian et al., 2008). Seeds typically become infected with *C. sojina* through the pod walls and display gray to black discolorations (Singh & Sinclair, 1985). *C.sojina* infected seeds will not germinate as readily and tend to produce seedlings with FLS lesions on the cotyledons which can lead to additional infections across a field (Mian et al., 2008; Singh & Sinclair, 1985).

Wind and splashing rain aid in the dispersal of the pathogen (Mian et al., 2008). As of yet, the main source of natural inoculum for FLS disease are the conidia produced directly from the necrotic lesions (Lehman, 1928). Conidia are asexual spores and are produced as early as two days after FLS lesions appear (Mian et al., 2008). A conidium can land on a soybean plant, provided that there is adequate moisture, may germinate within the hour to infect the plant; foliar symptoms may not be visible until two weeks after the initial infection (Mian et al., 2008). FLS is considered a polycyclic disease since lesions may continue to produce conidia throughout the season, thus, contributing to continued infection cycles (Mengistu et al., 2011; Mian et al., 2008). The disease reduces the green photosynthetic area of the plant, and yield losses as high as 60% have been reported (Dashiell & Akem, 1991). In the U.S. alone, a loss of almost 8 million soybean bushels was attributed to FLS in 2009 (Koenning & Wrather, 2010). Soybean is one of the most planted crops in the U.S., for use in industrial processes or as a protein source, and account for 90% of oilseed production (Compendium of Soybean Diseases, 1999; USDA-ERS, 2012).

Soybean growth stage and the presence or absence of inoculum contribute significantly to the development of frogeye leaf spot disease, but ambient conditions also play a crucial role. In Tennessee, the soybean growing season may span from late April to early November, depending on planting and harvest dates. Soybean growth rate is dictated by temperature and maturity group and, with the exception of double-crop soybeans, the majority of soybeans will enter the reproductive growth stage by late-June or early-July in Tennessee. Soybeans reach the reproductive growth stage when the first flowers begin to appear. FLS symptoms tend to manifest around the time which soybeans begin to produce flowers, hence the weather around that time period will greatly impact the progression, or lack, of FLS development in the field.

Planting resistant soybean cultivars and a two-year rotation to a non-host crop are typical recommendations for managing FLS disease (Mian et al., 1998). Foliar fungicide applications are also a suitable alternative to control disease, but are generally only recommended between the R1-R5 growth stages of soybean development (beginning flower to beginning seed) depending on the level of FLS disease pressure, which may not warrant fungicide application at all (Mian et al., 2008). There are a multitude of fungicides labeled for the control of FLS on soybean, with varying degrees of efficacy. QoI, also known as “strobilurin”, fungicides target the cytochrome bc_1 complex of the fungal mitochondria and prevent ATP production by mitochondrial respiration, thereby inhibiting fungal growth and spore germination (Bartlett et al., 2002). Strobilurin fungicides were first marketed in 1996 and quickly gained prevalence due to their broad spectrum activity (Staub & Morton, 2008). Since their introduction to the

fungicide market, various phytopathogens have been reported as QoI-fungicide resistant (Fisher & Meunier, 2008). The first incidence of QoI-fungicide resistant *C.sojina* was in 2010 from a Tennessee soybean field being treated with pyraclostrobin; however, by the end of the 2014 season QoI-resistant FLS had been reported in 10 states and over 106 countries (Zhang et al., 2012; http://frogeye.ipmpipe.org/cgi-bin/sbr/public.cgi).

While mutations conferring fungicide resistance tend to occur naturally in microbial populations, use and misuse of fungicides aides in continued selection for resistant organisms. Studies sometimes provide seemingly conflicting results with regards to the best strategy for reducing selection for fungicide resistance. Currently, it appears that while mixtures of high and low-risk fungicides may select for resistance less than a single application of a high-risk fungicide, the fitness costs attributed to the fungus as a result of acquiring resistance have a greater impact on whether such a fungicide mixture will be beneficial (Hobbelen et al. 2011;Mikaberidze, McDonald, & Bonhoeffer, 2014).

The variation in fungicide sensitivity of an organism within a geographic area is not entirely understood; the detection of resistance is not necessarily indicative of resistance in the entire population. The objectives of this test were to: 1) determine the epidemiology associated with QoI-resistance in the state of Tennessee via soybean sentinel plots, and 2) evaluate the variation in *C. sojina* QoI fungicide sensitivity across a smaller scale.

Materials and Methods

Sentinel Plot Study

A national soybean sentinel plot program was established in 2005 for the purposes of monitoring soybean rust in the United States via the Integrated Pest Management– Pest Information Platform for Extension and Education (ipm-PIPE: sbr.ipmpipe.org). After the emergence of QoI-fungicide resistant frogeye leaf spot disease, many states utilized the same program to monitor the development of QoI resistance in various counties. In Tennessee sentinel plots have been utilized as a means of monitoring for QoI-resistance since 2013; however, only the data from 2014 and 2015 will be discussed. In collaboration with Tennessee county extension agents, sentinel plots were established within farmers' fields planted with a FLS susceptible soybean cultivar. The exact location, planting date, row spacing, and previous crop of soybean sentinel plots were dependent upon agronomic utilization and land availability in each county. Sentinel plot area ranged from 50 to 4050 m^2 (500 ft^2 to 1 acre) and was marked with bicycle flags to avoid any fungicide applications occurring in the area. Soybean planting dates ranged from May 5th to July 2nd, for full season soybean, and June 19th to June 23rd for soybean planted behind wheat (Table 1 and 2). Sentinel plots may have been set-up in the same county both years, but they were not in the exact same field. There were a total of 15 sentinel plots in 2014 (Table 1) and 12 in 2015 (Table 2), scattered across west and middle Tennessee, which encompassed 3 different climate divisions (Figure 1). According to the National Oceanic and Atmospheric Administration (NOAA), west Tennessee is categorized as climate division 4, while the

middle Tennessee region is divided into climate divisions 3 (east of west Tennessee, but west of the Cumberland plateau) and 2 (encompasses the Cumberland plateau). Climate divisions are established by assessing variations in temperature, precipitation, and heating/cooling degree days. Monthly weather data for the 2014 and 2015 soybean-growing season was obtained from the National Oceanic and Atmospheric Administration's (NOAA) "climate at a glance" weather service.

Extension county agents were responsible for collecting approximately 50 soybean leaves weekly, commencing at growth stage R1 (beginning-bloom) and ending at or near maturity (R8), and shipping them overnight to the West Tennessee Research and Extension Center (WTREC) for processing and evaluation. In order to capture the diversity within a field, county agents randomly collected leaves from fields in a diamond or "w" pattern. Leaf samples were collected in plastic bags, and stored at room temperature (~25°C) or in the refrigerator (~4°C) until shipping and processing.

Weekly samples were used to evaluate FLS disease incidence and severity. Incidence and severity data was recorded for each individual sentinel plot and averaged for each sampling date. FLS severity was evaluated as percent leaf surface area affected with FLS lesions and FLS incidence was based on the number of leaflets with FLS symptoms. FLS incidence and severity was used to calculate the area under the disease progress curve (AUDPC) for each county over the course of the season. The AUDPC was calculated using the formula:

$$\text{AUDPC} = \sum_{i}^{n-1} \left[\left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i) \right]$$

where y_i percentage of leaf area affected (disease severity)or incidence of disease at the *i*th observation, *t* = time (days), and *n* = total number of observations. ∑ is the sum of areas of all of the individual trapezoids or areas from *i* to *n* – 1. I and *i* + 1 represent observations from 1 to *n*. Sampling dates were converted into Julian days, such that January 1 is now Julian day 1. In order to compare disease development across the various sentinel plots, AUDPC values were standardized by dividing the AUDPC by the duration of the disease epidemic from onset of disease symptoms to the last sampling date for each county.

Weekly sentinel plot samples were also used to evaluate fluctuations in QoI-sensitivity over the course of the soybean-growing season. When present, FLS lesions were isolated from affected leaves and stored at -80°C for future analysis with quantitative PCR as described below. Additionally, a germination assay, as described below, was conducted to evaluate *C.sojina* conidia for QoI-sensitivity based on ability to germinate on fungicide-amended media. The percentage of QoI-resistance refers to the percentage of conidia which were QoI-resistant; such that, if 95% QoI-resistance is reported, the implication is that 5% of the conidia were QoI-sensitive. For the QPCR results, the same principle applies when the percentage of the G143A allele is discussed because the percentage of the wildtype allele is implied. In 2015 the Giles

county sentinel plot was moved to a different field with the same soybean variety in the middle of the season due to a fungicide application over original sentinel plot area.

Directional Study

Directional studies were conducted in Milan, TN (Gibson County) and Dyersburg, TN (Dyer County) in 2014 and in Jackson, TN (Madison County) in 2015. Leaf samples were collected in late August or September. The purpose of the directional study was to evaluate the diversity in *C. sojina* QoI fungicide sensitivity across a smaller scale. An area of a field was selected and soybean leaves were randomly collected from the upper, middle, and lower canopy across 3 to 5 plants. This collection was repeated approximately 1.5 m (5 ft) to the north, south, east, and west regions of the original central sampled area. FLS lesions were collected from the different directions and stored at -80°C pending additional processing.

Assessing QoI Fungicide Resistance

Conidial Germination Assay

FLS infected soybean leaf samples were incubated overnight in a “moist chamber” to facilitate sporulation. The moist environment was generated by incubating leaves in a plastic bag containing a damp paper towel. Using a 20 µl pipette outfitted with sterile pipette tips, conidia were suctioned off of lesions in sterile, deionized water. Conidia were dislodged by depositing a 10 µl droplet of water onto a sporulating lesion and pipetting up and down until the conidia were no longer attached to the lesions, but were instead floating freely in the water drop. A dissecting scope was used to better observe the conidia. The conidia-enriched droplets were transferred to additional lesions and those conidia collected, as necessary. The number of leaves and lesions

from which conidia were collected varied depending on the leaflet severity, but an average of 9 leaves were used from each sample. More leaves were sampled from sources with low levels of disease severity or low levels of sporulation, so as to increase the chances of obtaining the 50 required conidia.

A total of 50-60 µl of sterile water was utilized to generate the *C.sojina* spore suspension from each sample; half of the composite spore suspension was deposited onto control potato dextrose agar (PDA) plates (non-amended plated), while the remaining half of the spore suspension was deposited onto PDA plates amended with a discriminatory dose of azoxystrobin (0.1µg/µl). Both fungicide-amended and non-amended plates were supplemented with salicylhydroxamic acid (SHAM) to prevent the alternative oxidase respiratory pathway from allowing QoI-sensitive conidia to overcome the inhibitory effect of the fungicide in the media. Only assays with at least 50 conidia on each plate were included in the analysis. Spore suspensions were spread onto the plates using sterilized glass rods. Plates remained on the laboratory benchtop at room temperature (~25°C) until evaluation. Conidia were allowed 14-18 hours for germination before assessment. The number of germinated and non-germinated conidia from each plate was recorded using a compound light microscope. If a germ tube exceeding half of the length of the conidium the conidium was considered “germinated”. Only assays with at least 70% germination on non-amended plate were included in the analysis In order to account for nonviable or dead conidia, the % germination on the azoxystrobin-amended plates was adjusted as follows:

$$\frac{\#\ spores\ germinated\ on\ amended\ plate}{total\ \#\ spores\ counted\ on\ the\ amended\ plate} \div \frac{\#\ spores\ germinated\ on\ the\ non-amended\ plate}{total\ \#\ of\ spores\ counted\ on\ the\ non-amended\ plate} * 100$$

DNA Extraction

FLS lesions, approximately 5 to 10mm in diameter, were stored at -20°C in microcentrifuge tubes until DNA could be extracted. Total genomic DNA was extracted using the Qiagen DNeasy plant mini kit (QIAGEN, Valencia, CA, USA). Previous attempts to extract adequate amounts of DNA from single lesions proved difficult and inconsistent; therefore, FLS lesions from 2014 were pooled by sample for DNA extraction. The exact number of lesions, as well as the quality of the lesions, was recorded for each sample to determine the number of lesions that were needed to obtain a sufficient amount of DNA to perform the qPCR protocol, DNA was extracted from 1, 5, 10, 15, and 20 lesion(s). While DNA extracted from single lesions did result in successful QPCR reactions a couple of times, a greater degree of success was observed using DNA extracted from 15 good quality lesions. For this study, DNA was extracted from, on average, 15 lesions per sample. DNA was eluted in the AE buffer provided by Qiagen. Initially, elutions were conducted using 50 or 100µl of buffer; however, eventually samples were eluted in 2, 60 µl elutions to maximize the amount of DNA extracted without significantly increasing the volume. The DNA was maintained at 4°C.

Quantitative real-time PCR

The quantitative real-time PCR protocol used to assess the samples for the presence and/or lack of the G143A mutation was developed by Zeng et al., 2014. They generated *C.sojina*- specific PCR primers, designed to amplify the region of the mitochondrial cytochrome bc1 gene where the G143A point mutation occurs, as well as

a set of TaqMan® (Life Technologies, city, state, country) hybridization probes specific for both the wildtype (QoI-sensitive) allele and the mutant (QoI-resistant) allele (Zeng et al., 2014). The quantitative PCR TaqMan™ assay allows for the detection and quantification of both the G143A mutation conferring QoI-resistance and the wildtype allele which leaves the pathogen QoI-sensitive. Because the *C.sojina* DNA tested from the 2014 sentinel plots was a compilation of multiple lesions from the same area, it would not be unexpected for the assay to detect both the sensitive and the resistant alleles for the cytochrome bc1 gene within a reaction; thus, the "%G143A" or the "%WT" which will be referred to throughout this paper refers to the percentage of the total *C.sojina* DNA estimated by the qPCR which was determined to be either the mutant(G143A) or the wildtype(WT) allele. Similarly, because the germination assay is performed using a compilation of *C.sojina* conidia from multiple FLS lesions within an area, it would not be surprising for a mixture of QoI-sensitive and QoI-resistant conidia to be detected within a sample.

The qPCR platform utilized was the IQ™5 from Bio-Rad (Bio-Rad, Hercules, CA, USA). PCR reactions were performed in 25µl volumes, comprised of 10µl TaqMan® Master Mix (2X), 1.25µl TaqMan® Custom SNP genotyping assay (20X), 9.25 µl molecular grade water (ThermoScientific, Waltham, MA, USA) , 0.5 µl (20µg/µl), Bovine Serum Albumin (ThermoScientific, Waltham, MA, USA) and 4 µl of template DNA. The qPCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 sec and 62°C for 1 min. The TaqMan® Master Mix contains the AmpliTaq Gold®DNA polymerase. The TaqMan® probe labeled with

the VIC fluorophore hybridizes to the mutant or QoI-resistant allele, while the FAM-labeled fluorophore hybridizes to the wildtype or QoI-sensitive allele. Standard curve quantitation was utilized to quantify the DNA concentrations of the unknown FLS samples by comparing their cycle threshold (Ct) values to the Ct values of the standards.

DNA was extracted from pure *C.sojina* cultures of known QoI-sensitivity by scraping off the mycelium into 2 ml screw cap tubes supplemented with sterile glass bead and freezing the specimens at -80°C for at least 3 hours prior to rupturing the cellular tissue in the FastPrep® FP 120 (ThermoSavant, Carlsbad, CA, USA). DNeasy Plant mini kit (QIAGEN, Valencia, CA, USA) was used to extract DNA. Nanodrop 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA) was used to quantify DNA. Samples were diluted 6-fold (10 ng/µl, 1.0 ng/ µl, 0.1 ng/µl, 0.01 ng/µl, 0.001 ng/µl, and 0.0001 ng/µl) in molecular biology grade water (FisherScientific, Waltham, MA, USA). In order to reduce the adherence of *C.sojina* DNA to the plastic tubes, the stock elution of each extraction was amended with 1µl of (1 mg/ml) salmon sperm DNA (Invitrogen, Carlsbad, CA, USA). Each qPCR reaction involved 2 standard curves: one for a QoI-resistant and one for a QoI-sensitive *C.sojina* isolate to act as controls for the FAM and VIC probes, respectively. The expected QoI-sensitivity of the isolates was confirmed using qPCR before the isolates could be used as DNA/probe standards. All samples, including the standards and the non -template controls (NTCs) were run in duplicate.

Results

Sentinel Plots

Weather

There were no significant differences in temperature and precipitation across the three Tennessee climate divisions encompassed in this study when averaged for July, August, and September (Table 3). Climate division 4 was, on average, 1 to 4 degrees warmer than divisions 3 or 2. July was the warmest month for all three climate divisions in 2015, while August was the warmest month for all three climate divisions in 2014. In 2014, August was the month with the greatest precipitation (4.5-6.1”) for all three climate divisions. In 2015, climate divisions 2 and 3 experienced the greatest precipitation in July (8.2 and 6.2”, respectively), while climate division 4 experienced approximately 0.2 inches more of precipitation in August than in July. September was the month with the lowest temperature and precipitation for all climate divisions in both 2014 (20.9-21.9°C; 1.2-3.47”) and 2015 (20.5-22.8°C; 1.87-3.32”).

AUDPC

During both years, the majority of sentinel plots experienced an onset of FLS disease during the R2 growth stage. Sentinel plots which never developed FLS included Rutherford (2014 and 2015) and Hardin Counties (2014) and are excluded from the AUDPC analysis. The AUDPC was calculated for both FLS incidence and FLS severity. In 2014 the AUDPC for incidence ranged from 6-93 while the AUDPC for severity ranged from 0.1 to 8 (Table 7). In 2015 the AUDPC for incidence ranged from 1-98 while the AUDPC for severity ranged from 0.01 to 3 (Table 7). In many of the sentinel plots (Robertson, Giles, Perry, Tipton, Hardeman, and Fayette) a reduction was observed in both AUDPC-incidence and AUDPC-severity from 2014 to 2015. Franklin

and Lake Counties demonstrated a substantial increase in both AUDPC-incidence and AUDPC-severity from 2014 to 2015. Coffee and Canon Counties both demonstrated a moderate increase in AUDPC-incidence from one year to the other; however, AUDPC-severity was not significantly altered.

When AUDPC values were averaged by climate division instead of by individual county, climate divisions 3 and 4 often demonstrated similar levels of accumulation in the AUDPC for FLS incidence and AUDPC for severity (Table 7). When AUDPC values were averaged by climate division, the greatest average AUDPC for incidence and severity in 2014 was across climate division 3 (52.8) while the greatest average AUDPC for incidence and severity for 2015 was climate division 2 (70.99) (Table 7). Both the AUDPC averages for incidence and severity decreased for climate divisions 3 and 4 from 2014 to 2015.Climate division 2 was the anomaly which demonstrated an increase in average AUDPC incidence and average AUDPC severity from 2014 to 2015. When evaluated by individual county from 2014 to 2015, six of eleven counties experienced a decrease in AUDPC incidence and nine of eleven counties displayed a reduction in AUDPC severity. In 2014 incidence of FLS in the sentinel plots began to escalate at the end of July, while simultaneously displaying a steady rise in FLS disease severity throughout the month of August (Figure 2 and 3). In 2015 incidence began to increase the first week of August, but FLS disease severity did not begin to increase until mid-August (Figure 4 and 5).

Quantification of QoI Resistance

A multivariate correlation analysis in JMP suggested that the correlation between the qPCR and the germination assay was very low (r=0.135); however, it is

important to recall that only 39 sentinel samples from the 2014 soybean season were compared for this analysis because they were the only samples with data for both QoI assessment methods. When %G143A and %QoI-resistance were averaged by county over the course of the season (Table 4), the qPCR assay typically detected greater levels of resistant DNA than the resistant conidia detected by germination assay. Using Least Squares means in JMP, analysis of variance identified significant differences in the presence of the G143A mutation across Tennessee climate divisions when detected using qPCR. The %G143A was significantly lower in climate division 2 than in divisions 3 and 4. However, the percentage of QoI-resistance, as detected using the germination assay, did not vary significantly by climate division. According to the qPCR assessment, climate divisions 4 and 3 *C.sojina* infections were on average 97-100% QoI resistant (i.e. the majority of *C.sojina* DNA harbors the G143A mutation), while only 69% of the *C.sojina* DNA in climate divisions 2 harbors the G143A mutation (Table 4).

Conidial germination assays were conducted on weekly sentinel plot samples throughout the season with varying levels of success. Germination assays were often hindered by lack of conidia and/or bacterial contamination present on soybean leaves. Nevertheless, many of the assays were successful and yielded at least 100 *C.sojina* conidia. There was no significant difference in the detected level of QoI-resistance between 2014 and 2015 when averaged across the sentinel plots in all counties (Figure 6). When the level of QoI-resistant conidia was compared across the sentinel plots in all counties for both years, there were no obvious patterns. Some of the counties (Cannon) demonstrated greater resistance the following year while others exhibited the same

(Coffee, Giles, Lake, Perry, Robertson), or a reduced (Franklin, Tipton, Weakley), level of resistance the following year (Figure 6). The average level of QoI-resistance in the sentinel plots was 65% in 2015 and 69% in 2014. When averaged for both years, the QoI-resistance for climate division 2 (48%) was significantly lower than the QoI-resistance for climate division 4 (77%); the level of resistance in climate division 3 (67%) did not differ significantly from the other two divisions (Table 8).

Directional Study

Low levels of sporulation on FLS meant that many of the conidial germination assays yielded less than 50 conidia and were, thus, excluded from the results (Table 6). Because the germination assays could not be replicated or completed successfully for all directions at all three canopy heights, the data obtained is not likely to be representative of the true level of variation in the *C.sojina* population. Nevertheless, one interesting pattern was that the percentage of QoI-resistant conidia was often greater in samples taken from higher in the canopy than lower in canopy. Dyer County produced the most “complete” directional study, with 10 of the 15 direction/canopy-height combinations completed successfully. It is interesting to note, that even within this small area the percentage of QoI-resistant conidia varied from 50 to 100%. Similarly, in Madison county conidial QoI-resistance varied from 5 to 85% within one canopy. Gibson demonstrated the narrowest range of QoI-resistance (23-31%), but also the fewest number of successful germination assays. Even within a canopy level the range of QoI-resistance was as much as 34%.

Discussion

Sentinel Plots

While FLS disease is favored by warm and wet ambient conditions, there may not have been sufficient variation in average weather data to correlate to any differences in FLS incidence and severity based on geographic distribution within the state of Tennessee. Wind patterns are likely to play an important role in dispersal of *C.sojina* inoculum and, even if optimal disease conditions are occurring, if the pathogen is absent then no disease will occur. While it was not uncommon for a sentinel plot to reach 100% incidence of frogeye leaf spot before the soybean field reached maturity. FLS did not typically manifest prior to mid-July and the greatest levels of disease incidence and severity occurred between mid-August and mid-September. The average disease severity within a field did not typically exceed 30% and many fields never averaged more than 10% disease severity. The sentinel plot data seems to suggest that while FLS is present, and causing disease in Tennessee, conditions in the state may not be extremely favorable for disease progression since severity tends to remain low, at least in the two years of this study. Lower disease severity means that growers may not have to spray fungicides to manage the disease.

The qPCR data seems to suggest that the cooler weather in Tennessee climate division 2 is not as favorable for mutant *C.sojina* to cause infection. The counties in climate divisions 2 and 3 are in relatively close proximity to each other, but the difference in QoI-resistance between the two divisions might be explained by the fact that only two counties from division 2 participated in the sentinel plot study. Incorporating additional counties from climate division 2 and even climate division 1

(east TN) may provide additional information regarding the level of QoI-resistance, as well as the prevalence and onset of FLS across the state.

Fluctuations in QoI-resistant conidia demonstrated no discernable pattern either within a sentinel plot or within climate divisions. Without the selection pressure exerted by a fungicide application, these variations in QoI-resistance in a field may simply be the natural fluctuations resulting from intraspecies competition. Additional years of sentinel plot studies would have to be conducted in order to be able to assess how weather may or may not be influencing selection for QoI-resistance in the *C.sojina* population of Tennessee. Alternatively, it is possible that the *C.sojina* isolates responsible for the majority of FLS infections in Tennessee are those which have the G143A allele, which would suggest that the mutation may confer increased aggressiveness compared to wildtype *C.sojina*. More than 90% of the *C.sojina* isolates from soybean fields evaluated in Mississippi already carry the G143A allele (Standish et al., 2015).

Because PCR assess genetic material, which has a significant impact on phenotype of an organism, it may provide a more accurate assessment of QoI-sensitivity than the germination assay which is more dependent on having viable or living conidia. Assuming that the qPCR data is more accurate than the spore germination assay at assessing QoI sensitivity, it would appear that while the germination assay results are often fairly close to the qPCR results, the conidia germination assay may underestimate the level of QoI resistance in the field. This is in contrast to a similar study conducted on the causal agent of almond scab, *Fusicladium carpophilum,* where the frequency of QoI-resistance was underestimated using qPCR

compared to the conidial germination assay (Luo et al., 2013). This study also cited heteroplasmy, the presence of mitochondria both with and without the G143A mutation within the same isolate, as an explanation for the lack of correlation between germination assays and the qPCR results because the phenotypic and genotypic data might not manifest the same way.

The discrepancy in results between the two methods of evaluating QoI-sensitivity may also be explained by the use of different FLS lesions to complete the assessments. Because the *C.sojina* infections across a field are not expected to be the result of infection from genetically identical conidia, it would not be surprising for a study conducted on different FLS lesions from the same area to yield different proportions of the QoI-sensitive and QoI-resistant alleles, as is the case with the qPCR and germination assays which were conducted using approximately 15 lesions and 9 entire leaves (> 15 individual lesions), respectively. Additionally, the PCR assay will amplify DNA from the lesions regardless of whether the fungal tissue is living or dead; however, the germination assay is only meant to assess living cells and is dependent on the conidia still being attached to the lesion in order to be successful.

The AUDPC data initially appeared to suggest that the development of FLS was less pronounced when moving from west to middle Tennessee because the average AUDPC values for 2014 were greater in climate divisions 4 and 3 than they were in climate division 2. There was an anomaly, however, in climate division 2 due to Franklin County experiencing a much greater level of FLS incidence in 2015 than in 2014 which resulted in the average 2015 AUDPC incidence being greater for climate division 2 than

for climate division 3. It is important to recall that the sentinel plots are neither planted in the same location nor using the same soybean variety both years. Discrepancies in the level of QoI-resistance and in the AUDPC for FLS incidence and severity are not unexpected, because the inoculum potential of the field and the FLS-susceptibility of the varieties may differ. It is still possible, however, to compare levels of QoI-resistance and disease onset within a county because they indicate potential reservoirs of *C.sojina* infection and could help explain disease epidemics the current or following years.

The AUDPC for incidence and severity was observed to have decreased in the majority of the sentinel plot counties from 2014 to 2015. The winter between those two years may have reduced the inoculum potential in those counties; but differences in ambient conditions during the soybean-growing season may also have influenced FLS disease progression. The change in field and in soybean variety for each sentinel plot county from 2014 to 2015 is likely to have significantly influenced the incidence and severity ratings and hence sentinel plots comparisons across years may be misleading.

Because the majority of the sentinel plot soybean fields did not reach the reproductive growth stage until August, the temperature in August is likely to have a more significant impact on disease progression. In 2014, August averaged the highest temperature and precipitation; however, July averaged the highest temperature and precipitation for 2015 in two of the three climate divisions. Ambient conditions in July may have been favorable for disease in 2015 but less disease may have been observed since the soybean were not at the appropriate growth stage for disease. The increase in average AUDPC values from 2014 to 2015 for climate division 2 might be attributed to

the prematurely warm weather that occurred in July which was unusual for that climate. The warm, wet weather may have stimulated FLS disease progression to occur sooner in that region.

Directional Study

Samples taken from higher in the canopy, where leaves are youngest and where infections are most recent, exhibited greater levels of QoI-resistant conidia than those isolated from lower in the canopy where both leaves and lesions are older, which suggests that younger leaves are either more susceptible to the mutants, or that the mutants may not be causing infections in a field until later in the season. The lesions higher in the canopy also have greater exposure to wind-blown conidia, which increases the chances for genetic recombination amongst isolates to occur and, thus, aids in proliferation of QoI-resistant conidia. Alternatively, while the samples for the directional studies are taken from areas not treated with a fungicide, there is the possibility for fungicides to drift over from neighboring fields where they have been applied which could potentially exert selection for fungicide resistance. *C.sojina* isolates exposed to fungicide selection pressure in adjacent fields may also be blown into fields with no previous history of fungicide application, thus, increasing the proportion of QoI-resistance in the upper canopy of the unsprayed field. Canopy height is likely to influence the amount of light available to any germinating conidia which land on the plant; greater exposure to light could also lead to warmer temperatures in that zone. In laboratory conditions, *C.sojina* sporulates most under 12 hours of light and 12 hours of darkness at 25±2°C (Gomez & Reis, 2013). For *Cercospora kikuchii* grown in lab,

sporulation is reduced by 60% when grown in darkness at 13°C compared to when grown under 12 hours of light and 12 hours of darkness, but between 22-28°C the influence of light on sporulation disappears (Chen et al., 1979).

It can be hypothesized that this selection for fungicide resistance would be more pronounced in the FLS lesions from the upper canopy, because it would be more difficult for unintentional fungicide applications to penetrate the lower canopy. This might indicate that QoI-sensitivity levels across a typical agronomic field might exhibit even greater levels of variation. FLS samples taken from the same canopy height in a county might be expected to demonstrate similar levels of QoI-resistance since those infections likely occurred at approximately the same time; however, this trend was not observed so perhaps the inoculum originates in areas with different selections for QoI-fungicide resistance.

This also demonstrates potential flaws in the conidial germination assay as a means of assessing QoI-sensitivity because it illustrates how subsets of a field sample may not be representative of the overall population. Future germination assays should be conducted at least in duplicate with sampling conducted at various canopy heights and directions within a field to improve accuracy. Pooled FLS lesion DNA will be evaluated using the previously discussed TaqMan qPCR assay and compared to the germination assay results.

References

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. Pest Management Science, 58(7), 649-662. doi: 10.1002/ps.520

Chen, M. D., Lyda, S. D., & Halliwell, R. S. (1979). Environmental Factors Influencing Growth and Sporulation of Cercospora kikuchii. *Mycologia, 71*(6), 1150-1157. doi: 10.2307/3759101

Chupp, C. (1954). A monograph of the fungus genus Cercospora. Ithaca, New York: Chupp, Charles. Compendium of Soybean Diseases. (1999). (G. L. Hartman, J. B. Sinclair, & J. C. Rupe Eds. fourth edition ed.): The American Phytopathological Society.

Cruz, C. D., & Dorrance, A. E. (2009). Characterization and Survival of Cercospora sojina in Ohio. Plant Health Progress. doi:10.1094/php-2009-0512-03-rs

Dashiell, K., & Akem, C. (1991). Yield losses in soybeans from frogeye leaf spot caused by Cercospora sojina. Crop Protection, 10(6), 465-468. doi:10.1016/S0261-2194(91)80134-2

Fisher, N., & Meunier, B. (2008). Molecular basis of resistance to cytochrome bc1 inhibitors. Federation of European Microbiological Societies, 8, 183-192. doi:DOI:10.1111/j.1567-1364.2007.00328.x

Gomez, D.E., & Reis, E.M. (2013). Influence of substrates, light, filter paper and pH on the sporulation of Cercospora sojina. *Summa Phytopathologica, 39*(2), 126-129.

Goodwin, S. B., Dunkle, L. D., & Zismann, V. L. (2001). Phylogenetic Analysis of Cercospora and Mycosphaerella Basedon the Internal Transcribed Spacer Region of Ribosomal DNA. Phytopathology, 11.

Hobbelen, P. H. F., Paveley, N. D., & van den Bosch, F. (2011). Delaying Selection for Fungicide Insensitivity by Mixing Fungicides at a Low and High Risk of Resistance Development: A Modeling Analysis. Phytopathology, 101(10), 1224-1233. doi: 10.1094/PHYTO-10-10-0290

Kim, H., Newell, A., Cota-Sieckmeyer, R., Rupe, J., Fakhoury, A., & Bluhm, B. (2013). Mating-Type Distribution and Genetic Diversity of Cercospora sojina Populations on Soybean from Arkansas: Evidence for Potential Sexual Reproduction. Phytopathology, 103(10), 1045-1051. doi:10.1094/PHYTO-09-12-0229-R

Koenning, S., & Wrather, J. (2010). Suppression of soybean yield potential in the continental United States by plant diseases from 2006 to 2009. Plant Health Progress. doi:10.1094/PHP-2010-1122-01-RS

Lehman, S. G. (1928). Frogeye Leaf Spot of Soy Bean caused by Cercospora Diazu Miura. Journal of Agricultural Research, 36(9), 811-833Lehman, S. G. (1934). Frog-eye (Cercospora diazu Miura) on stems, pods, and seeds of Soybean, and the relation of these infections to recurrence of the disease. Journal of Agricultural Research, 48(2), 131-147 pp.

Luo, Y., Hou, L., Förster, H., & Adaskaveg, J. E. (2013). QoI Resistance in Fusicladium carpophilum Populations from Almond in California and Evaluation of Molecular

Resistance Mechanisms. Plant Disease, 97(10), 1322-1330. doi:10.1094/PDIS-12-12-1211-RE

Mengistu, A., Bond, J., Mian, R., Nelson, R., Shannon, G., & Wrather, A. (2011). Identification of Soybean Accessions Resistant to by Field Screening, Molecular Markers, and Phenotyping. Crop Science, 51(3), 1101. doi:10.2135/cropsci2010.09.0529

Mengistu, A., Kurtzweil, N. C., & Grau, C. R. (2002). First Report of Frogeye Leaf Spot (Cercospora sojina) in Wisconsin. Plant Disease, 86(11), 1272-1272. doi:10.1094/PDIS.2002.86.11.1272B

Mian, M. A. R., Boerma, H. R., Phillips, D. V., Kenty, M. M., Shannon, G., Shipe, E. R., Weaver, D. B. (1998). Performance of Frogeye Leaf Spot-Resistant and -Susceptible Near-Isolines of Soybean. Plant Disease, 82(9), 1017-1021. doi:10.1094/PDIS.1998.82.9.1017

Mian, M. A. R., Missaoui, A. M., Walker, D. R., Phillips, D. V., & Boerma, H. R. (2008). Frogeye Leaf Spot of Soybean: A Review and Proposed Race Designations for Isolates of Hara. Crop Science, 48(1), 14. doi:10.2135/cropsci2007.08.0432

Mikaberidze, A., McDonald, B. A., & Bonhoeffer, S. (2014). Can High-Risk Fungicides be Used in Mixtures Without Selecting for Fungicide Resistance? Phytopathology, 104(4), 324-331. doi: 10.1094/PHYTO-07-13-0204-R

Singh, T., & Sinclair, J. B. (1985). Histopathology of Cercospora sojina in Soybean Seeds. Phytopathology, 75(2), 185-189.

Standish, J. R., Tomaso-Peterson, M., Allen, T. W., Sabanadzovic, S., & Aboughanem-Sabanadzovic, N. (2015). Occurrence of QoI Fungicide Resistance in Cercospora sojina from Mississippi Soybean. Plant Disease, 99(10), 1347-1352. doi:10.1094/PDIS-02-15-0157-RE

Staub, T., & Morton, V. (2008). A short history of fungicides. APS net Features. doi:10.1094/APSnetFeature-2008-0308

USDA-ERS. (2012). Soybeans & Oil Crops:Background. 1-3. Retrieved from http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/background.aspx#.Uv7GdxBdUwB

USDA-Integrated Pest Management Pest Information Platform for Extension and Education (PIPE). (2014). http://frogeye.ipmpipe.org/cgi-bin/sbr/public.cgi

Zeng, F., Arnao, E., Zhang, G., Olaya, G., Wullschleger, J., Sierotzki, H., Bradley, C. A. (2014). Characterization of Quinone Outside Inhibitor Fungicide Resistance in Cercospora sojina and Development of Diagnostic Tools for its Identification. Plant Disease, 99(4), 544-550. doi:10.1094/PDIS-05-14-0460-RE

Zhang, G. (2012). Cercospora sojina: overwinter survival and fungicide resistance. (Doctor of Philosophy in Crop Sciences Dissertation), University of Illinois.

Zhang, G. R., Newman, M. A., & Bradley, C. A. (2012). First Report of the Soybean Frogeye Leaf Spot Fungus (Cercospora sojina) Resistant to Quinone Outside Inhibitor Fungicides in North America. Plant Disease, 96(5), 767-767. doi:10.1094/PDIS-10-11-0915-PDN

CHAPTER II

ASSESSING EFFICACY OF FUNGICIDES AGAINST FROGEYE LEAF SPOT DISEASE AND SELECTION FOR STROBILURIN FUNGICIDE RESISTANCE

Abstract

In an effort to understand the efficacy of different fungicides in light of QoI fungicide resistance, field trials were conducted in a randomized complete block design in four locations in Tennessee and one in Illinois during the 2013 and 2014 soybean growing seasons. A minimum of six foliar fungicides comprising QoI, DMI, DMI+QoI, SDHI+QoI, MBC, and chlorothalonil chemical groups were evaluated on a FLS-susceptible soybean variety using a R3 application time. Additional combination-chemical-group fungicides evaluated only in 2014 included: SDHI+QoI+DMI and MBC+DMI products. FLS disease severity (%) and soybean yield (bu/a) were recorded. The negative correlation between yield and increasing FLS disease severity was demonstrated during both growing seasons. In general, combination fungicides were within the top three highest-yielding treatments and conferred the greatest disease control; however, solo-DMI and solo-MBC fungicides also demonstrated adequate FLS disease control and yield protection. QPCR and conidial germination assay data indicated that not only are solo-QoI-fungicides ineffective at controlling FLS, and fungicides with combination multiple modes of action, while effective, still select for QoI-resistance if they contain a QoI-component.

Keywords: strobilurin, fungicide mixtures, frogeye leaf spot, QoI-resistance, *C.sojina*

Introduction

The first report of *Cercospora sojina* Hara, the causal agent of frogeye leaf spot (FLS) disease on soybean, originated in Japan in 1914(Chupp, 1954). In the early 1920s the pathogen was reported in the southern United States, where it remains prevalent; however, it has since spread to more northern states, such as Wisconsin and Ohio (Cruz & Dorrance, 2009; Lehman, 1928; Mengistu, et al., 2002). Although the pathogen can withstand below-zero overwintering conditions, FLS disease progression is favored by warm (25-30°C) and humid (>90%) conditions which enable spore production to occur as early as 48 hours after symptoms manifest (Cruz & Dorrance, 2009; Mian, et al., 2008). The fungus will overwinter and sporulate on infested soybean debris, and remains viable on debris even after two years in the field (Cruz & Dorrance, 2009; G. Zhang, 2012).

FLS disease is characterized by the angular to circular lesions which typically appear on soybean leaves (Lehman, 1928). Initially, lesions appear as small, dark brown spots but eventually develop into larger spots, with discolored gray or beige centers, surrounded by a dark red or brown border (Lehman, 1928). Lesions may coalesce as the disease progresses and contribute to premature defoliation (Lehman, 1928). While foliar FLS symptoms are the most commonly observed, additional parts of the soybean plant may also be affected: seeds, stems, and pods (Lehman, 1934). Seeds typically become infected with FLS through the pod walls and display gray to black discolorations (Singh & Sinclair, 1985). FLS-infected seeds experience delayed

germination and produce weaker seedlings with FLS lesions present on the cotyledons (Mian et al., 2008; Singh & Sinclair, 1985).

The sexual reproductive lifecycle of the *C.sojina* has never been observed, but there is speculation that cryptic sexual reproduction may be occurring and many *Cercospora* species have teleomorphs in the *Mycosphaerella* genus (Goodwin et al., 2001; Kim et al., 2013). As of yet, the main source of natural inoculum for FLS disease are the conidia produced directly from the necrotic lesions which are wind and rain dispersed (Lehman, 1928). A conidium can land on a soybean plant and provided with adequate moisture, may germinate within the hour to infect the plant; however, foliar symptoms may not be visible until two weeks after the initial infection (Mian et al., 2008). FLS lesions may continue to produce conidia throughout the season, thus, allowing for multiple infection cycles (Mengistu et al., 2011; Mian et al., 2008). The disease reduces the photosynthetic area of the plant, and yield losses exceeding 60% have been reported (Dashiell & Akem, 1991). In the U.S. alone, a loss of almost 8 million soybean bushels was attributed to FLS in 2009 (Koenning & Wrather, 2010). Soybeans are one of the most planted crops in the U.S., for use in industrial processes or as a protein source, and account for 90% of oilseed production (Compendium of Soybean Diseases, 1999; USDA-ERS, 2012).

Planting resistant soybean cultivars and rotating to a non-host crop for at least two years are typical recommendations for managing FLS disease (Mian et al., 1998). Foliar fungicide applications are also utilized to control disease, but are generally only recommended between the R1-R5 growth stages of soybean development, depending

on the level of FLS disease pressure, which may not warrant fungicide application at all (Mian et al., 2008). Multiple chemical companies have synthesized strobilurin compounds for fungicide use; however, the fungicides may differ in how they move through the plant. Even within the strobilurin-chemical group, the degree to which some compounds are, or are not, xylem-mobile varies, which can have an impact on overall fungicide efficacy at controlling a specific disease (Bartlett et al., 2002). For example, azoxystrobin and pyraclostrobin are both strobilurin fungicides with the same mode of action; however, the former is xylem-mobile while the latter is not (Bartlett et al., 2002). Strobilurin or quinone outside inhibitor (QoI) fungicides were first marketed in 1996 and quickly gained prevalence due to their broad spectrum activity (Staub & Morton, 2008). Unfortunately, their rise in popularity was also associated with a rise in reports of QoI-fungicide resistance in many plant pathogens (Fisher & Meunier, 2008)).

The first incidence of QoI-fungicide resistant *C.sojina* was in 2010 from a Tennessee soybean field being treated with a strobilurin fungicide (Zhang et al., 2012). Fungicide development programs are expensive and often require 10 years of testing prior to receiving EPA approval; therefore, it is essential to maximize the life of existing fungicides. Regardless of whether or not the majority of the *C.sojina* population in the United States is QoI-resistant, QoI fungicides remain effective at controlling a multitude of plant diseases (Vincelli, 2012). In light of current and continued selection for fungicide resistance in the *C.sojina* population, it becomes increasingly important to identify fungicides and/or fungicide combinations, which are not only effective at controlling FLS but also minimize selection for QoI-fungicide resistance.

Materials and Methods

Field Conditions and Treatment Application

Soybean foliar fungicide trials were evaluated across 4 locations in Tennessee and 1 in Illinois in 2013 and 2014. Tennessee trials were planted in 9 m long, four-row plots (76 cm row spacing) using Asgrow 4832 planted on 29 May 2013 and 5 May 2014 (Milan), 29 June 2013 and 12 May 2014 (Jackson), 29 May 2013 and 27 May 2014 (Dyersburg), and with Armor 53Z5 on 5 Jun 2014 (Knoxville), while the Illinois trial was planted in 7.6 m long, four-row plots (76 cm row spacing) using Armor 4744 on 5 September 2013 and 27 May 2014 (Dixon Springs) (Table 9). Foliar fungicides were applied to 4 row plots at the R3 (beginning pod) stage of soybean development, with the exception of the Knoxville trial where treatment was applied at R4 (full pod). Treatments included solo and combination mode of action products encompassing QoI, QoI+DMI, DMI, SDHI+QoI, MBC, MBC+DMI, SDHI+QoI+DMI and Chlorothalonil fungicide groups (Table 10). Including the non-treated control, there were a total of 7 treatments in 2013 and anywhere from 8 to 12 treatments in 2014 (table 10). In 2014, all trials included the original 6 fungicides (Headline-QoI, Topguard-DMI, QuadrisTop-QoI+DMI, Priaxor-SDHI+QoI, TopsinM-MBC, and Bravo-Chlorothalonil), which were also utilized in 2013, plus an additional treatment combination consisting of Priaxor+Domark (SHI+QoI+DMI). In addition to the 7 fungicides applied at all of the 2014 trials, certain trials included Overrule-MBC+DMI, (Dixon Springs, Milan, Dyersburg, and Jackson), AproachPrima-QoI+DMI (Dyersburg, Milan, Jackson), StrategoYLD-QoI+DMI (Jackson), and TopsinXTR –DMI+MBC (Jackson).

Treatments were replicated four times in a randomized complete block design. The Knoxville trial was sprayed on 29 Aug using a carbon- dioxide pressurized backpack sprayer outfitted with FF 80015 nozzles with 15-inch nozzle spacing, set to 30 psi and delivering 116.0 L/ha. The remaining Tennessee trials were sprayed using a Lee Spider Sprayer with T-jet 8002 flat fan nozzles spaced on 20- inch centers set to deliver 140.4 L/ha at 30 psi on 8 Aug 2013 and 29 Jul 2014 (Milan), 28 Aug 2013 and 30 Jul 2014 (Jackson), 5 Aug 2013 and 24 Jul 2014 (Dyersburg). The Illinois trials were sprayed using a carbon dioxide-powered backpack sprayer outfitted with four Twin Jet TJ60-8002 nozzles and delivering 187.1 L/ha at 40 psi on 15 Sept 2013 and 29 Jul 2014 (Dixon Springs).

Frogeye leaf spot disease severity, as a value of percent leaf area affected, was rated within the center two rows of each plot 2-3 weeks after fungicide application. The 2014 Knoxville trial has such low FLS incidence and severity (<5%) that leaf samples were combined by treatment instead of by plot number and disease ratings were not taken. Soybeans were harvested at maturity, and yield weight (bu/a) and moisture data were collected and adjusted to 13.5% moisture.

Data were analyzed in JMP Pro 10.0.2 (SAS Institute Inc., Cary, NC, 2012). FLS severity (%) and disease control (%) values were transformed using the *arcsine* transformation method to help normalize the distributions. Least squares means were separated using Tukey's honestly significant difference (HSD) test *(P*≤0.05) in JMP. To simplify the statistical analysis the six core fungicide treatments encompassing five different fungicide groups (QoI, DMI, QoI+DMI, MBC, and Chlorothalonil) utilized in all

trials both years were evaluated together. Each year was analyzed individually. Dixon Springs yield data from 2014 was questionable and excluded from the analysis.

Assessing QoI Resistance

Germination Assay

When possible, leaf samples were taken 2-3 weeks after fungicide application to evaluate *C.sojina* QoI-fungicide resistance. Knoxville was the only trial where FLS samples were collected from the area prior to treatment application; but due to the low incidence of disease only one sample was collected from the fungicide trial area and two samples were collected from the adjacent fields before fungicide application. Soybean leaves displaying FLS symptoms were collected and incubated overnight in a "moist environment" created by incubating leaves in a plastic bag with a damp paper towel to facilitate sporulation. Using a 20 µl pipette outfitted with sterile pipette tips, conidia were suctioned off of lesions in sterile, deionized water. Conidia were dislodged by depositing a 10 µl droplet of water onto a sporulating lesion and pipetting up and down until the conidia were suspended in the water drop. A dissecting scope was utilized to better observe conidia collection. The droplet could be transferred to multiple lesions to collect additional conidia, as necessary. The number of leaves and lesions from which spores were collected varied depending on leaflet disease severity. Samples with low levels of disease severity and/or sporulation often required more leaves to successfully complete the germination assay. A total of 50-60 µl of sterile water would be utilized to generate the *C.sojina* conidial suspension from each sample; half of the composite conidial suspension would be deposited onto control potato

dextrose agar (PDA) plates, while the remaining half of the suspension would be deposited onto azoxystrobin (QoI-fungicide)-amended PDA plates.

PDA media was prepared by combining 23.4 g PDA in 600 ml distilled water and autoclaving at 121°C. Separate flasks of media were prepared for the amended and unamended plates. Both fungicide-amended and unamended plates were supplemented with salicylhydroxamic acid (SHAM) to prevent the alternative oxidase respiratory pathway from allowing QoI-sensitive conidia to overcome the inhibitory effect of the fungicide once plated onto the media. The SHAM stock solution consisted of 0.2 g of SHAM and 2.0 ml Methanol. The azoxystrobin stock fungicide solution consisted of 100µg of technical grade azoxystrobin in 1 ml Acetone. The fungicide stock solution was serially diluted in acetone to 1 µg/ml and applied 600 µl were applied to the fungicide-amended flask only.

The goal of the germination assay was to assess at least 50 conidia for germination on both the fungicide-amended and the control plate. Assays with less than the required number of conidia excluded from the analysis. Spore suspensions were spread onto the plates using sterilized glass rods. Plates remained on the laboratory benchtop at room temperature (~25°C) until evaluation. Conidia were allowed 14-18 hours for germination before assessment. The number of germinated and non-germinated conidia from each plate was recorded using a compound light microscope. A germ tube exceeding half of the length of the conidia was considered germinated. Assays which had less than 70% germination on non-amended plates were excluded from analysis so as to minimize inaccurately assessing the level of QoI-resistance on

the amended plates. In order to account for dead conidia, the % germination on the azoxystrobin-amended plates was adjusted as follows:

$$\frac{\#\ spores\ germinated\ on\ amended\ plate}{total\ \#\ spores\ counted\ on\ the\ amended\ plate} \div \frac{\#\ spores\ germinated\ on\ the\ non-amended\ plate}{total\ \#\ of\ spores\ counted\ on\ the\ non-amended\ plate} * 100$$

Quantitative PCR

The quantitative PCR assay was conducted in the exact same manner as that described in the preceding chapter. DNA was extracted from an average of 14 FLS lesions from every plot of each field using the Qiagen DNeasy plant mini kit (QIAGEN, Valencia, CA, USA). After the initial extraction, *C.sojina* DNA was maintained at 4°C until the QPCR could be performed.

The quantitative PCR TaqMan® assay allows for the detection and quantification of both the G143A mutation conferring QoI-resistance and the wildtype allele which leaves the pathogen QoI-sensitive. Because the *C.sojina* DNA tested from the 2014 sentinel plots was a compilation of multiple lesions from the same area, it would not be unexpected for the assay to detect both the sensitive and the resistant alleles for the cytochrome bc_1 gene within a reaction; thus, the "%G143A" or the "%WT" which will be referred to throughout this paper refers to the percentage of the total *C.sojina* DNA estimated by the qPCR which was determined to be either the mutant(G143A) or the wildtype(WT) allele. Similarly, because the germination assay is performed using a compilation of *C.sojina* conidia from multiple FLS lesions within an area, it would not be surprising for a mixture of QoI-sensitive and QoI-resistant conidia to be detected within a sample.

The qPCR platform utilized was the IQ™5 from Bio-Rad (Bio-Rad, Hercules, CA, USA). PCR reactions were performed in 25µl volumes, comprised of 10µl TaqMan®

Master Mix (2X), 1.25µl TaqMan® Custom SNP genotyping assay (20X), 9.25 µl molecular grade water (ThermoScientific, Waltham, MA, USA) , 0.5 µl (20µg/µl), Bovine Serum Albumin (ThermoScientific, Waltham, MA, USA) and 4 µl of template DNA. The qPCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 sec and 62°C for 1 min. The TaqMan® Master Mix contains the AmpliTaq Gold®DNA polymerase. The TaqMan® probe labeled with the VIC fluorophore hybridizes to the mutant or QoI-resistant allele, while the FAM-labeled fluorophore hybridizes to the wildtype or QoI-sensitive allele. Standard curve quantitation was utilized to quantify the DNA concentrations of the unknown FLS samples by comparing their cycle threshold (Ct) values to the Ct values of the standards.

DNA was extracted from pure *C.sojina* cultures of known QoI-sensitivity by scraping off the mycelium into 2 ml screw cap tubes supplemented with sterile glass bead and freezing the specimens at -80°C for at least 3 hours prior to rupturing the cellular tissue in the FastPrep® FP 120 (ThermoSavant, Carlsbad, CA, USA). DNeasy Plant mini kit (QIAGEN, Valencia, CA, USA) was used to extract DNA. Nanodrop 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA) was used to quantify DNA. Samples were diluted 6-fold (10 ng/µl, 1.0 ng/ µl, 0.1 ng/µl, 0.01 ng/µl, 0.001 ng/µl, and 0.0001 ng/µl) in molecular biology grade water (FisherScientific, Waltham, MA, USA). In order to reduce the adherence of *C.sojina* DNA to the plastic tubes, the stock elution of each extraction was amended with 1µl of (1 mg/ml) salmon sperm DNA (Invitrogen, Carlsbad, CA, USA). Each qPCR reaction involved 2 standard curves: one for a QoI-

resistant and one for a QoI-sensitive *C.sojina* isolate to act as controls for the FAM and VIC probes, respectively. The expected QoI-sensitivity of the isolates was confirmed using qPCR before the isolates could be used as DNA/probe standards. All samples, including the standards and the non -template controls (NTCs) were run in duplicate.

Data were analyzed in JMP Pro 10.0.2 (SAS Institute Inc., Cary, NC, 2012). FLS severity data (%) was transformed using the *arcsine* transformation method to help normalize the distributions. LSMeans were separated using Tukey's honestly significant difference (HSD) test *(P*≤0.05) in JMP.

Results

Fungicide Trial Data

Statistical analysis indicated that soybean yield differed significantly ($p<0.001$) by year, location, and fungicide and that FLS severity also differed significantly ($p<0.0001$) by year, location, fungicide, and fungicide*location interaction. Pairwise comparisons between 2013 trial locations demonstrated that there were differences in how the six fungicide treatments influenced yield and disease severity. Pairwise comparisons between 2014 trials also demonstrated significant differences between locations and treatment effects on yield and disease severity.

A negative correlation ($r= -0.61$) was observed between yield and FLS severity in both years. Locations with greater FLS disease severity (%) typically had lower yields than those with lower FLS disease severity (Figures 7 and 8). In 2013, both Milan trials experienced 50% disease severity and, thus, had significantly lower yields (40 bu/a)

than Dyersburg which experienced only 9% disease severity and produced 61 bu/a in yield. Because Dixon Springs was planted after wheat and experienced a shorter season, in a different climate, the yield from there was lower than any of the TN trials, even though it averaged only 1% FLS severity in the field.

When averaged across all trials in 2013, the solo-QoI and solo-chlorothalonil treatments were associated with the greatest levels of FLS severity (24-27%) and did not differ significantly from the non-treated control in terms of yield (Figure 9 and 10). In 2013 the yield in Dyersburg (61 bu/a) was significantly greater than in Jackson (51 bu/a) which was significantly greater than Milan-A4 (40 bu/a) and Milan-A8 (40 bu/a) which were also significantly greater than Dixon Springs (28 bu/a). The QoI+DMI, SDHI+QoI, DMI, and MBC treatments did not differ statistically from each other in terms of yield and the DMI, MBC, and QoI+DMI did not differ significantly in FLS severity. The QoI+DMI combination treatment averaged the highest yield (48 bu/a) and the lowest disease severity (18%) (Figure 9). All of the fungicide treatments were associated with significantly less FLS disease severity than the non-treated, but only the QoI+DMI treatment had significantly greater yield than the non-treated (Figure 9 and 10). The solo-DMI, solo-MBC, and QoI+DMI combination were associated with significantly less disease severity than the solo-QoI (Figure 10).

Mean FLS disease severity for the Tennessee trials was significantly lower in 2014 (4%) than it was in 2013 (31%). Dixon Springs, IL was the exception because it demonstrated greater overall FLS severity in 2014 (31%) than 2013 (1% (Figure 12). Across all of the 2014 trials, FLS severity was typically highest amongst QoI and

Chlorothalonil-treated plots which displayed similar levels of disease severity as non-treated plots (Figure 14). In 2014 yield was significantly greater in Jackson and Knoxville than it was in Milan or Dyersburg, but when averaged across all locations, there were no differences in yield by fungicide group (Figure 11 and 13).

Although FLS disease was lower in 2014, when evaluating only the 8 treatments used in all the trials this year, the solo-QoI treatment was associated with 12% disease severity which, as expected, did not differ significantly from the untreated (14%).The solo-DMI, solo-MBC, QoI+DMI combo, and the three-way combination SDHI+QoI+DMI did not differ significantly from each other in terms of the average disease severity they were associated with (8-9%). When evaluating 2014, using the 10 treatments that were used in Milan, Dyersburg, and Jackson, there were no significant differences in soybean yield by treatment when averaged across locations; however, the treatments containing at least two or more fungicide groups were all associated with higher yields than the solo treatments. Additionally, the top four fungicide treatment groups, in the 10 treatment trials, associated with the lowest FLS severity were the solo-DMI, the solo-MBC, the DMI+MBC combo, and the SDHI+QoI+DMI three- way combination treatment. When evaluating only the 12 treatment Jackson trial, the DMI, MBC, DMI+MBC, and SDHI+QoI+DMI treatments all averaged significantly lower, by 1-3%, FLS severity than the untreated, which averaged 8%, and the solo-QoI, which averaged 7%.

Quantitative PCR and Germination Assay Data

When the percentage of the G143A allele, as detected using QPCR, was averaged across all of the 2014 trials by treatment (post-application), there were no statistically significant differences; but, there were some interesting patterns. In 2014, the Milan trial was the only location where the QPCR detected any of the wildtype allele (4-47%); however, there were no significant differences in %G143A across treatments (Table 12). *C.sojina* DNA from the non-treated plots was 38% wildtype (WT), or QoI-sensitive allele, and the two fungicide group treatments which contained the most QoI-sensitive DNA were the DMI+MBC and Chlorothalonil-treated plots, with 47 and 43% WT DNA detected, respectively. For the Milan trial, the SDHI+QoI, SDHI+QoI+DMI, and solo-QoI treatments were associated with the greatest levels of the G143A allele (97-100%).

When averaged across all 2014 trial locations, the top four treatments (QoI, QoI+DMI, SDHI+QoI, and SDHI+QoI+DMI) associated with greater than, or equal to, 90% detection of the QoI-resistant allele all had a QoI-component as part of the treatment, regardless of whether the QoI fungicide was applied alone or in combination with a fungicide with a different mode of action (Table 11). The two fungicide treatments with the lowest percentage of QoI-resistant conidia, when averaged across the 2014 trials, were the DMI+MBC and Chlorothalonil treatments, with 79 and 75% resistance, respectively (Table 11). The non-treated plots averaged 79% QoI-resistance.

FLS samples taken from the Knoxville fungicide trial, and the neighboring area, prior to fungicide application exhibited an average of 67% G143A using the QPCR, but

samples collected after fungicide application all exhibited 100% G143A. Following treatment application, leaf samples were collected from the Dixon Springs, IL trial in early August and again in late September. QPCR results from both sampling dates consistently identified all of the samples as being 100% G143A, regardless of the treatment applied. Additionally, the 2014 germination assay results for Dixon Springs indicated that QoI-resistance ranged from 80-100% with the average being 94%.

According to the germination assay, in 2013 QoI-resistance existed at less than 15% of the conidia population in Dixon Springs, Dyersburg, Jackson, and Milan; however, according to the QPCR assay, in 2014 100% of the *C.sojina* tested from Dixon Springs, Dyersburg, Jackson, Knoxville, and 81% of the DNA tested from Milan had the G143A allele conferring QoI-fungicide resistance. Although the germination assay detected very low levels of QoI-resistant conidia overall in 2013, the solo-QoI (7%) and the SDHI+QoI (11%) treatments were associated with the highest levels for that year (Table 13).

Discussion

Fungicide Trial

Differences in 2013 yield by location could be attributed to the varying levels of FLS severity; locations with greater FLS severity, such as Milan,) experienced more negative impacts on yield, as a result of FLS, than locations with lower FLS severity. Because FLS severity was less than 10% in the majority of the 2014 trials, there were no significant differences in soybean yield by treatment since the disease was not significantly reducing the green photosynthetic area of the plant and, thus, had little

impact on yield this year. When FLS disease pressure is low in a field, it may not be necessary for growers to apply a fungicide to control disease. The rise in FLS severity (1% to 30%) from 2013 to 2014 in Dixon Springs might also explain the sudden increase in QoI-resistance: the infecting *C.sojina* inoculum must have been predominantly resistant the second year of the trial.

Quantitative PCR and Germination Assay

Whether or not the increase in detection of the G143A mutation in the Knoxville trial after treatment application was the result of fungicide selection remains unclear, especially since the isolates from the non-treated, which theoretically should not have been under any fungicide selection pressure, did not amplify.

The increased selection for QoI-resistance in plots receiving treatments with QoI active ingredients as part of the treatment was not unexpected; however, the lack of reduced selection for QoI-resistance from plots treated with a combination product such as a QoI+ DMI or a SDHI+QoI was interesting. Combination products are often recommended to reduce selection for resistance, but the QPCR assay suggests that is not the case with QoI-resistance in *C. sojina* if a QoI-component is included in the treatment. An inoculation study conducted using *Phytophthora infestans* (late blight of tomato and potato) and *Plasmopora viticola* (downy mildew of grape) suggested that the initial percentage of the pathogen population demonstrating fungicide-resistance has a significant impact on the rate of selection for resistance (Samoucha & Gisi, 1987). If 10% of the pathogen population was resistant to phenylamide fungicides, the majority of the population demonstrated resistance after a handful of generations, even if a two-

way combination fungicide (containing a phenylamide component) was applied; however, the use of a three-way combination fungicide delayed selection for resistance to the extent that even if 50% of the original pathogen population was fungicide-resistant that proportion of resistance in the population remained relatively stable post-fungicide application.

The consistency in the allele identified via the QPCR for the Dixon Springs trial from August to September may suggest that once selection pressure has been exerted, then additional fluctuations in QoI-sensitivity are likely to be minimal, at least within a single season. Applying two fungicides with different mode of action(s) at separate times in a season, however, may significantly alter levels of QoI-sensitivity within a season as the pathogen population responds to each fungicide.

The discrepancy in these values may be the result of increased selection for QoI resistance manifesting in 2014. For example, in Dixon Springs where the germination assay indicated that the average QoI-resistance was 8% one year, but averaged 90% resistance the next year. This may simply be because the field where the trial was conducted in 2015 harbored more QoI-resistant *C.sojina* inoculum than the 2014 field. An alternative explanation is that the proportion of the conidia in the field which are QoI-resistant is not necessarily representative of the amount of FLS infections within that field which will ultimately be QoI-fungicide resistant.

Assessment of Fungicide Efficacy

Ultimately, it is important to control FLS disease severity because yields diminish as severity increases. While products with QoI-components appear to be selecting for

resistance, that does not necessarily diminish their usefulness against other fungal plant pathogens. As a product with multiple target sites, Chlorothalonil is less prone to selecting for resistance; however, it is not effective at controlling FLS meaning that it is not a practical recommendation to producers. This study indicates that QoI and Chlorothalonil fungicides, when applied alone, are ineffective at controlling FLS disease. Fungicide products with active ingredients belonging to the DMI and MBC chemical groups remain effective at controlling FLS disease of soybean whether they are applied alone or in combination with each other and/or fungicides of alternative chemical groups. Applying dual and/or three-way combination fungicides is an important strategy for maintaining efficacy of existing fungicides and reducing selection for fungicide resistance; however, this study seems to indicate that while combination products are amongst the most effective and controlling FLS disease in the field, they still exert selection for QoI-resistance, if containing a QoI-component.

Although FLS-resistant soybean varieties are available, producers may be unwilling to plant them due to higher-yields or cheaper prices associated with the FLS-susceptible varieties. Foliar fungicide trials are relevant today in order to supply producers with recommendations in order to minimize yield loss associated with uncontrolled plant disease. Untreated susceptible varieties may experience as much as 31% yield loss (Akem & Dashiell, 1994).

References

Akem, C.N. & Dashiell, K.E. (1994). Effect of planting date on severity of frogeye leaf spot and grain yield by soybeans. Crop Protection, 13(8), 607-610. doi: http://dx.doi.org/10.1016/0261-2194(94)90006-X

Chupp, C. (1954). A monograph of the fungus genus Cercospora. Ithaca, New York: Chupp, Charles.

Compendium of Soybean Diseases. (1999). (G. L. Hartman, J. B. Sinclair, & J. C. Rupe Eds. fourth edition ed.): The American Phytopathological Society.

Cruz, C. D., & Dorrance, A. E. (2009). Characterization and Survival of Cercospora sojina in Ohio. Plant Health Progress. doi:10.1094/php-2009-0512-03-rs

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. Pest Management Science, 58(7), 649-662. doi: 10.1002/ps.520

Dashiell, K., & Akem, C. (1991). Yield losses in soybeans from frogeye leaf spot caused by Cercospora sojina. Crop Protection, 10(6), 465-468. doi:10.1016/S0261-2194(91)80134-2

Fisher, N., & Meunier, B. (2008). Molecular basis of resistance to cytochrome bc1 inhibitors. Federation of European Microbiological Societies, 8, 183-192. doi:DOI:10.1111/j.1567-1364.2007.00328.x

Goodwin, S. B., Dunkle, L. D., & Zismann, V. L. (2001). Phylogenetic Analysis of Cercospora and Mycosphaerella Based on the Internal Transcribed Spacer Region of Ribosomal DNA. Phytopathology, 11.

Kim, H., Newell, A., Cota-Sieckmeyer, R., Rupe, J., Fakhoury, A., & Bluhm, B. (2013). Mating-Type Distribution and Genetic Diversity of Cercospora sojina Populations on Soybean from Arkansas: Evidence for Potential Sexual Reproduction. Phytopathology, 103(10), 1045-1051. doi:10.1094/PHYTO-09-12-0229-R

Koenning, S., & Wrather, J. (2010). Suppression of soybean yield potential in the continental United States by plant diseases from 2006 to 2009. Plant Health Progress. doi:10.1094/PHP-2010-1122-01-RS

Lehman, S. G. (1928). Frogeye Leaf Spot of Soy Bean caused by Cercospora Diazu Miura. Journal of Agricultural Research, 36(9), 811-833

Lehman, S. G. (1934). Frog-eye (Cercospora diazu Miura) on stems, pods, and seeds of Soybean, and the relation of these infections to recurrence of the disease. Journal of Agricultural Research, 48(2), 131-147 pp.

Mengistu, A., Bond, J., Mian, R., Nelson, R., Shannon, G., & Wrather, A. (2011). Identification of Soybean Accessions Resistant to by Field Screening, Molecular Markers, and Phenotyping. Crop Science, 51(3), 1101. doi:10.2135/cropsci2010.09.0529

Mengistu, A., Kurtzweil, N. C., & Grau, C. R. (2002). First Report of Frogeye Leaf Spot (Cercospora sojina) in Wisconsin. Plant Disease, 86(11), 1272-1272. doi:10.1094/PDIS.2002.86.11.1272B

Mian, M. A. R., Boerma, H. R., Phillips, D. V., Kenty, M. M., Shannon, G., Shipe, E. R., . . . Weaver, D. B. (1998). Performance of Frogeye Leaf Spot-Resistant and -

Susceptible Near-Isolines of Soybean. Plant Disease, 82(9), 1017-1021. doi:10.1094/PDIS.1998.82.9.1017

Mian, M. A. R., Missaoui, A. M., Walker, D. R., Phillips, D. V., & Boerma, H. R. (2008). Frogeye Leaf Spot of Soybean: A Review and Proposed Race Designations for Isolates of Hara. Crop Science, 48(1), 14. doi:10.2135/cropsci2007.08.0432

Samoucha, Y., and Gisi, U. (1987). Use of two-and three-way mixtures to prevent buildup of resistance to phenylamide fungicides against Phytophthora and Plasmopara. Phytopathology 77: 1405-1409.

Singh, T., & Sinclair, J. B. (1985). Histopathology of Cercospora sojina in Soybean Seeds. Phytopathology, 75(2), 185-189.

Staub, T., & Morton, V. (2008). A short history of fungicides. APS net Features. doi:10.1094/APSnetFeature-2008-0308

USDA-ERS. (2012). Soybeans & Oil Crops:Background. 1-3. Retrieved from http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/background.aspx#.Uv7GdxBdUwB

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

Zeng, F., Arnao, E., Zhang, G., Olaya, G., Wullschleger, J., Sierotzki, H.,Bradley, C. A. (2014). Characterization of Quinone Outside Inhibitor Fungicide Resistance in Cercospora sojina and Development of Diagnostic Tools for its Identification. Plant Disease, 99(4), 544-550. doi:10.1094/PDIS-05-14-0460-RE

Zhang, G. (2012). Cercospora sojina: overwinter survival and fungicide resistance. (Doctor of Philosophy in Crop Sciences Dissertation), University of Illinois.

Zhang, G. R., Newman, M. A., & Bradley, C. A. (2012). First Report of the Soybean Frogeye Leaf Spot Fungus (Cercospora sojina) Resistant to Quinone Outside Inhibitor Fungicides in North America. Plant Disease, 96(5), 767-767. doi:10.1094/PDIS-10-11-0915-PDN

CONCLUSION

The level of QoI-fungicide resistant *C.sojina* appears to vary across Tennessee, not only across counties, but also within the same county. The average level of QoI resistance across Tennessee sentinel plots was 69% in 2014 and 65% in 2015. The level of QoI-resistance was significantly lower in climate division 2 counties and suggests that ambient conditions in climate division 2 may be less favorable for QoI-resistant *C.sojina* than climate divisions 3 and 4. Disease control is essential to preventing yield loss; however, the disease pressure within a field must be considered prior to fungicide application to receive maximum benefits. Fungicides with only QoI mode of action are displaying selection pressure for resistance. Combination-QoI treatments provide better disease control than solo strobilurin treatments, but may exhibit different selections for resistance. QoI+DMI and MBC mode of actions consistently provide high levels of disease control. Chlorothalonil and the SDHI+QoI treatments may not be as effective as alternate modes of action at controlling FLS when there is a high proportion of resistance in the field.

APPENDIX

Table 1: 2014 Tennessee sentinel plot information

County	Climate division	Soybean variety	Planting date	Irrigated	Previous crop
Franklin	2	Pioneer 49T97R	5/29/2014	No	N/A
Coffee	2	N/A*	N/A	N/A	N/A
Cannon	3	Pioneer Group 4	6/4/2014	No	N/A
Giles	3	Asgrow 4232	5/5/2014	Yes	N/A
Hardin	3	N/A	N/A	N/A	N/A
Rutherford	3	Pioneer P53T51LL	7/2/2014	No	N/A
Robertson	3	Armor 48R40	5/20/2014	No	soybean
Perry	3	SCS 9474RR	6/21/2014	No	soybean
Fayette	4	Dyna-Grow 31ry45	5/21/2014	No	soybean
Tipton	4	Asgrow 5632	N/A	N/A	N/A
Lauderdale	4	AG 4832	5/22/2014	No	soybean
Hardeman	4	AG 5332 GENRR2Y	6/10/2014	No	soybean
Henry	4	N/A	N/A	N/A	N/A
Lake	4	N/A	N/A	N/A	N/A
Weakley	4	Warren Seed DS4850	5/22/2014	No	Corn

*Information not available

Table 2: 2015 Tennessee sentinel plot information

County	Climate division	Variety	Planting date	Irrigated	Previous crop
Franklin	2	Becks 485	6/25/2015	No	canola
Coffee	2	N/A	N/A	N/A	N/A
Perry	3	Croplan R2C 4752S GENRR2Y?STS	6/20/2015	No	soybean
Robertson	3	Croplan R2C 4752	6/23/2015	N/A	wheat
Cannon	3	Asgrow 5233	6/23/2015	No	wheat
Giles1*	3	39T67	5/16/2015	Yes	soybean
Giles2*	3	39T67	5/16/2015	No	soybean
Rutherford	3	Pioneer P53T51LL	5/22/2015	No	soybean
Fayette	4	Asgrow 3934	5/5/2015	No	corn
Weakley	4	Asgrow 4934	6/22/2015	No	wheat
Hardeman	4	Croplan 4752	6/12/2015	No	cotton
Tipton	4	Ozarks	6/19/2015	N/A	wheat
Lake	4	N/A	N/A	N/A	N/A

*Giles sentinel plot was moved to a new field on 7/20/15

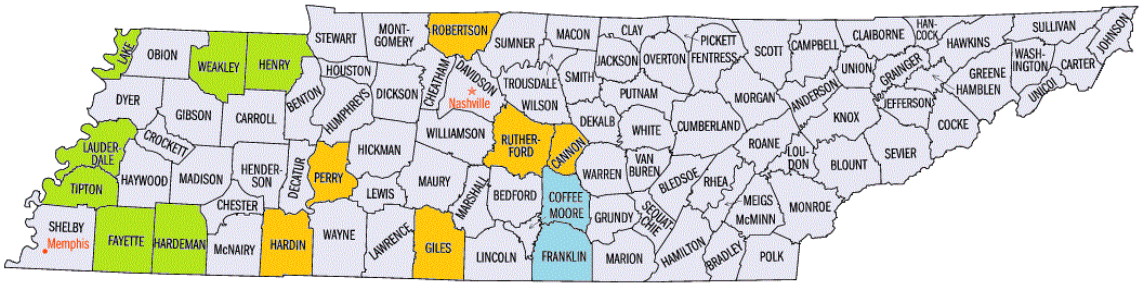

Figure 1: Tennessee county map

*Sentinel plots colored by climate division (green=4, orange=3, and blue=2)

Table 3: Tennessee weather data by climate divisions

		Climate Division					
		2		3		4	
Month	Year	Avg. Temperature (°C)	Avg. Precipitation (inches)	Avg. Temperature (°C)	Avg. Precipitation (inches)	Avg. Temperature (°C)	Avg. Precipitation (inches)
July	2014	22.2	4.62	23.2	3.31	23.7	3.94
August	2014	23.3	6.06	24.9	5.07	25.8	4.45
September	2014	20.9	2.26	21.6	1.2	21.9	3.47
July	2015	24.6	8.15	26.1	6.19	27.3	4.62
August	2015	22.5	5.54	23.6	4.05	24.4	4.76
September	2015	20.5	3.32	21.9	2.15	22.8	1.87

*No significant differences (p>0.05) in average monthly temperature and precipitation by climate division or by year

Table 4: 2014 sentinel plot average QoI-assessment by climate division

Assay Detection (p-value)	Climate Division 2	Climate Division 3	Climate Division 4
%G143A* (p=0.0013)	68.7[b]	99.9[a]	96.5[a]
%QoI-resistant (p=0.258)	54.5	67.9	77.4

*Means within a row followed by the same letter are not different according to Tukey's HSD (p≤0.05)

Table 5: 2014 sentinel plot mean QoI-assessment by county

Sentinel Plot	Mean %G143A (QPCR)	Mean %QoI-resistant (Germination Assay)
Canon	100	21
Coffee	61	48
Franklin	100	85
Giles	100	64
Hardeman	98	46
Henry	91	76
Lake	100	78
Lauderdale	100	75
Perry	99	80
Robertson	100	68
Tipton	100	88
Weakley	87	100
Fayette	100	-*

*Excluded counties for which germination assay was not conducted and those which demonstrated no incidence of FLS throughout the season

Table 6: Percentage of QoI-resistant *C.sojina* conidia in TN counties by canopy height and direction sampled

County	Canopy Height	Direction Sampled				
		Center	North	East	South	West
Dyer (9/24/14)[1]	High	71.46	100	95.69	84.35	90.26
	Middle	71.12	-[2]	77.7	89.67	100
	Low	49.61	-	-	-	-
Gibson (9/8/14)	High	-	-	31.44	-	-
	Middle	-	-	23	30.7	-
	Low	-	-	-	-	-
Madison (8/26/14)	High	-	51.1	62.86	-	85.11
	Middle	-	-	21.27	-	5.39
	Low	-	32.92	-	-	-

[1] Sample collection date,
[2] Data from assays with less than 50 conidia excluded

Table 7: Sentinel plot AUDPC data

Climate Division	Sentinel Plots	2014 AUDPC Incidence	2014 AUDPC Severity	2015 AUDPC Incidence	2015 AUDPC Severity	2014 Avg. AUDPC Incidence by climate division	2014 Avg. AUDPC Severity by climate division	2015 Avg. AUDPC Incidence by climate division	2015 Avg. AUDPC Severity by climate division
2	Franklin	6.07	0.12	88.82	1.42	26.79	0.97	70.99	1.37
2	Coffee	47.50	1.82	53.16	1.33	-	-	-	-
3	Canon	36.70	1.79	50.07	0.88	52.75	2.96	32.82	0.51
3	Robertson	73.69	7.18	25.02	0.20	-	-	-	-
3	Giles	91.47	2.66	48.05	0.93	-	-	-	-
3	Perry	9.15	0.20	8.13	0.04	-	-	-	-
4	Fayette	8.90	0.11	1.04	0.01	46.20	2.66	42.88	0.77
4	Lake	9.13	0.19	97.93	3.15	-	-	-	-
4	Weakley	19.70	0.32	43.02	0.20	-	-	-	-
4	Henry	52.47	1.93	N/A	N/A	-	-	-	-
4	Lauderdale	63.37	4.95	N/A	N/A	-	-	-	-
4	Tipton	77.12	3.40	29.84	0.22	-	-	-	-
4	Hardeman	92.68	7.70	42.56	0.28	-	-	-	-

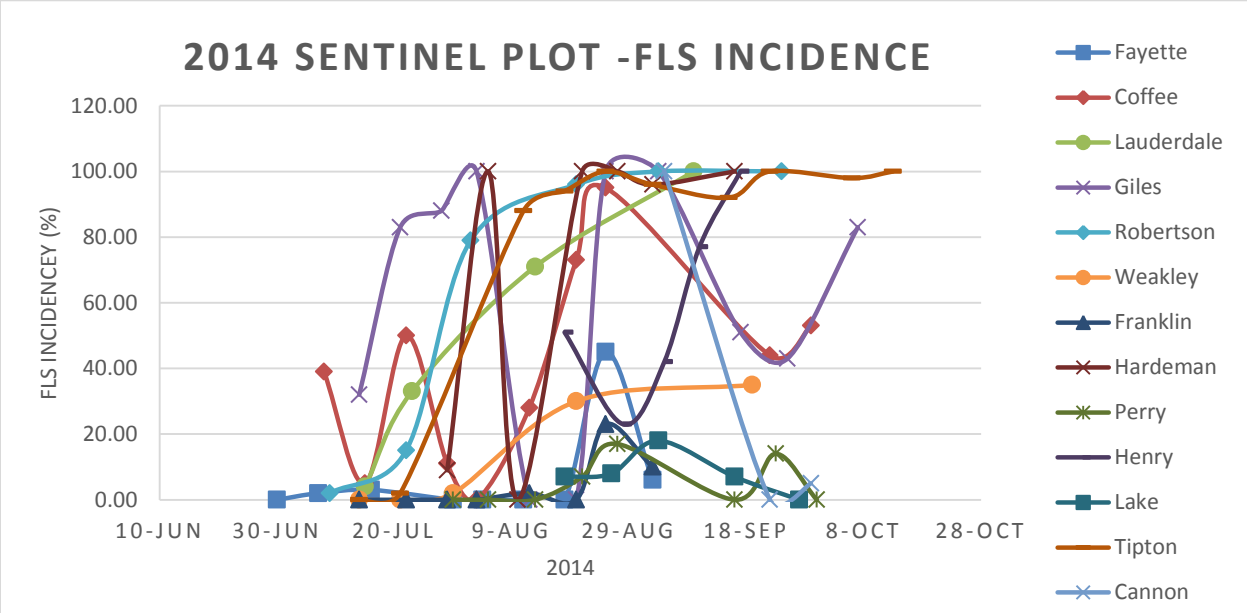

Figure 2: 2014 sentinel plot-FLS incidence

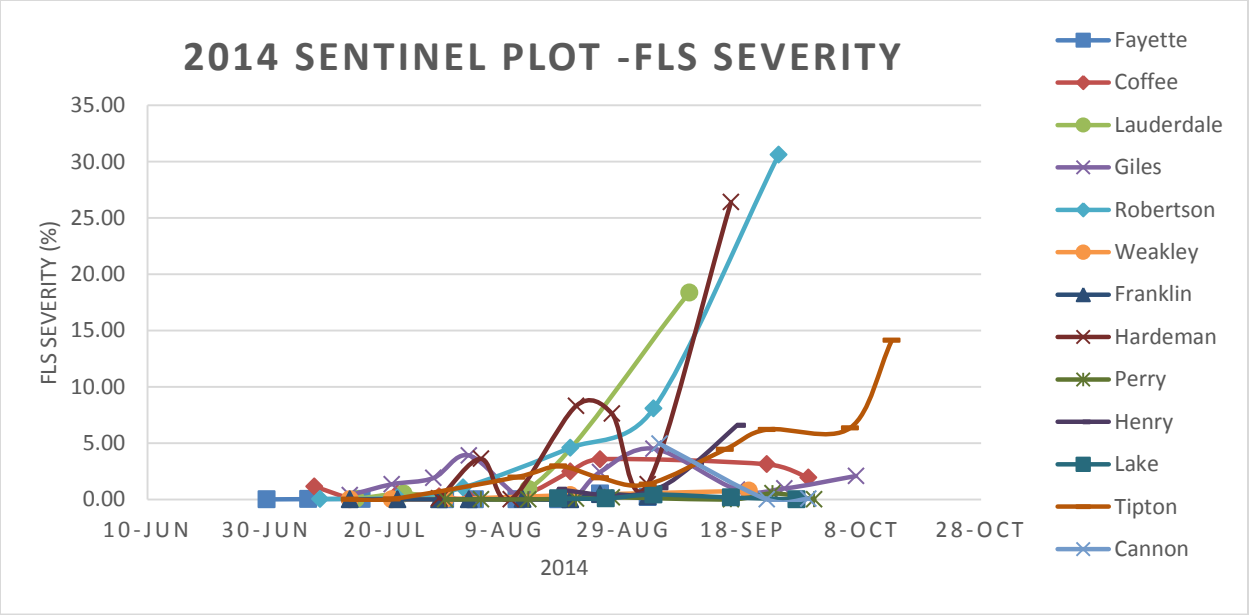

Figure 3: 2014 sentinel plot-FLS severity

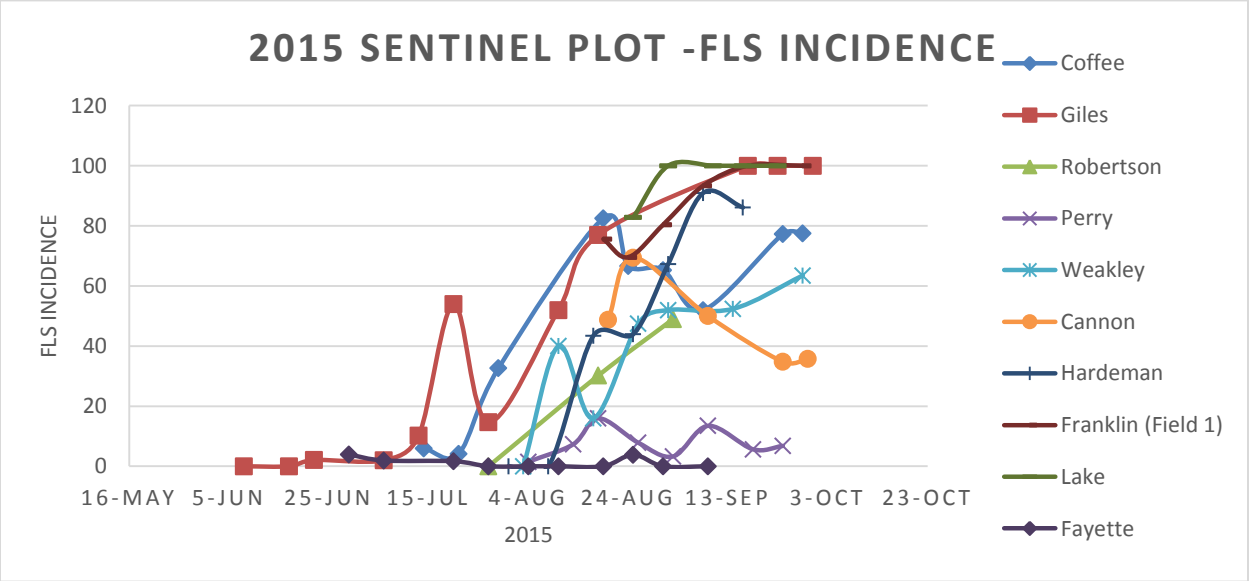

Figure 4: 2015 sentinel plot-FLS incidence

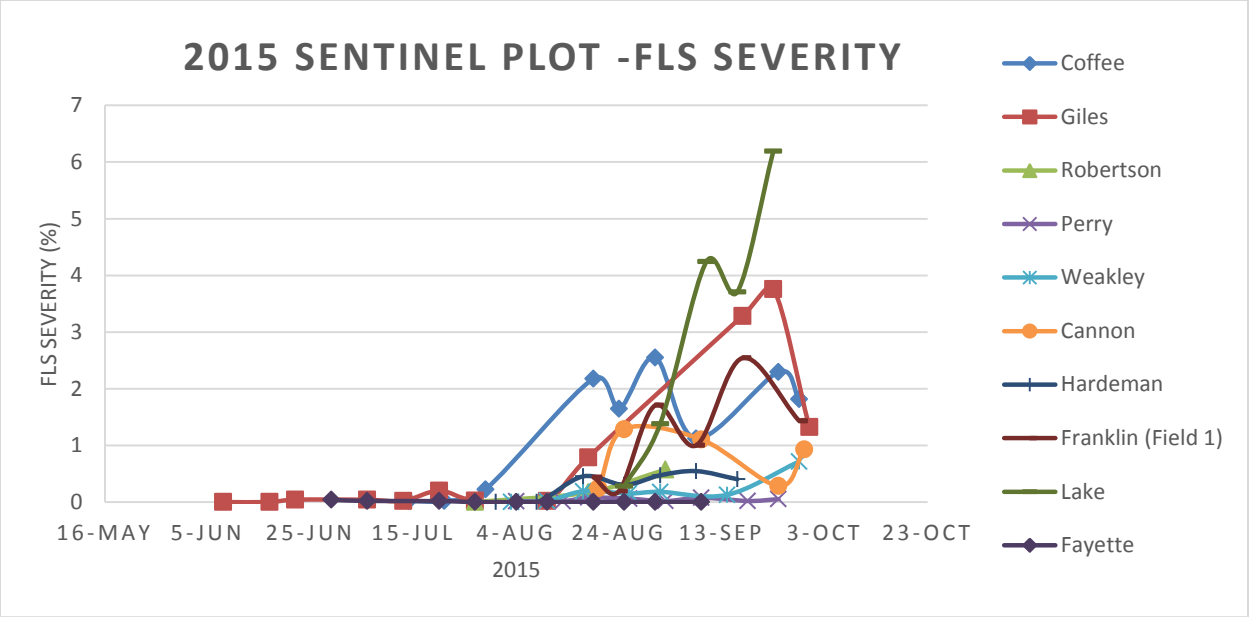

Figure 5: 2015 sentinel plot-FLS severity

Table 8: Germination Assay-detected mean QoI-resistant (%) *C.sojina* conidia in TN sentinel plots by climate division

Year (p-value)	Climate Division 2	Climate Division 3	Climate Division 4
2014 (p=0.258)	55	68	77
2015 (p=0.009)	44[b]*	67[ab]	77[b]
Average (p=0.008)	48[b]	67[ab]	77[a]

*Means followed by the same letter within a row are not different according to Tukey's HSD (p≤0.05)

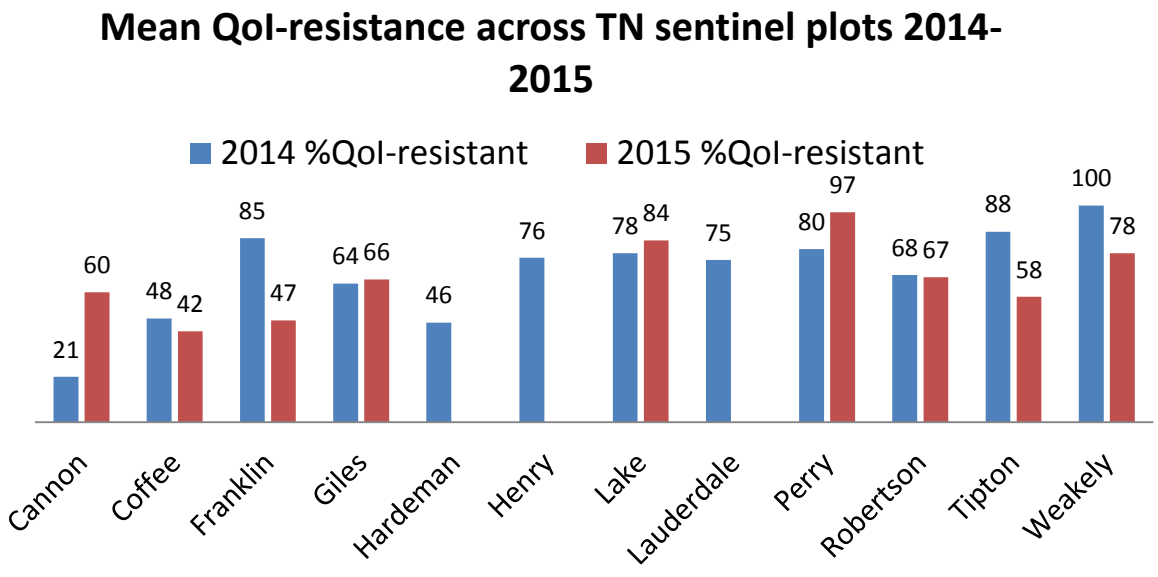

Figure 6: Mean QoI-resistance across TN sentinel plots (2014-2015)

* No significant differences within the 2014 (p=0.3026) and 2015 (p=0.1435) sentinel plots

Table 9: Field trial location information

Trial Location (city, state)	Soybean Variety	FLS Pressure	2013 Planting Date	2014 Planting Date	Plot Dimensions	Application Method	2013 Spray Date/ soybean growth stage		2014 Spray Date/ soybean growth stage	
Dixon Springs, IL	Armor 4744	Low	-[1]	May 27[1]	76 cm row spacing , 7.6 m long	CO_2-backpack sprayer	Sept. 5	R3	July 29	R3
Dyersburg, TN	Asgrow 4832	Low	May 29	May 27	76 cm row spacing , 9 m long	Lee Spider Sprayer	Aug. 5	R3	July 24	R3
Jackson, TN	Asgrow 4832	Moderate	June 29[1]	May 12	76 cm row spacing , 9 m long	Lee Spider Sprayer	Aug. 28	R3	July 30	R3
Milan, TN (A4)	Asgrow 4832	High	May 29	May 5	76 cm row spacing , 9 m long	Lee Spider Sprayer	Aug. 8	R3	July 29	R3
Milan, TN (A8)	Asgrow 4832	High	May 29	N/A	76 cm row spacing , 9 m long	Lee Spider Sprayer	Aug. 8	R3	N/A	N/A
Knoxville, TN	Armor 53Z5	Low	N/A	June 5	76 cm row spacing , 9 m long	CO_2-backpack sprayer	N/A	N/A	Aug. 29	R4

[1]Planted after wheat was harvested

Table 10: Fungicide treatments

Product Name	Rate (fl. oz/a)	Active Ingredient	Group Name	FRAC Code[2]	Mode of Action and Target Site
Headline	6	Pyraclostrobin	Quinone Outside Inhibitor (QoI/Strobilurin)	11	QoI - prevents respiration within the fungal mitochondria
Topguard	7	Flutriafol	Demethylation Inhibitor (DMI/Triazole)	3	DMI - inhibits the enzyme C14-demethylase involved in sterol biosynthesis
QuadrisTop	8	Azoxystrobin & Difenoconazole	QoI/Strobilurin & DMI/Triazole	11 + 3	QoI - prevents respiration DMI - inhibits sterol biosynthesis
Priaxor	4	Fluxapyroxad & Pyraclostrobin	Succinate Dehydrogenase Inhibitor (SDHI) & QoI/Strobilurin	7 + 11	SDHI- targets complex II in fungal respiration complex QoI - prevents respiration
TopsinM	20	Thiophanate-methyl	Methyl Benzimidazole Carbamate (MBC)	1	MBC: inhibit β tubulin biosynthesis and interfere with cell division
Bravo	6	Chlorothalonil	Chlorothalonil (phthalonitriles)	M5	Multisite activity
Priaxor & Domark[1]	4 4	Fluxapyroxad & Pyraclostrobin & Tetraconazole	SDHI & QoI/Strobilurin & DMI/Triazole	7+11+3	SDHI- targets complex II in fungal respiration complex QoI - prevents respiration DMI-demethylation inhibitor
Overrule[1]	20	Thiophanate-methyl & Tebuconazole	DMI/Triazole & MBC	3+1	DMI-demethylation inhibitor MBC-inhibits tubulin biosynthesis
AproachPrima[1]	6.8	Picoxystrobin & Cyproconazole	QoI/Strobilurin & DMI/Triazole	11+3	QoI - prevents respiration DMI - inhibits sterol biosynthesis
StrategoYLD[1]	4	Prothioconazole & Trifloxystrobin	DMI/Triazole & QoI/Strobilurin	3+11	QoI - prevents respiration DMI - inhibits sterol biosynthesis
TopsinXTR[1]	20	Thiophanate-methyl & Tebuconazole	MBC & DMI/Triazole	1+3	DMI-demethylation inhibitor MBC-inhibits tubulin biosynthesis

[1]Treatments in certain 2014 trials only

[2]FRAC codes are designated by the Fungicide Resistance Action Committee (FRAC) as a means of identifying active ingredients with the potential for cross resistance. Go to http://www.frac.info/ for additional information.

Table 11: Mean G143A% associated with each treatment for 2014 trials

	QoI	QoI+DMI	SDHI+QoI+DMI	SDHI+QoI	MBC	DMI	DMI+MBC	Chlorothalonil	Nontreated
%G143A	91	91	91	90	89	88	79	75	79

*No significant differences (p>0.05)

Table 12: G143A-detection in 2014 Milan trials by treatment fungicide group

	Non-treated	DMI+MBC	Chlorothalonil	QoI+DMI	DMI	MBC	QoI	SDHI+QoI	SDHI+QoI+DMI
%G143A	62%	53%	57%	86%	88%	90%	97%	100%	100%

*No significant differences (p>0.05)

Table 13: Mean QoI-resistance (%) by fungicide group for 2013 trials via conidial germination assay

	Non-treated	DMI+ MBC	Chlorothalonil	QoI+ DMI	DMI	MBC	QoI	SDHI+ QoI	SDHI +QoI+DMI
%QoI-resistant	4%	N/A	5%	5%	2%	4%	7%	11%	N/A

*No significant differences (p>0.05)

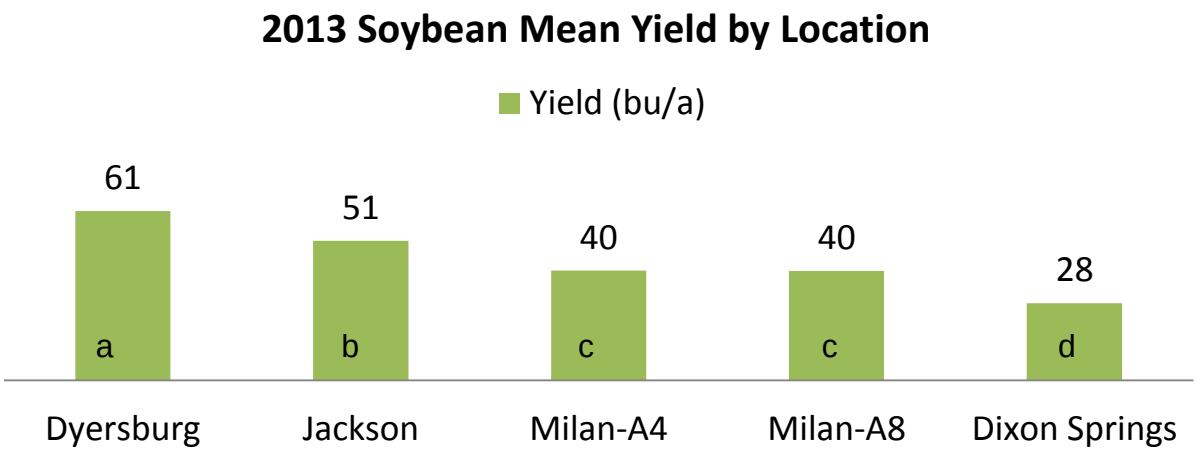

Figure 7: 2013 Mean yield by location
(p<0.0001)

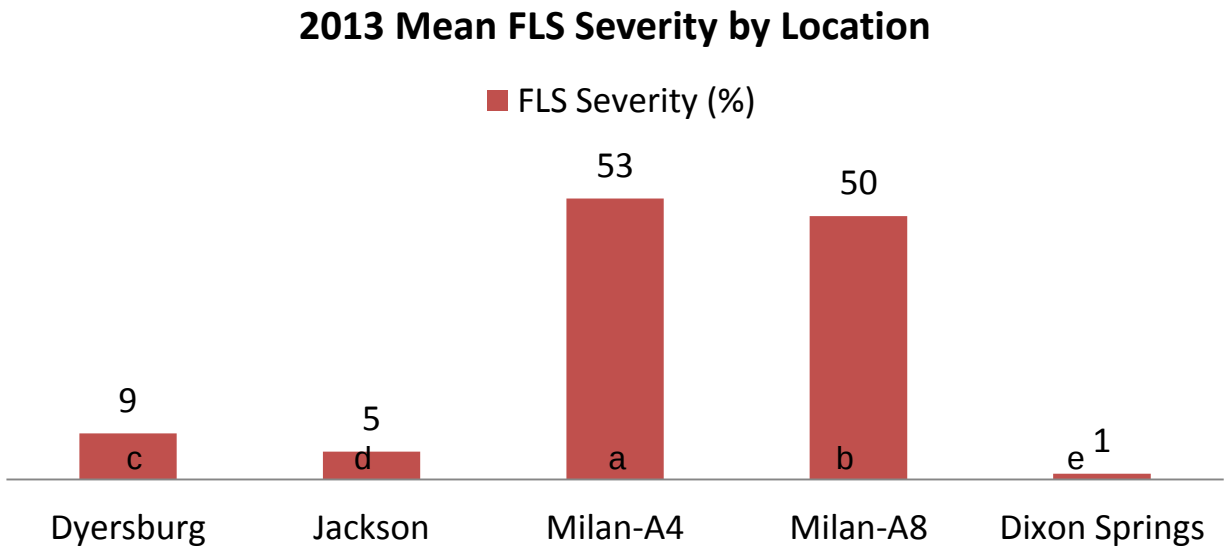

Figure 8: 2013 mean FLS severity by location
(p<0.0001)

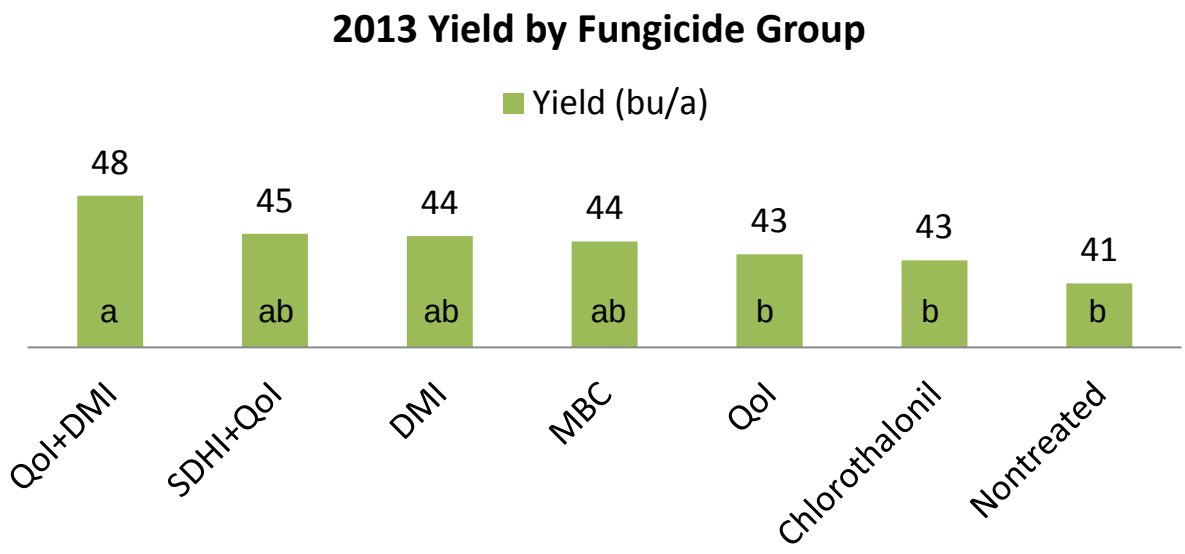

Figure 9: 2013 mean yield by fungicide group
(p=0.0008)

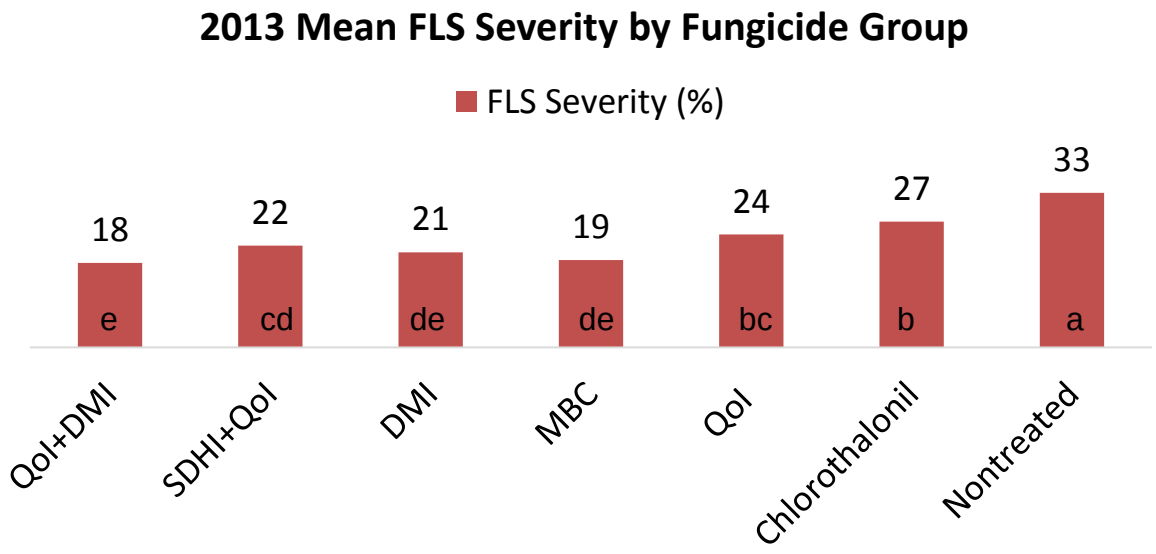

Figure 10: 2013 mean FLS severity by fungicide group
(p<0.0001)

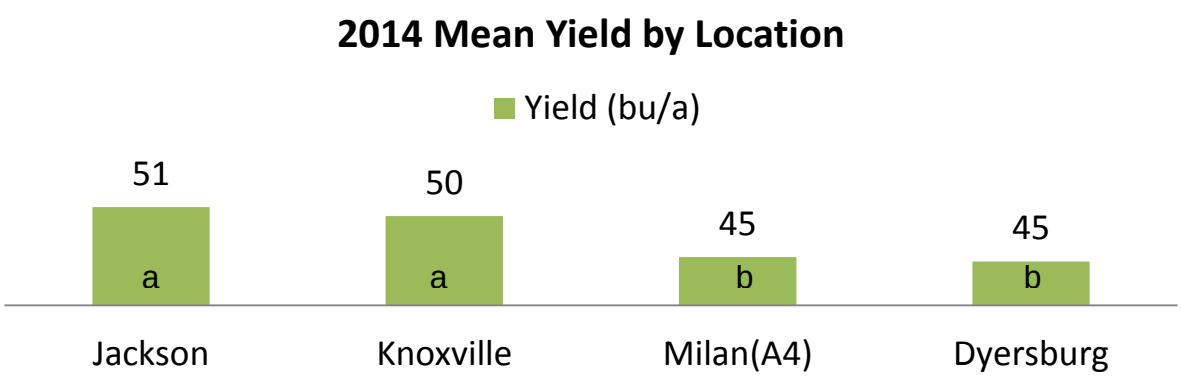

Figure 11: 2014 mean yield by location (p<0.0005)

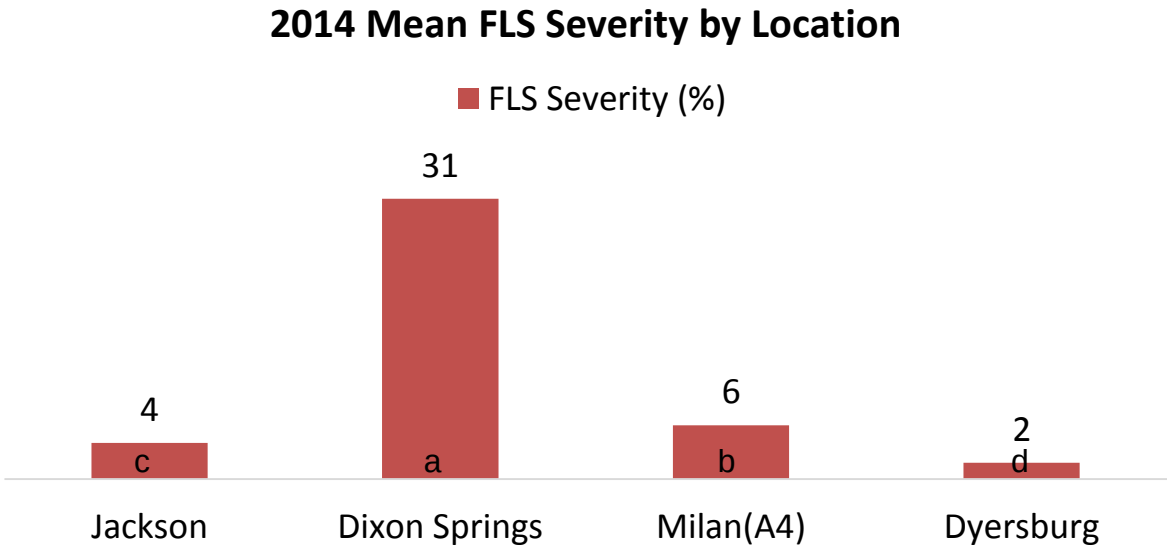

Figure 12: 2014 mean FLS severity by location (p<0.0001)

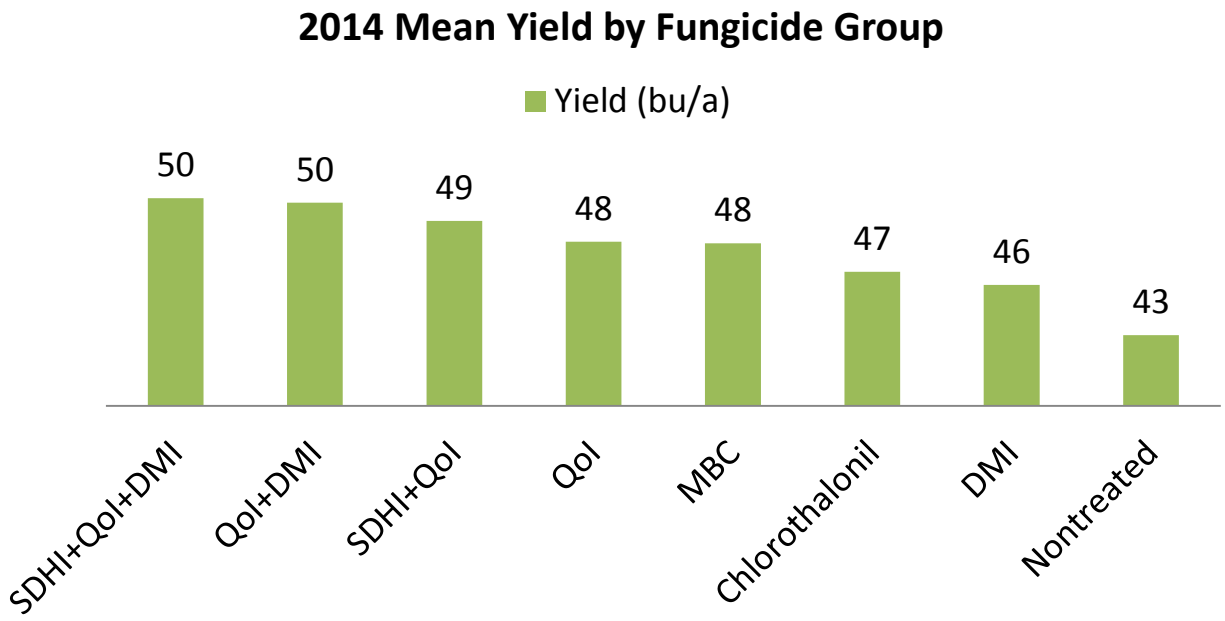

Figure 13: 2014 mean yield by fungicide group
(p=0.1061)

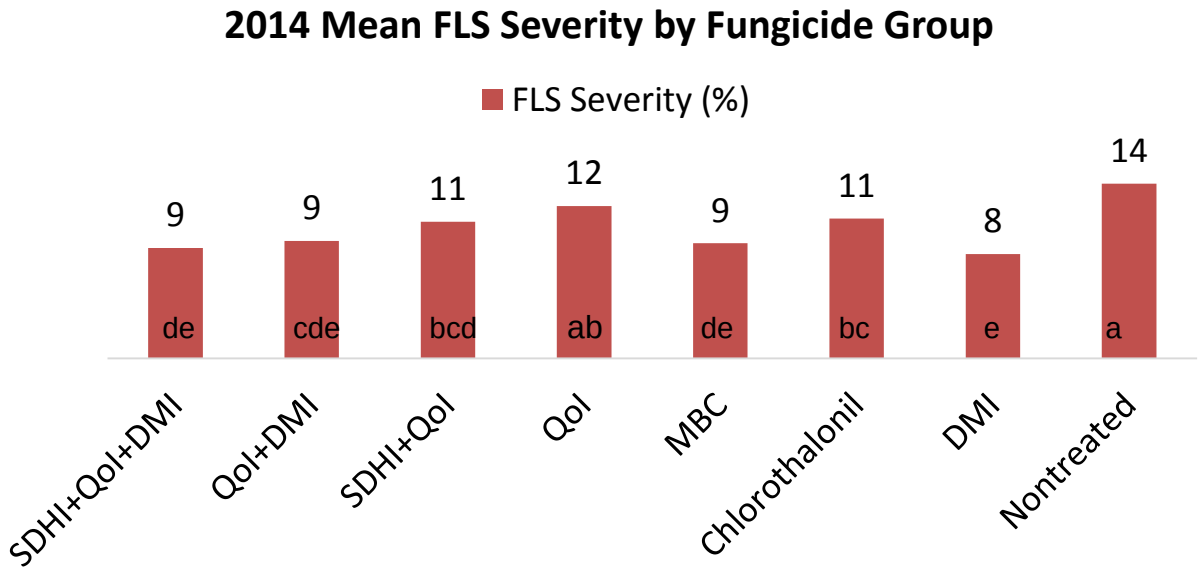

Figure 14: 2014 mean FLS severity by fungicide group
(p<0.0001)

VITA

Alicia M. Cochran graduated cum laude from the University of Arizona in 2013 with a Bachelor of Science in Microbiology and a minor in Plant Sciences. During her undergraduate career she studied microbial interactions with plant root tip border cells, and worked in a plant virology lab .In 2014 she received the Charles Wheeler Most Outstanding Master's Student award from the Department of Entomology and Plant Pathology at the University of Tennessee-Knoxville.