

2013

Sensitivity and resistance of *Cercospora kikuchii*, causal agent of *Cercospora* leaf blight and purple seed stain of soybean, to selected fungicides

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SENSITIVITY AND RESISTANCE OF *CERCOSPORA KIKUCHII*, CAUSAL AGENT OF CERCOSPORA
LEAF BLIGHT AND PURPLE SEED STAIN OF SOYBEAN, TO SELECTED FUNGICIDES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of
Plant Pathology and Crop Physiology

by

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August 2013

ACKNOWLEDGEMENTS

First and foremost, I thank God for a modest amount of talent and ample gumption to complete the requirements for this degree. Secondly, I thank my family. In particular, I acknowledge my loving wife, Beth, who has supported me through every endeavor. Without her, none of our successes would be possible. I credit my children, Camryn and Keegan, for providing the motivation to achieve something more and their unconditional love. I hope that I have provided a good example for them to follow.

I am thankful for O. D. Johnson and John Stapp teaching me the value of agriculture at a young age.

Thanks are extended to H. Lynn Walker for being an enlightening teacher, mentor, and friend. He is solely responsible for my interest in Plant Pathology. I would also like to express gratitude to Michael Tilley and Lawrence Higginbotham for their friendship, expertise, and support at Louisiana Tech University.

For the past ten years at LSU, I acknowledge the support from countless farm managers, research technicians, mechanics, and student workers. Without these unheralded people, applied research would be impossible. Specifically, I thank Myra Purvis, Ralph Sheppard, and Todd Yelverton for their assistance, advice, friendship, and expertise. I thank all of the other graduate students that paved the way. Special thanks are extended to Josh Temple, Jarrod Hardke, and Kyle Fontenot for their friendship and uncanny abilities to provide quality food and entertainment.

Thanks are extended to Ray Schneider for agreeing to serve as my Committee Chairman, supplying research suggestions, and offering kind words of encouragement throughout this process. I also acknowledge Jeffery Hoy, Charles Overstreet, and Zhijun Liu for manuscript reviews and for making time to serve on my committee. Thanks also are extended to Clayton Hollier for helpful advice and encouragement.

I would like to express my sincere gratitude to B. Rogers Leonard and G. Boyd Padgett for my employment, for providing a means by which to obtain this degree, and for setting an unparalleled example for me and many others. Their lessons of how to work hard, treat others, and conduct quality research have served me well.

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ABSTRACT

Isolates of *Cercospora kikuchii*, the causal agent of Cercospora leaf blight (CLB) and purple seed stain (PSS), were used to determine baseline sensitivities to selected quinone outside inhibitor (QoI) and demethylation inhibitor (DMI) fungicides by conducting radial growth assays on fungicide-amended media. The effective concentration to inhibit 50% radial growth (EC_{50}) for each isolate was calculated by linear interpolation of the dose-response relationship. All baseline distributions were non-normal with outliers towards the less sensitive ends of the spectra, and median EC_{50} values for azoxystrobin, pyraclostrobin, trifloxystrobin, flutriafol, propiconazole, and tetraconazole were 0.081, 0.013, 0.012, 0.273, 0.143, 1.47 $\mu\text{g/ml}$, respectively. When compared to baseline sensitivities, median EC_{50} values for isolates exposed to azoxystrobin, pyraclostrobin, and trifloxystrobin in 2011/2012 were significantly higher at 37.2/57.6, 10.1/12.2, and 20.1/29.1 $\mu\text{g/ml}$, respectively. Cross-resistance to all three QoI fungicides was observed in the 2011 and 2012 populations. Discriminatory doses of 10 $\mu\text{g/ml}$ were developed for all three QoI fungicides to distinguish between sensitive and resistant isolates. Approximately 83% of all isolates screened in 2011 and 2012 were resistant to QoI fungicides, and isolates from 21 of 27 parishes tested positive for resistance. Median EC_{50} values for isolates exposed to flutriafol, propiconazole, and tetraconazole in 2011/2012 were 0.41/0.54, 0.33/0.24, and 0.75/0.73 $\mu\text{g/ml}$. Significant shifts from the baseline towards less sensitivity were detected in isolates exposed to flutriafol and propiconazole. Additionally, outliers towards less DMI sensitivity were detected for all three DMI fungicides 2012. Strong, positive, and significant cross-sensitivity was observed among all three DMI fungicides. At a discriminatory dose of 5 $\mu\text{g/ml}$ thiophanate methyl, methyl benzimidazole carbamate (MBC) resistance was detected in the 2000, 2011, and 2012 populations at 23.3, 44.8, and 35.7%, respectively, with resistant isolates in 19 of 27 parishes. Isolates exhibiting multiple resistance to QoI and MBC fungicides also were detected in 15 of 27 parishes. Ninety-eight percent of MBC-resistant isolates also were resistant to QoI fungicides. Based on results from this research, CLB/PSS management strategies with QoI and MBC fungicides should be reconsidered in areas where resistance has been confirmed, and *C. kikuchii* populations should be further monitored for shifts in DMI sensitivity.

CHAPTER 1 INTRODUCTION AND REVIEW OF LITERATURE

Soybean, [*Glycine max* (L.) Merr.], is the leading oilseed crop consumed in the world (Wilcox 2004). It is also an important crop in the United States, with 31.3 million hectares planted in 2012 that produced approximately 81 million metric tons. Total crop value from 1996 to 2012 has increased from \$12.5 billion to \$43.2 billion with prices increasing from \$0.26 to \$0.51 per kg, respectively. Soybean yields have steadily increased in the United States since record keeping began. From 1924 to 1933, soybean yields averaged 813 kg per hectare; however, over the last ten years, yields averaged 2,600 kg per hectare. Louisiana is one of 31 states that produce soybean in the United States and is ranked 18th in planted hectarage for 2012 (460,500), a 48,600-hectare increase from 2011. Soybeans were the most abundant field crop in Louisiana from 2008 to 2012, with yields averaging from 2,100 to 2,900 kg per hectare (NASS 2013).

Soybean is affected by diseases caused by viruses, bacteria, fungi, and nematodes. Several economically-important fungal diseases affect leaves, upper stems, pods, and seeds of soybean plants. Many of these diseases such as pod and stem blight, *Diaporthe phaseolorum* (Cooke and Ellis) var. *sojae* (S. G. Lehman) Wehmeyer; brown spot, *Septoria glycines* Hemmi; Phomopsis seed decay, *Phomopsis longicolla* T. W. Hobbs; frogeye leaf spot, *Cercospora sojae* K. Hara; Cercospora leaf blight/purple seed stain, *Cercospora kikuchii* (Matsumoto and Tomoyasu) M. W. Gardner; downy mildew, *Peronospora manshurica* (Naumov) Syd. in Gaum; aerial blight, *Rhizoctonia solani* Kuhn; southern blight, *Sclerotium rolfsii* (Sacc.); and soybean rust, *Phakopsora pachyrhizi* Syd. & P. Syd.; affect soybeans at various stages of development in the Southern U. S. (Hartman et al. 1999; Wrather and Koennig 2013). Among these, Cercospora leaf blight/purple seed stain caused average losses of 140,500 metric tons in soybean annually from 1996 to 2012 (Wrather and Koennig 2013). Disease incidence and severity has increased over the past 10 years in Louisiana (Schneider et al 2003, Cai et al 2009).

Historical Perspective and Symptoms on Soybean

In 1921, purple seed stain (PSS), purple “speck”, or “Shihan”, of soybean was reported in Korea (Suzuki). Matsumoto and Tomayasu first described the pathogen infecting seeds, seedlings, stems, pods, and leaves. Infected seed coats were colored purple and frequently had gaping cracks. Upon germination, white tufts of mycelia were described affecting seedlings often resulting in plant death. Foliar symptoms were described by Matsumoto and Tomayasu as angular, purplish-red leaf spots irregularly limited by veins occurring on the lower leaves. Stem and pod symptoms were not described in detail, and the organism was dubbed *Cercosporina kikuchii*. (Matsumoto and Tomayasu 1925).

The disease was first observed and similarly described in the United States in 1924 by Gardner and 1951 by Murakishi. In inoculation experiments where Koch’s postulates were completed, Murakishi (1951) further described symptoms on stems and petioles as slightly sunken, irregular, reddish-purple areas. In addition, symptoms were described on pods as minute, reddish to reddish-purple areas later becoming purplish-black (Murakishi 1951).

The first field description of *Cercospora* leaf blight (CLB) was published in 1980 by H. J. Walters. The author provided an excellent description of foliar symptoms, paraphrased as follows: exposed, upper leaves having a light purple, leathery appearance with angular and irregular lesions occurring on upper and lower leaf surfaces; lesions may coalesce to form necrotic areas; numerous infections cause rapid chlorosis and necrosis resulting in defoliation starting with the uppermost leaves and moving downward; premature blighting of younger, uppermost leaves is the most obvious symptom. Symptoms were reproduced with inoculation experiments (Walters 1980), and the causal agent is currently identified as *Cercospora kikuchii* (Matsumoto and Tomayasu) Gardner.

Isolation, Culture, and Sporulation of *Cercospora kikuchii*

Isolation of *C. kikuchii* from seeds, stems, pods, and leaves may be achieved by using many techniques. Generally, isolation of the fungus from seed is easily accomplished by surface sterilization and subsequent placement on an agar-based growth medium (personal observation). Isolation of *C. kikuchii* from stems, pods,

and leaves is usually accomplished by single spore isolation from diseased tissue after incubation at high relative humidity (Cai and Schneider 2005, Imazaki et al 2006a, 2006b).

Cercospora kikuchii may be cultured on a variety of solid media including: potato dextrose agar (PDA), V-8 juice agar, carrot-leaf decoction agar (CLDA), malt extract agar (MEA), turnip leaf agar (TLA), soybean leaf agar (SLA), seed coat agar (SCA), and senescent soybean plant agars (SSPA) (Kilpatrick and Johnson 1956, Crane and Crittenden 1967, Chen et al 1979, Lyda et al 1979, Vathakos and Walters 1979, El-Gholl et al 1982, Roy 1982). Colony characteristics vary among isolates, but are typically characterized by white mycelial growth changing to olive/gray with culture age. The substrata of most colonies are dark maroon to purple. Most isolates produce a characteristic pigment that diffuses throughout the growth medium, which may vary in amount and color, depending on the isolate (Murakishi 1951, Roy 1982, Pathan et al 1989, Almeida et al 2005). Colony growth rates also vary among isolates, and optimal conditions for culture of *C. kikuchii* are 25°C with a 12h light: dark cycle (Almeida et al 2005). The fungus also thrives in a liquid growth medium containing sucrose, soy flour, and corn meal yielding dense, tan-colored mycelial growth after 72-120 h, which turns purplish with culture age (Boyette and Walker 1985, personal observations).

Sporulation of *C. kikuchii* in culture was first achieved on CLDA, TLA, and SLA in 1956 (Kilpatrick and Johnson). In 1958, a technique utilizing selective sub-culturing was developed to obtain stable, sporulating isolates of the fungus (Jones). Results from later studies were inconsistent with regard to sporulation on CLDA and V8 (Vathakos and Walters 1979, Lyda et al 1979, Yeh and Sinclair 1979, 1980). Growth on SSPA induced sporulation of *C. kikuchii*, which suggested a possible nutritional factor in soybean plants that initiates sporulation (Vathakos and Walters 1979, Yeh and Sinclair 1980, Roy 1982). Boyette and Walker (1985) induced abundant sporulation among four isolates of the fungus using the above-described liquid medium and a pelletizing technique involving sodium alginate. Their technique offered a relatively inexpensive alternative to petri dish inoculum production and an option for viable storage. Variability among isolates of *C. kikuchii* also is an important factor affecting sporulation (Chen et al 1979, Yeh and Sinclair 1982, Roy 1982, Boyette and Walker 1985), and isolates may lose the ability to sporulate in culture over time (El-Gholl et al 1982).

Conidia range in size from 1.3 to 6.1 by 40 to 445 μm and are hyaline, acicular, septate (2 to 49 septations), and truncate at the bases. Conidial size and numbers may be variable based on isolate, inoculum type, substrate, light, temperature, pH, and environmental factors (Matsumoto and Tomoyasu 1925, Murakishi 1951, Chen et al 1979, Yeh and Sinclair 1980).

Cercosporin Production

The previously-mentioned purplish pigment was isolated, purified, and identified as cercosporin in 1957 (Kuyama and Tamura). This compound appears to play a significant role in pathogenicity, symptom expression, colonization of seed coats, and virulence (Kilpatrick and Johnson 1956, Ilyas et al 1975, Fajola 1978, Upchurch et al 1991, Velichetti and Sinclair 1994). In Brazil, a positive correlation has been shown between cercosporin content and disease severity (Almeida et al 2005). Additionally, isolates exhibiting purple to red halos in culture have been more virulent when compared to “off-color” isolates. As with many other factors involving *C. kikuchii*, the amount of cercosporin production may be highly variable depending on the isolate (Almeida et al 2005).

Infection

Optimum temperatures and leaf wetness periods for infection by *C. kikuchii* are 20 to 30°C and 8 to 24h, respectively (Walters 1980, Martin and Walters 1982, Boyette and Walker 1985, Schuh 1991). Schuh also reported that conidia have the ability to germinate without the presence of a water film and at a relative humidity as low as 92.5% (1991). Hyphae of *C. kikuchii* were observed entering through stomata without forming appressoria, and directly penetrating the cuticle by appressorial formation (Fujita 1990). Infection of flowers by *C. kikuchii* has not been observed (Kilpatrick 1956, Roy and Abney 1976). Infection has been documented in young, developing pods (Roy and Abney 1976, Fujita 1990), and *C. kikuchii* appears to primarily infect the seed coat of soybean by direct hyphal penetration of the cuticle (Murakishi 1951, Chen et al 1979, Singh and Sinclair 1984, Fujita 1990, Velichetti et al 1992). The fungus appears to have the ability to enter seeds via hyphae through seed coat pores, which suggests that seed pore size and density may affect cultivar susceptibility (Chen et al 1979). The formation of microsclerotia-like structures, or hyphal aggregates,

was observed within the hourglass cell layer of seeds (Singh and Sinclair 1984, Fujita 1990). Mycelia of *C. kikuchii* have been observed within the hourglass and parenchyma cell layers of seed with higher concentrations in the hilum region than in the palisade region. The fungus was occasionally observed in the cotyledon and embryo (Ilyas et al 1975).

Effect on Seed

Based on results from previous studies, *C. kikuchii* was recovered from 87 to 99% of purple-stained seed (PSS) and 3 to 11% of apparently healthy seed (AHS) (Murakishi 1951, Wilcox and Abney 1973, Imazaki et al 2007). In seed lots with high levels of PSS, Kulik (1957) observed higher levels of infected AHS. Another possible explanation of this phenomenon is that AHS are infected by isolates that do not produce cercosporin (Kilpatrick and Johnson 1956, Velichetti and Sinclair 1994). Results from previous studies indicated reductions ranging from 0 to 49% in germinability of PSS (Murakishi 1951, Sherwin and Kreitlow 1952, Wilcox and Abney 1973, Roy and Abney 1976, Chen et al 1979, Hepperly and Sinclair 1981, Yeh and Sinclair 1982). Reduced germination of PSS may occur as the total affected area of seed increases (Yeh and Sinclair 1982, Pathan et al 1989), and *C. kikuchii* may reduce seedling emergence by 0 to 15% (Sherwin and Kreitlow 1952, Wilcox and Abney 1973).

***Diaporthe/Phomopsis* Complex**

There are many seedborne fungi associated with soybean. As many as 12 genera were isolated from soybean seed (Kilpatrick and Hartwig 1955, Roy and Abney 1976, Pathan et al 1989). An inverse relationship in seed colonization by *C. kikuchii* and *Diaporthe/Phomopsis* spp. was reported (Roy and Abney 1976, 1977, Hepperly and Sinclair 1981, Yeh and Sinclair 1982). One possible explanation is an antagonistic relationship between the two genera of fungi (Roy and Abney 1977, Pathan et al 1989). In other studies where no antagonism was observed, it was hypothesized that the two species may compete for nutrients and space (Yeh and Sinclair 1982, Pathan et al 1989, Jackson et al 2006). Recent studies described relationships between oleic and linoleic acid content of seed, *C. kikuchii*, and the *Diaporthe/Phomopsis* complex (Xue et al 2008).

Infection Timing

Results from some studies showed that an increased flowering period and delayed maturity may increase occurrence of PSS (Crane and Crittenden 1962, 1966, Fujita 1990). Other studies found no relationship between length of flowering period and PSS incidence. Inoculation with *C. kikuchii* from first flower (R1) to young pod (R3) caused the most infection, and inoculation at R2-R5 may cause a reduction in seed weight (Lavolette and Athow 1972, Roy and Abney 1976, Chen et al 1979). Reduced infection was observed as plants were inoculated at successively later growth stages, and seeds did not appear to be infected when mature plants were inoculated (Roy and Abney 1976).

Latent Infection

Based on evidence presented by Orth and Schuh (1992), latent infections in soybean by *C. kikuchii* result from penetration of the epidermal cell wall of leaves by appressoria followed by colonization of only one to a few cells resulting in non-visible symptoms. After penetration, haustoria were restricted in growth because the neighboring cells collapse, which was a plant reaction. The authors further suggested that latent infecting hyphae resumed growth and sporulation following the death of host tissue (Orth and Schuh 1992), and contributed to inoculum load after senescence and leaf drop (1994). High humidity during dew period interruption increased disease severity and latent infection (Schuh 1993).

Pathogen Survival

Cercospora kikuchii has the ability to produce chlamydospores (Matsumoto 1928, Murakishi 1951). Another survival mechanism in *C. kikuchii* is microcycle conidiation, a process where recapitulation of conidiation occurs after conidial germination without an intervening phase of mycelial growth (Fernandez et al 1991). The fungus survived for up to 42 months on dead soybean stems, pods, and leaves (Kilpatrick 1956, Kilpatrick and Johnson 1956, Fujita 1990), and may overwinter on soil surface debris, contributing to the initial inoculum for the next growing season (Jones 1968, Almeida et al 2001). Additionally, dispersal of *C. kikuchii* could be favored by infected seeds sown in different production areas (Almeida et al 2005).

Alternative Hosts

In 1992 and 1994, Orth and Schuh suggested the possibility of alternative inoculum sources based on “abundant” spores and favorable environmental conditions early and throughout the growing season. Isolates of *Cercospora* species from other plant species were shown to cause purple seed stain by injecting pods with inoculum (Kilpatrick and Johnson 1956). In 1982, Roy showed that several *Cercospora* spp. caused PSS by injecting developing pods and inoculating mature seed. Additionally, Roy (1982) concluded that several crop and weed species may serve as alternative hosts and inoculum sources for PSS. In 1988, McLean and Roy claimed that six weed species (cocklebur, sicklepod, smallflower morningglory, pitted morningglory, prickly sida, and spotted spurge) were symptomless hosts for *C. kikuchii* based on results from greenhouse tests.

Variability of the Pathogen

Genetic variability of *C. kikuchii* was demonstrated in Brazil in 2005 by random amplified polymorphic DNA analysis, where 72 isolates were divided into seven distinguishable lineages. The seven groups were not correlated with cercosporin production, virulence, or geographic origin (Almeida et al 2005). Imazaki et al analyzed 160 isolates from South America and 245 isolates from Japan and also found seven lineages; however, only one lineage was common between the two areas (2006a). Cai and Schneider (2005) showed diversity in the population of *C. kikuchii* from Louisiana by segregation of isolates of the fungus into many different vegetative compatibility groups (VCG), suggesting a covert or recently lost sexual stage. Evidence also was presented indicating leaf and seed isolates may be better suited to respectively infect leaves and seed (Cai and Schneider 2005). Based on further research, VCG was not an efficient indicator of evolutionary lineage (Cai and Schneider 2005). Additionally, when comparing a newly-arisen lineage to an old lineage, Cai et al found that the new lineage was less aggressive (2009).

Resistant Varieties

Since 1999, *C. kikuchii* has generally become more of a problem, particularly in the Mid-Southern United States (Schneider et al 2003). This may be caused, in part, by lack of varietal resistance. Sources of genetic resistance have been identified in the past (Wilcox et al 1975, Srisombun and Supapornhemin 1993,

Orth and Schuh 1994); however, more sources may prove useful. There is evidence that a single, dominant gene, not linked with *Phomopsis* resistance, controls resistance to PSS in PI 80837 (Jackson et al 2006, 2008). In 2002, 59 of 62 varieties included in Louisiana trials were determined to be susceptible to CLB (Schneider et al 2003). From 2007 to 2012, no varieties in Louisiana were identified as resistant to purple seed stain and *Cercospora* leaf blight (Anonymous 2012a).

Fungicide Application Efficacy and Timing

In Louisiana, fungicide applications during the reproductive stages of development of soybean are recommended for management of CLB/PSS. (Anonymous 2012b). In recent years, many available fungicides were ineffective on CLB (Padgett and Purvis 2005, 2007a, 2007b, Price and Padgett 2008). Additionally, fungicide application timing studies were conducted with varying results (Padgett and Purvis 2007a, 2007b, Price and Padgett 2008, Price et al 2011, 2013, Delaney et al 2012).

Recommended Fungicides for Management of Cercospora Leaf Blight/Purple Seed Stain

Quinone outside inhibitor (QoI) fungicides, such as azoxystrobin (Quadris™), pyraclostrobin (Headline™), and trifloxystrobin (Gem™) are recommended for management of CLB/PSS in Louisiana (Anonymous 2012b). These fungicides interfere with mitochondrial respiration, and in turn, affect spore germination and hyphal growth (Bartlett et al 2002). According to the Fungicide Resistance Action Committee (FRAC) (2013), QoI fungicides are considered to be “high-risk” for resistance development. This chemistry type was introduced into the commercial market in 1996, and resistance in fungal pathogens was documented in 1998 (FRAC 2013). In 2010, a QoI-resistant population of *C. sojina*, the causal agent of frogeye leaf spot in soybean, was documented in Tennessee (Zhang and Bradley 2011).

Demethylation inhibitor (DMI) fungicides, such as flutriafol (Topguard™), tetraconazole (Domark™), and propiconazole (Tilt™) applied alone have not been recommended for management of CLB/PSS in Louisiana (Anonymous 2010). However, recently these fungicides were registered and are recommended alone for management of CLB/PSS in soybean, with the exception of propiconazole (Anonymous 2012b). These fungicides inhibit sterol synthesis, which is important in the cell wall structure of fungi (FRAC 2013).

According to the Fungicide Resistance Action Committee (FRAC), DMI fungicides pose a “medium-risk” for resistance development (2013). Resistance in *C. beticola*, the sugar beet leaf spot pathogen, to DMI fungicides was documented in Greece and the United States (Karaoglanidis et al 2000, Secor et al 2010, Kirk et al 2013).

Thiophanate-methyl (Topsin™) also is recommended for management of *Cercospora* leaf blight and purple seed stain in Louisiana (Anonymous 2012b). This fungicide belongs to the methyl benzimidazole carbamate (MBC) class, which inhibits growth of fungi by interfering with microtubule assembly during mitosis (Howard and Aist 1977). FRAC considers thiophanate-methyl “high-risk” for inducing resistance in fungal plant pathogens (FRAC 2013). Resistance of *C. kikuchii* to thiophanate-methyl has been documented in Japan since the late 1980s, and the fungicide was shown to aid in selection of resistant isolates of the fungus in field studies (Sakai 1999, Imazaki et al 2006b). This resistance arises from a mutation in the β -tubulin gene (Monma et al 2003) leading to high levels of resistance *in vitro* and *in vivo* (Ishii et al 2001).

Cultural Techniques

Jones and Almeida et al (1968 and 2001, respectively) showed that burying crop residues may reduce survival of *C. kikuchii*. It was also suggested that paraquat applications prior to harvest may increase the number of infective propagules of *C. kikuchii* (Cerkauskas and Sinclair 1980).

Biological Control Agents

To date, no successful biological control agents for *C. kikuchii* have been developed. However, a bacterium, *Chromobacterium violaceum*, was recently discovered in the Brazilian Amazon that is inhibitory to *C. kikuchii* growth *in vitro* (Barreto et al 2008).

Project Objectives

- I. To determine baseline sensitivity of *Cercospora kikuchii* to quinone outside inhibitor and demethylation inhibitor fungicides.
- II. To determine if shifts in sensitivities to quinone outside inhibitor and demethylation inhibitor fungicides have occurred in *Cercospora kikuchii*.

III. To determine the existence and extent of methyl benzimidazole carbamate fungicide resistance in *Cercospora kikuchii*.

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CHAPTER 2

BASELINE SENSITIVITY OF *CERCOSPORA KIKUCHII* TO QUINONE OUTSIDE INHIBITOR AND DEMETHYLATION INHIBITOR FUNGICIDES

Introduction

Cercospora leaf blight (CLB) and purple seed stain (PSS) are significant diseases of soybean in the United States causing average losses of 140,500 metric tons annually from 1996 to 2012 (Wrather and Koennig 2013). Soybean is the most prevalent field crop grown in Louisiana with 460,000 planted hectares in 2012 (NASS 2013). Disease incidence and severity of CLB/PSS has markedly increased within the past 10 to 15 years in Louisiana (Schneider et al 2003, Cai et al 2009). Recently, many commercially available fungicides have proven inefficacious on CLB/PSS (Padgett and Purvis 2005, 2007a, 2007b, Price and Padgett 2008, Price et al 2013). Additionally, fungicide type, application rate, and timing studies indicated little or no effect on CLB/PSS (Padgett and Purvis 2007a, 2007b; Price and Padgett 2008; Price et al 2011; Delaney et al 2012; Price et al 2013).

Fungicide applications during the reproductive growth stages of soybean are recommended by the Louisiana State University Agricultural Center (LSU AgCenter) for management of CLB/PSS in Louisiana (Anonymous 2012). Historically, two quinone outside inhibitor (QoI) fungicides, azoxystrobin (Quadris™) and pyraclostrobin (Headline™) were recommended by LSU AgCenter for CLB/PSS management (Anonymous 2010). Currently, Gem™ RC (trifloxystrobin), Headline™ 2.08EC (pyraclostrobin), Headline™ SC (pyraclostrobin), and Quadris™ 2.08SC (azoxystrobin) are QoI fungicides recommended by LSU AgCenter for CLB/PSS management. Demethylation inhibitor (DMI) fungicides applied alone have not been recommended by the LSU AgCenter for CLB/PSS management until recently (Anonymous 2012). Topguard™ (flutriafol), Domark™ (tetraconazole) and Tilt™ (propiconazole) are DMI fungicides that were recently added to the LSU AgCenter list of recommended fungicides to manage CLB/PSS. Mixtures containing one QoI and one DMI fungicide that are currently LSU AgCenter-recommended for management of CLB/PSS are Quadris Xtra™

(azoxystrobin + cyproconazole), Quilt™ (azoxystrobin + propiconazole), Quilt Xcel™ (azoxystrobin + propiconazole), Stratego™ (trifloxystrobin + propiconazole), and Stratego YLD™ (trifloxystrobin + prothioconazole) (Anonymous 2012).

QoI fungicides were introduced to the market around 1996, and resistance of fungal pathogens to this chemistry class was documented in 1998 (FRAC 2013). These fungicides interfere with mitochondrial respiration by blocking electron transport at the quinol-oxidizing site of the cytochrome bc_1 complex (III), which subsequently affects spore germination and hyphal growth (Bartlett et al 2002). This mode of action is highly specific and can be overcome by a single-step mutation (Gisi et al 2002). Consequently, these fungicides are considered high risk for resistance development (FRAC 2013). Establishment of baseline sensitivities to QoI fungicides is necessary to determine if sensitivity shifts occur in the future.

Some fungi have the ability to utilize an alternative respiration pathway, bypassing complex III in the respiration cycle and, consequently, avoiding the effects of QoI fungicides *in vitro*, which significantly affects effective concentration (EC) values (Ziogas et al 1997). This phenomenon is thought to only occur *in vitro*, and to be prevented by plant flavones *in vivo* (Bartlett et al 2002). Previous research indicates that alternative oxidation (AOX) occurs in some *Cercospora* species, and salicylhydroxamic acid (SHAM) or propyl gallate (PG) may be used to inhibit this alternative pathway (Bradley and Pedersen 2011, Zhang et al 2012a, 2012b). Other studies indicated that SHAM and PG may be toxic to fungal isolates *in vitro* (Seyran et al 2010). Previous fungicide sensitivity research utilized SHAM at rates of 60-100 $\mu\text{g/ml}$ (Wise et al 2007, Wise et al 2009, Bradley and Pedersen 2011, Zhang et al 2012a, 2012b), while PG was used at approximately 50 $\mu\text{g/ml}$ (Seyran et al 2010).

DMI fungicides were commercially introduced in 1975, and resistance of fungal pathogens to this chemistry class was first observed in 1982 (FRAC 2013). These fungicides interact with cytochrome P450s at the site of the 14 α -demethylase (CYP51) and C-22 desaturase (CYP61) blocking 14 α -demethylation (Kelly et al 1995). Later in the sterol biosynthesis pathway, an accumulation of 5-hydroxy sterol is the toxic component of the fungicide (Watson et al 1989). Several changes throughout the sterol biosynthesis pathway must occur in

plant pathogenic fungi in order for resistance to occur with DMI fungicides, which results in a lower rate of resistance development (“medium risk”) than QoI fungicides (FRAC 2013). As with QoI fungicides, baseline sensitivities to DMI fungicides must be determined to detect shifts in population sensitivities over time (FRAC 2013).

Cross-sensitivity/resistance is defined as similar sensitivity/resistance to two or more fungicides where sensitivity/resistance is controlled by the same genetic factor. Cross-sensitivity/resistance is known to commonly occur with QoI and DMI fungicides across many species of plant pathogenic fungi including other *Cercospora* species (Reynolds et al 1996, Erickson and Wilcox 1997, Karaoglanidis et al 2000, Pasche et al 2004, Rebollar-Alviter et al 2007, Wong and Midland 2007, Wise et al 2009, Sombardier et al 2010). Variability in cross sensitivity/resistance to QoI and DMI fungicides also has been documented (Henry and Trivellas 1989, Karaoglanidis et al 2001, 2003, Pasche et al 2004, Wise et al 2011, Bolton et al 2012).

The primary objective of this study was to establish *in vitro* baseline sensitivity to selected QoI and DMI fungicides. Ancillary objectives were to determine alternative respiration occurrence in *C. kikuchii*, and to examine cross-sensitivity patterns for both fungicide types.

Materials and Methods

Isolate Sources. *C. kikuchii* isolates, 176 total, were originally obtained in Louisiana by G. Cai in 2000, and provided by R. W. Schneider for this study in 2010 (Cai and Schneider 2005). The isolates were maintained on one-half strength V8-agar and stored at 4°C. Isolates included 115 foliar and 30 seed isolates from the Macon Ridge Research Station (MRRS) near Winnsboro and 12 foliar and 16 seed isolates from the Dean Lee Research Station (DLRS) near Alexandria. Three isolates of unknown plant tissue origin were obtained from the Ben Hur Research Station (BHRS) near Baton Rouge. Stock cultures were maintained on V8 agar (20% juice) at 25°C with a 12h light: dark cycle, and cultures 21-35 days old were used in all assays. Radial growth assays were chosen as the method for determining toxicity because of sparse sporulation in culture.

Toxicity of Alternative Respiration Inhibitors to *Cercospora kikuchii*. Serial dilutions of SHAM were made in ethanol (95%) then added to sterilized, molten potato dextrose agar (PDA) (Cole-Parmer, Inc.) cooled to ~50°C to achieve final concentrations of 0, 20, 40, 60, 80, 100, and 120 µg/ml. PDA amended with PG was prepared in the same manner, but concentrations were adjusted to 0, 33, 66, 100, 133, 166, and 200 µg/ml. The media were aseptically dispensed into sterile petri dishes (100 x 15mm, ~20ml/dish) and allowed to solidify. Twenty isolates were selected at random (RAND function, Microsoft Excel) for exposure to each AOX inhibitor. Mycelial discs (6mm diameter) were cut from stock cultures using a #3 cork borer, inverted, transferred to the media, and placed in incubator at 25°C with a 12h light: dark cycle. Treatments were arranged in a completely randomized design (CRD) with two replicates per isolate. After incubation for 5 (SHAM) or 7 (PG) days, colony diameter was measured using a circular inking template (Pickett Industries, Inc., No. 1304I). Data were analyzed using mixed model analysis (PROC MIXED, SAS Institute), and mean colony diameters were compared with the Tukey-Kramer post hoc adjustment ($P < 0.05$).

Effect of Propyl Gallate on Azoxystrobin Sensitivity in *Cercospora kikuchii*. Because SHAM was toxic to isolates in the previous assay, propyl gallate was included in assays to determine if AOX occurs in *C. kikuchii*. Serial dilutions of a technical formulation of azoxystrobin (99.5%) were made in acetone (95%) and added to sterilized, molten PDA to achieve final concentrations that ranged ten-fold from 0.001 to 10 µg/ml. A non-amended control was included, and propyl gallate (dissolved in ethanol) was either added for a final concentration of 200 µg/ml or omitted for each treatment. The media were aseptically dispensed into sterile petri dishes (100 x 15mm, ~20ml/dish) and allowed to solidify. Twenty isolates were selected, transferred, and incubated as previously described. Treatments were arranged in a randomized complete block (RCB) design with incubator shelves serving as blocks. There were four isolates per dish and four replicates per isolate. After 5 days, colony diameter was measured as previously described, and data were converted to % inhibition by comparison to respective controls. The effective concentration to inhibit 50% of fungal radial growth (EC_{50}) was determined for each treatment by linear interpolation of a regression of log concentration by % inhibition (GraphPad Prism 5.0). The equation for the dose-response model is as follows: $Y=0+(100-$

$0)/(1+10^{((\text{LogEC}_{50}-X)*1))}$). Mean EC_{50} values for each isolate (with and without PG) were compared using a two-sample t-test with $\alpha = 0.05$ (PROC TTEST, SAS Institute).

Baseline Sensitivities to Selected Fungicides. Ten-fold dilutions of technical formulations (ChemService, Inc.) of azoxystrobin (99.5%), pyraclostrobin (99.5%), trifloxystrobin (99.3%), flutriafol (98.2%), propiconazole (97.5%), and tetraconazole (98.7%) were performed in acetone, added to sterilized, molten PDA, and dispensed as previously described. Final concentrations for the QoI and DMI fungicides ranged from 0.001 to 10 $\mu\text{g}/\text{ml}$. PDA amended with only acetone was included as a control. For each baseline sensitivity assay, 50 isolates were arbitrarily chosen. Isolates were transferred and incubated as previously described. Colony diameters were measured 5 days after transfer, data were converted to % inhibition, and EC_{50} values were calculated as previously described. Mean EC_{50} values were determined for each isolate, and sensitivity profiles were constructed for each fungicide (GraphPad Prism 5.0). Tests for normality were conducted using the Shapiro-Wilks method (GraphPad Prism 5.0).

Determining Cross-Sensitivity Patterns to Fungicides. Isolates from 2000 that were exposed to azoxystrobin and trifloxystrobin in baseline sensitivity assays were available for QoI cross-sensitivity analysis, while isolates exposed *in vitro* to flutriafol and propiconazole were available for DMI cross sensitivity analysis. Mean EC_{50} values for each isolate for each fungicide were compared. Because data violated the assumption of normality before and after transformation, a non-parametric form of correlation, Spearman's rank correlation test, was performed. Correlation coefficients (ρ) of 1, 0, and -1 were interpreted as perfect-positive, none, and perfect-negative correlation, respectively.

Results

Toxicity of Alternative Respiration Inhibitors to *Cercospora kikuchii*. Five days after transfer, PDA amended with SHAM significantly inhibited growth of *C. kikuchii*. Mean colony growth for the non-amended control was 8.6 mm while colonies averaged 2.1, 1.7, 1.6, 1.2, 0.9, and 0.3 mm at SHAM concentrations of 20, 40, 60, 80, 100, and 120 $\mu\text{g}/\text{ml}$, respectively. SHAM reduced colony diameter by 76 to 97% across all concentrations (Figure 2.1). Propyl gallate did not significantly reduce *C. kikuchii* growth after seven days.

Mean colony growth of the non-amended control was 11.2 mm compared to 11.0, 10.8, 10.6, 10.6, 10.6, and 10.5 mm at PG concentrations of 33, 66, 100, 133, 166, and 200 µg/ml, respectively. Growth inhibition ranged from 1.5 to 6.0% across PG concentrations (Figure 2.2).

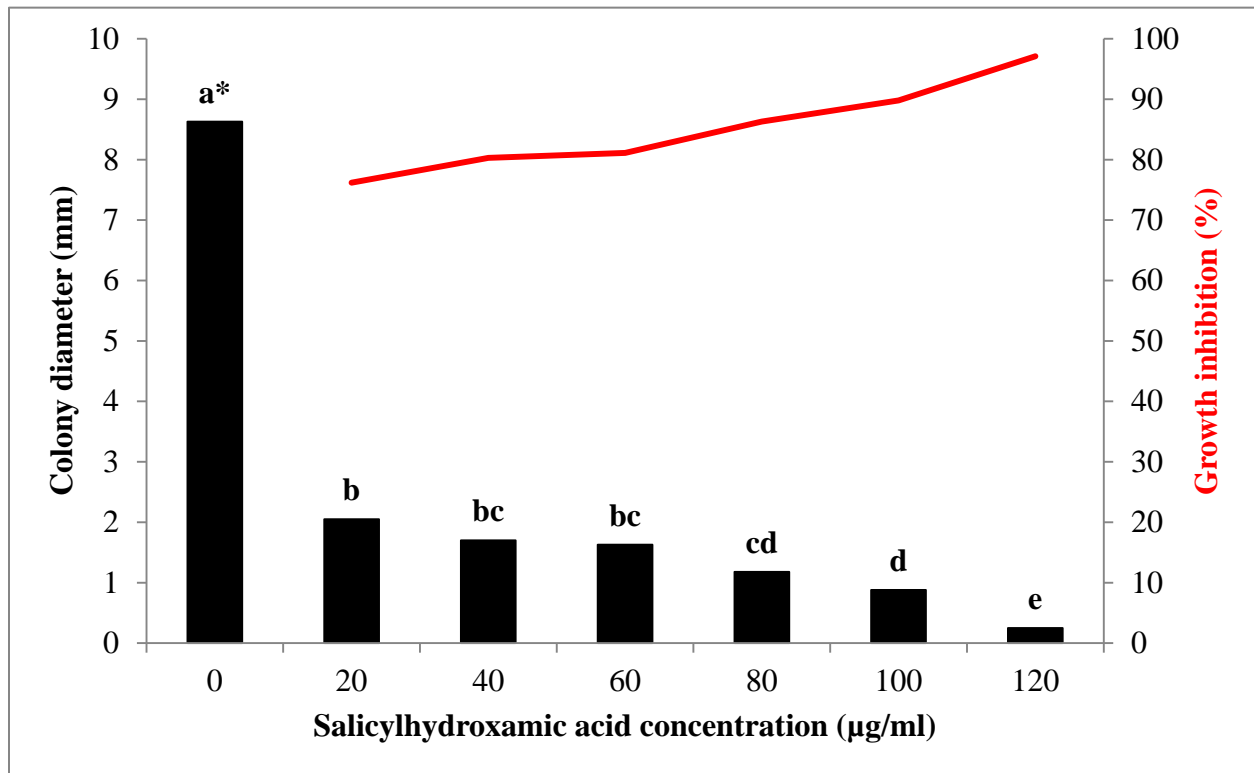


Figure 2.1. Effect of salicylhydroxamic acid on radial growth of isolates of *Cercospora kikuchii* collected in 2000 on potato dextrose agar (PDA).

*Means followed by the same letter do not differ significantly (PROC MIXED, $\alpha=0.05$, Tukey-Kramer adjustment).

Effect of Propyl Gallate on Azoxystrobin Sensitivity in *Cercospora kikuchii*. Mean EC_{50} values for individual isolates did not significantly differ when propyl gallate was added to PDA amended with azoxystrobin. Azoxystrobin sensitivities of isolates not exposed to propyl gallate ranged from 0.035 to 0.215 µg/ml with an overall mean of 0.106 µg/ml. Overall mean azoxystrobin sensitivity for isolates exposed to propyl gallate was 0.076 µg/ml with a range of 0.034 to 0.222 µg/ml (Table 2.1).

Baseline Sensitivity of *Cercospora kikuchii* to Azoxystrobin. EC_{50} values of isolates exposed to azoxystrobin ranged from 0.026 to 0.356 µg/ml with a median of 0.081 µg/ml. The 95% confidence interval of the distribution was [0.083, 0.121] with a mean of 0.102. The distribution was non-normal ($W = 0.8454$, $P < 0.0001$) with outliers towards the less-sensitive end of the spectrum (Figure 2.3).

Baseline Sensitivity of *Cercospora kikuchii* to Pyraclostrobin. EC₅₀ values of isolates exposed to pyraclostrobin ranged from 0.0003 to 0.103 µg/ml with a median of 0.013 µg/ml. The 95% confidence interval of the distribution was [0.012, 0.023] with a mean of 0.017 µg/ml. The distribution was non-normal (W = 0.7258, P < 0.0001) with outliers towards the less-sensitive end of the spectrum (Figure 2.4).

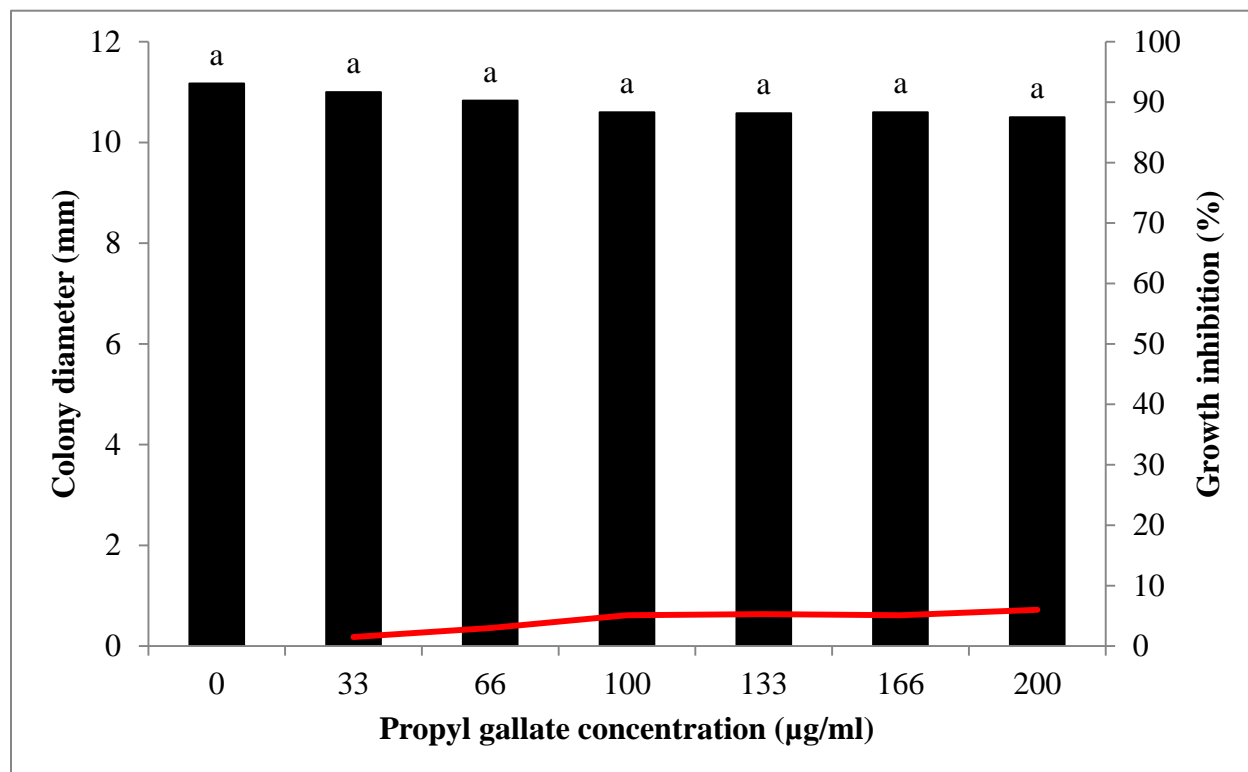


Figure 2.2. Effect of propyl gallate on radial growth of isolates of *Cercospora kikuchii* collected in 2000 on potato dextrose agar (PDA).

*Means followed by the same letter do not differ significantly (PROC MIXED, $\alpha=0.05$, Tukey-Kramer adjustment).

Table 2.1. Effect of propyl gallate on azoxystrobin sensitivities of selected isolates of *Cercospora kikuchii* collected in 2000.

Isolate Number	Mean EC ₅₀ without PG ¹	Mean EC ₅₀ with PG	t-value	p-value
3	0.035 ²	0.034	0.05	0.9582
9	0.059	0.038	0.92	0.3935
26	0.093	0.132	-0.55	0.6022
32	0.114	0.093	0.37	0.7275
33	0.031	0.040	-0.72	0.4976
47	0.102	0.054	1.35	0.2634
49	0.066	0.058	0.22	0.8335
65	0.042	0.040	0.12	0.9090

Isolate Number	Mean EC ₅₀ without PG ¹	Mean EC ₅₀ with PG	t-value	p-value
78	0.215 ²	0.104	0.68	0.5389
85	0.182	0.222	-0.16	0.8783
97	0.102	0.124	-0.2	0.8496
100	0.107	0.098	0.09	0.9320
111	0.181	0.041	1.08	0.3515
142	0.126	0.056	0.85	0.4273
143	0.142	0.100	0.4	0.7063
150	0.107	0.111	-0.05	0.9592
153	0.080	0.036	2.01	0.1291
155	0.053	0.045	0.49	0.6393
166	0.191	0.058	0.94	0.4143
176	0.084	0.041	1.47	0.1925

¹ Propyl gallate.

² Mean EC₅₀ values (with or without propyl gallate) for each isolate compared with a two-sample t-test (PROC TTEST, $\alpha=0.05$).

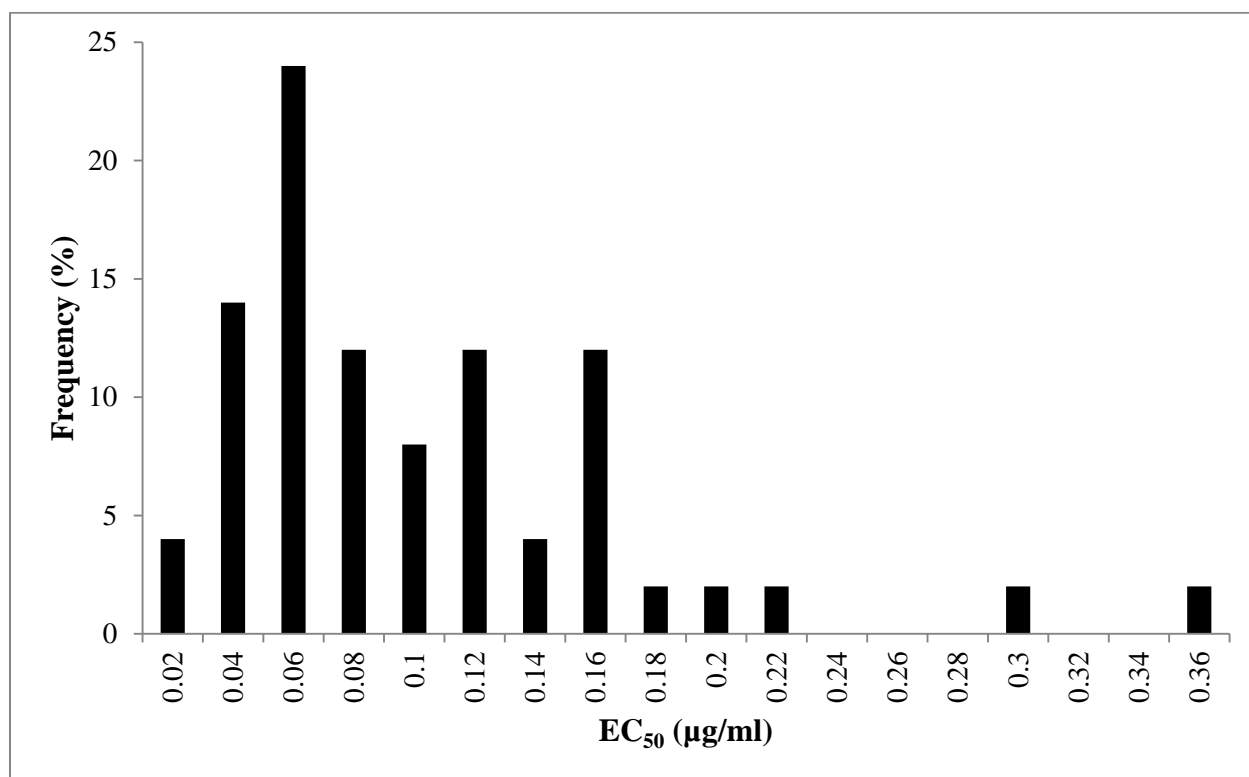


Figure 2.3. Baseline sensitivity of *Cercospora kikuchii* to azoxystrobin as determined by the effective concentration that inhibited 50% of radial growth (EC₅₀ values) of 50 isolates.

Baseline Sensitivity of *Cercospora kikuchii* to Trifloxystrobin. EC₅₀ values of isolates exposed to trifloxystrobin ranged from 0.004 to 0.063 µg/ml with a median of 0.012 µg/ml. The 95% confidence interval

of the distribution was [0.012, 0.018] with a mean of 0.015. The distribution was non-normal ($W = 0.7475$, $P < 0.0001$) with outliers towards the less-sensitive end of the spectrum (Figure 2.5).

Cross-Sensitivity of Isolates to Azoxystrobin and Trifloxystrobin. A Spearman rank correlation test was used to determine relationships between EC_{50} values of isolates from 2000 exposed in sensitivity assays to azoxystrobin and trifloxystrobin. A significant, positive correlation was found ($\rho(47) = 0.62$, $n = 49$, $P < 0.0001$) between sensitivities of isolates exposed to these fungicides (Figure 2.6).

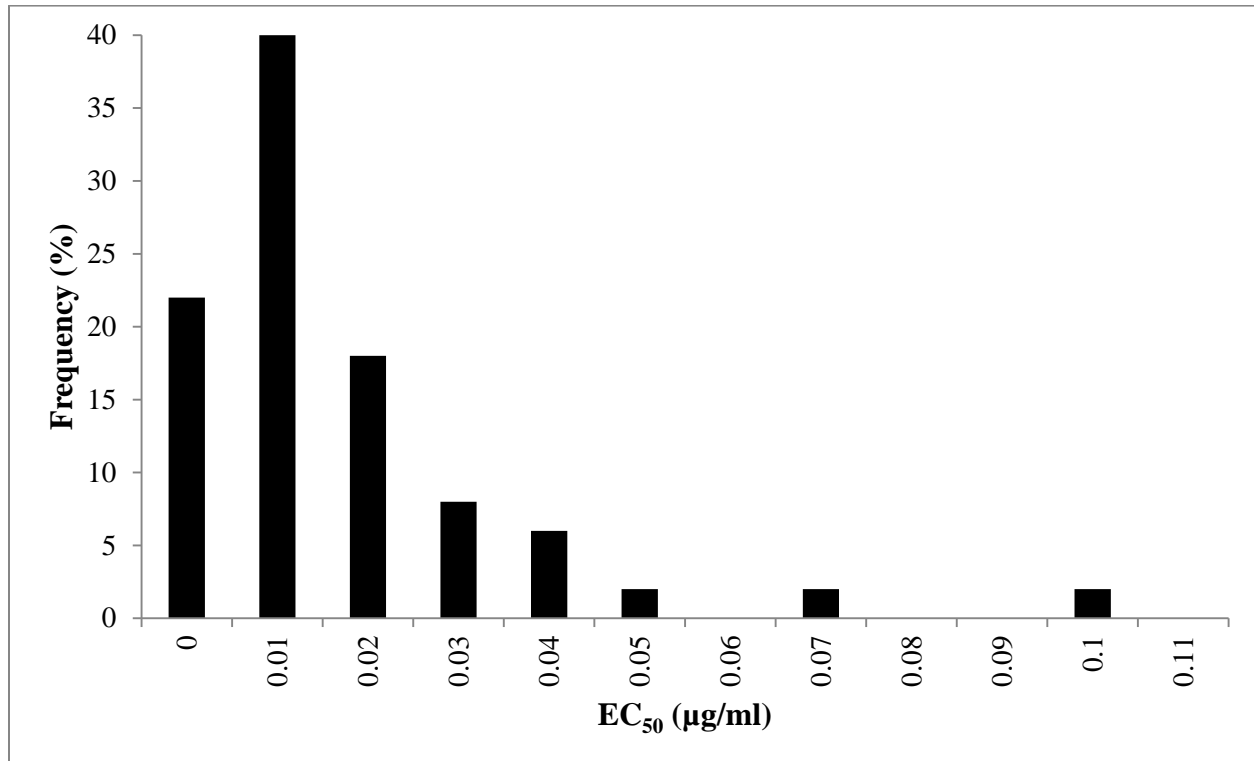


Figure 2.4. Baseline sensitivity of *Cercospora kikuchii* to pyraclostrobin as determined by the effective concentration that inhibited 50% of radial growth (EC_{50} values) of 50 isolates.

Baseline Sensitivity of *Cercospora kikuchii* to Flutriafol. EC_{50} values of isolates exposed to flutriafol ranged from 0.096 to 1.46 µg/ml with a median of 0.273 µg/ml. The 95% confidence interval of the distribution was [0.333, 0.571] with a mean of 0.452. A non-normal distribution with outliers towards the less-sensitive end was observed ($W = 0.7640$, $P < 0.0001$) (Figure 2.7).

Baseline Sensitivity of *Cercospora kikuchii* to Propiconazole. EC_{50} values of isolates exposed to propiconazole ranged from 0.022 to 0.954 µg/ml with a median of 0.143 µg/ml. The 95% confidence interval

of the distribution was [0.133, 0.232] with a mean of 0.182 $\mu\text{g/ml}$. A non-normal distribution was observed with outliers towards the less-sensitive end ($W = 0.6861$, $P < 0.0001$) (Figure 2.8).

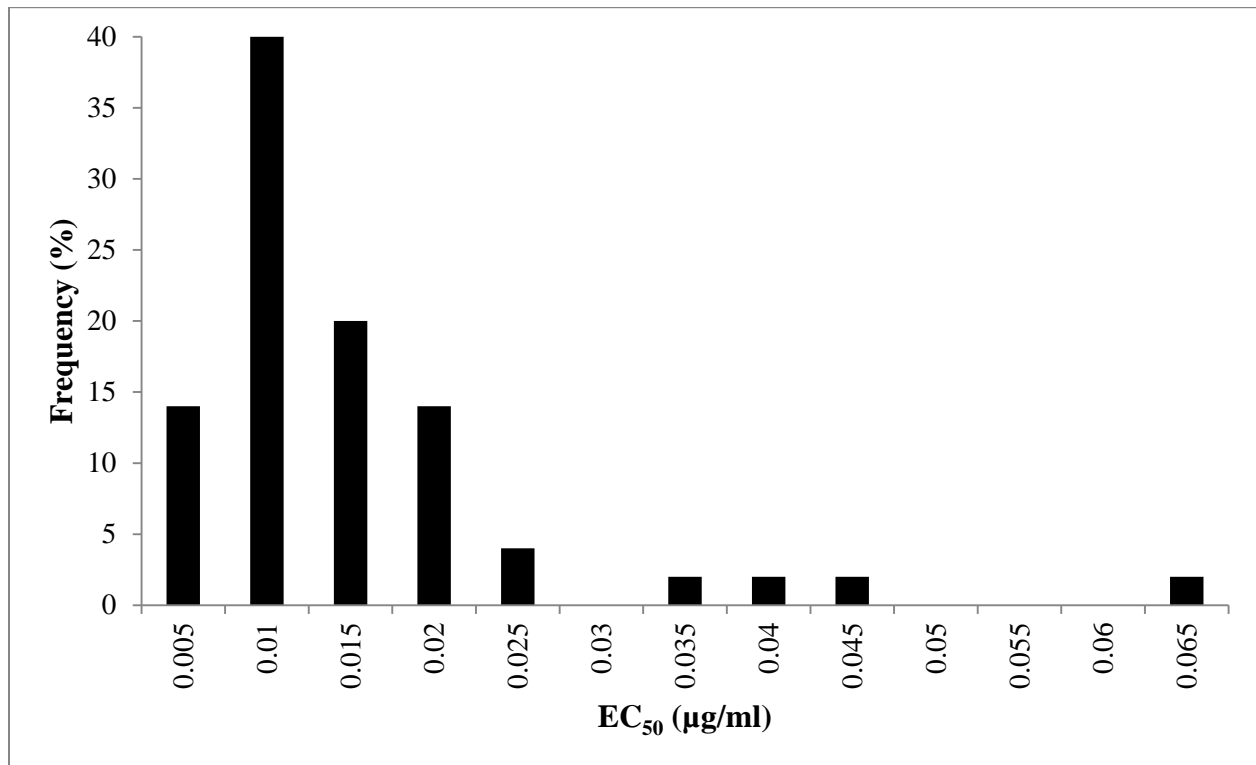


Figure 2.5. Baseline sensitivity of *Cercospora kikuchii* to trifloxystrobin as determined by the effective concentration that inhibited 50% of radial growth (EC_{50} values) of 50 isolates.

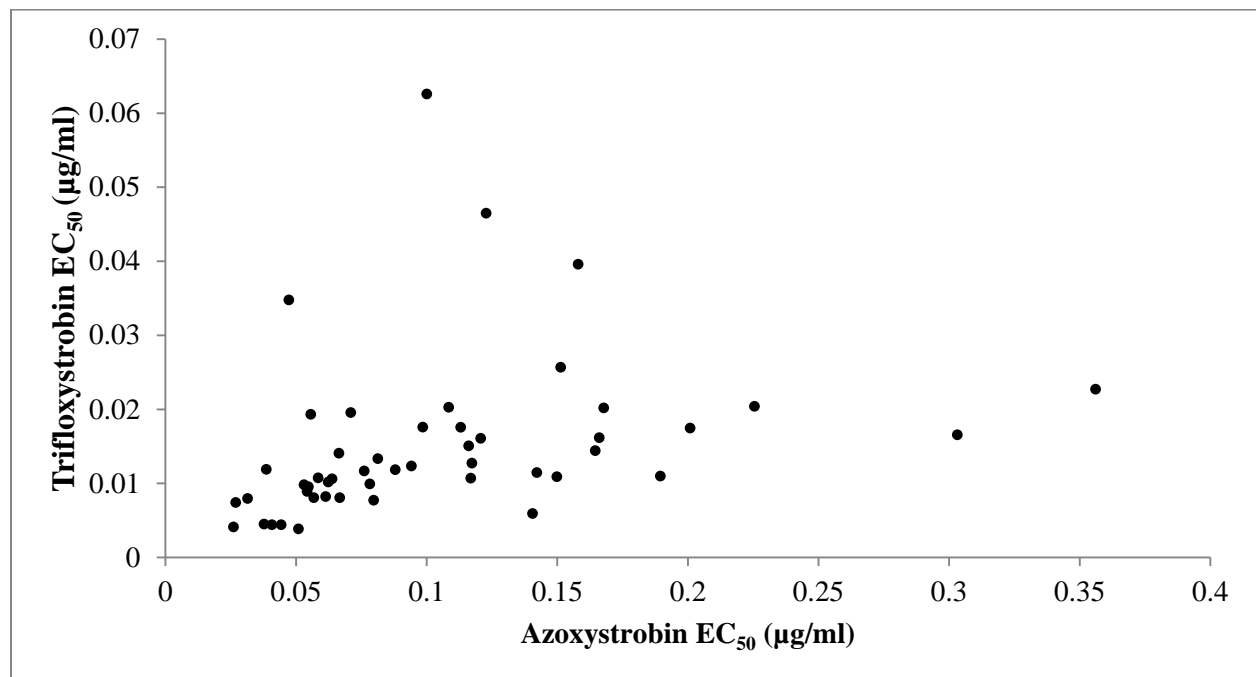


Figure 2.6. Spearman rank correlation between azoxystrobin and trifloxystrobin sensitivities of *Cercospora kikuchii* in 2012. $\rho(47) = 0.62$, $n = 49$, $P < 0.0001$.

Baseline Sensitivity of *Cercospora kikuchii* to Tetraconazole. EC₅₀ values of isolates exposed to tetraconazole ranged from 0.109 to 5.76 µg/ml with a median of 1.47 µg/ml. The 95% confidence interval of the distribution was [1.33, 1.98] with a mean of 1.66 µg/ml. A non-normal distribution with outliers towards the less-sensitive end was observed (W=0.9130, P = 0.0015) (Figure 2.9).

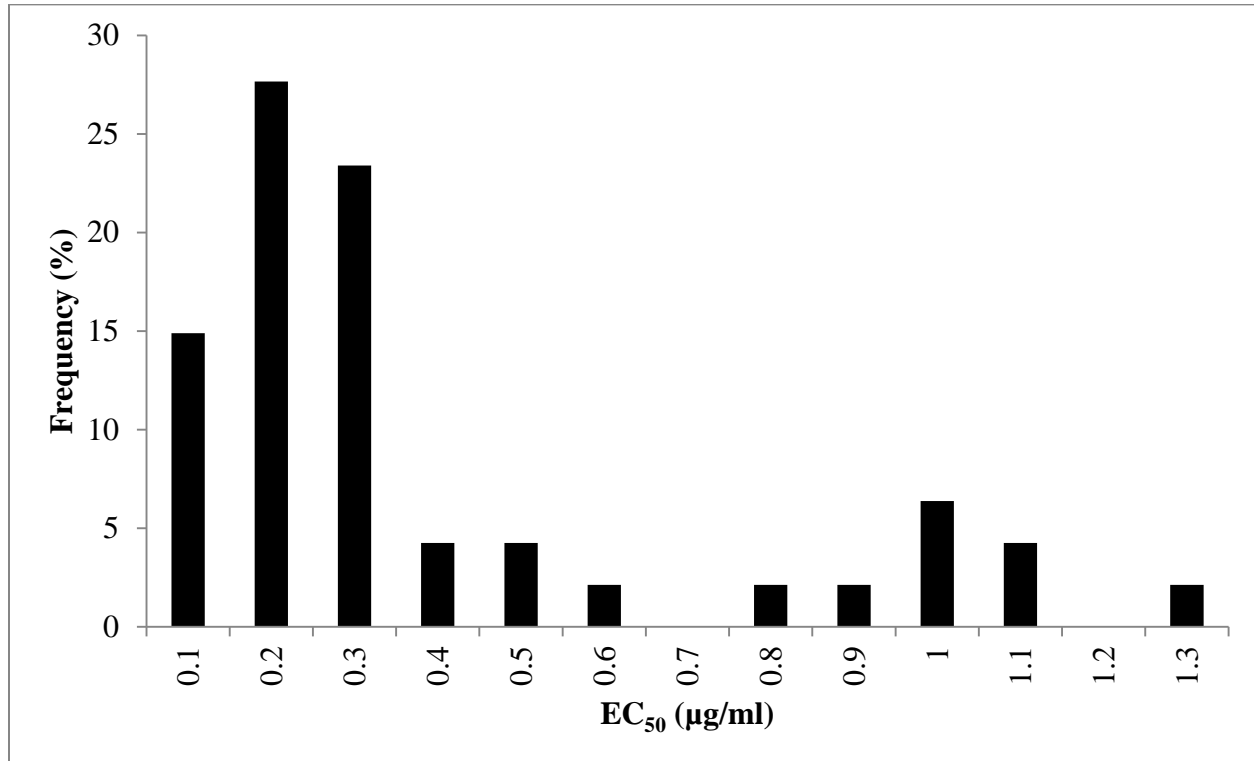


Figure 2.7. Baseline sensitivity of *Cercospora kikuchii* to flutriafol as determined by the effective concentration that inhibited 50% of radial growth (EC₅₀ values) of 47 isolates.

Cross-Sensitivity of Isolates to Flutriafol and Propiconazole. Spearman’s rank correlation test was used to determine significant relationships between EC₅₀ values of isolates from 2000 exposed to flutriafol and propiconazole. A weak, positive correlation was found (ρ (18) = 0.25, n = 20, P = 0.1464) between sensitivities of isolates exposed to these fungicides (Figure 2.10).

Discussion

Results from this study indicate SHAM is toxic to *C. kikuchii* *in vitro*. Therefore, SHAM was not used in subsequent assays to inhibit the alternative respiration pathway. Results from an earlier study also indicated SHAM toxicity to *Fusicladium effusum* in radial growth assays (Seyran et al 2010). An alternative AOX inhibitor, propyl gallate, did not appear to significantly affect radial growth of *C. kikuchii* and was used in

assays to inhibit AOX. Although an overall increase in isolate sensitivity was observed with the addition of propyl gallate to the azoxystrobin amended medium, there were no significant differences when comparing individual isolate sensitivities. Therefore, it was surmised that AOX did not occur in these isolates of *C. kikuchii* in radial growth assays, and AOX inhibitors were not utilized in subsequent experiments. These results are contrary to other research with *Cercospora* species in which *C. beticola*, *C. zea-maydis*, and *C. sojina* tested positive for AOX, and SHAM was used as an inhibitor in conidial germination inhibition assays (Malandrakis et al 2006, Bradley and Pedersen 2011, Zhang et al 2012a, 2012b). However, these results agree with other studies with plant pathogenic fungi where SHAM toxicity was observed (Seyran et al 2010), and propyl gallate was used to inhibit AOX (Miguez et al 2004).

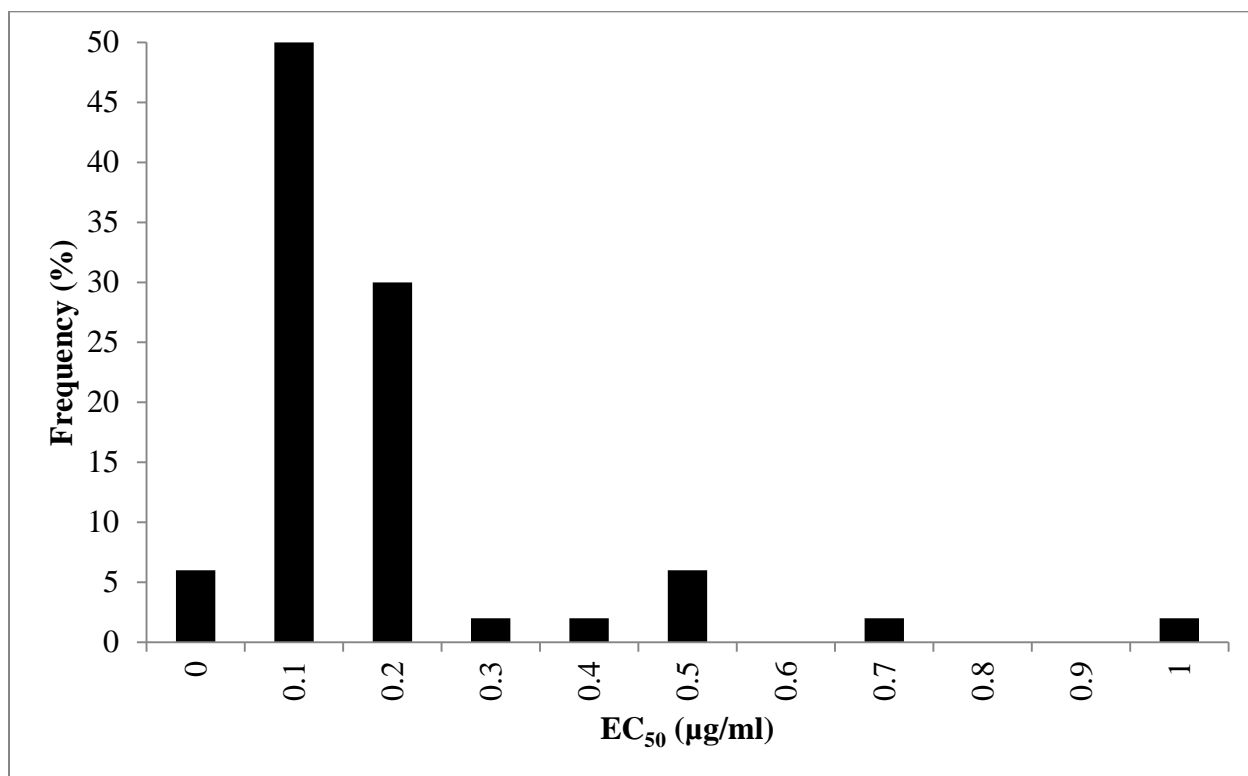


Figure 2.8. Baseline sensitivity of *Cercospora kikuchii* to propiconazole as determined by the effective concentration that inhibited 50% of radial growth (EC₅₀ values) of 50 isolates.

The baseline sensitivity of *C. kikuchii* for azoxystrobin had a 14-fold range of EC₅₀ values, and the majority of isolates had sensitivities to azoxystrobin ranging from 0.04 to 0.16 µg/ml, a four-fold span. Sensitivity values for pyraclostrobin had a much higher 1000-fold range overall; however, the presence of outliers on both ends of the spectrum may explain this phenomenon. The bulk of isolates represented in

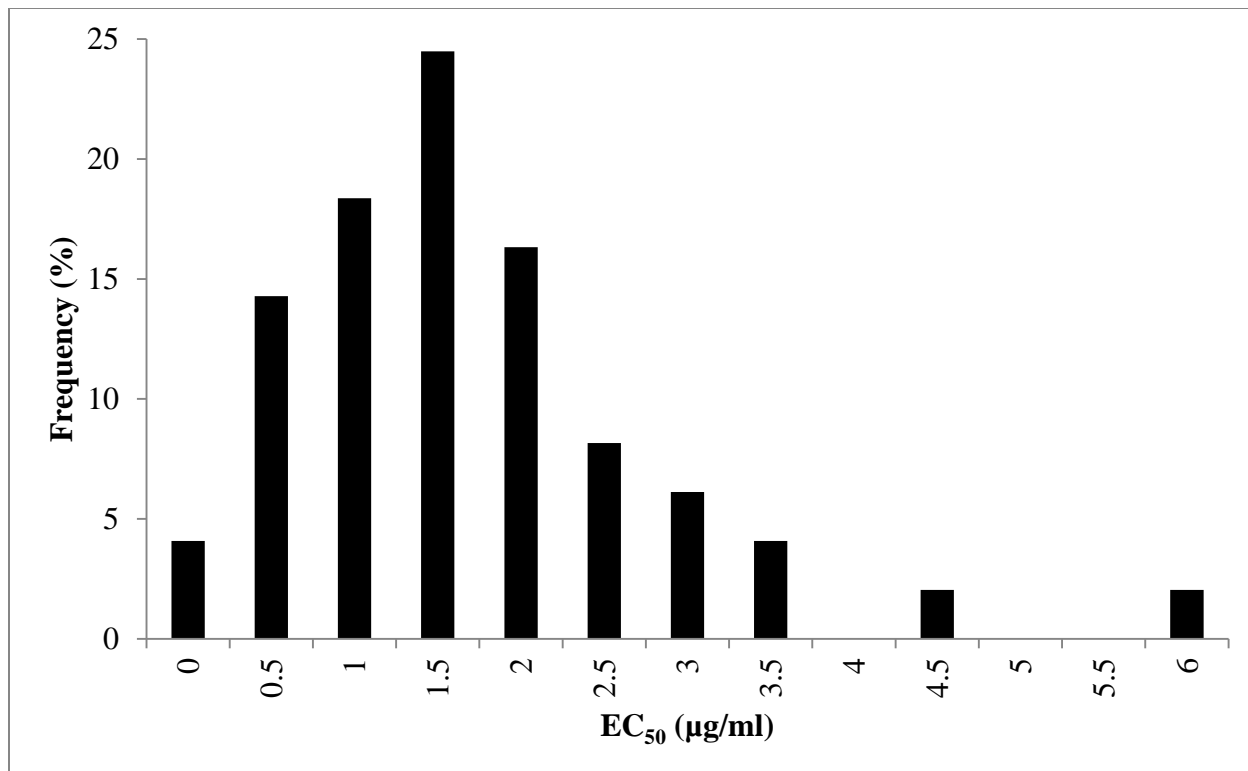


Figure 2.9. Baseline sensitivity of *Cercospora kikuchii* to tetraconazole as determined by the effective concentration that inhibited 50% of radial growth (EC₅₀ values) of 49 isolates.

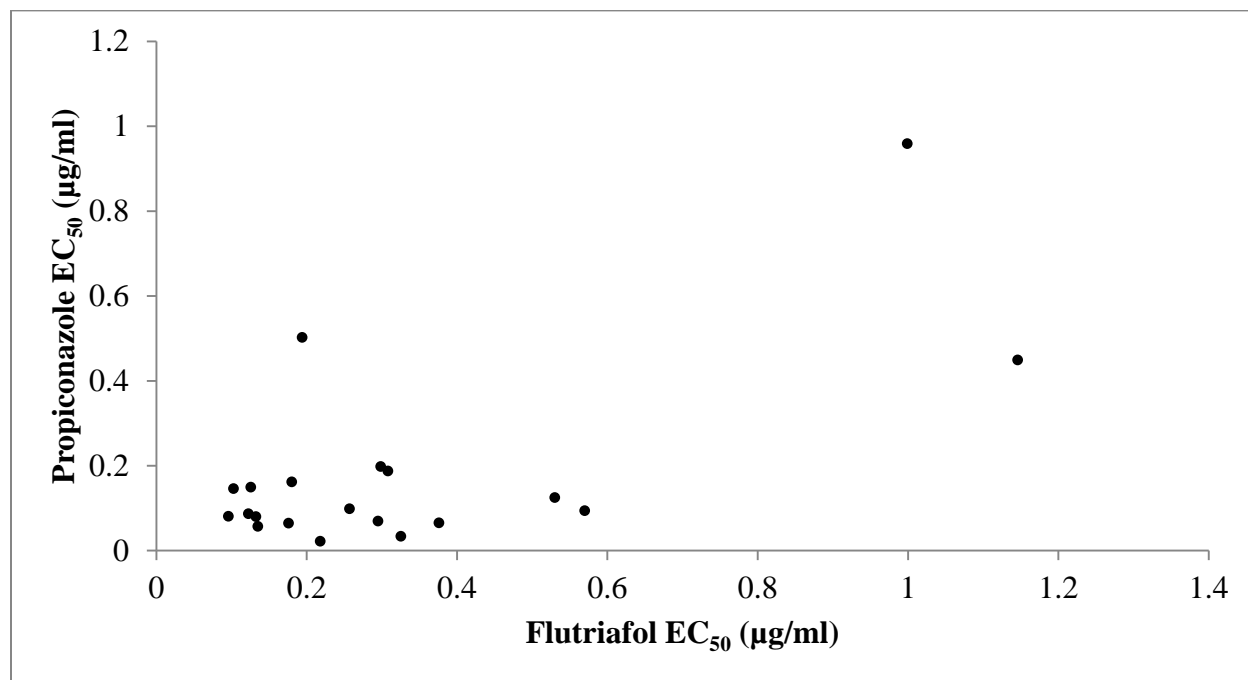


Figure 2.10. Spearman rank correlation between flutriafol and propiconazole sensitivities of *Cercospora kikuchii* in 2012. $\rho(18) = 0.25$, $n = 20$, $P < 0.2885$.

the pyraclostrobin sensitivity profile had EC₅₀ values ranging from 0.003 to 0.02 µg/ml, a 17-fold range. The sensitivity profile for trifloxystrobin covered a 13-fold range with the majority of isolates categorized between

0.005 and 0.02 µg/ml, a four-fold span. Other studies using conidial germination to determine baseline sensitivities to QoI fungicides in *Cercospora* species indicated ranges of 5 to 17-fold (Bradley and Pedersen, 2011, Zhang et al 2012a). Overall sensitivity to azoxystrobin *in vitro* was approximately six-fold less when compared to pyraclostrobin and trifloxystrobin, which indicated that the former was less effective on *C. kikuchii*. Similar phenomena have been observed in other studies with overall sensitivities of azoxystrobin ranging from 3 to 10-fold less when compared to pyraclostrobin and trifloxystrobin (Wong and Wilcox 2000, Pasche et al 2004, Wise et al 2008, Bradley and Pedersen 2011). Two studies featuring *C. zea-maydis* and *C. sojae* indicated differences of 46 to 180-fold in toxicity among azoxystrobin and pyraclostrobin or trifloxystrobin *in vitro* (Bradley and Pedersen 2011, Zhang et al 2012a). However, the difference in toxicity *in vitro* does not appear to translate to field efficacy, with numerous studies indicating similar efficacy on CLB/PSS among all three QoI fungicides *in vivo* (Padgett et al 2003, Padgett and Purvis 2005, 2007a, Delaney et al 2012).

Sensitivity profiles for DMI fungicides had EC₅₀ values ranging from 10 to 13-fold. The majority of isolate sensitivities for flutriafol ranged from 0.1 to 0.3 µg/ml, a three-fold span. Other research with a related species, *C. beticola*, indicated sensitivity ranges to flutriafol from 10 to 156-fold (Karaoglanidis et al 2000, 2001, 2003). A one-fold sensitivity range was observed in the majority of isolates of *C. kikuchii* exposed to propiconazole with EC₅₀ values from 0.1 to 0.2 µg/ml. Sensitivity ranges for other *Cercospora* species (*C. arachidiola* and *C. beticola*) to propiconazole were 7-fold and 266-fold, respectively (Hancock and Weete 1985, Karaoglanidis and Thanassoulopoulos 2003). For tetraconazole, most isolates of *C. kikuchii* were categorized from 0.5 to 2.5, a 5-fold designation. Bolton et al (2012) indicated wide (>100-fold) tetraconazole sensitivity ranges for *C. beticola*. Studies with plant pathogens other than *Cercospora* species also revealed varying ranges (1 to 900-fold) for *in vitro* sensitivity to DMI fungicides (Romero and Sutton 1997, Reynolds et al 1996, Zehr et al 1999, Holb and Schnabel 2007, Wong and Midland 2007, Fang et al 2009, Seyran et al

2010). This variability could be explained by diversity within the pathogen population, slight differences in sensitivity mechanisms, or slight differences among fungicide modes of action (Hildebrand et al 1988, Kendall et al 1993).

A strong, positive correlation was observed between azoxystrobin and trifloxystrobin when comparing EC₅₀ values with Spearman's rank correlation test. Other research has demonstrated a similar correlation between EC₅₀ values of other fungal pathogens when exposed to QoI fungicides (Pasche et al 2004, Rebollar-Alviter et al 2007, Wise et al 2009), which is indicative of the similar mode of action between the two fungicides. Therefore, it would not be advisable to replace one QoI fungicide with another in a disease management situation.

Lesser correlation was found between flutriafol and propiconazole. Variations in *in vitro* cross-sensitivity to DMI fungicides were previously observed for other plant pathogenic fungi (Kendall 1986, Hildebrand et al 1988, Peever and Milgroom 1993, Hsiang et al 1997, Robbertse et al 2001, Karaoglanidis and Thanassoulopoulos 2003). Differences in cross-sensitivity could be explained by varying genetic factors controlling DMI sensitivity, slight differences in the mode-of-action among DMIs, slight differences in resistance/sensitivity mechanisms, or small sample size (Hildebrand et al 1988, Kendall et al 1993). In this case, a low number of isolates, 20, were available that were exposed to both fungicides.

All six sensitivity profiles defined in this study were non-normal with outliers towards the less-sensitive ends of the spectra. According to FRAC, non-normal baseline distributions could be a "clear warning of resistance" (2013). Previous research with other *Cercospora* species detailed similar baseline distributions to QoI fungicides (Bradley and Pedersen 2011, Zhang et al 2012a). In some cases, when these non-normal distributions are observed, resistance in other *Cercospora* species has been confirmed to QoI fungicides in subsequent years (Secor et al 2010, Bradley and Pedersen, 2011, Zhang et al 2012b). Limited information concerning baseline sensitivities for DMI fungicides is available for *Cercospora* species; however, resistance to DMI fungicides has been confirmed in *C. beticola* in Greece and the United States (Karaoglanidis et al 2001, Secor et al 2010).

Over the past 10-15 years, QoI fungicide use has increased in Louisiana soybean production. Fungicide use statistics are limited, but the first indication of use of QoI fungicides in Louisiana soybean was in 2000 with approximately 5% of planted hectares treated (NASS 2013). From 2000-2006 coverage increased to approximately 38% of planted hectares with QoI fungicides comprising the majority of applications (NASS 2013). Current fungicide application estimates from scientists in the Southern United States range from 40 to 75% of planted hectares (personal communication, G. B. Padgett, C. Hollier, E. J. Sikora, and R. W. Schneider). Recently, fungicide use has been encouraged by industry for “yield bumps” or “plant health effects”, which has likely resulted in an increased number of applications (BASF 2013, Bayer Crop Science 2013, DuPont 2013). Two QoI fungicides historically have been recommended by the LSU AgCenter for management of CLB/PSS in Louisiana: azoxystrobin and pyraclostrobin (Anonymous 2010). Over the past 10 years, efficacy of these two QoI fungicides appears to have decreased. Over the past few years, field trial results from southern Louisiana indicated high levels of efficacy with the DMI fungicides, flutriafol and tetraconazole, on CLB/PSS (Schneider et al 2013). Since that time, LSU AgCenter recommendations for management of CLB/PSS have been changed to include several DMI fungicides, consequently increasing DMI applications to soybean throughout Louisiana. With the increased use of QoI fungicides and recent interest in DMI fungicides in soybean, it is prudent to monitor *C. kikuchii* populations for shifts in sensitivities to these fungicide chemistries.

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CHAPTER 3

QUINONE OUTSIDE INHIBITOR RESISTANCE IN LOUISIANA POPULATIONS OF *CERCOSPORA KIKUCHII*

Introduction

Cercospora leaf blight (CLB) and purple seed stain (PSS) are significant diseases of soybean produced in the United States causing estimated average losses of 140,500 metric tons annually from 1996 to 2012 (Wrather and Koennig 2013). The foliar phase of the disease affects soybean during reproductive phases of development with symptoms first appearing in the upper canopy eventually causing premature defoliation and subsequent yield loss (Matsumoto and Tomayasu 1925, Murakishi 1951, Walters et al 1980). The seed phase of the disease is marked by seed colonization from the pathogen usually resulting in purple staining of mature seed (Matsumoto and Tomayasu 1925, Murakishi 1951, Walters et al 1980). Disease incidence and severity of CLB/PSS have markedly increased within the past 10 to 15 years in Louisiana (Schneider et al 2003, Cai et al 2009).

Historically, two quinone outside inhibitor (QoI) fungicides, azoxystrobin (Quadris™) and pyraclostrobin (Headline™), applied during reproductive stages of development have been recommended by the Louisiana State University Agricultural Center (LSU AgCenter) for management of CLB/PSS (Anonymous 2010). Currently, trifloxystrobin (Gem RC™), pyraclostrobin (Headline 2.08EC™ & Headline SC™), and azoxystrobin (Quadris 2.08SC™) are QoI fungicides recommended by the LSU AgCenter for CLB/PSS management. Mixtures containing one QoI and one demethylation inhibitor (DMI) fungicide that are currently LSU AgCenter-recommended for management of CLB/PSS are azoxystrobin + cyproconazole (Quadris Xtra™), azoxystrobin + propiconazole (Quilt™, Quilt Xcel™), trifloxystrobin + propiconazole (Stratego™), and trifloxystrobin + prothioconazole (Stratego YLD™) (Anonymous 2012).

Over the past 10-15 years, QoI fungicide use has increased in Louisiana soybean. Fungicide use statistics are limited, but the first documentation of use of QoI fungicides in Louisiana soybean was in 2000 with approximately 5% of planted hectares treated (NASS 2013). From 2000-2006 coverage increased to approximately 38% of planted hectares with QoI fungicides comprising the majority of applications (NASS

2013). It is estimated that 40 to 75% of planted hectareage receives a minimum of one fungicide application per season (personal communications, G. B. Padgett, C. Hollier, E. J. Sikora, and R. W. Schneider). Recently, fungicide use has been encouraged by industry for “yield bumps” or “plant health effects”, which has likely resulted in an increased number of applications (BASF 2013, Bayer Crop Science 2013, DuPont 2013).

Many commercially available fungicides have proven inefficacious on CLB/PSS (Padgett and Purvis 2005, 2007a, 2007b, Price and Padgett 2008, Price et al 2013). Based on results from field studies, fungicide type, application rate, and timing have little or no effect on CLB/PSS (Padgett and Purvis 2007a, 2007b; Price and Padgett 2008; Price et al 2011; Delaney et al 2012; Price et al 2013).

QoI fungicides interfere with mitochondrial respiration by blocking electron transport at the quinol-oxidizing site of the cytochrome bc_1 complex (III), which subsequently affects spore germination and hyphal growth (Bartlett et al 2002). This mode of action is site-specific and can be overcome by a single-step mutation (Gisi et al 2002). Consequently, these fungicides are at high risk of resistance development in fungal populations (FRAC 2013). Some fungi have the ability to utilize an alternative respiration pathway, bypassing complex III in the respiration cycle and, consequently, avoiding the effects of QoI fungicides *in vitro* (Ziogas et al 1997). This phenomenon is thought to only occur *in vitro*, and to be prevented by plant flavones *in vivo* (Bartlett et al 2002). Alternative respiration was not detected in isolates of *C. kikuchii* used in this study when comparing EC_{50} values of isolates exposed to an AOX inhibitor and azoxystrobin (See Chapter 2).

QoI fungicides were introduced to the market around 1996, and resistance of fungal pathogens to this class of chemistry was documented in 1998 (FRAC 2013). Since that time, 56 species of fungi pathogenic to 20 horticultural and agronomic crops were identified as resistant to QoI fungicides (FRAC 2013). *Cercospora* species confirmed resistant to QoI fungicides include *C. beticola* and *C. sojina* (Bolton et al 2012, Zhang et al 2012b, respectively).

Establishment of baseline sensitivities to QoI fungicides is necessary to determine if sensitivity shifts are occurring or may occur in the future (Wong and Wilcox 2000, Avila-Adame et al 2003, Pasche et al 2004, Mondal et al 2005, Wise et al 2009, Secor et al 2010, Kirk et al 2012, Zhang et al 2012a, Zhang et al 2012b).

Baseline sensitivities to azoxystrobin, pyraclostrobin, and trifloxystrobin have previously been determined for isolates of *C. kikuchii* from 2000, which represent a true baseline population because of collection prior to the widespread use of QoI fungicides by Louisiana soybean producers (See Chapter 2).

The primary objective of this study was to determine if shifts in sensitivities to azoxystrobin, pyraclostrobin, and trifloxystrobin have occurred in *C. kikuchii* since 2000. Ancillary objectives were to determine discriminatory doses for all three fungicides, to delineate the extent of fungicide resistance in Louisiana, to detect QoI-resistant isolates in field trials, and to illustrate the effect of QoI fungicides on QoI-resistant isolates *in vivo*.

Materials and Methods

Isolate Sources. In 2011 and 2012, isolates of *C. kikuchii* were obtained from symptomatic soybean leaves from producer fields throughout Louisiana. Sixty-five locations in 21 parishes were sampled in 2011 while 36 locations in 27 parishes were sampled in 2012 (Figure 3.1). Symptomatic soybean leaflets were collected, approximately 10 per location, placed in sealable Ziploc™ plastic bags, and transported to the laboratory in an ice chest. Leaves were stored at 4°C until they were processed. Samples were removed within 72 h, and 5 symptomatic leaf sections, measuring 2 cm², were cut using scissors then placed in sterile test tubes (20 x 125 mm). Three ml of sterile, distilled water was added to each test tube, which was immediately capped and shaken vigorously for 30 s. Five drops of the resulting suspension were placed on a glass slide and observed with a stereomicroscope for conidia matching previously reported characteristics (Hartman, et al 1999) of *C. kikuchii*. Conidia were singly removed from the suspension with the aid of a glass needle fashioned from a micro pipette. Because of the length and flexible nature of the conidia, single spore isolation was readily achieved when a single spore wrapped around the tip of the glass needle. Single spores were transferred to water agar (1.5%) amended with chloramphenicol (75 µg/ml) and streptomycin sulfate (125 µg/ml) then allowed to incubate at room temperature for 5 to 7 days. Colonies that appeared to be producing cercosporin were selected, transferred via hyphal tips to V8 agar (20% juice), and maintained at 25°C with a 12h light: dark cycle. Resulting isolates received a numerical designation, and pools of 160 from 20 parishes and 82 from 18

parishes were available for analysis in 2011 and 2012, respectively (Tables 3.1 and 3.2). Unless otherwise indicated, cultures 21-35 days old were used in all assays and incubated as previously described.

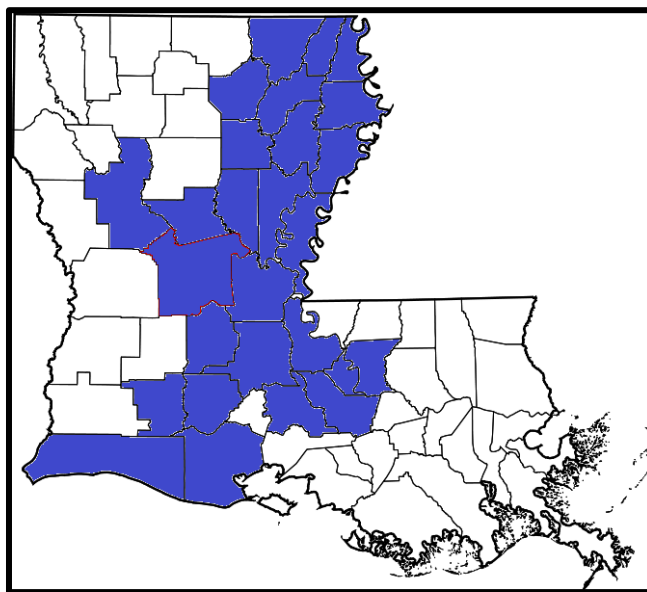


Figure 3.1. Parishes sampled in 2011 and 2012 for soybean affected with *Cercospora* leaf blight.

Table 3.1. Designations of isolates of *Cercospora kikuchii* and parishes of origin, 2011.

Isolate Designations	Parish of Origin
84, 86-88, 95, 99-102, 107-109, 118, 119, 140-142	Avoyelles
127,128	Caldwell
1-14, 55, 56	Catahoula
42-50, 57-60, 62, 63, 67-80, 89	Concordia
144-147	East Carroll
81, 82	East Baton Rouge
33, 34, 66	Evangeline
51, 52, 131,132	Franklin
35, 36	Jefferson Davis
15-26	LaSalle
83, 124-126, 129	Morehouse
130	Ouachita
61, 85, 103-106, 110, 113-115	Pointe Coupee
27-32, 53, 54, 90, 91, 96-98	Rapides
65	Richland
40, 92-94, 116, 117, 120-123, 155-160	Saint Landry
133-139, 143, 150-154	Saint Martin
37-39, 41, 64	Vermilion
148, 149	West Carroll
111, 112	West Baton Rouge

Table 3.2. Designations of isolates of *Cercospora kikuchii* and parishes of origin, 2012.

Isolate Designations	Parish of Origin
6, 7, 15, 47, 48	Avoyelles
59	Cameron
26-28, 41-45	Catahoula
16, 22-25, 33-35, 52	Concordia
38-40, 53	East Carroll
17-20	Evangeline
54	Franklin
77, 78	Grant
64, 65	Jefferson Davis
29, 30	Madison
79-82	Natchitoches
8-10, 46	Ouachita
1-5, 51	Pointe Coupee
13, 14, 56, 57	Rapides
11, 12, 21, 31, 32, 58, 61-63, 66-72	Saint Landry
60	Saint Martin
36, 37, 55	Tensas
73-76	Vermilion

Determining Sensitivity to Quinone Outside Inhibitor Fungicides. Radial growth assays for assessing % inhibition were utilized instead of spore germination assays as the method for determining fungicide toxicity because of sparse sporulation in culture. Ten-fold dilutions of technical formulations (ChemService, Inc.) of azoxystrobin (99.5%), pyraclostrobin (99.5%), and trifloxystrobin (99.3%) were performed in acetone, added (1.0 ml/1 L) to sterilized, molten PDA (50°C), and aseptically dispensed into sterile petri dishes (15 x 100 mm, ~20 ml/dish). Final concentrations for the QoI fungicides ranged from 0.001 to 10 µg/ml with PDA amended with only acetone included for comparison. For each sensitivity profile, about 50 isolates were chosen at random (RAND function, Microsoft Excel), transferred to amended PDA, and incubated as previously described. Colony diameter was calculated as previously described (See Chapter 2) 5 days after transfer, and data were converted to % inhibition by comparison to respective non-amended controls. The effective concentration to inhibit 50% of fungal radial growth (EC₅₀) was determined for each treatment by linear interpolation of a regression of log concentration by % inhibition (GraphPad Prism 5.0). The equation for the dose-response model is as follows: $Y=0+(100-0)/(1+10^{((\text{LogEC}_{50}-X)*1)})$. Mean EC₅₀ values were

determined for each isolate, and sensitivity profiles were constructed for each fungicide and year (GraphPad Prism 5.0). Tests for normality were conducted using the Shapiro-Wilk method (GraphPad Prism 5.0). In most cases data violated the assumption of normality, so the Kruskal-Wallis test was used to determine differences in distributions among years. Dunn's multiple comparison test was used to compare distributions between years (GraphPad Prism 5.0).

Determining Cross-Sensitivity Patterns. Isolates from 2011 that were exposed to both azoxystrobin and trifloxystrobin in sensitivity assays and isolates from 2012 that were exposed in sensitivity assays to all three QoI fungicides were available for comparison. Mean EC₅₀ values for each isolate and each fungicide were compared in four separate tests. Isolate sensitivities were compared for azoxystrobin and trifloxystrobin for 2011, while azoxystrobin and pyraclostrobin, azoxystrobin and trifloxystrobin, and pyraclostrobin and trifloxystrobin were compared for 2012 isolates. Because data violated the assumption of normality before and after transformation, a non-parametric form of correlation, Spearman's rank correlation test, was performed. Correlation coefficients (ρ) of 1, 0, and -1 translate to perfect-positive, none, and perfect-negative correlation.

Establishment of Discriminatory Doses. Obvious, logical separation spans were evident in all QoI distributions from 2011 and 2012. The midpoint of these separation spans were used to distinguish between sensitive and resistant isolates. Radial growth inhibition (%), relative to non-amended controls, at 0.001, 0.01, 0.1, 1, and 10 $\mu\text{g/ml}$ azoxystrobin, pyraclostrobin, and trifloxystrobin was determined for all QoI-sensitive isolates from 2000, 2011, and 2012 and then compared to all QoI-resistant isolates from 2011 and 2012 to establish discriminatory doses.

Field Trials. In 2011 (Test 1), soybean ('TV 49R22') was planted (23 seed/row m) on May 9 in a Gigger-Gilbert silt loam soil at the Macon Ridge Research Station near Alexandria, LA. In 2012 (Tests 2, 3, and 4), three different varieties of soybean ('AG 4303', 'AG 5532', and 'PI 96M60') were planted at the same rate and same soil type on Apr 15, May 16, and Jun 18, respectively. Standard pest control and cultural practices for soybean production were followed according to recommendations of the LSU AgCenter. Treatments were replicated three times in randomized complete blocks for each test, and plots were separated

by 1.5 to 3.0 m alleys. Plot size was 4.0 m by 6 m (4 rows). Successive fungicide applications at V6, R1, R3, R5, and R6 created treatments that received 0, 1, 2, 3, 4, or 5 total applications of Headline 2.08 EC (pyraclostrobin) each time at a rate of 840 ml/ha. The center two rows were treated, while the outside two rows served as buffer zones between treatments. Treatments were applied with a CO₂-charged spray boom configured with ULD 120-02 flat fan nozzles spaced on 51 cm centers delivering 180 L/ha at 207 kPa. Fungicide application and harvest dates for all tests are included in Table 3.3. Plots were harvested using a small-plot combine, and seed samples were saved for each plot. Ten purple-stained seed were kept from each plot for analysis. In the laboratory, seed were halved, taking care to split purple lesions. One half of each split seed was surface sterilized in 1:10 bleach: distilled water for 45-75 s, rinsed in sterile, distilled water for 15 s, and placed on PDA amended with chloramphenicol (75 µg/ml) and streptomycin sulfate (125 µg/ml) either containing 0 or 10 µg/ml pyraclostrobin. Cultures were allowed to incubate for 5 days, and the number of viable *C. kikuchii* colonies was enumerated to determine the percentage of resistant isolates per plot. Data were subjected to mixed model analysis, and treatment means were compared using the Tukey-Kramer post hoc adjustment ($\alpha=0.05$) (SAS Institute).

Table 3.3. Pertinent dates for Tests 1-4 of discriminatory dose soybean field studies for *Cercospora kikuchii*.

	Fungicide Application Dates	Harvest Date
Test 1 (2011)	6/7, 6/16, 6/27, 8/3	9/17
Test 2 (2012)	5/22, 6/1, 6/22	7/26
Test 3 (2012)	6/14, 6/22, 7/19, 8/7, 8/23	10/17
Test 4 (2012)	7/26, 8/7, 8/23, 9/6	11/1

Results

2000, 2011, and 2012 Azoxystrobin Sensitivity profiles. Baseline sensitivity of *C. kikuchii* to azoxystrobin ranged from 0.026 to 0.356 µg/ml with a median of 0.081 µg/ml (See Chapter 2). In 2011, EC₅₀ values of *C. kikuchii* isolates exposed to azoxystrobin ranged from 0.028 to 77.9 µg/ml with a median of 37.2

µg/ml. The 95% confidence interval of the distribution was [26.1, 39.1] with a mean of 32.6 µg/ml azoxystrobin. The overall sensitivity profile was not normally distributed ($W = 0.9156$, $P = 0.0012$) (Figure 3.2). In 2012, overall sensitivity ranged from 0.122 to 83.7 µg/ml with a median of 57.6 µg/ml azoxystrobin. The 95% confidence interval of the distribution was [46.9, 58.4] with a mean of 52.7 µg/ml. Significant differences between the medians of the baseline sensitivity profile and distributions from 2011 and 2012 were detected ($KW = 87.97$, $P = <0.0001$) (Figure 3.3).

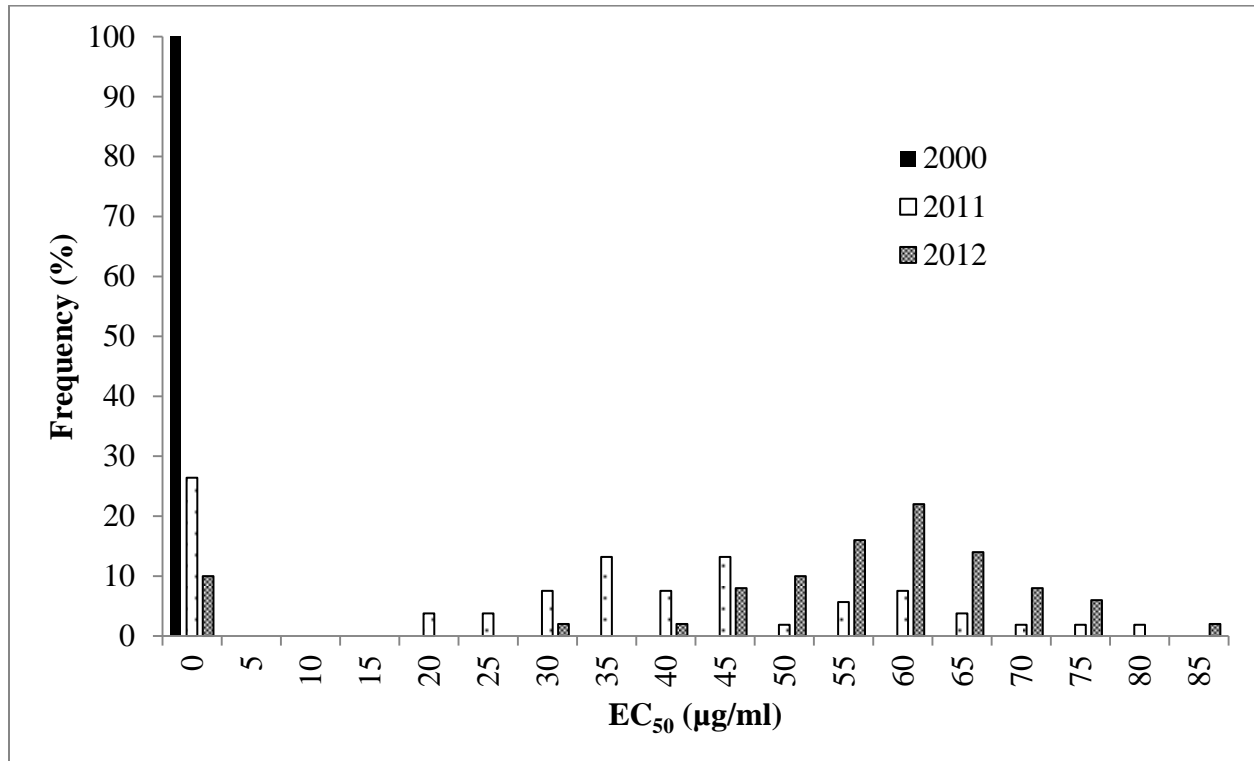


Figure 3.2. Sensitivity profiles of *Cercospora kikuchii* to azoxystrobin for 50, 53, and 50 isolates from 2000, 2011, and 2012.

2000, 2011, and 2012 Profiles for Azoxystrobin-Sensitive Isolates. All isolates from 2000 were sensitive to azoxystrobin and are included for comparison (See Chapter 2). A logical separation point between n sensitive and resistant isolates in 2011 was 9.3 µg/ml. Approximately 26% of isolates from 2011 had EC₅₀ values < 9.3 µg/ml ranging from 0.028 to 0.883 with a median of 0.095 µg/ml azoxystrobin. The 95% confidence interval for the distribution was [0.031, 0.280] with a mean of 0.156 µg/ml, and the profile for sensitive isolates from 2011 was not normally distributed ($W = 0.4946$, $P < 0.0001$) (Figure 3.4). The logical separation point between sensitive and resistant isolates in 2012 was 15.1 µg/ml. Ten percent of isolates from

2012 had EC₅₀ values < 15.1 µg/ml azoxystrobin ranging from 0.122 to 0.150 with a median of 0.138 µg/ml azoxystrobin. The 95% confidence interval for the distribution was [0.124, 0.150] with a mean of 0.137 µg/ml, and normality was incalculable due to low isolate numbers (Figure 3.4).

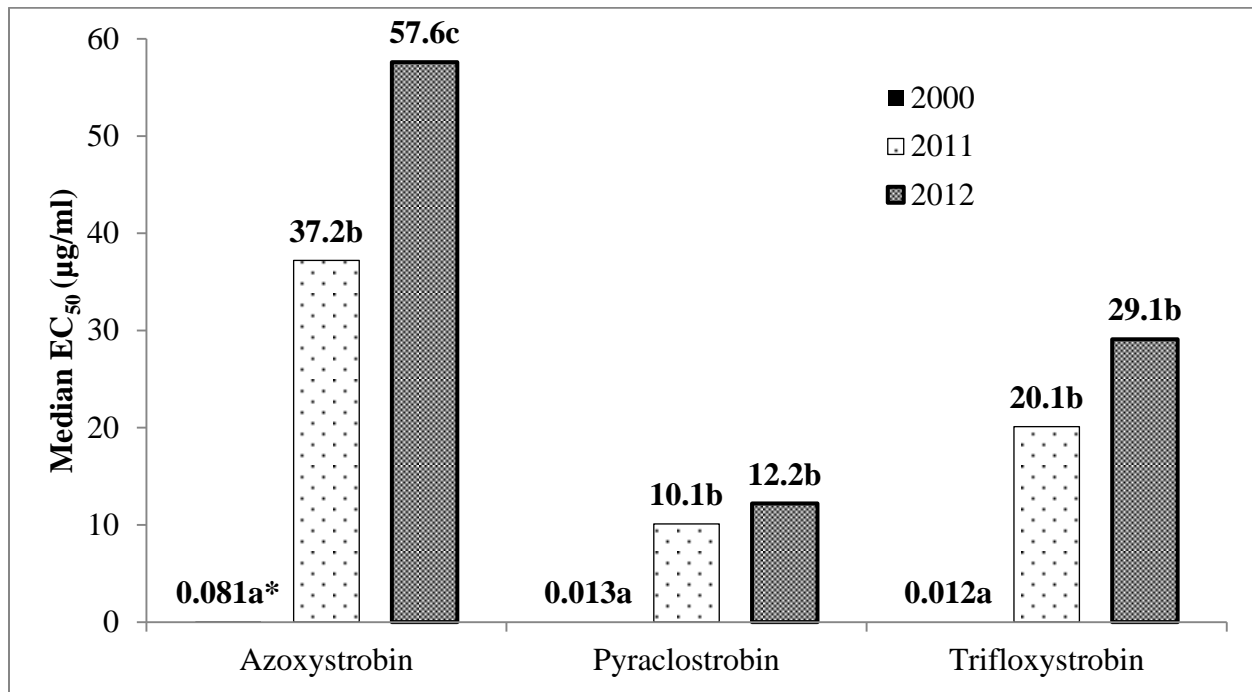


Figure 3.3. Sensitivity distribution comparison for *Cercospora kikuchii* to azoxystrobin, pyraclostrobin, and trifloxystrobin for 2000, 2011, and 2012.

*Medians followed by the same letter are not significantly different (Kruskal-Wallis, Dunn's multiple comparison test, $\alpha=0.05$)

2011 and 2012 Profiles for Azoxystrobin-Resistant Isolates. In 2011, 74% of isolates had EC₅₀ values > 9.3 µg/ml azoxystrobin ranging from 17.5 to 77.9 with a median of 42.7 µg/ml. The 95% confidence interval was [39.3, 49.2] with a mean of 44.3 µg/ml, and the distribution of resistant isolates was normal ($W = .9731$, $P = 0.4659$) (Figure 3.4). In 2012, 90% of isolates had mean EC₅₀ values > 15.1 µg/ml azoxystrobin ranging from 30.1 to 83.7 with a median of 59.4 µg/ml. The 95% confidence interval was [55.4, 61.6] with a mean of 58.5 µg/ml azoxystrobin, and the profile of resistant isolates was normally distributed ($W = .9916$, $P = 0.9837$) (Figure 3.5).

2000, 2011, and 2012 Pyraclostrobin Sensitivity Profiles. Baseline sensitivity of *C. kikuchii* to pyraclostrobin ranged from 0.0003 to 0.103 µg/ml with a mean of 0.017 µg/ml (See Chapter 2). In 2011, EC₅₀

values of *C. kikuchii* isolates exposed to pyraclostrobin ranged from 0.009 to 27.4 $\mu\text{g/ml}$ with a median of 10.1 $\mu\text{g/ml}$. The 95% confidence interval of the distribution was [8.9, 12.5] with a mean of 10.8 $\mu\text{g/ml}$, and

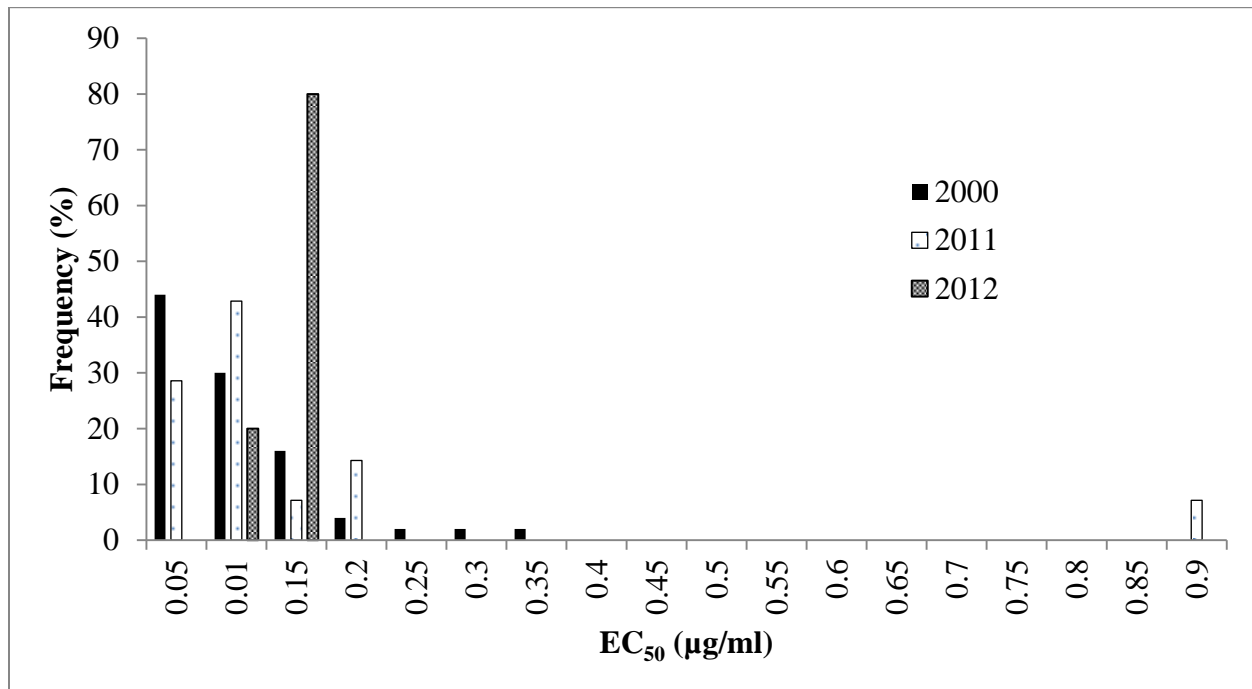


Figure 3.4. Profiles of 50, 14, and 5 azoxystrobin-sensitive isolates of *Cercospora kikuchii* from 2000, 2011, and 2012, respectively.

the overall sensitivity profile was not normally distributed ($W = 0.9313$, $P = 0.0069$) (Figure 3.6). In 2012, EC_{50} values of *C. kikuchii* isolates exposed to pyraclostrobin ranged from 0.101 to 33.7 $\mu\text{g/ml}$ with a median of 12.2 $\mu\text{g/ml}$. The 95% confidence interval of the distribution was [10.7, 15.9] with a mean of 13.3 $\mu\text{g/ml}$, and the overall sensitivity profile was not normally distributed ($W = 0.9274$, $P = 0.0040$) (Figure 3.5). Significant differences between the medians of the baseline sensitivity profile and distributions from 2011 and 2012 were detected ($KW = 92.82$, $P = <0.0001$) (Figure 3.3).

2000, 2011, and 2012 Profiles for Pyraclostrobin-Sensitive Isolates. All isolates of *C. kikuchii* from the baseline in 2000 were found to be sensitive to pyraclostrobin and are included for comparison (See Chapter 2). A logical separation point between sensitive and resistant isolates in 2011 was 0.8 $\mu\text{g/ml}$. In 2011, approximately 10% of isolates had EC_{50} values $< 0.8 \mu\text{g/ml}$ pyraclostrobin ranging from 0.009 to 0.045 with a median of 0.015 $\mu\text{g/ml}$. The 95% confidence interval for the distribution was [0.001, 0.037] with a mean of 0.019 $\mu\text{g/ml}$, and normality could not be determined because of too few sensitive isolates (Figure 3.6). A

logical separation point between sensitive and resistant isolates in 2012 was 1.6 $\mu\text{g/ml}$. In 2012, approximately 16% of isolates had EC_{50} values $< 1.6 \mu\text{g/ml}$ pyraclostrobin ranging from 0.100 to 0.300 with a median of 0.155 $\mu\text{g/ml}$. The 95% confidence interval for the distribution was [0.123, 0.232] with a mean of 0.018 $\mu\text{g/ml}$, and the profile of sensitive isolates was normally distributed ($W = 0.9170$, $P = 0.4059$) (Figure 3.7).

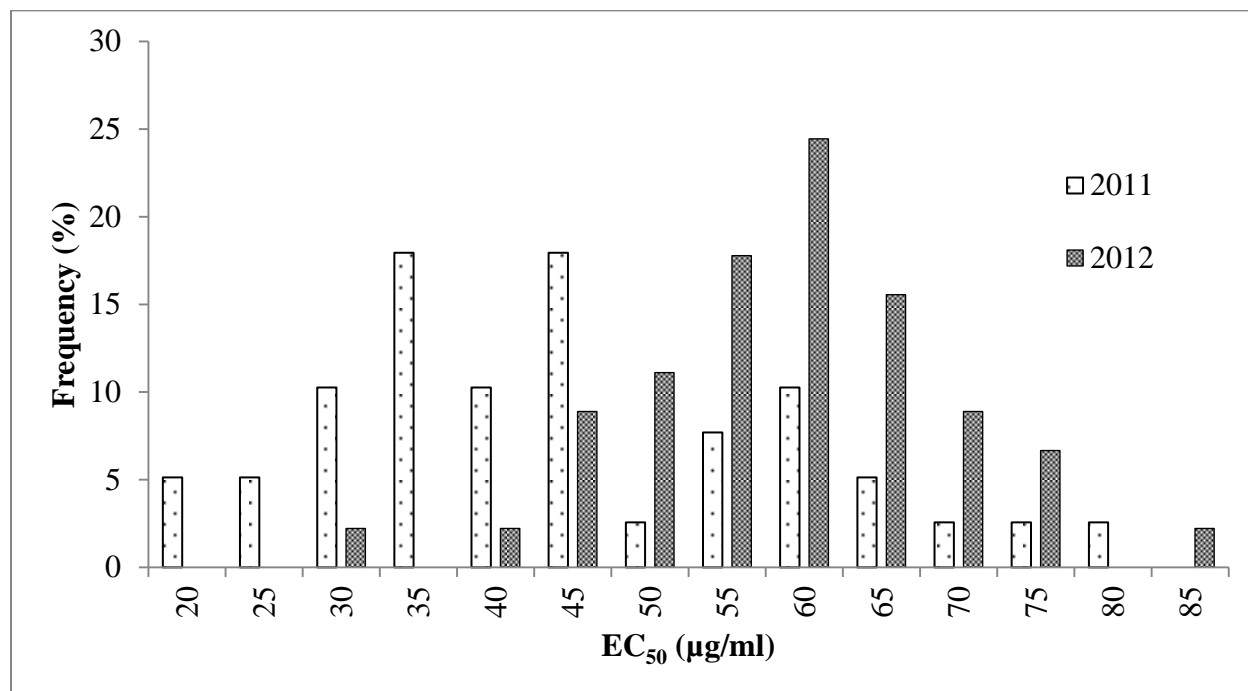


Figure 3.5. Profiles of 39 and 45 azoxystrobin-resistant isolates of *Cercospora kikuchii* from 2011 and 2012, respectively.

2011 and 2012 Profiles for Pyraclostrobin-Resistant Isolates. In 2011, 90% of isolates had EC_{50} values $> 0.8 \mu\text{g/ml}$ ranging from 1.5 to 27.4 with a median of 10.5 $\mu\text{g/ml}$. The 95% confidence interval was [10.3, 13.6] with a mean of 12.0 $\mu\text{g/ml}$ pyraclostrobin, and the profile of resistant isolates was not normally distributed ($W = 0.8851$, $P = 0.0004$) (Figure 3.7). In 2012, 84% of isolates had EC_{50} values $> 1.6 \mu\text{g/ml}$ ranging from 3.0 to 17.2 with a median of 10.6 $\mu\text{g/ml}$ pyraclostrobin. The 95% confidence interval was [9.9, 12.1] with a mean of 11.0 $\mu\text{g/ml}$ pyraclostrobin, and the profile of resistant isolates was normally distributed ($W = 0.9756$, $P = 0.4859$) (Figure 3.8).

2000, 2011, and 2012 Trifloxystrobin Sensitivity Profiles. Baseline sensitivity of isolates of *C. kikuchii* from 2000 to trifloxystrobin ranged from 0.004 to 0.063 $\mu\text{g/ml}$ with a mean of 0.015 $\mu\text{g/ml}$ (See Chapter 2). In 2011, EC_{50} values of *C. kikuchii* isolates exposed to trifloxystrobin ranged from 0.006 to 90.1

$\mu\text{g/ml}$ with a median of $20.1 \mu\text{g/ml}$. The 95% confidence interval of the distribution was [16.8, 28.4] with a mean of $22.6 \mu\text{g/ml}$ trifloxystrobin, and the overall sensitivity profile was not normally distributed ($W = 0.9044$, $P = 0.0008$) (Figure 3.8). In 2012, EC_{50} values of *C. kikuchii* isolates exposed to trifloxystrobin ranged from 0.005 to $111.7 \mu\text{g/ml}$ with a median of $29.1 \mu\text{g/ml}$. The 95% confidence interval of the distribution was [23.5, 35.0] with a mean of $29.2 \mu\text{g/ml}$ trifloxystrobin, and the overall sensitivity profile was not normally distributed ($W = 0.8990$, $P = 0.0004$) (Figure 3.9). Significant differences between medians of the baseline sensitivity profile and distributions from 2011 and 2012 were detected ($KW = 62.31$, $P = <0.0001$) (Figure 3.3).

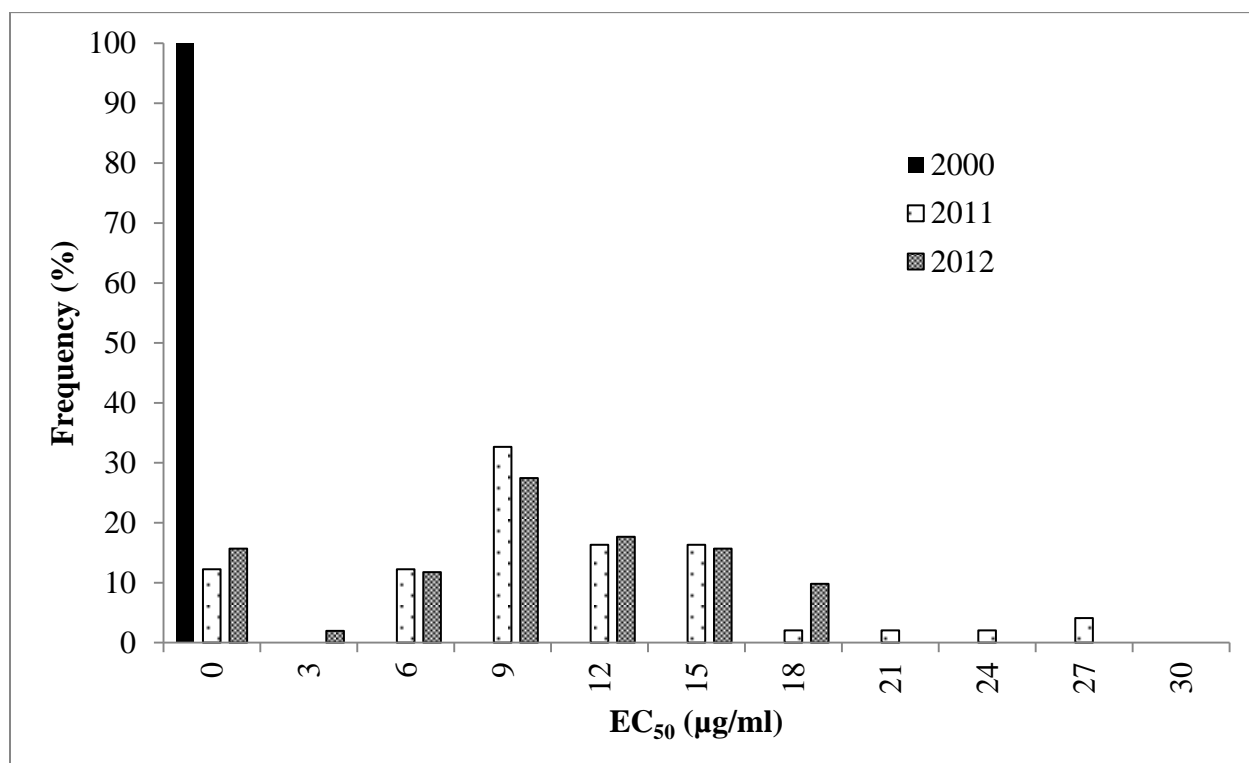


Figure 3.6. Sensitivity profiles of *Cercospora kikuchii* to pyraclostrobin for 50, 49, and 51 isolates from 2000, 2011, and 2012, respectively.

2000, 2011, and 2012 Profiles for Trifloxystrobin-Sensitive Isolates. All isolates included in the baseline from 2000 were sensitive to trifloxystrobin (See Chapter 2). A logical separation point between sensitive and resistant isolates in 2011 was $1.9 \mu\text{g/ml}$. Approximately 25% of isolates from 2011 had EC_{50} values $< 1.9 \mu\text{g/ml}$ trifloxystrobin ranging from 0.006 to $0.335 \mu\text{g/ml}$ with a median of $0.011 \mu\text{g/ml}$. The 95% confidence interval of the distribution was [0.000, 0.110] with a mean of $0.048 \mu\text{g/ml}$, and the profile of sensitive isolates was not normally distributed ($W = 0.4926$, $P < 0.0001$) (Figure 3.10). A logical separation

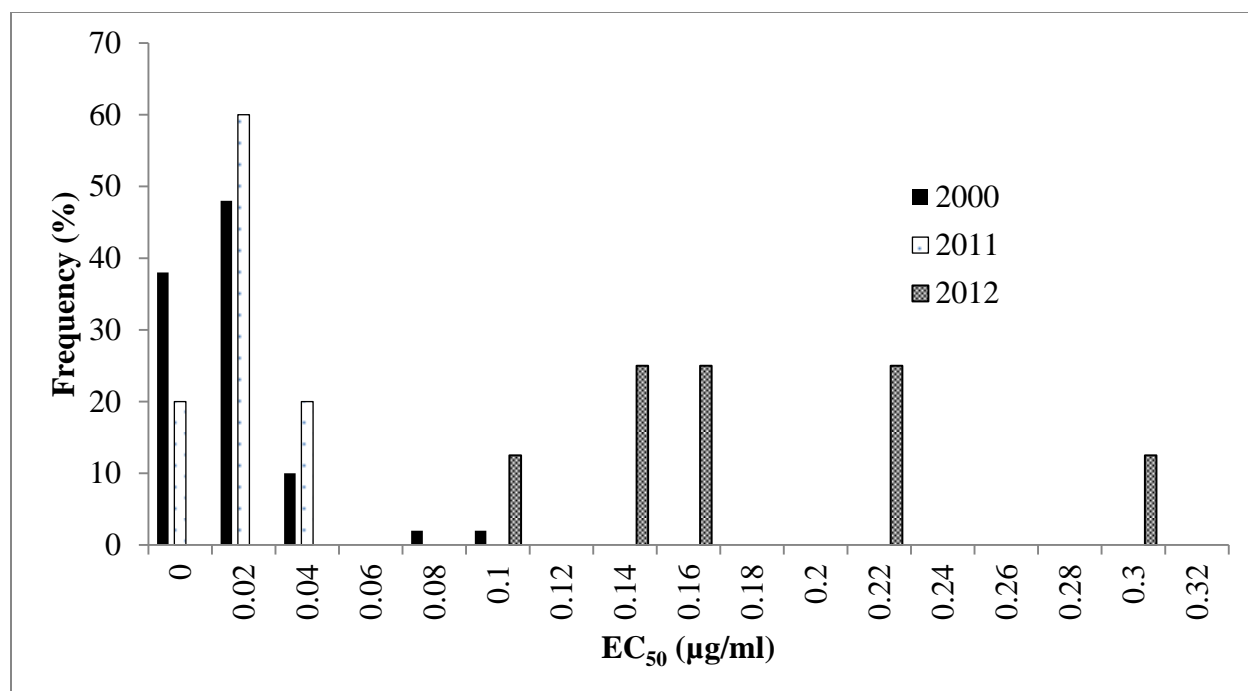


Figure 3.7. Profiles of 50, 5, and 8 pyraclostrobin-sensitive isolates of *Cercospora kikuchii* from 2000, 2011, and 2012, respectively.

point between sensitive and resistant isolates in 2012 was 5.7 µg/ml. In 2012, approximately 16% of isolates from 2012 had EC₅₀ values < 5.7 µg/ml trifloxystrobin ranging from 0.005 to 0.091 µg/ml with a median of 0.014. The 95% confidence interval of the distribution was [0.000, 0.047] with a mean of 0.023 µg/ml, and the profile of sensitive isolates was not normally distributed (W = 0.6516, P = 0.0006) (Figure 3.10).

2011 and 2012 Profiles of Trifloxystrobin-Resistant Isolates. In 2011, 77% of isolates had EC₅₀ values > 1.9 µg/ml ranging from 4.2 to 90.1 with a median of 25.8. The 95% confidence interval was [23.9, 35.8] with a mean of 29.9 µg/ml trifloxystrobin, and the profile of resistant isolates was not normally distributed (W = 0.9140, P = 0.0074) (Figure 3.11). In 2012, 84% of isolates had EC₅₀ values > 5.7 µg/ml ranging from 11.6 to 111.7 with a median of 31.5 µg/ml trifloxystrobin. The 95% confidence interval of resistant isolates was [29.4, 40.1] with a mean of 34.8 µg/ml trifloxystrobin, and the profile was not normally distributed (W = 0.8213, P < 0.0001) (Figure 3.11).

Cross-Sensitivity of Quinone Outside Inhibitor Fungicides in 2011 and 2012. A Spearman rank correlation test was used to determine significant relationships between EC₅₀ values of isolates exposed to two and three QoI fungicides in sensitivity assays in 2011 and 2012, respectively. A significant, positive correlation

was found for the 2011 isolates exposed to azoxystrobin and trifloxystrobin (ρ (48) = 0.62, n = 50, $P < 0.0001$) (Figure 3.12). Significant, positive correlations also were determined for azoxystrobin and pyraclostrobin (ρ (34) = 0.49, n = 36, $P = 0.0026$) and pyraclostrobin and trifloxystrobin (ρ (37) = 0.61, n = 39, $P < 0.0001$) in isolates exposed to these fungicides in 2012 sensitivity assays (Figures 3.13 and 3.15). A weak, positive correlation was determined for azoxystrobin and trifloxystrobin (ρ (39) = .30, n = 41, $P = 0.0582$) for 2012 isolates (Figure 3.14).

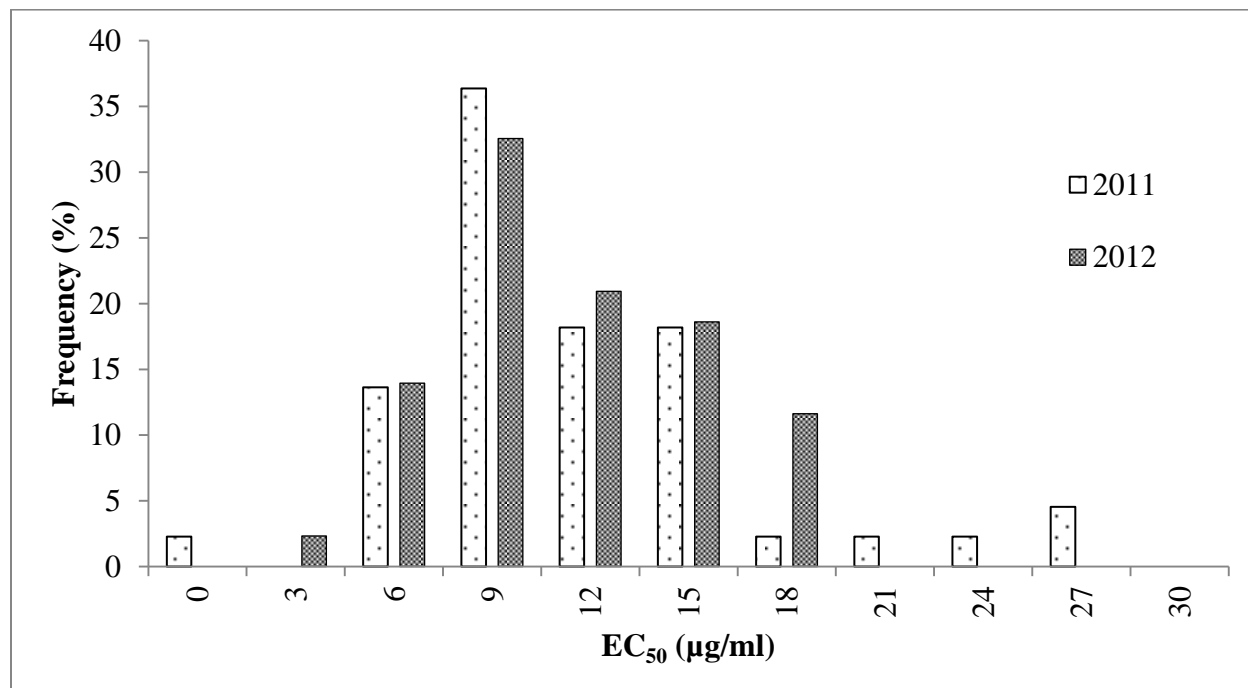


Figure 3.8. Profiles of 44 and 43 pyraclostrobin-resistant isolates of *Cercospora kikuchii* from 2011 and 2012, respectively.

Establishment of Discriminatory Doses. For each fungicide, mean growth inhibition (%) at concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml was calculated for sensitive and resistant isolates to determine discriminatory doses. For azoxystrobin at respective concentrations, mean radial growth inhibition was 0, 0, 7, 57, 81, and 99% for 71 sensitive isolates from 2000, 2011, and 2012. Radial growth inhibition for 84 resistant isolates from 2011 and 2012 was 0, 1, 2, 2, 4, and 19% at respective azoxystrobin concentrations (Figure 3.16). For pyraclostrobin at respective concentrations, mean radial growth inhibition was 0, 7, 37, 68, 95, and 100% for 62 sensitive isolates from 2000, 2011, and 2012. Mean radial growth inhibition for 87 resistant isolates from 2011 and 2012 was 0, 1, 3, 6, 17, and 45% at respective pyraclostrobin concentrations

(Figure 3.17). For trifloxystrobin at respective concentrations, mean radial growth inhibition was 0, 7, 51, 89, 97, and 99% for 69 sensitive isolates from 2000, 2011, and 2012. Mean radial growth inhibition for 80 resistant isolates from 2011 and 2012 was 0, 3, 4, 7, 19, and 30% at respective trifloxystrobin concentrations (Figure 3.18).

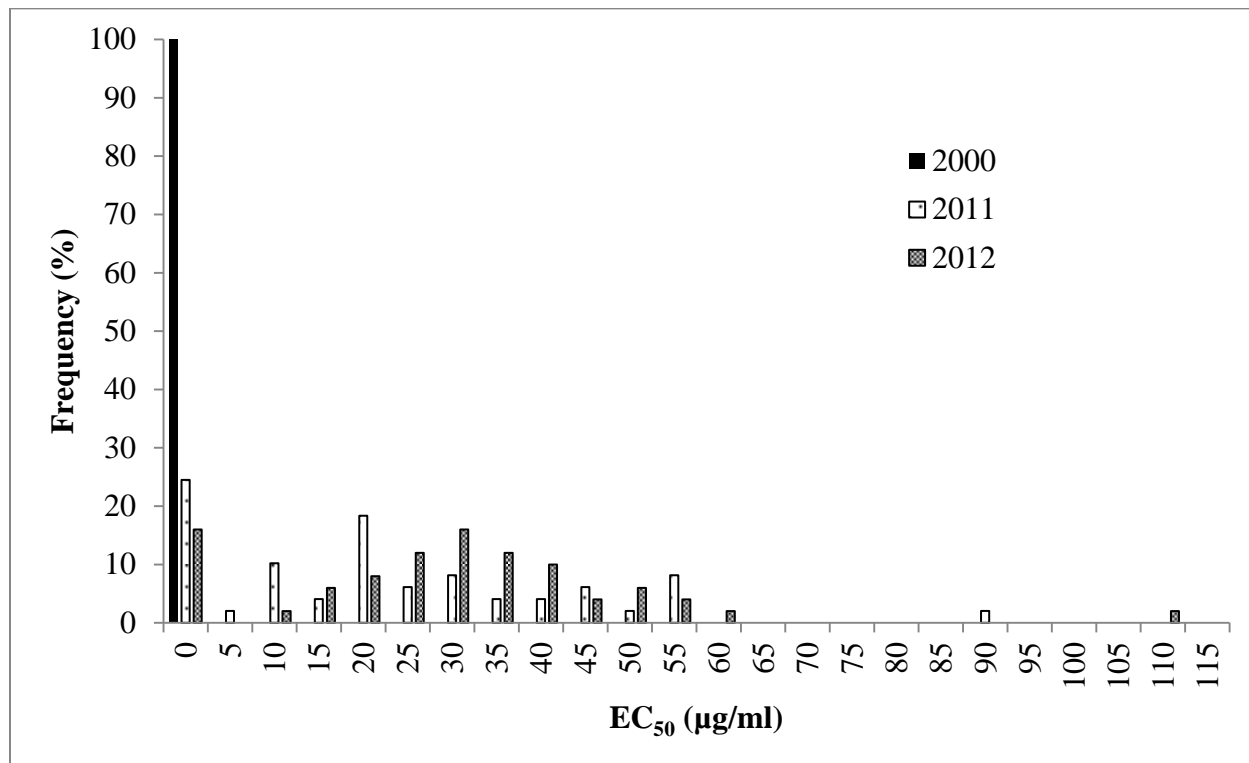


Figure 3.9. Sensitivity profiles of *Cercospora kikuchii* to trifloxystrobin for 49, 49, and 50 isolates from 2000, 2011, and 2012, respectively.

Extent of Quinone Outside Inhibitor Resistance in Louisiana. There were no QoI-resistant isolates of *C. kikuchii* in the 2000 collection. In 2011 and 2012, 80 and 86%, respectively, of isolates were QoI-resistant. The remainder of isolates in 2011 and 2012 were QoI-sensitive (Figure 3.19). In 2011 and 2012, isolates from 21 parishes throughout Louisiana were resistant to QoI fungicides (Figure 3.20). Individual parish information indicating the number of isolates tested and confirmed resistant for 2011 and 2012 are listed in Table 3.4.

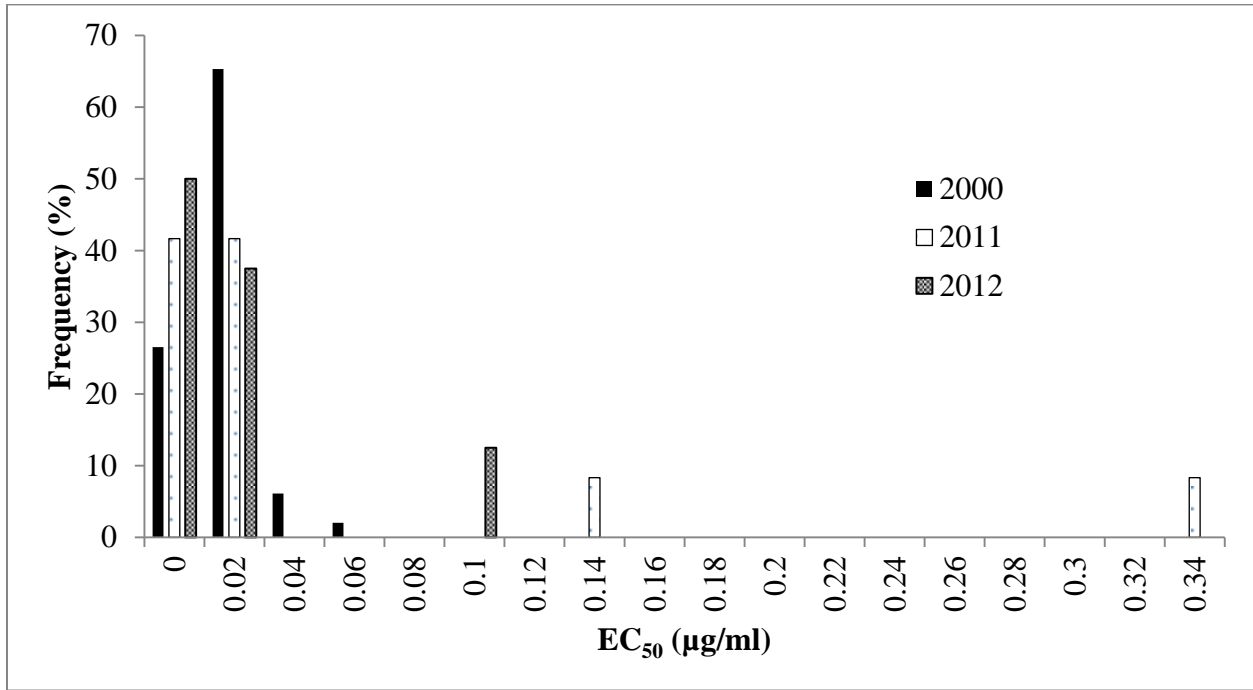


Figure 3.10. Profiles of 49, 12, and 8 trifloxystrobin-sensitive isolates of *Cercospora kikuchii* from 2000, 2011, and 2012, respectively.

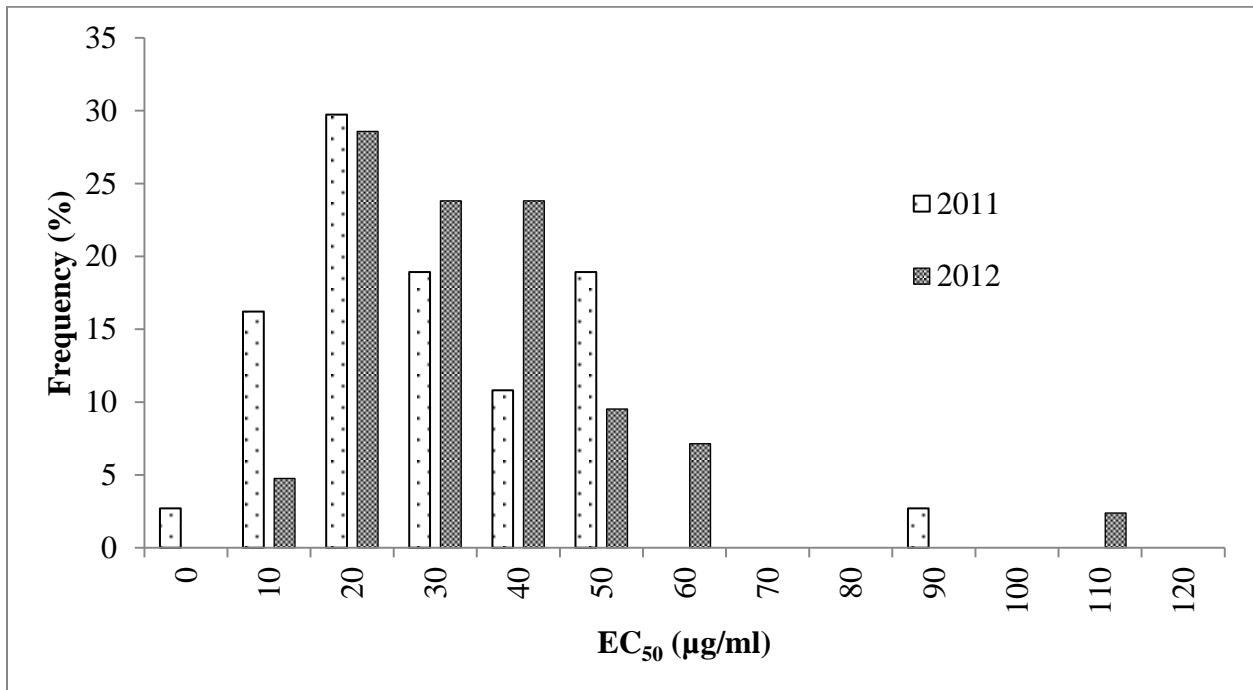


Figure 3.11. Profiles of 37 and 42 trifloxystrobin-resistant *Cercospora kikuchii* isolates from 2011 and 2012, respectively.

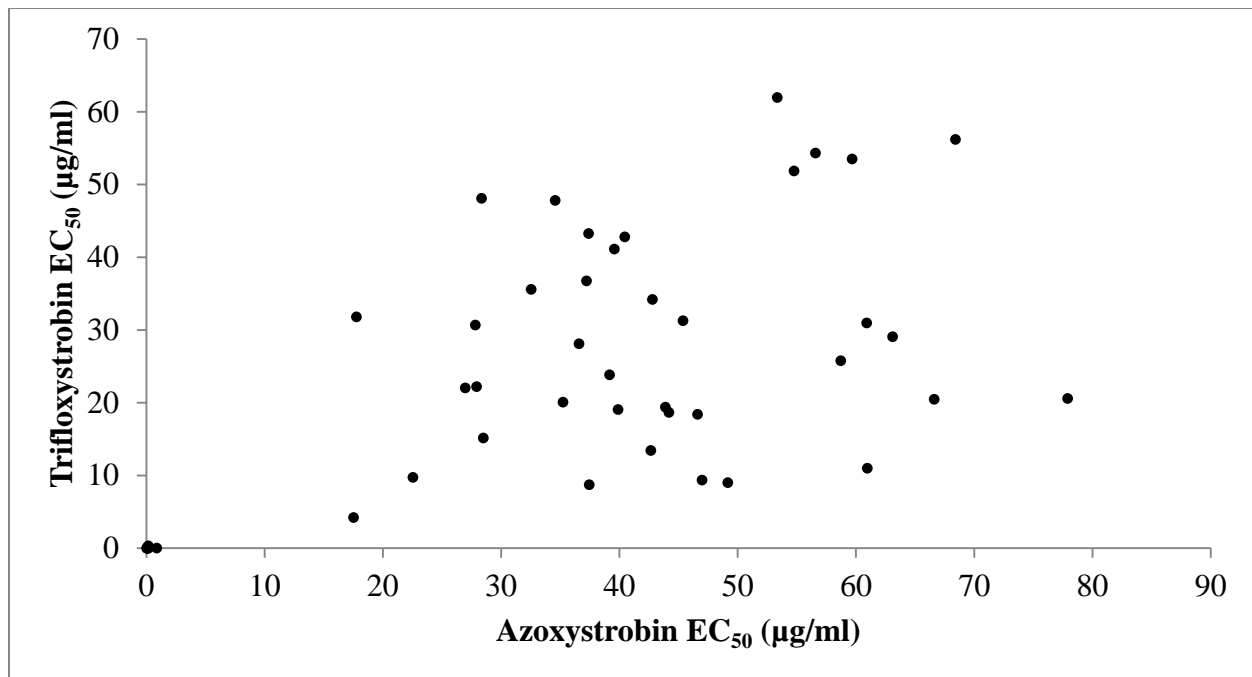


Figure 3.12. Spearman rank correlation between EC₅₀ values for isolates of *Cercospora kikuchii* exposed to azoxystrobin and trifloxystrobin in sensitivity assays in 2011. $\rho(48) = 0.62$, $n = 50$, $P < 0.0001$.

Results from Field Trials. A discriminatory dose of pyraclostrobin (10 µg/ml) was used to detect resistant seed isolates of *C. kikuchii* in four field trials conducted in 2011 and 2012. No significant differences were detected in the frequency of QoI-resistant isolates among treatments where plots received 0, 1, 2, 3, 4, or 5 applications of pyraclostrobin (Headline™ 840 ml/ha). The percentage of resistant isolates ranged from 74 to 91%, with a mean of 77% resistant isolates present in non-treated plots (Figure 3.21).

Discussion

Baseline sensitivity of *C. kikuchii* to azoxystrobin was approximately 10-fold lower than pyraclostrobin and trifloxystrobin (See Chapter 2), which is consistent with baseline sensitivities of *C. zea-maydis* and *C. sojae* in which conidial germination assays were conducted (Bradley and Pedersen 2011, Zhang et al 2012a). Although, EC₅₀ values in radial growth assays were higher in this study when compared with other studies utilizing conidial germination assays, relative values are similar to other studies illustrating QoI baselines or QoI resistance in fungal pathogens (Wong and Wilcox 2000, Avila-Adame et al 2003, Pasche et al 2004, Mondal et al 2005, Wise et al 2008, 2009, Secor et al 2010, Kirk et al 2012, Zhang et al 2012a, 2012b).

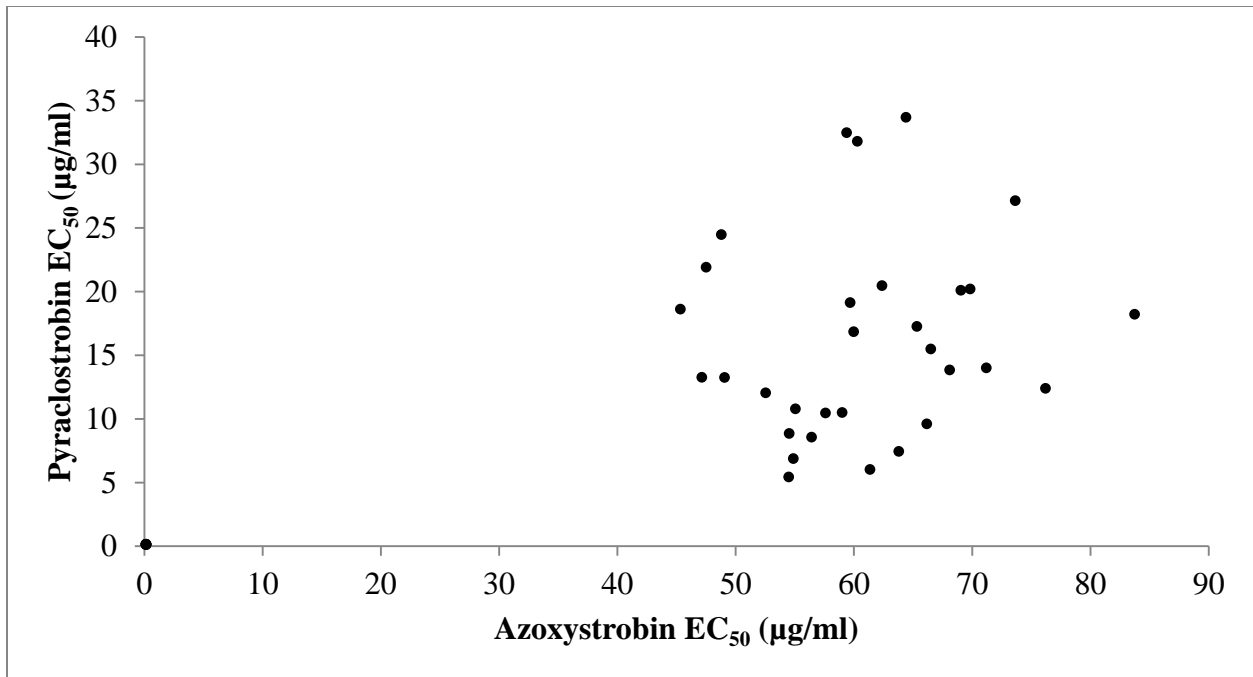


Figure 3.13. Spearman rank correlation between EC₅₀ values for isolates of *Cercospora kikuchii* exposed to azoxystrobin and pyraclostrobin in sensitivity assays in 2012. $\rho(34) = 0.49$, $n = 36$, $P = 0.0026$.

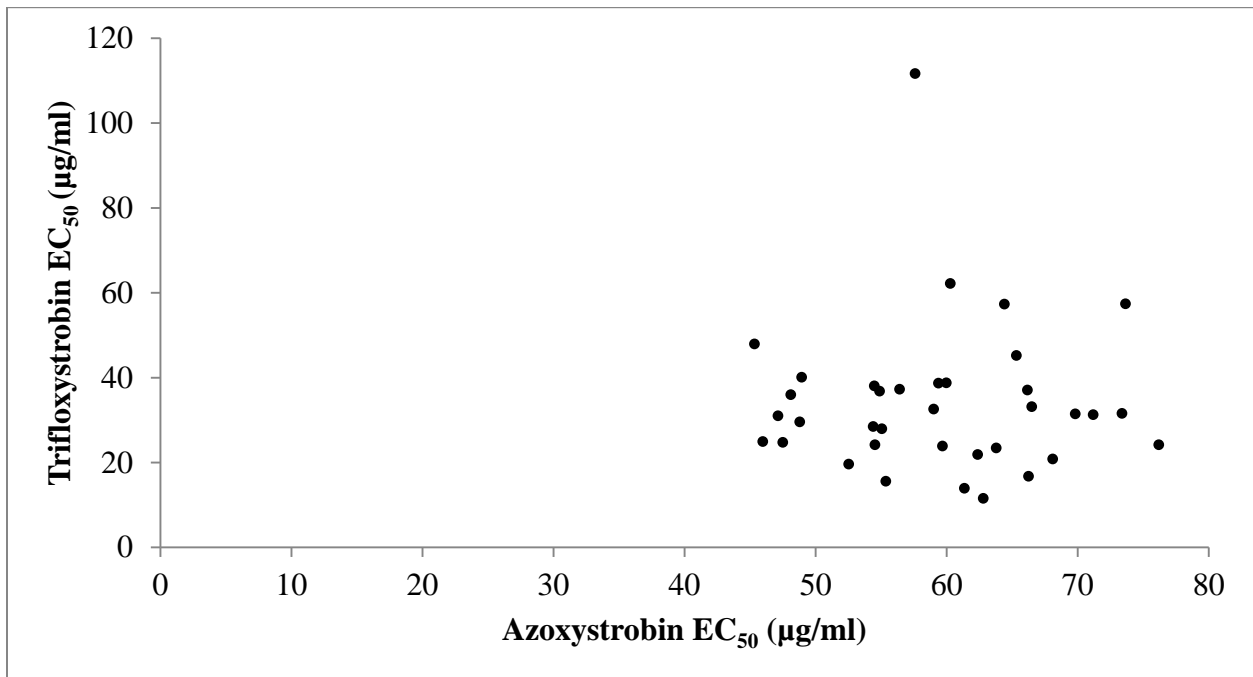


Figure 3.14. Spearman rank correlation between EC₅₀ values for isolates of *Cercospora kikuchii* exposed to azoxystrobin and trifloxystrobin in sensitivity assays in 2012. $\rho(39) = .30$, $n = 41$, $P = 0.0582$.

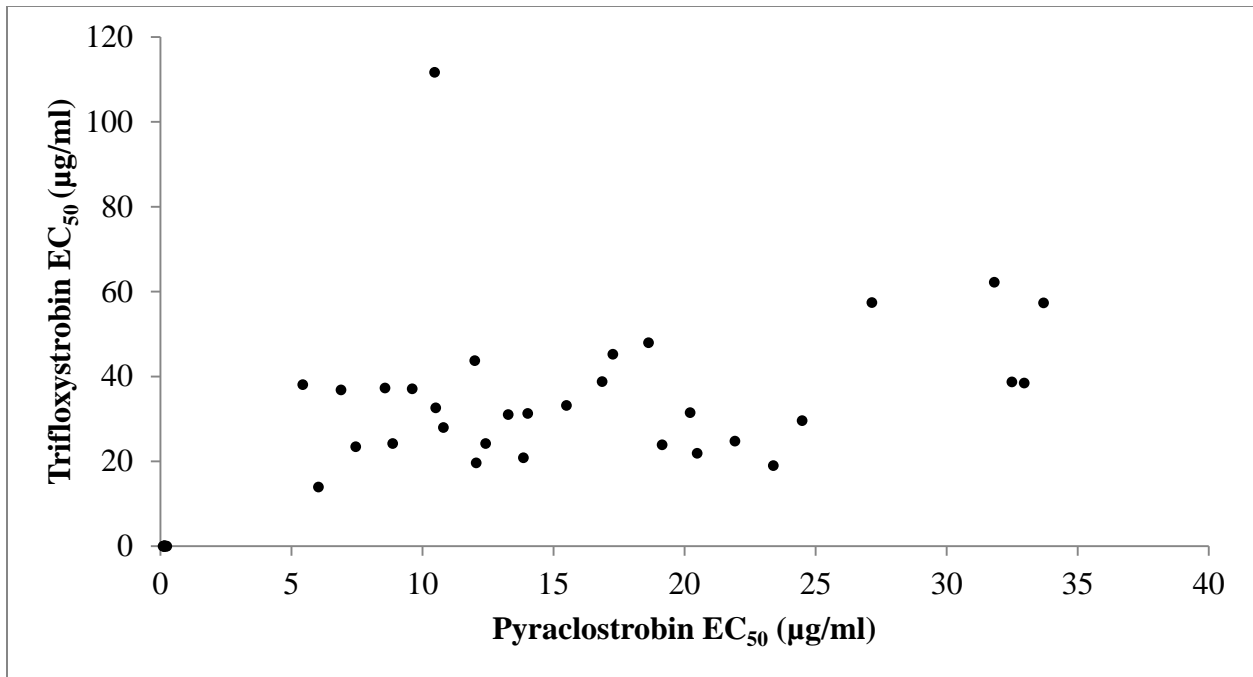


Figure 3.15. Spearman rank correlation between EC_{50} values of isolates of *Cercospora kikuchii* exposed to pyraclostrobin and trifloxystrobin in sensitivity assays in 2012. $\rho(37) = 0.61$, $n = 39$, $P < 0.0001$.

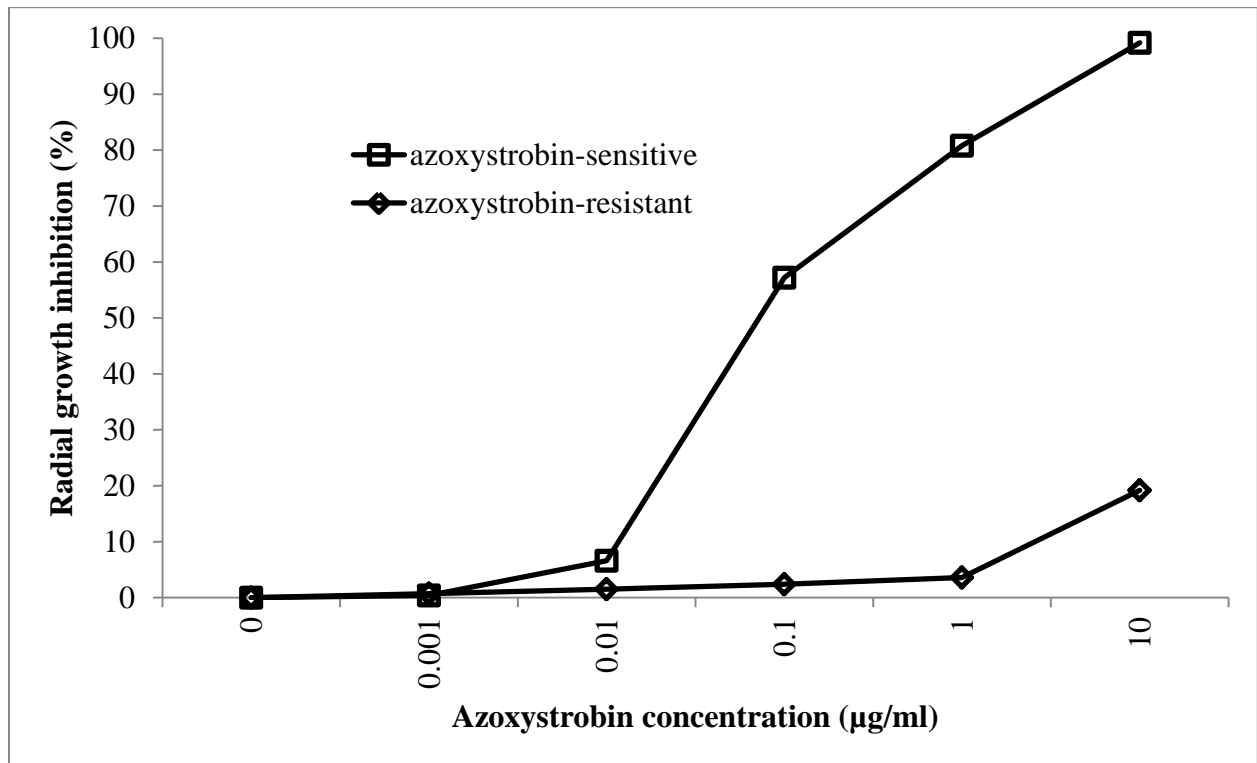


Figure 3.16. Radial growth inhibition of isolates of *Cercospora kikuchii* sensitive ($n = 71$) and resistant ($n = 84$) to azoxystrobin at a range of concentrations.

Therefore, radial growth assays were effective in delineating QoI resistance in *C. kikuchii*. Radial growth assays also have been successfully utilized to determine fungicide sensitivities of other plant pathogenic fungi including *Plasmopara viticola*, *Colletotrichum graminicola*, *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, *Mycosphaerella citri*, *Phytophthora cactorum*, and *Botrytis cinerea* (Wong and Wilcox 2000, Avila-Adame et al 2003, Mondal et al 2005, Rebollar-Alviter et al 2007, Myresiotis et al 2008).

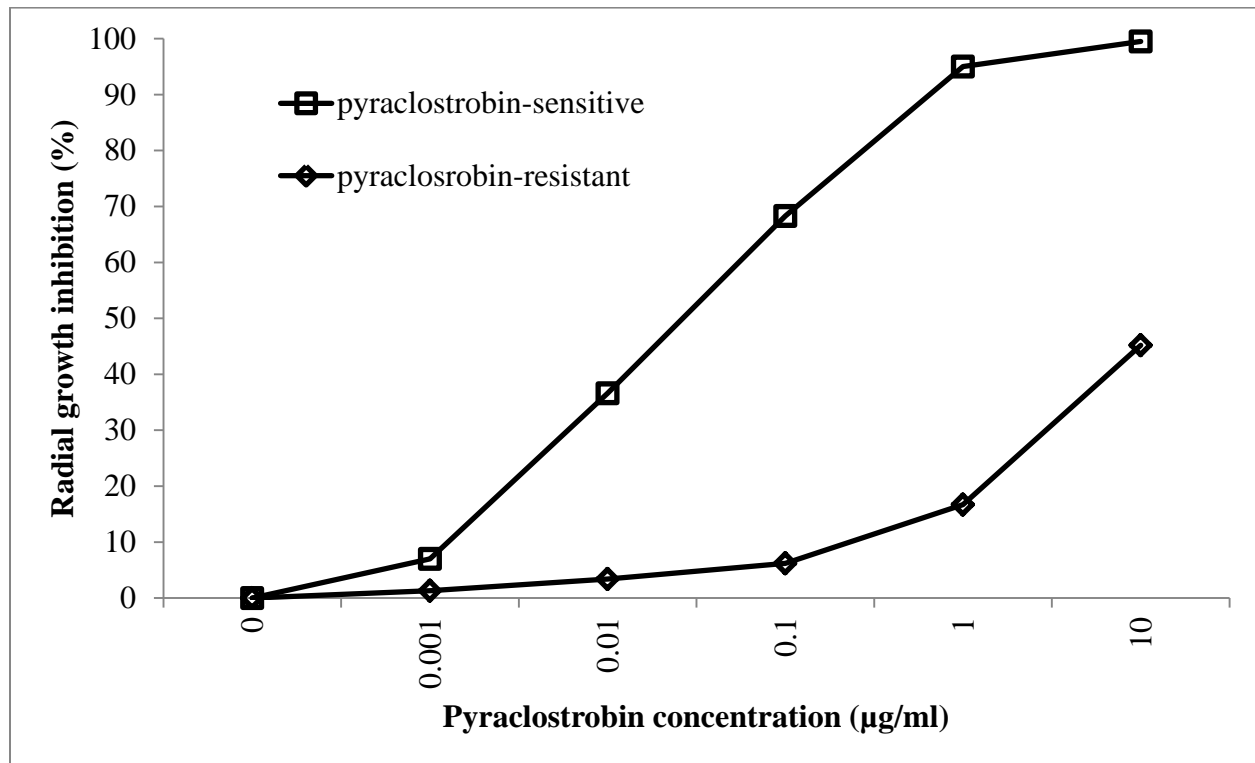


Figure 3.17. Radial growth inhibition of isolates of *Cercospora kikuchii* sensitive (n = 62) and resistant (n = 87) to pyraclostrobin at a range of concentrations.

In 2011 and 2012, azoxystrobin sensitivity profiles showed clear indications of resistance. Overall respective azoxystrobin sensitivities for 2011 and 2012 were 365 and 517-fold lower when compared to the baseline from 2000. Similarly, pyraclostrobin sensitivity profiles from 2011 and 2012 indicated QoI resistance with respective differences in sensitivities of 635 and 782-fold. Trifloxystrobin sensitivity profiles from 2011 and 2012 also revealed QoI resistance with respective differences in overall sensitivities of 1507 and 1947-fold lower. Other research indicated large sensitivity differences between QoI-sensitive and -resistant plant pathogenic fungi (Avila-Adame et al 2003, Pasche et al 2004, Wise et al 2009, Zhang et al 2012b). Generally,

isolates of *C. kikuchii* from 2012 appeared to be less-sensitive to QoI fungicides than 2011 isolates. Also, 2012 isolates were consistently less-sensitive for all three QoI fungicides included in the study, which could be attributed to variation in the pathogen populations or the smaller sample pool in 2012.

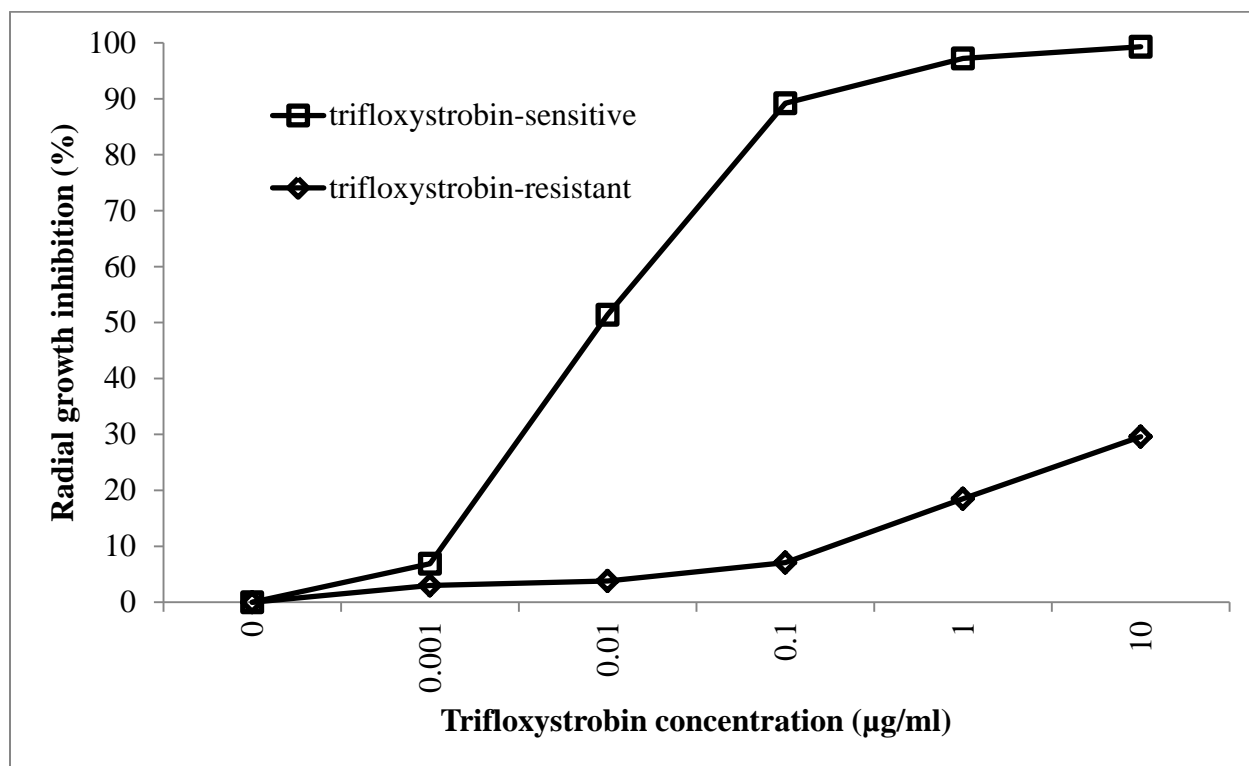


Figure 3.18. Radial growth inhibition of isolates of *Cercospora kikuchii* sensitive (n = 69) and resistant (n = 80) to trifloxystrobin at a range of concentrations.

Significant correlations were observed between QoI fungicides when comparing EC₅₀ values with a Spearman rank correlation test. Other research demonstrated similar correlations between QoI fungicides (Pasche et al 2004, Rebollar-Alviter et al 2007, Wise et al 2009), which is indicative of the similar mode of action between the two fungicides. Because of this similarity between QoI fungicides, it is not advisable to replace one QoI fungicide for another in a disease management situation, and rotation to a different mode-of-action is ideal. An obvious distinction was observed between QoI-sensitive and -resistant isolates, and they could easily be divided into two categories with an EC₅₀ value of the midpoint between sensitive and resistant isolates serving as a logical separation point. Azoxystrobin-resistant isolates were 20 to 200-fold less sensitive than azoxystrobin-sensitive isolates in 2011 and 2012, respectively. A sensitivity gap of 10 to 33-fold also was observed between 2011 and 2012 pyraclostrobin-sensitive and -resistant isolates, respectively. Similarly,

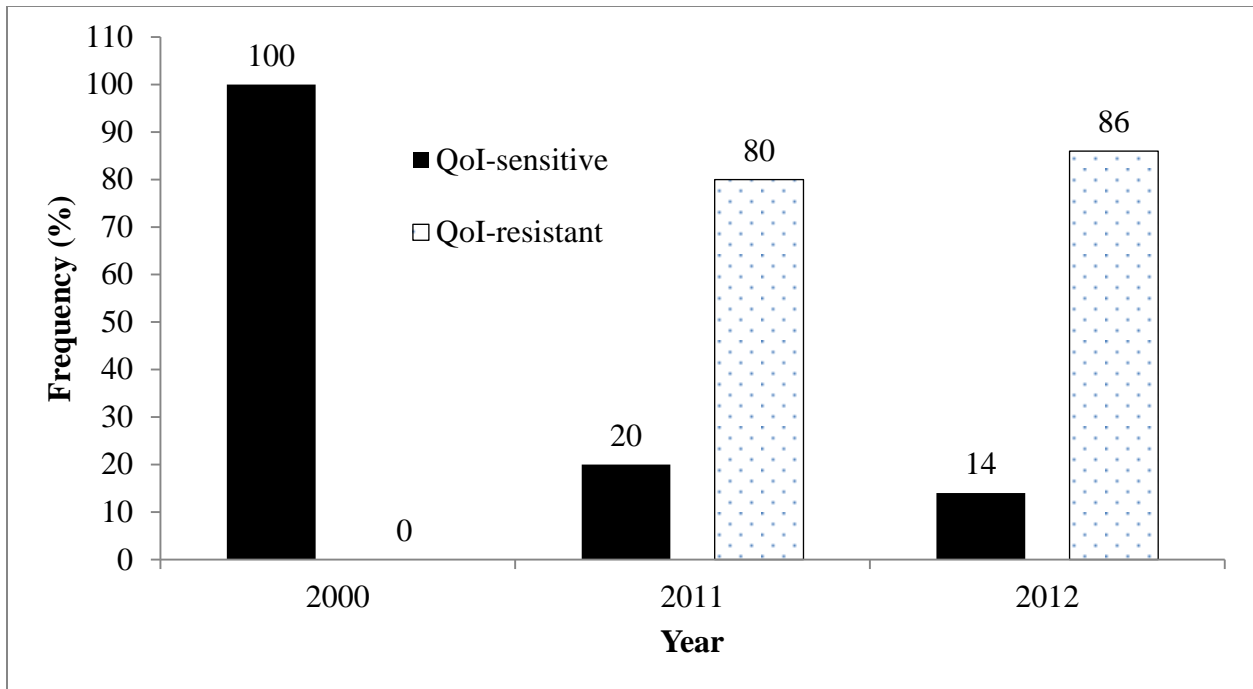


Figure 3.19. Frequency of quinone outside inhibitor-sensitive and -resistant isolates of *Cercospora kikuchii* in Louisiana from 2000, 2011, and 2012.

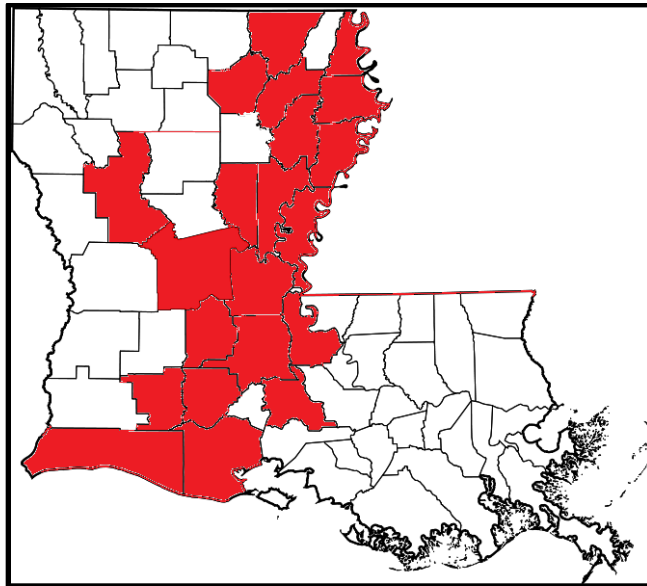


Figure 3.20. Parishes with confirmed quinone outside inhibitor resistance in Louisiana as determined from isolates of *Cercospora kikuchii* in 2011 and 2012.

Table 3.4. Quinone outside inhibitor-sensitive and –resistant isolates of *Cercospora kikuchii* in sampled Louisiana parishes in 2011 and 2012.

Parish	2011		2012	
	No. Tested Isolates	No. QoI-R*	No. Tested Isolates	No. QoI-R
Avoyelles	12	12	2	2
Caldwell	0	0	1	1
Cameron	0	0	1	1
Catahoula	14	13	8	7
Concordia	29	27	6	4
East Carroll	0	0	4	3
East Baton Rouge	2	0	0	0
Evangeline	3	3	5	4
Franklin	2	1	1	1
Jefferson Davis	0	0	1	1
LaSalle	9	5	0	0
Madison	0	0	2	2
Morehouse	1	1	0	0
Ouachita	0	0	1	1
Pointe Coupee	7	6	4	4
Rapides	11	8	3	3
Richland	1	1	0	0
Saint Landry	4	3	13	11
Saint Martin	0	0	1	1
Tensas	0	0	3	1
Vermilion	5	4	4	4

*Quinone outside inhibitor resistant isolates.

trifloxystrobin-resistant isolates were easily distinguished in 2011 and 2012 with respective differences of 13 and 127-fold. Large differences in sensitivities between sensitive and resistant isolates were commonly observed in situations where QoI resistance was identified (Avila-Adame et al 2003, Pasche 2004, Wise et al 2009, Secor et al 2010, Kirk et al 2012, Zhang et al 2012b).

Radial growth inhibition at previously-mentioned QoI fungicide concentrations was calculated for all sensitive isolates from 2000, 2011, and 2012 and then compared to that of resistant isolates from 2011 and 2012. According to the results, 10 µg/ml azoxystrobin inhibited 99.2% radial growth of sensitive *C. kikuchii* isolates as compared to 19.2% of resistant isolates. Pyraclostrobin at 10 µg/ml inhibited 99.5% and 45.2% of radial growth on sensitive and resistant isolates, respectively. Trifloxystrobin at 1 and 10 µg/ml inhibited 97.2 and 99.3% of sensitive isolates as compared to 18.5 and 29.6% of resistant isolates, respectively. Simply stated,

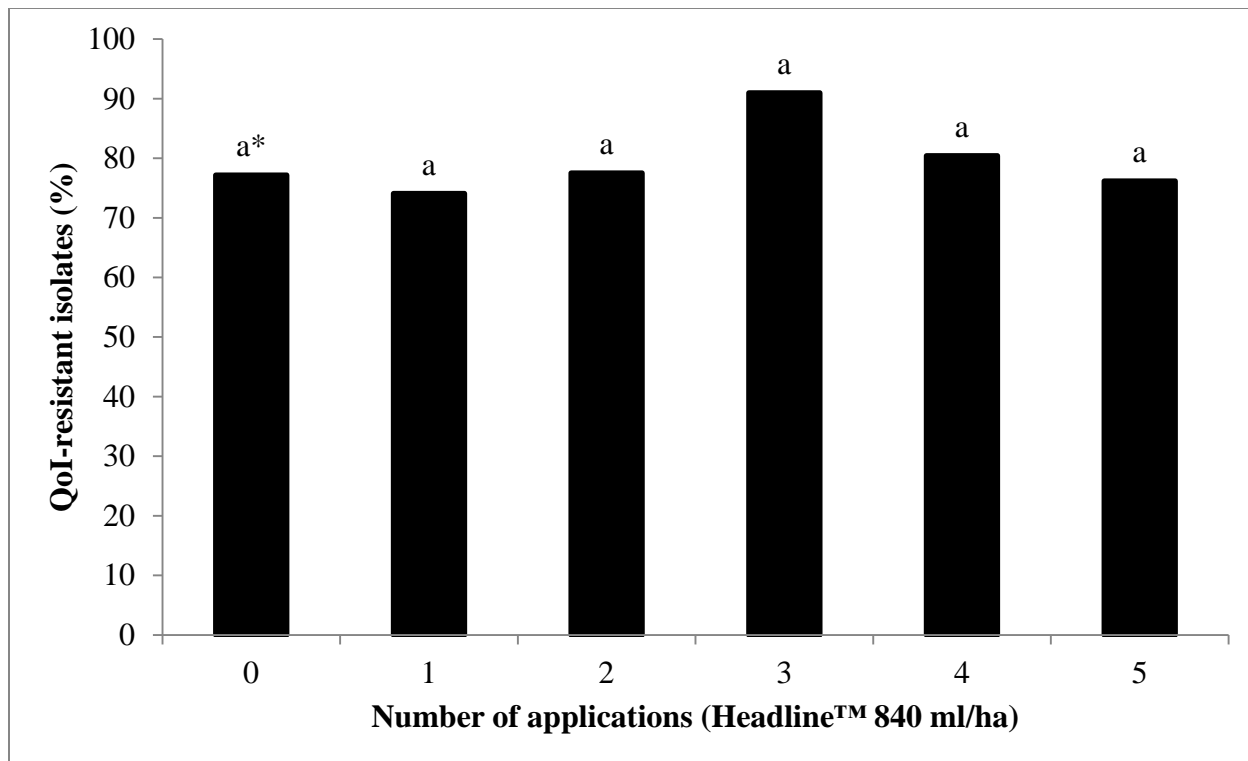


Figure 3.21. Percentage of pyraclostrobin-resistant isolates of *Cercospora kikuchii* in plots receiving successive applications of Headline™ (840 ml/ha) at V6, R1, R3, R5, and R6.

*Means followed by the same letter are not significantly different (PROC MIXED, SAS Institute followed by Tukey-Kramer post hoc adjustment, $\alpha = 0.05$).

sensitive isolates will not grow at the above concentrations. Therefore, azoxystrobin, pyraclostrobin, and trifloxystrobin at 10 $\mu\text{g/ml}$ may be used as discriminatory doses to detect resistant isolates of *C. kikuchii*.

Discriminatory doses also were used to determine QoI resistance in other plant pathogenic fungi after baseline sensitivity determination (Wise et al 2009, Secor et al 2010, Kirk et al 2012). Radial growth assays are tedious, time-consuming, and may be expensive. The development of discriminatory doses reduces effort, time consumption, and input costs of identifying QoI-resistant isolates of *C. kikuchii* from soybean. Discriminatory doses may prove useful in rapidly-identifying QoI-resistant isolates in field studies, and further delineating resistance in Louisiana and throughout the United States.

An experiment was devised to evaluate successive applications of pyraclostrobin to soybean to illustrate the effect of QoI fungicide applications on resistant isolates in the field. A discriminatory dose of 10 $\mu\text{g/ml}$ pyraclostrobin was successfully used to identify and quantify QoI-resistant isolates *C. kikuchii*. Results indicated that field applications of QoI-fungicides to soybean had no effect on the proportion of QoI-resistant

isolates of *C. kikuchii* that were recovered from seeds, and that QoI-resistant isolates comprised the majority of the pathogen population at the trial location. Approximately 77% of isolates from non-treated plots were resistant to pyraclostrobin, which is consistent with the frequencies of QoI-resistant isolates identified in assays in 2011 and 2012.

Over the past 10-15 years, QoI fungicide use has significantly increased in Louisiana soybean and comprises the majority of applications (NASS 2013). Current fungicide application estimates from scientists in the Southern United States range from 40 to 75% of planted hectares (personal communications, G. B. Padgett, C. Hollier, E. J. Sikora, and R. W. Schneider). Additionally, encouragement by industry has likely resulted in more QoI applications in Louisiana (BASF 2013, Bayer Crop Science 2013, DuPont 2013). According to these results, QoI resistance in *C. kikuchii* appears to be commonplace and widespread in soybean producing areas throughout the state. Additionally, results indicate that QoI applications have little to no effect on pathogen populations that contain a majority of resistant individuals. Consequently, applications of QoI fungicides to soybean for management of CLB/PSS in areas where resistant isolates have been found are not advisable at this time.

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CHAPTER 4

SENSITIVITY OF *CERCOSPORA KIKUCHII* TO DEMETHYLATION INHIBITOR FUNGICIDES IN LOUISIANA

Introduction

Cercospora leaf blight (CLB) and purple seed stain (PSS) are significant diseases of soybean in the United States causing estimated average losses of 140,500 metric tons annually from 1996 to 2012 (Wrather and Koennig 2013). The foliar phase of the disease affects soybean during reproductive phases of development with symptoms first appearing in the upper canopy eventually causing premature defoliation and subsequent yield loss (Matsumoto and Tomayasu 1925, Murakishi 1951, Walters et al 1980). The seed phase of the disease is marked by seed colonization by the pathogen usually resulting in purple staining of mature seed (Matsumoto and Tomayasu 1925, Murakishi 1951, Walters et al 1980). Disease incidence and severity of CLB/PSS have markedly increased within the past 10 to 15 years in Louisiana (Schneider et al 2003, Cai et al 2009).

Demethylation inhibitor (DMI) fungicides were commercially introduced the mid-1970's (FRAC 2013). These fungicides interact with cytochrome P450s at the site of the 14 α -demethylase (CYP51) and C-22 desaturase (CYP61) blocking 14 α -demethylation (Siegel 1981, Kelly et al 1995). Later in the sterol biosynthesis pathway, an accumulation of 5-hydroxy sterol is the toxic component of the fungicide (Watson et al 1989). Several changes throughout the sterol biosynthesis pathway must occur in plant pathogenic fungi for disease control problems to occur with DMI fungicides, which results in slower development of resistance ("medium risk") than other fungicides (FRAC 2013). Therefore, shifts in DMI sensitivities are usually detected by monitoring populations over time and comparing to baseline sensitivities (Stanis and Jones 1985, Henry and Trivellas 1989, Eckhert et al 1994, Romero and Sutton 1997, Erickson and Wilcox 1997, Karaoglanidis 2000, 2001, 2002, 2003a, Holb and Schnabel 2006, Wong and Midland 2007, Secor et al 2010, Sombardier et al 2010, Wise et al 2011, Bolton et al 2012). Field resistance of fungal plant pathogens to DMI fungicides was first observed in 1981 (Fletcher and Wolfe, FRAC 2013). Since that time, 29 plant pathogenic species in

approximately 20 horticultural and agronomic crops have been confirmed resistant to DMI fungicides.

Cercospora species confirmed resistant to DMI fungicides include *C. beticola* (Henry and Trivellas 1989, Karaoglanidis 2000, Secor et al 2010).

Fungicide applications during the reproductive stage of development of soybean are recommended by the Louisiana State University Agricultural Center (LSU AgCenter) for management of CLB/PSS in Louisiana (Anonymous 2012). Historically, DMI fungicides have not been recommended by the LSU AgCenter for CLB/PSS management (Anonymous 2010). Topguard™ (flutriafol), Domark™ (tetraconazole) and Tilt™ (propiconazole) are demethylation inhibitor (DMI) fungicides that were recently added to the LSU AgCenter list of recommended fungicides to manage CLB/PSS. Fungicide mixtures containing one quinone outside inhibitor (QoI) and one DMI fungicide that are currently LSU AgCenter-recommended for management of CLB/PSS are Quadris Xtra™ (azoxystrobin + cyproconazole), Quilt™ (azoxystrobin + propiconazole), Quilt Xcel™ (azoxystrobin + propiconazole), Stratego™ (trifloxystrobin + propiconazole), and Stratego YLD™ (trifloxystrobin + prothioconazole) (Anonymous 2012).

Over the past ten years, many commercially available QoI fungicides have proven ineffective on CLB/PSS (Padgett and Purvis 2005, 2007a, 2007b, Price and Padgett 2008, Delaney et al 2012, Price et al 2013). Additionally, previous research also indicates that QoI resistance (azoxystrobin, pyraclostrobin, and trifloxystrobin) in *C. kikuchii* is widespread in soybean-producing areas of Louisiana with confirmation in 21 parishes (See Chapter 3). Recent field trials in southern Louisiana have indicated efficacy of DMI fungicides on CLB/PSS (Schneider et al 2013) resulting in recommendation of DMI fungicides for management of CLB/PSS (Anonymous 2012). The decline in efficacy of QoI fungicides, finding of QoI resistance, promising DMI efficacy in southern Louisiana, and changes in CLB/PSS management recommendations likely have increased applications of DMI fungicides to Louisiana soybean. Therefore, it is prudent to monitor DMI sensitivities in *C. kikuchii* for changes over time.

Baseline sensitivities to DMI fungicides must be determined to detect shifts in sensitivities over time (FRAC 2013), which have been well documented for many plant pathogenic fungi (Fletcher and Wolfe 1981,

Stanis and Jones 1985, Hancock and Weete 1985, Romero and Sutton 1997, Reynolds et al 1996, Erickson and Wilcox 1997, Zehr et al 1999, Karaoglanidis et al 2000, 2002, and 2003a, Wong and Midland 2007, Fang et al 2009, Wise et al 2011, Yuan et al 2013). Baseline sensitivities of *C. kikuchii* to flutriafol, propiconazole, and tetraconazole have been determined (See Chapter 2).

Resistance to DMI fungicides occurs slowly with the elimination of sensitive individuals and gradual increase of resistant isolates over time (FRAC 2013). Incidences of DMI resistance in plant pathogenic fungi and other *Cercospora* species have been well documented (Stanis and Jones 1985, Henry and Trivellas 1989, Eckert et al 1994, Erickson and Wilcox 1997, Zehr et al 1999, Karaoglanidis et al 2000, Hold and Schnabel 2006, Sombardier et al 2010). To our knowledge, DMI-sensitivity has not been documented in *C. kikuchii*.

Cross-sensitivity/resistance is defined as similar sensitivity to two or more fungicides where sensitivity/resistance is controlled by the same genetic factor. Cross-sensitivity/resistance is known to commonly occur with DMI fungicides across many species of plant pathogenic fungi (Reynolds et al 1996, Erickson and Wilcox 1997, Karaoglanidis et al 2000, Wong and Midland 2007, Wise et al 2009, Sombardier et al 2010). Variability in cross sensitivity/resistance to DMI fungicides also was documented (Henry and Trivellas 1989, Karaoglanidis et al 2001, 2003b, Wise et al 2011, Bolton et al 2012).

The primary objective of this study was to determine sensitivities of *C. kikuchii* to flutriafol, propiconazole, and tetraconazole using isolates collected in Louisiana in 2011 and 2012 and to detect potential shifts in sensitivity since 2000. An ancillary objective was to examine cross-sensitivity patterns among the three fungicides.

Materials and Methods

Isolate Sources. In 2011 and 2012, isolates of *C. kikuchii* were obtained from symptomatic soybean leaves from producer fields throughout Louisiana. Sixty-five locations in 21 parishes were sampled in 2011 while 36 locations in 27 parishes were sampled in 2012 (Figure 4.1). Symptomatic soybean leaflets were collected, approximately 10 per location, placed in sealable Ziploc™ plastic bags, and transported to the laboratory in an ice chest. Leaves were stored at 4°C until they were processed. Samples were removed within

72 h, and 5 symptomatic leaf sections, measuring 2 cm², were cut using scissors then placed in sterile test tubes (20 x 125 mm). Three ml of sterile, distilled water was added to each test tube, which was immediately capped and shaken vigorously for 30 s. Five drops of the resulting suspension were placed on a glass slide and observed with a stereomicroscope for conidia matching previously reported characteristics (Hartman, et al 1999) of *C. kikuchii*. Conidia were singly removed from the suspension with the aid of a glass needle fashioned from a micro pipette. Because of the length and flexible nature of the conidia, single spore isolation was readily achieved when a single spore wrapped around the tip of the glass needle. Single spores were transferred to water agar (1.5%) amended with chloramphenicol (75 µg/ml) and streptomycin sulfate (125 µg/ml) then allowed to incubate at room temperature for 5 to 7 days. Colonies that appeared to be producing cercosporin, a perylenequinone photo activated toxin that plays a significant role in pathogenicity, symptom expression, colonization of seed coats, and virulence (See Chapter 1), were selected, transferred via hyphal tips to V8 agar (20% juice), and maintained at 25°C with a 12h light: dark cycle. Resulting isolates received a numerical designation, and pools of 160 from 20 parishes and 82 from 18 parishes were available for analysis in 2011 and 2012, respectively (Tables 4.1 and 4.2). Unless otherwise indicated, cultures 21-35 days old were used in all assays and incubated as previously described.

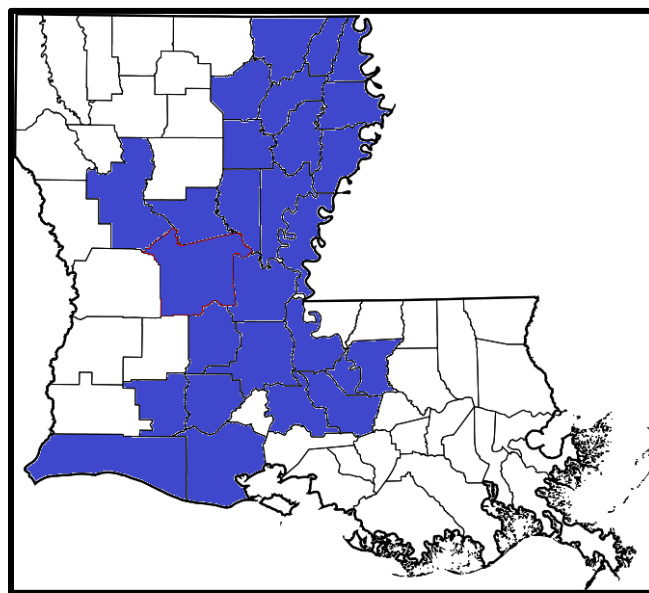


Figure 4.1. Louisiana parishes sampled for *Cercospora kikuchii* in 2011 and 2012.

Determining Sensitivity to Demethylation Inhibitor Fungicides. Ten-fold dilutions of technical formulations (ChemService, Inc.) of flutriafol (98.2%), propiconazole (97.5%), and tetraconazole (98.7%) were performed in acetone, added (1.0 ml/1 L) to sterilized, molten PDA (50°C), and aseptically dispensed into sterile petri dishes (15 x 100 mm, ~20 ml/dish). Final concentrations ranged from 0.0001 to 10 µg/ml with PDA amended with acetone-only included for comparison. For each sensitivity profile, 50 isolates were chosen at random (RAND function, Microsoft Excel), transferred to amended PDA, and incubated as previously described. Colony diameter was determined 5 days after transfer as previously described (See Chapter 2), and data were converted to % inhibition by comparison to respective non-amended controls. The effective concentration to inhibit 50% of fungal radial growth (EC₅₀) was determined for each treatment by linear interpolation of a regression of log concentration by % inhibition (GraphPad Prism 5.0). The equation for the dose-response

Table 4.1. Designations of isolates of *Cercospora kikuchii* and parishes of origin, 2011.

Isolate Designations	Parish of Origin
84, 86-88, 95, 99-102, 107-109, 118, 119, 140-142	Avoyelles
127,128	Caldwell
1-14, 55, 56	Catahoula
42-50, 57-60, 62, 63, 67-80, 89	Concordia
144-147	East Carroll
81, 82	East Baton Rouge
33, 34, 66	Evangeline
51, 52, 131,132	Franklin
35, 36	Jefferson Davis
15-26	LaSalle
83, 124-126, 129	Morehouse
130	Ouachita
61, 85, 103-106, 110, 113-115	Pointe Coupee
27-32, 53, 54, 90, 91, 96-98	Rapides
65	Richland
40, 92-94, 116, 117, 120-123, 155-160	Saint Landry
133-139, 143, 150-154	Saint Martin
37-39, 41, 64	Vermilion
148, 149	West Carroll
111, 112	West Baton Rouge

Table 4.2. Designations of isolates of *Cercospora kikuchii* and parishes of origin, 2012.

Isolate Designations	Parish of Origin
6, 7, 15, 47, 48	Avoyelles
59	Cameron
26-28, 41-45	Catahoula
16, 22-25, 33-35, 52	Concordia
38-40, 53	East Carroll
17-20	Evangeline
54	Franklin
77, 78	Grant
64, 65	Jefferson Davis
29, 30	Madison
79-82	Natchitoches
8-10, 46	Ouachita
1-5, 51	Pointe Coupee
13, 14, 56, 57	Rapides
11, 12, 21, 31, 32, 58, 61-63, 66-72	Saint Landry
60	Saint Martin
36, 37, 55	Tensas
73-76	Vermilion

model is as follows: $Y=0+(100-0)/(1+10^{((\text{LogEC}_{50}-X)*1)})$. Mean EC_{50} values were determined for each isolate, and sensitivity profiles were constructed for each fungicide and year (GraphPad Prism 5.0). Baseline sensitivity profiles for *C. kikuchii* were previously constructed using the same methods described above for the same three DMI fungicides (See Chapter 2). Tests for normality were conducted using the Shapiro-Wilk method (GraphPad Prism 5.0). In most instances data violated the assumption of normality even after transformation. As a result, a non-parametric form of analysis of variance, the Kruskal-Wallis test, was used to determine significant differences among sensitivity profiles. Dunn's multiple comparison tests were used to compare differences between years (GraphPad Prism 5.0).

Determining Cross-Sensitivity Patterns. Isolates from 2012 that were exposed *in vitro* to all three fungicides, flutriafol, propiconazole, and tetraconazole, were selected for comparison. Because data violated the assumption of normality before and after transformation, a non-parametric form of correlation, Spearman's rank correlation test was performed. Data were compared for flutriafol and propiconazole, flutriafol and

tetraconazole, and propiconazole and tetraconazole. Correlation coefficients (ρ) of 1, 0, and -1 translate to perfect-positive, none, and perfect-negative correlation.

Results

2000, 2011, and 2012 Flutriafol Sensitivity Profiles. Baseline sensitivity in 2000 of *C. kikuchii* to flutriafol ranged from 0.096 to 1.46 $\mu\text{g/ml}$ with a median of 0.273 $\mu\text{g/ml}$ (See Chapter 2, Figure 4.2). In 2011, EC_{50} values of *C. kikuchii* isolates exposed to flutriafol ranged from 0.009 to 0.906 $\mu\text{g/ml}$ with a median of 0.409 $\mu\text{g/ml}$. The 95% confidence interval of the distribution was [0.344, 0.451] with a mean of 0.398 $\mu\text{g/ml}$ flutriafol. The overall sensitivity profile was normally distributed ($W = 0.9755$, $P = 0.4077$) (Figure 4.2). In 2012, sensitivity ranged from 0.130 to 5.48 $\mu\text{g/ml}$ with a median of 0.542 $\mu\text{g/ml}$ flutriafol (Figure 4.2). The 95% confidence interval of the distribution was [0.570, 1.080] with a mean of 0.826 $\mu\text{g/ml}$ flutriafol, and the profile was not normally distributed ($W = 0.6623$, $P < 0.0001$) (Figure 4.2). A significant shift was detected in the median of the 2012 population when compared to the baseline. ($KW = 13.71$, $P = 0.0011$) (Figure 4.3).

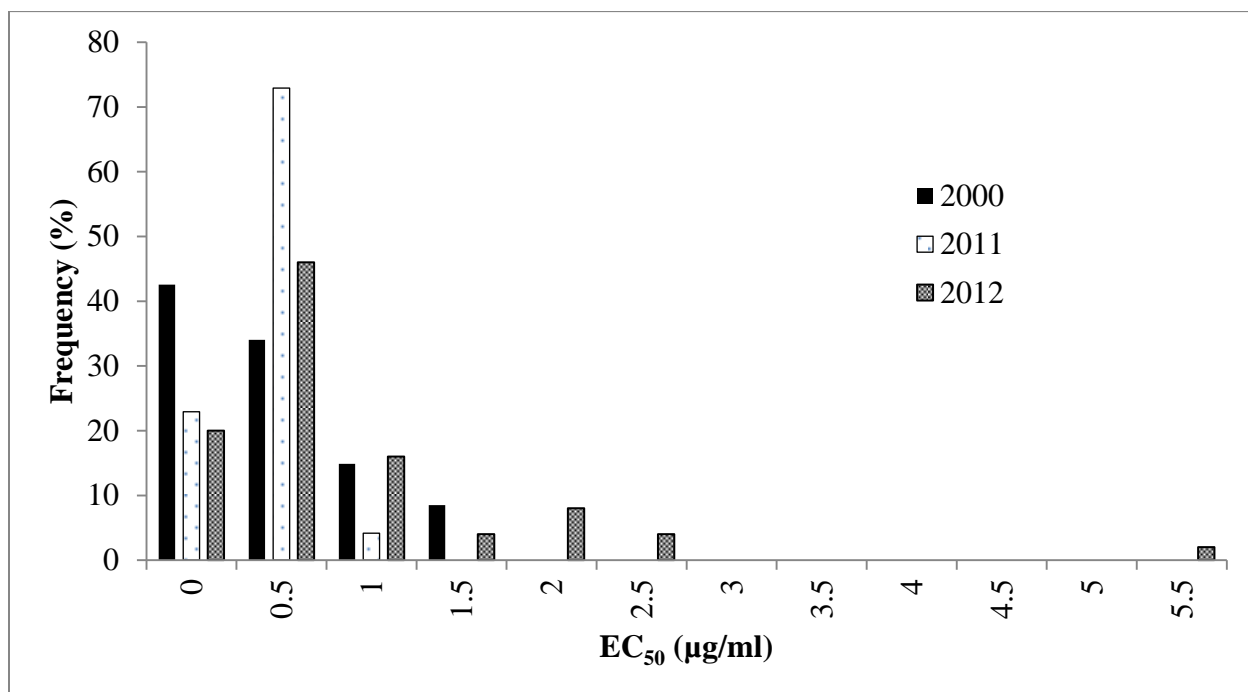


Figure 4.2. Sensitivity profiles of *Cercospora kikuchii* to flutriafol for 47, 48, and 50 isolates from 2000, 2011, and 2012, respectively.

2000, 2011, and 2012 Propiconazole Sensitivity Profiles. Baseline sensitivity of *C. kikuchii* to propiconazole ranged from 0.022 to 0.954 µg/ml with a median of 0.143 µg/ml (Chapter 2, Figure 4.4). In 2011, EC₅₀ values of *C. kikuchii* isolates exposed to propiconazole ranged from 0.038 to 1.21 µg/ml with a median of 0.335 µg/ml (Figure 4.4). The 95% confidence interval of the distribution was [0.304, 0.517] with a mean of 0.411 µg/ml propiconazole. The overall sensitivity profile was not normally distributed (W = 0.8917, P = 0.0038) (Figure 4.4). In 2012, sensitivity ranged from 0.052 to 2.84 µg/ml with a median of 0.245 µg/ml propiconazole (Figure 4.3). The 95% confidence interval of the distribution was [0.258, 0.634] with a mean of 0.446 µg/ml propiconazole, and the profile was not normally distributed (W = 0.6254, P = < 0.0001) (Figure 4.4). A significant shift in medians towards less sensitivity was detected with Kruskal-Wallis one-way analysis of variance in 2011 and 2012 sensitivity profiles when compared to the baseline. (KW = 21.87, P < 0.0001) (Figure 4.3).

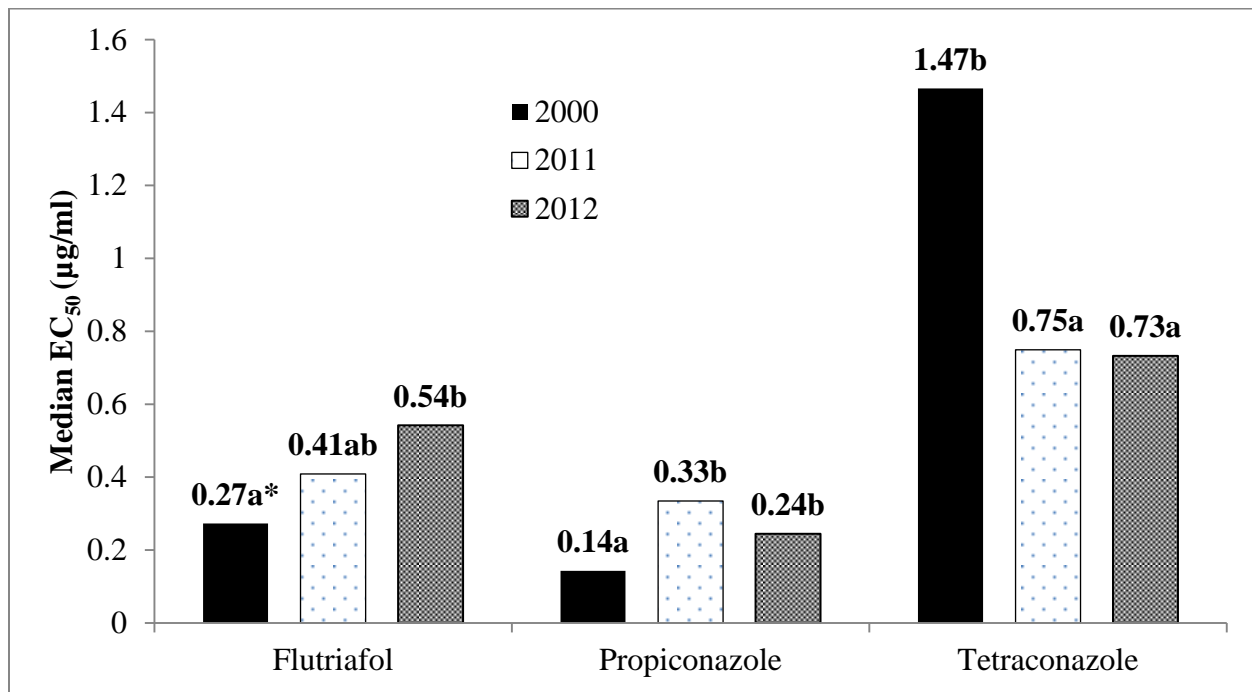


Figure 4.3. Sensitivity distribution comparison for flutriafol, propiconazole, and tetraconazole in *Cercospora kikuchii* for 2000, 2011, and 2012.

*Medians followed by the same letter are not significantly different (Kruskal-Wallis, Dunn's multiple comparison test, $\alpha=0.05$).

2000, 2011, and 2012 Tetraconazole Sensitivity Profiles. Baseline sensitivity of *C. kikuchii* to tetraconazole ranged from 0.109 to 5.76 µg/ml with a median of 1.46 µg/ml (See Chapter 2, Figure 4.5). The 95% confidence interval of the distribution was [1.33, 2.0] with a mean of 1.66 µg/ml tetraconazole. In 2011, EC₅₀ values of *C. kikuchii* isolates exposed to tetraconazole ranged from 0.103 to 3.60 µg/ml with a median of 0.335 µg/ml (Figure 4.5). The 95% confidence interval of the distribution was [0.732, 1.2] with a mean of 0.960 µg/ml tetraconazole. The overall sensitivity profile was not normally distributed (W = 0.8325, P < 0.0001) (Figure 4.5). In 2012, sensitivity ranged from 0.161 to 5.66 µg/ml with a median of 0.732 µg/ml tetraconazole (Figure 4.5). The 95% confidence interval of the distribution was [0.864, 1.58] with a mean of 1.22 µg/ml tetraconazole, and the profile was not normally distributed (W = 0.7256, P = < 0.0001) (Figure 4.4). A significant shift towards more sensitivity was detected in 2011 and 2012 sensitivity profiles when compared to the baseline. (KW = 21.87, P < 0.0001) (Figure 4.3).

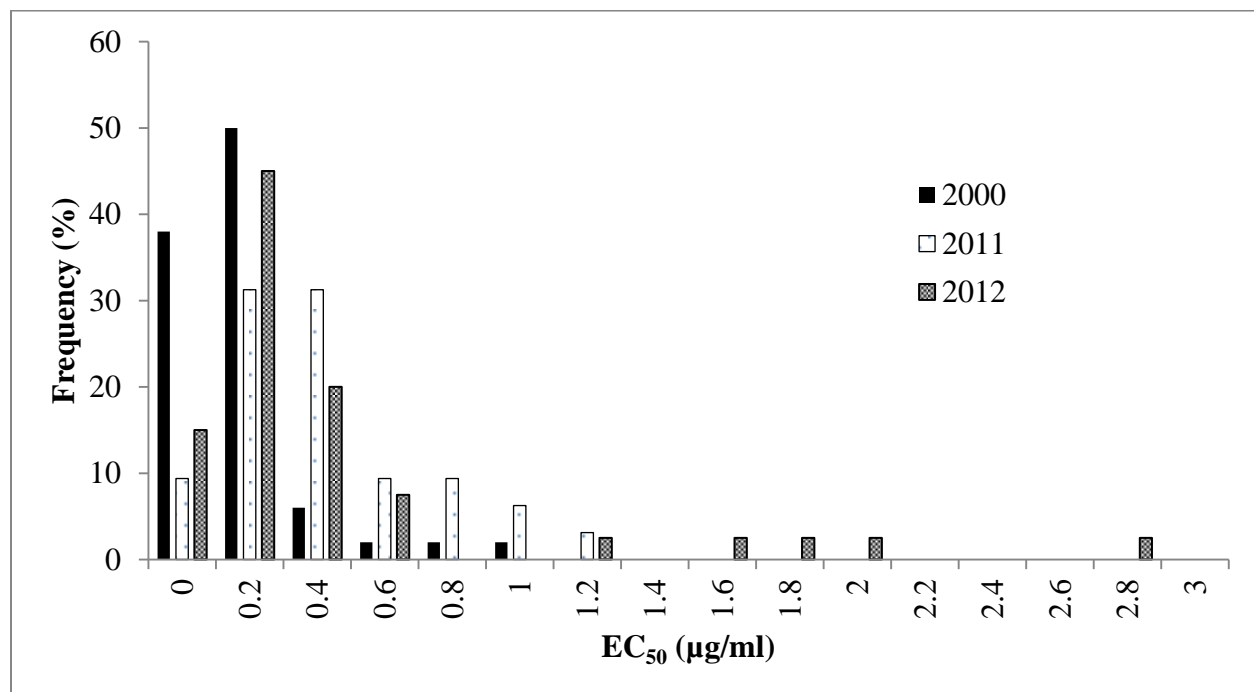


Figure 4.4. Sensitivity profiles of *Cercospora kikuchii* to propiconazole for 50, 32, and 40 isolates from 2000, 2011, and 2012, respectively.

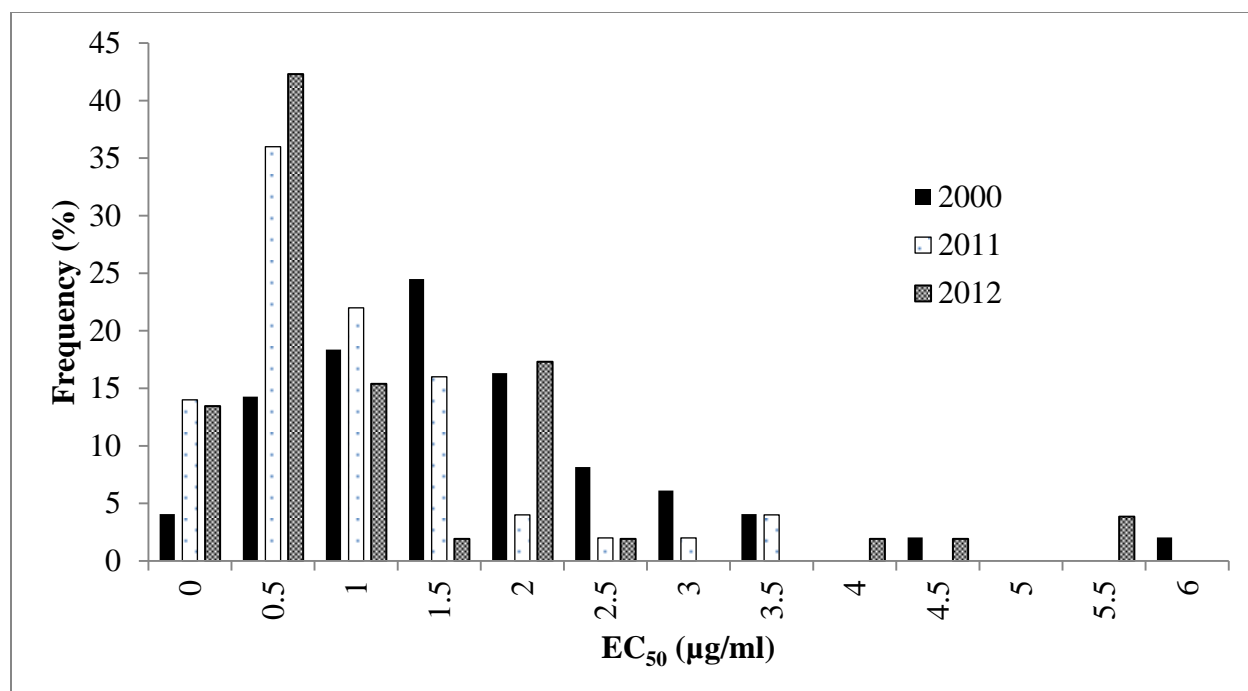


Figure 4.5. Sensitivity profiles of *Cercospora kikuchii* to tetraconazole for 49, 50, and 52 isolates from 2000, 2011, and 2012, respectively.

Cross-Sensitivities to Demethylation Inhibitor Fungicides in 2012. A Spearman rank correlation test was used to determine significant relationships between EC₅₀ values of DMI-resistant isolates in 2012. Significant correlations were found between all possible two-way comparisons of flutriafol, propiconazole, and tetraconazole (Figures 4.6, 4.7, and 4.8). Strong, positive correlations were evident between flutriafol and propiconazole ($\rho (25) = 0.80, n = 27, P < 0.0001$) and flutriafol and tetraconazole ($\rho (39) = 0.81, n = 41, P < 0.0001$) (Figures 4.6 and 4.7). A significant, positive correlation was observed between propiconazole and tetraconazole ($\rho (24) = 0.61, n = 26, P = 0.0008$) (Figure 4.8).

Discussion

In this study, radial growth assays were used to determine sensitivity of *C. kikuchii* isolates to DMI fungicides. Methods similar to those used in the present study were used in other studies to determine DMI fungicide toxicity to plant pathogenic fungi (Stanis and Jones 1985, Hancock and Weete 1985, Koller and Wubben 1989, Henry and Trivellas 1989, Eckert et al 1994, Romero and Sutton 1997, Reynolds et al 1996,

Erickson and Wilcox 1997, Zehr et al 1999, Karaoglanidis et al 2000, 2001, 2003a, Holb and Schnabel 2006, Wong and Midland 2007, Fang et al 2009, Sombardier et al 2010, Wise et al 2011, Kenaith and Hansen 2012, Yuan et al 2013).

Isolates of *C. kikuchii* exposed to flutriafol in 2000, 2011, and 2012 had sensitivities ranging from 15, 101, and 42-fold, respectively. Other research with a related species, *C. beticola*, indicated sensitivity ranges to flutriafol from 10 to 156-fold (Karaoglanidis et al 2000, 2001, 2003a). Respective sensitivity ranges for isolates of *C. kikuchii* exposed to propiconazole for the three populations were 43, 32, and 55-fold. Sensitivity ranges for other *Cercospora* species including *C. arachidiola* and *C. beticola* to propiconazole were 7-fold and 266-fold, respectively (Hancock and Weete 1985, Karaoglanidis et al 2003a). Isolates exposed to tetraconazole in the present study showed sensitivity ranges of 53, 35, and 35-fold in populations from 2000, 2011, and 2012, respectively. Bolton et al found wide (>100-fold) tetraconazole sensitivity ranges for *C. beticola* (2012). Studies with plant pathogens other than *Cercospora* species showed varying ranges (1 to 900-fold) in *in vitro* sensitivity to DMI fungicides (Reynolds et al 1996, Romero and Sutton 1997, Zehr et al 1999, Holb and Schnabel 2006, Wong and Midland 2007, Fang et al 2009, Seyran et al 2010). Additionally, it was demonstrated that isolates of *Mycosphaerella fijiensis*, *Monilinia fructicola*, and *C. beticola* not exposed to DMI fungicides generally have smaller sensitivity ranges when compared to isolates that have been previously exposed to DMI fungicides (Romero and Sutton 1996, Zehr et al 1999, Karaoglanidis et al 2001). Sensitivity ranges described in the present study confirm findings from other studies; however, prior field exposure of the isolates from 2000, 2011, and 2012 to DMI fungicides is unknown.

When comparing *C. kikuchii* sensitivity distributions to flutriafol, outliers towards the less-sensitive end appeared in the 2012 profile. A comparison of the medians of the 2000, 2011, and 2012 sensitivity profiles also indicated a shift towards less sensitivity to flutriafol. Similar outliers were observed in the 2011 and 2012 sensitivity profiles for propiconazole. Additionally, a significant shift in the medians of the 2011 and 2012

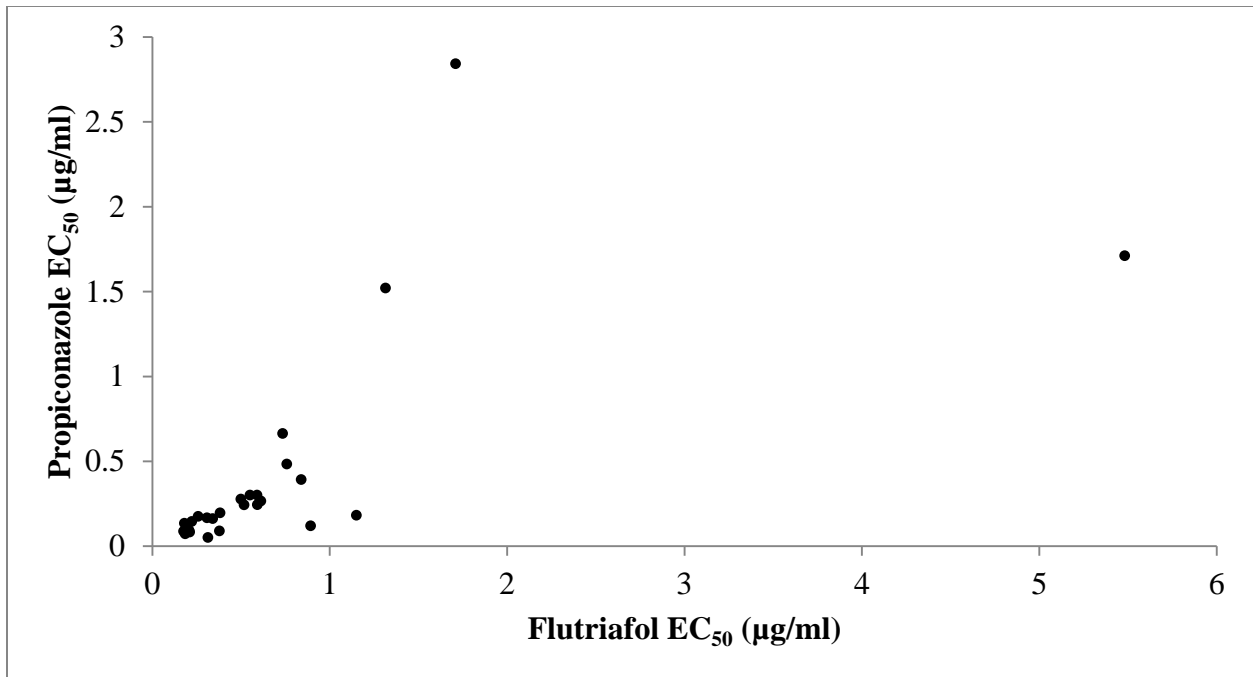


Figure 4.6. Spearman rank correlation between EC_{50} values of isolates of *Cercospora kikuchii* exposed to flutriafol and propiconazole in sensitivity assays in 2012. $\rho(25) = 0.80$, $n = 27$, $P < 0.0001$.

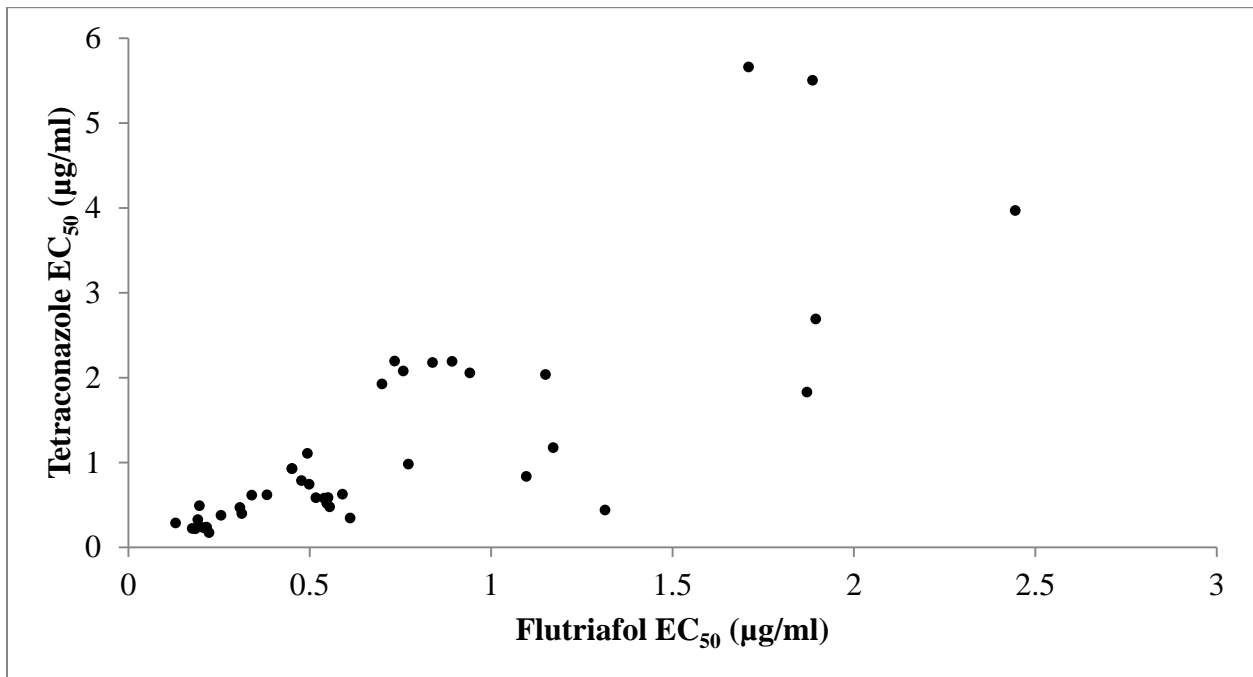


Figure 4.7. Spearman rank correlation between EC_{50} isolates of *Cercospora kikuchii* exposed to flutriafol and tetraconazole in sensitivity assays in 2012. $\rho(39) = 0.81$, $n = 41$, $P < 0.0001$.

profiles for propiconazole when compared to the 2000 sensitivity profile. Conversely, a significant increase in tetraconazole sensitivity appears to have occurred in *C. kikuchii* populations since 2000. However, similar outliers towards the less-sensitive end are present in the 2000 and 2012 tetraconazole sensitivity profiles which

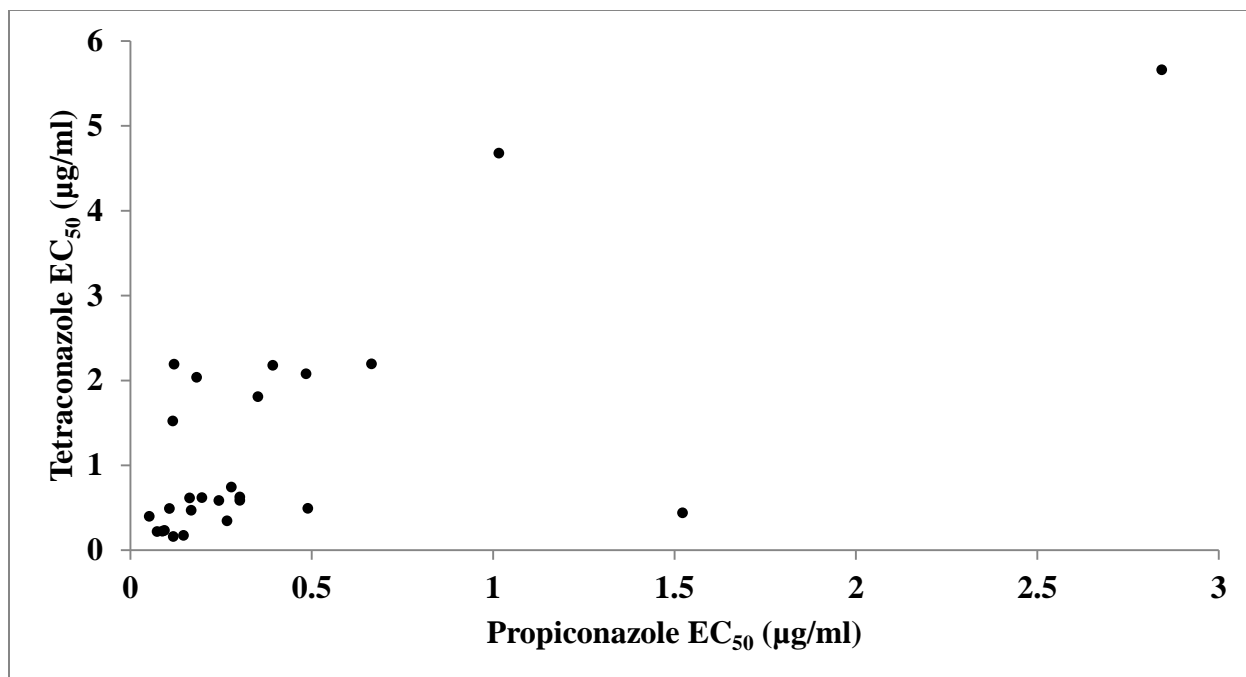


Figure 4.8. Spearman rank correlation between EC₅₀ values of isolates of *Cercospora kikuchii* exposed to propiconazole and tetraconazole in sensitivity assays in 2012. ρ (24) = 0.61, n= 26, P = 0.0008.

could be indicative of a shift in sensitivity. Many other examples of shifts toward less-sensitivity to DMI fungicides have been documented in other species of plant-pathogenic fungi including *Venturia inaequalis*, *Cercospora beticola*, *Penicillium digitatum*, *Mycosphaerella fijiensis*, *Uncinula necator*, *Monilinia fructicola*, *Colletotrichum cereale*, *Podosphaera aphanis*, and *Ascochyta rabiei* (Stanis and Jones 1985, Henry and Trivellas 1989, Eckhert et al 1994, Romero and Sutton 1997, Erickson and Wilcox 1997, Karaoglanidis 2000, 2001, 2002, 2003a, Holb and Schnabel 2006, Wong and Midland 2007, Sombardier et al 2010, Secor et al 2010, Wise et al 2011, Bolton et al 2012). Specifically, DMI resistance was confirmed in *C. beticola* in Greece and in the United States by detecting shifts among *in vitro* sensitivity profiles (Karaoglanidis et al 2000, Secor et al 2010).

Overall toxicity of flutriafol and propiconazole were similar in this study, while tetraconazole was less inhibitory). Studies with plant pathogens other than *Cercospora* species including *Cladosporidium caryigenum*, *Mycosphaerella fijiensis*, *Monilinia fructicola*, *Colletotrichum cereale*, *Magnaporthe grisea*, and *Fusicladium effusum* also showed varying ranges of sensitivity to DMI fungicides (Reynolds et al 1996, Romero and Sutton 1997, Zehr et al 1999, Holb and Schnabel 2006, Wong and Midland 2007, Fang et al 2009, Seyran et al 2010).

This variability could be explained by diversity within the pathogen population, slight differences in sensitivity mechanisms, or slight differences between fungicide modes of action (Hildebrand et al 1988, Kendall et al 1993). However, variation in intrinsic activity may or may not translate to field efficacy of DMI fungicides. For example, studies with *Uncinula necator* and *Colletotrichum cereale* indicated a positive correlation between *in vitro* and *in vivo* DMI activity (Erickson and Wilcox 1997, Ypema et al 1997, Wong and Midland 2007). On the contrary, other research indicated little or no relationship between *in vitro* studies and field efficacy of DMI fungicides with *C. beticola* (Secor et al 2010).

Strong correlations were observed between flutriafol and propiconazole and flutriafol and tetraconazole when comparing EC₅₀ values with a Spearman rank correlation test. Other studies indicated strong, positive relationships between DMI fungicides *in vitro* (Reynolds et al 1996, Erickson and Wilcox 1997, Karaoglanidis et al 2000, 2002, 2003b, Wong and Midland 2007, Sombardier et al 2010). A weaker correlation was found between tetraconazole and propiconazole. This variation in cross-sensitivity was observed for other plant-pathogenic fungi (Kendall 1986, Hildebrand et al 1988, Peever and Milgroom 1993, Hsiang et al 1997, Robbertse et al 2001, Karaoglanidis et al 2003b). Differences in cross-sensitivity could be explained by varying genetic factors controlling DMI sensitivity, slight differences in the mode-of-action among DMIs, differences of *in vitro* activity, or slight differences in resistance mechanisms (Hildebrand et al 1988, Kendall et al 1993). Significant, positive correlations were found between all two-way combinations of all three fungicides, which were expected because all three DMIs belong to the same sub-group of sterol biosynthesis inhibitors (FRAC 2013). This correlation likely is a result of the similar mode-of-action for the three fungicides, and indicates that replacing one DMI with another for field application would not be advisable.

Results from this study generally define DMI *in vitro* sensitivity of *C. kikuchii* in Louisiana. Isolates with reduced sensitivity were observed, and significant shifts in sensitivity profiles were determined indicating a possible shift in DMI sensitivity in isolates of *C. kikuchii*. Further monitoring over time is required to determine if this trend continues. Confirmation of cross-sensitivity between DMI fungicides in *C. kikuchii* has

practical implications by discouraging repeated applications and encouraging rotation to fungicides with different modes of action.

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CHAPTER 5

MULTIPLE RESISTANCE OF *CERCOSPORA KIKUCHII* TO THIOPHANATE-METHYL AND QUINONE OUTSIDE INHIBITOR FUNGICIDES IN LOUISIANA

Introduction

Cercospora leaf blight (CLB) and purple seed stain (PSS) are significant diseases of soybean in the United States causing estimated average losses of 140,500 metric tons annually from 1996 to 2012 (Wrather and Koennig 2013). Foliar symptoms of the disease appear during reproductive phases of soybean development in the upper canopy and eventually causing premature defoliation and subsequent yield loss (Matsumoto and Tomayasu 1925, Murakishi 1951, Walters et al 1980). The seed phase of the disease is marked by seed colonization by the pathogen usually resulting in purple staining of mature seed (Matsumoto and Tomayasu 1925, Murakishi 1951, Walters et al 1980). Disease incidence and severity of CLB/PSS have markedly increased within the past 10 to 15 years in Louisiana (Schneider et al 2003, Cai et al 2009).

Fungicide applications during the reproductive stage of development of soybean are recommended by the Louisiana State University Agricultural Center (LSU AgCenter) for management of CLB/PSS in Louisiana. Currently, Topsin™ (thiophanate-methyl), a methyl benzimidazole carbamate (MBC) fungicide, and two quinone outside inhibitor fungicides, Quadris™ (azoxystrobin) and Headline™ (pyraclostrobin), have been recommended by the LSU AgCenter for management of CLB/PSS (Anonymous 2012). Over the past ten years, many commercially-available MBC and QoI fungicides have proven inefficacious on CLB/PSS (Padgett and Purvis 2005, 2007a, 2007b, Price and Padgett 2008, Anonymous 2012, Delaney et al 2012, Price et al 2013). Additionally, fungicide type, application rate, and timing studies have indicated little or no effect on CLB/PSS (Padgett and Purvis 2007a, 2007b; Price and Padgett 2008; Price et al 2011; Delaney et al 2012; Price et al 2013).

Thiophanate-methyl belongs to the MBC class of fungicides, which inhibit growth of fungi by interfering with microtubule assembly during mitosis (Howard and Aist 1977). The fungicide resistance action committee (FRAC) considers thiophanate-methyl “high-risk” for inducing resistance in fungal plant pathogens (FRAC 2013). This resistance arises from a mutation in the β -tubulin gene (Upchurch et al 1991, Monma et

al 2003) leading to high levels of resistance *in vitro* and *in vivo* (Ishii et al 2001). Resistance to MBC fungicides was first documented soon after their introduction (Schroeder and Provvidenti 1969), and since this time, resistance to MBC fungicides of nearly 130 fungal pathogens of fruit, vegetable, turf grass, horticultural, and agronomic crops has been confirmed (FRAC 2013). Six *Mycosphaerella* species, four *Cercospora* species, and one *Cercosporidium* species, all closely-related to *C. kikuchii*, were confirmed resistant to MBC fungicides (FRAC 2013). Resistance of *C. kikuchii* to thiophanate-methyl was documented in Japan beginning in the late 1980s (Sakai 1999, Imazaki et al 2006b).

Several QoI fungicides are recommended for management of CLB/PSS in Louisiana (Anonymous 2012). These fungicides interfere with mitochondrial respiration by blocking electron transport at the quinol-oxidizing site of the cytochrome bc_1 complex (III), which subsequently affects spore germination and hyphal growth (Bartlett et al 2002). This mode of action is highly specific and can be overcome by a single-step mutation (Gisi et al 2002). Consequently, these fungicides are also considered to have a high risk of resistance development (FRAC 2013). QoI fungicides were introduced around 1996, and resistance of fungal pathogens to this class of chemistry was documented in 1998 (FRAC 2013). Since that time, 56 species of fungi pathogenic to 20 important horticultural and agronomic crops have been identified as resistant to QoI fungicides (FRAC 2013). *Cercospora* species confirmed resistant to QoI fungicides include *C. beticola*, *C. sojina*, and *C. kikuchii* (Bolton et al 2012, Zhang et al 2012b, See Chapter 3, respectively).

Multiple fungicide resistance occurs when single fungal isolates are determined to be resistant to two or more fungicides having differing modes of activity (FRAC 2013). Many cases of multiple resistance to MBC and other fungicide types (from 2 to 5 modes of activity) in plant pathogens have previously been reported in situations where fungicides are extensively used, such as orchards, vegetable production, and greenhouse applications (Elad et al 1992, Moorman and Lease 1992, Raposo et al 1996, Myresiotis et al 2007, Sun et al 2010, Weber 2011, Chapman et al 2011). Multiple resistance to MBC and QoI fungicides also was documented in *C. beticola*, a major pathogen of sugar beet that is closely related to *C. kikuchii* (Karaoglanidis et al 2000, Secor et al 2010). Additionally, MBC-resistant fungal isolates have exhibited cross-resistance to MBC

fungicides in every documented case. That is, if the isolate is resistant to one MBC fungicide, it is highly likely that the pathogen will be resistant to all MBC fungicides (Kenaith and Zitter 1998, Cuna and Rizzo 2003, Wong et al 2008).

Previous research indicates that in fungal isolates where MBC resistance has occurred, there are usually no major fitness costs of the mutation (Ruppel 1975, Dovas et al 1976, Ruppel et al 1980, Henry and Trivellas 1989, Raposo et al 1996, Yoshimura et al 2004, Secor et al 2010, Chapman et al 2011). Research also showed that MBC-resistant fungal isolates remain somewhat stable *in vitro* and *in vivo* (Dovas et al 1976, Ruppel et al 1980, Yoshimura et al 2004, Secor et al 2010). Similarly, there does not appear to be a competitive disadvantage associated with QoI-resistant fungal isolates (Luo and Schnabel 2008, Corio-Costet et al 2011, Karaoglanidis et al 2011, Kim and Xiao 2011).

Field applications of MBC fungicides were determined to be highly selective for MBC-resistant isolates of fungal plant pathogens (Dovas et al 1976, Ruppel et al 1980, Ishii et al 1985, Culbreath et al 2002, Karaoglanidis et al 2003, Imazaki et al 2006, Karaoglanidis and Bardas 2006). Once selected, MBC-resistant isolates tend to persist in field populations for long periods of time, slowly declining after discontinuation of MBC applications (Dovas et al 1976, Ruppel et al 1980, Ishii et al 1985, Karaoglanidis et al 2003, Yoshimura et al 2004, Secor 2010).

The objective of this study was to investigate sensitivity to thiophanate-methyl in *C. kikuchii* and to assess multiple resistance to QoI and MBC fungicides in isolates collected in Louisiana in 2000, 2011, and 2012.

Materials and Methods

Isolate Sources. One hundred seventy-six isolates of *C. kikuchii* isolates were originally obtained in Louisiana by G. Cai in 2000, and provided by R. W. Schneider for this study in 2010 (Cai and Schneider 2005). Isolates were maintained in one-half strength V8 agar at 4°C. Isolates included 115 foliar and 30 seed isolates from the Macon Ridge Research Station (MRRS) near Winnsboro and 12 foliar and 16 seed isolates from the

Dean Lee Research Station (DLRS) near Alexandria. Three isolates of unknown plant tissue origin were obtained from the Ben Hur Research Station (BHRS) near Baton Rouge.

In 2011 and 2012, isolates of *C. kikuchii* were obtained from symptomatic soybean leaves from producer fields throughout Louisiana. Sixty-five locations in 21 parishes were sampled in 2011 while 36 locations in 27 parishes were sampled in 2012 (Figure 5.1). Symptomatic soybean leaflets were collected, approximately 10 per location, placed in sealable Ziploc™ plastic bags, and transported to the laboratory in an ice chest. Leaves were stored at 4°C until they were processed. Samples were removed within 72 h, and 5 symptomatic leaf sections, measuring 2 cm², were cut using scissors then placed in sterile test tubes (20 x 125 mm). Three ml of sterile, distilled water was added to each test tube, which was immediately capped and shaken vigorously for 30 s. Five drops of the resulting suspension were placed on a glass slide and observed with a stereomicroscope for conidia matching previously reported characteristics (Hartman, et al 1999) of *C. kikuchii*. Conidia were singly removed from the suspension with the aid of a glass needle fashioned from a micro pipette. Because of the length and flexible nature of the conidia, single spore isolation was readily achieved when a single spore wrapped around the tip of the glass needle. Single spores were transferred to water agar (1.5%) amended with chloramphenicol (75 µg/ml) and streptomycin sulfate (125 µg/ml) then allowed to incubate at room temperature for 5 to 7 days. Colonies that appeared to be producing cercosporin were selected, transferred via hyphal tips to V8 agar (20% juice), and maintained at 25°C with a 12h light: dark cycle. Resulting isolates received a numerical designation, and pools of 160 from 20 parishes and 82 from 18 parishes were available for analysis in 2011 and 2012, respectively (Tables 5.1 and 5.2). Unless otherwise indicated, cultures 21-35 days old were used in all assays and incubated as previously described.

Determining Sensitivity to Methyl Benzimidazole Carbamate Fungicides and Resistance

Probabilities. In preliminary and other research isolates of *C. kikuchii* were transferred to PDA amended with a commercial formulation of thiophanate-methyl at final concentrations of 0, 1.56, 6.25, 25, 100, 400, and 1600 µg/ml. Sensitive isolates exhibited no growth at 1.56 µg/ml, while resistant isolates were uninhibited at 1600 µg/ml (data not shown, Imazaki et al 2006). Therefore, isolates were either highly sensitive or highly resistant.

Based on these preliminary results, and previous research with *C. kikuchii* and other *Cercospora* species, a discriminatory dose of 5 µg/ml thiophanate-methyl was chosen to distinguish between MBC-sensitive and -resistant isolates (Henry and Trivellas 1989, Bugbee 1995, Campbell et al 1998, Weiland and Halloin 2001, Imazaki et al 2006). Dilution of a technical formulation (ChemService, Inc.) of thiophanate-methyl (99.1%) was performed in acetone, added (1.0 ml/1 L) to sterilized, molten PDA (50°C), and aseptically dispensed into sterile petri dishes (15 x 100 mm, ~20 ml/dish). PDA amended with only acetone was included for comparison. All isolates from 2000 (n = 176), 2011 (n = 160), and 2012 (n = 82) were tested for MBC sensitivity. With the aid of a cork borer, 6 mm mycelial discs were cut from stock cultures, inverted, and aseptically transferred to PDA containing 0 or 5 µg/ml thiophanate-methyl. Cultures were incubated as previously described for 5 days then observed for radial growth. Isolates were considered sensitive if no radial growth was observed on 5 µg/ml thiophanate-methyl and resistant if growth was observed. Resistant isolates were scored with a 1, while sensitive isolates were scored with a 0. Because data had a binomial distribution, logistic regression was performed to determine probabilities of MBC resistance for each year, and odds ratios were used to make comparisons between years (PROC LOGISTIC, SAS Institute). Odds ratios were subsequently converted to probability ratios (Osborne 2006) to compare the relative risk of MBC resistance between years.

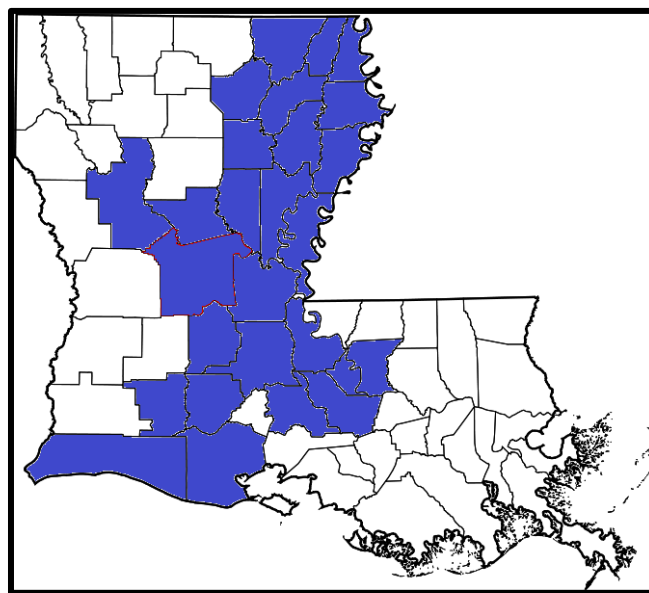


Figure 5.1. Louisiana parishes sampled for *Cercospora kikuchii* in 2011 and 2012.

Table 5.1. Designations of isolates and parishes of origin, 2011.

Isolate Designations	Parish of Origin
84, 86-88, 95, 99-102, 107-109, 118, 119, 140-142	Avoyelles
127,128	Caldwell
1-14, 55, 56	Catahoula
42-50, 57-60, 62, 63, 67-80, 89	Concordia
144-147	East Carroll
81, 82	East Baton Rouge
33, 34, 66	Evangeline
51, 52, 131,132	Franklin
35, 36	Jefferson Davis
15-26	LaSalle
83, 124-126, 129	Morehouse
130	Ouachita
61, 85, 103-106, 110, 113-115	Pointe Coupee
27-32, 53, 54, 90, 91, 96-98	Rapides
65	Richland
40, 92-94, 116, 117, 120-123, 155-160	Saint Landry
133-139, 143, 150-154	Saint Martin
37-39, 41, 64	Vermilion
148, 149	West Carroll
111, 112	West Baton Rouge

Table 5.2. Designations of isolates and parishes of origin, 2012.

Isolate Designations	Parish of Origin
6, 7, 15, 47, 48	Avoyelles
59	Cameron
26-28, 41-45	Catahoula
16, 22-25, 33-35, 52	Concordia
38-40, 53	East Carroll
17-20	Evangeline
54	Franklin
77, 78	Grant
64, 65	Jefferson Davis
29, 30	Madison
79-82	Natchitoches
8-10, 46	Ouachita
1-5, 51	Pointe Coupee
13, 14, 56, 57	Rapides
11, 12, 21, 31, 32, 58, 61-63, 66-72	Saint Landry
60	Saint Martin
36, 37, 55	Tensas
73-76	Vermilion

Determining Sensitivity to Quinone Outside Inhibitor Fungicides and Resistance Probabilities.

Isolates from 2000 (n = 98), 2011 (n = 102), and 2012 (n = 62) were subjected to discriminatory doses of 10 µg/ml azoxystrobin, pyraclostrobin, or trifloxystrobin with non-amended PDA serving as a control (See Chapter 3). Cultures were incubated as previously described then observed for radial growth, and isolates were considered sensitive if no radial growth was observed and resistant if growth was observed. Isolates resistant and sensitive to QoI fungicides were scored in the same manner as MBC isolates (1 = resistant, 0 = sensitive). Probabilities and odds ratios of the occurrence of QoI resistance were determined as previously described for QoI resistant isolates and compared among years. Since there were no QoI-resistant isolates detected from 2000, 5% resistant scores were weighted in the analysis (Hosmer and Lemeshow 1989) to avoid quasi separation of data points, which would result in grossly-exaggerated parameter estimates and confidence intervals (PROC LOGISTIC, SAS Institute). Odds ratios were converted to probability ratios (Osborne 2006) to compare the relative risk of QoI resistance between years.

Determining Multiple-Resistance Probabilities. Isolates from 2000 (n = 98), 2011 (n = 102), and 2012 (n = 62) were tested for MBC and QoI sensitivity as previously described. Isolates resistant to both MBC and QoI fungicides were scored with a 1, while isolates that were not resistant to both fungicide types were scored with a 0. Probabilities and odds ratios of the occurrence of multiple resistance were determined as previously described and compared among years. As described above, there were no resistant isolates detected in 2000, and 5% resistant scores were weighted in the analysis (Homer and Lemeshow 1989). Odds ratios were converted to probability ratios (Osborne 2006) to compare the relative risk of multiple resistance between years. Chi-square analysis also was performed comparing overall proportions of MBC and QoI resistant isolates from the 2011 and 2012 populations.

Determining the Proclivity of Methyl Benzimidazole Carbamate-Resistant Isolates of *Cercospora kikuchii* to Exhibit Resistance to Quinone Outside Inhibitor Fungicides. Isolates resistant to MBC fungicides from 2000 (n = 41), 2011 (n = 45), and 2012 (n = 14) were selected for analysis. MBC-resistant isolates that were also resistant to QoI fungicides were scored with a 1, while MBC-resistant isolates sensitive

to QoI fungicides were scored with a 0. Probabilities and odds ratios of the occurrence of QoI resistance in MBC-resistant isolates were determined as previously described and compared among years. As described above, there were no QoI-resistant isolates detected in 2000, and 5% resistant scores were weighted in the analysis. Also, the same statistical procedure was followed with MBC-resistant isolates collected in 2012 because there were no QoI-sensitive isolates in this group. Odds ratios were converted to probability ratios (Osborne 2006) to compare the relative risk of QoI resistance in MBC-resistant isolates.

Results

Methyl Benzimidazole Carbamate Resistance. Isolates of *C. kikuchii* resistant to thiophanate-methyl were detected in 2000, 2011, and 2012 with 23, 45, and 36% MBC-resistant isolates, respectively (Figure 5.2). Estimated probabilities of isolates resistant to MBC fungicides were determined by logistic regression to be 0.23, 0.39, and 0.30 for 2000, 2011, and 2012, respectively (Figure 5.2). When comparing populations from 2000 and 2011, isolates from 2011 were 1.7 (0.5, 4.0) times more likely to exhibit resistance to MBC fungicides (Figure 5.3). There were no significant differences in the likelihood of MBC resistance with other population comparisons of 2000 to 2012 [1.3 (0.5, 3.4)] and 2011 to 2012 [0.8 (0.4, 2.0)] (Figure 5.3).

In the 2000 population, 41 of 176 *C. kikuchii* isolates from three parishes were confirmed MBC-resistant. In 2011, 62 of 160 isolates from 14 parishes were determined to be resistant to MBC fungicides. For 2012, 24 of 82 isolates from 19 parishes were MBC-resistant (Table 5.3). To date, MBC resistance has been found in a total of 19 parishes throughout soybean producing areas of Louisiana (Figure 5.4).

Quinone Outside Inhibitor Resistance. Isolates of *C. kikuchii* resistant to QoI fungicides were not detected in 2000 population. In 2011 and 2012, 84.0 and 85.5% of isolates were resistant to QoI fungicides, respectively (Figure 5.5). Estimated probabilities of isolates resistant to QoI fungicides were determined by logistic regression to be 0.05, 0.82, and 0.84 for 2000, 2011, and 2012, respectively (Figure 5.5). When comparing the population from 2000 to 2011 and 2012, isolates were approximately 16 times more likely to exhibit resistance to QoI fungicides in 2011 and 2012, a highly significant result (Figure

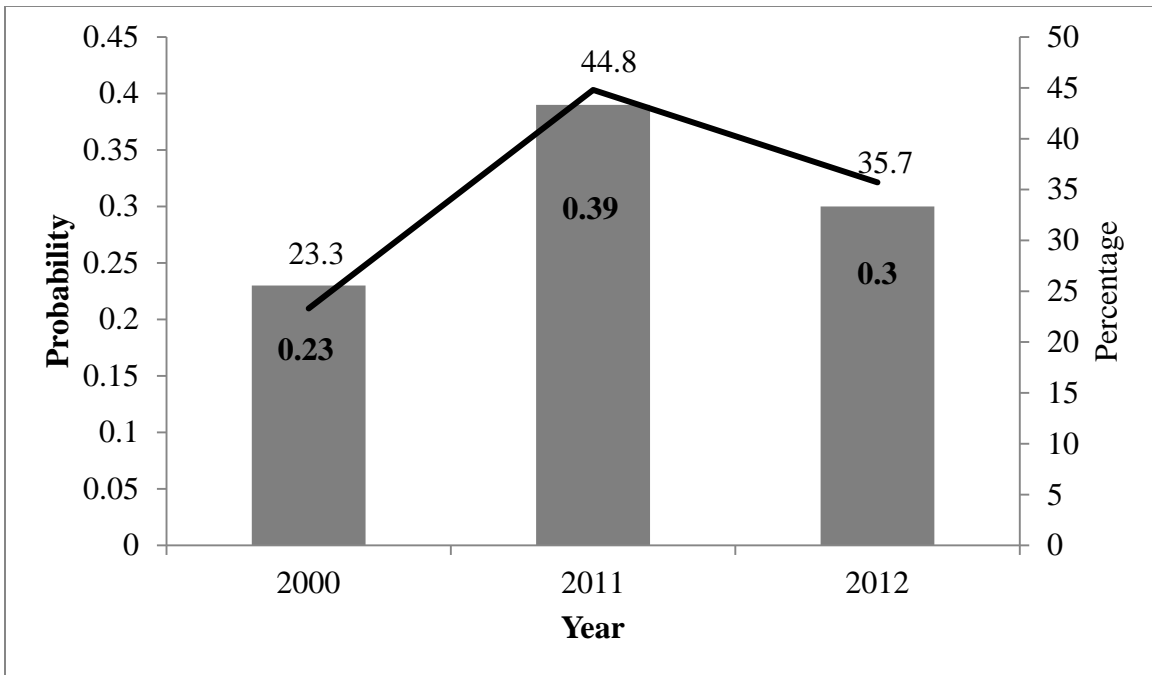


Figure 5.2. Probabilities and percentages of methyl benzimidazole carbamate resistance in isolates of *Cercospora kikuchii* from 2000, 2011, and 2012.

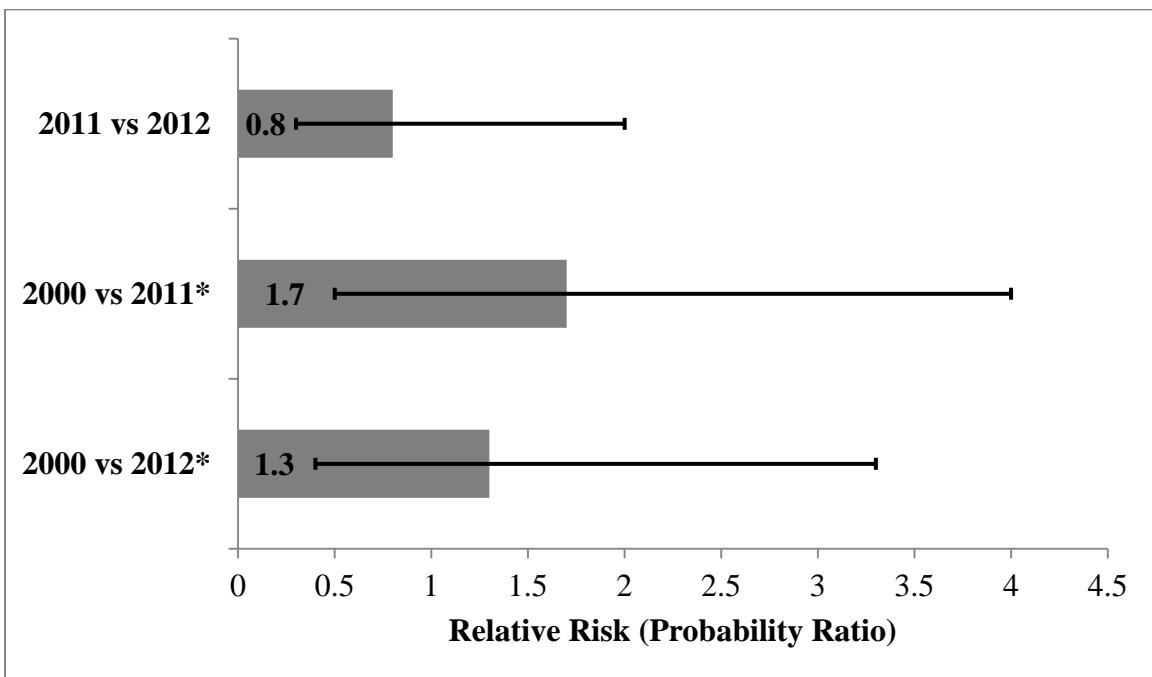


Figure 5.3. Relative risk and 95% confidence limits of methyl benzimidazole carbamate resistance in isolates of *Cercospora kikuchii* from 2000, 2011, and 2012.

*Denotes statistical significance. (PROC LOGISTIC, n = 418, Wald $X^2 = 9.261$, $P < 0.0001$).

5.6). There was no significant difference in the likelihood of QoI resistance between the 2011 and 2012 populations (Figure 5.6).

Table 5.3. Detailed parish information indicating number of tested and confirmed methyl benzimidazole carbamate-resistant isolates of *Cercospora kikuchii* in 2000, 2011, and 2012.

Parish	2000		2011		2012	
	No. Tested	MBC-R*	No. Tested	MBC-R	No. Tested	MBC-R
Avoyelles	0	0	17	7	5	1
Caldwell	0	0	2	0	0	0
Cameron	0	0	0	0	1	0
Catahoula	0	0	16	3	8	4
Concordia	0	0	30	17	9	4
East Carroll	0	0	4	4	4	1
East Baton	3	3	2	0	0	0
Evangeline	0	0	3	1	4	0
Franklin	145	20	4	2	1	1
Grant	0	0	0	0	2	0
Jefferson Davis	0	0	2	2	2	1
LaSalle	0	0	12	0	2	1
Madison	0	0	0	0	2	2
Morehouse	0	0	5	2	0	0
Natchitoches	0	0	0	0	4	0
Ouachita	0	0	1	0	4	0
Pointe Coupee	0	0	10	9	6	0
Rapides	28	18	13	5	4	2
Richland	0	0	1	0	0	0
Saint Landry	0	0	16	4	16	3
Saint Martin	0	0	13	0	1	1
Tensas	0	0	0	0	3	2
Vermilion	0	0	5	2	4	2
West Baton	0	0	2	2	0	0
West Carroll	0	0	2	2	0	0

*Methyl benzimidazole carbamate (MBC) resistant.

In the 2000 population, none of the 98 isolates from three parishes were QoI-resistant. In 2011, 84 of 100 isolates from 14 parishes were determined to be resistant to QoI fungicides. For 2012, 53 of 62 isolates from 17 parishes were QoI-resistant (Table 5.4). To date, QoI resistance in *C. kikuchii* has been found in a total of 21 parishes throughout soybean producing areas of Louisiana (Figure 5.7).

Multiple Resistance. Isolates of *C. kikuchii* resistant to both MBC and QoI fungicides were not detected in the 2000 population. In 2011 and 2012, 42.2 and 25.8% of isolates were multiple-resistant to MBC and QoI fungicides, respectively (Figure 5.8). Estimated probabilities of multiple resistance were determined by logistic regression to be 0.05, 0.43, and 0.34 for 2000, 2011, and 2012, respectively (Figure 5.8). When comparing the population from 2000 to the 2011 and 2012 populations, respective isolates were approximately 8 and 7 times

more likely to exhibit multiple resistance in 2011 and 2012, a significant result (Figure 5.9). There was no significant difference in relative risk of multiple resistance between the 2011 and 2012 populations (Figure 5.9).

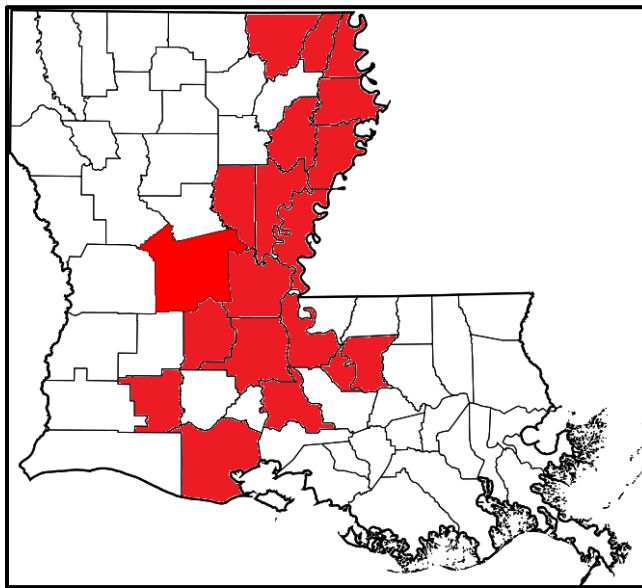


Figure 5.4. Parishes with methyl benzimidazole carbamate-resistant isolates of *Cercospora kikuchii* in Louisiana in 2000, 2011, and 2012.

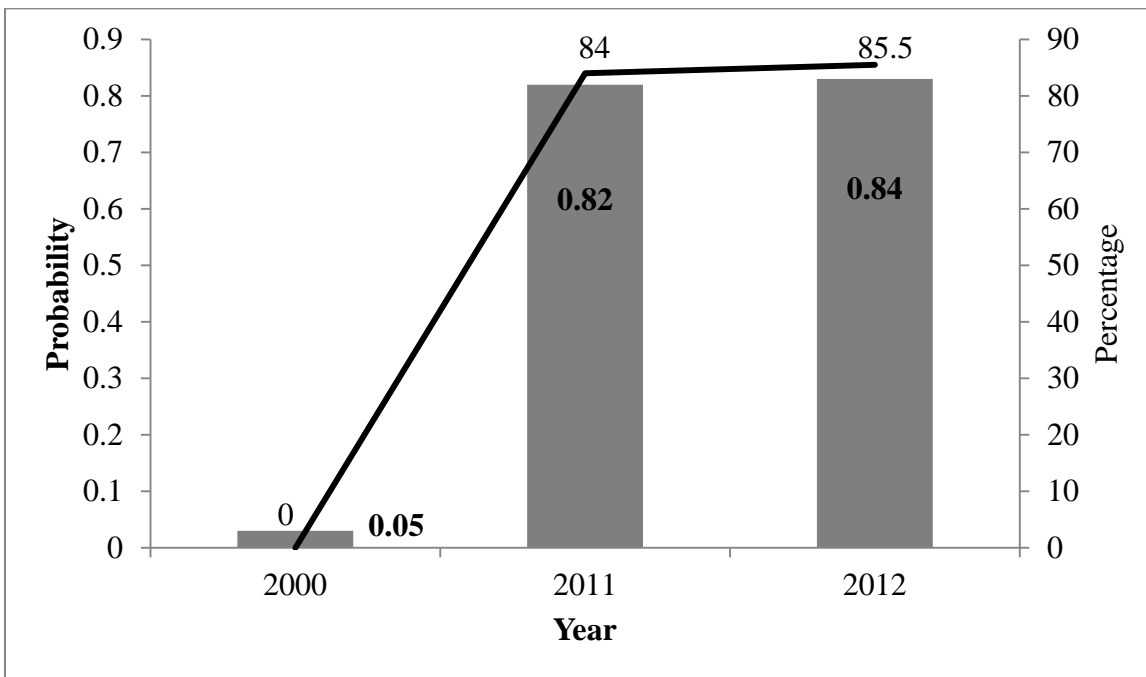


Figure 5.5. Probabilities and percentages of quinone outside inhibitor resistance in isolates of *Cercospora kikuchii* collected in 2000, 2011, and 2012.

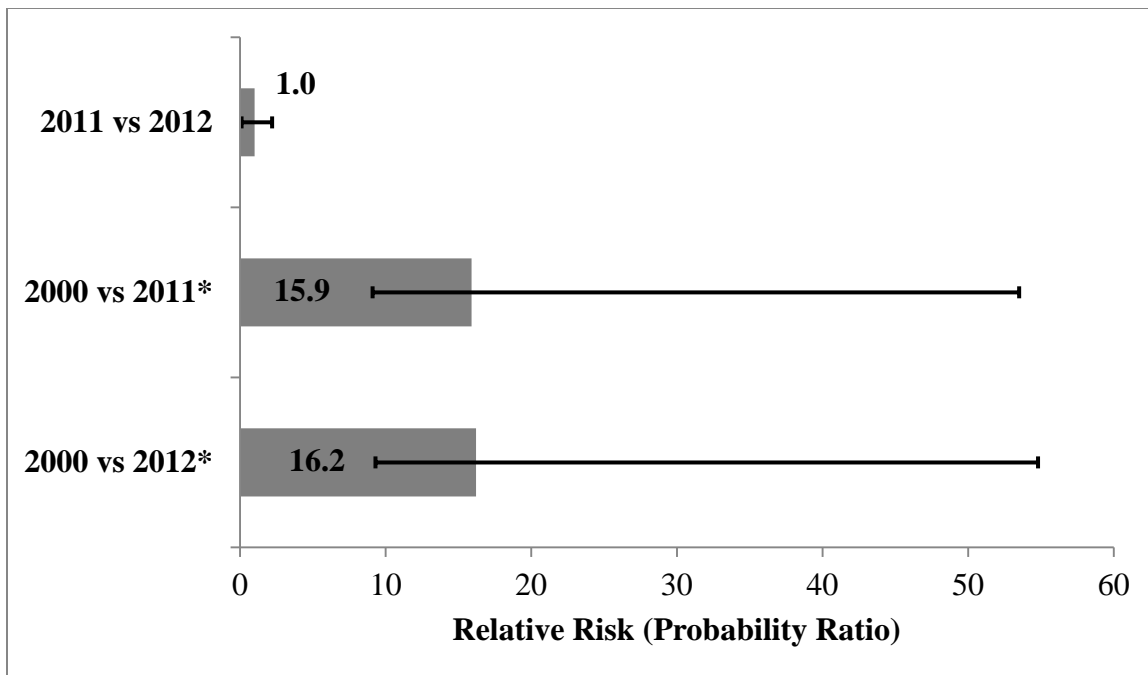


Figure 5.6. Relative risk and 95% confidence limits of quinone outside inhibitor resistance in isolates of *Cercospora kikuchii* collected in 2000, 2011, and 2012. *Denotes statistical significance (PROC LOGISTIC, n = 262, Wald $X^2 = 78.845$, $P < 0.0001$).

In the 2000 population, 0 out of 98 isolates from three parishes were multiple resistant to MBC and QoI fungicides. In 2011, 43 of 102 isolates from 11 parishes were determined to be resistant to both MBC and QoI fungicides. For 2012, 16 of 62 isolates from 12 parishes exhibited multiple resistance (Tables 5.3 and 5.4). To date, multiple resistance to MBC and QoI fungicides in *C. kikuchii* has been found in a total of 15 parishes throughout soybean producing areas of Louisiana (Figure 5.10).

Proclivity of Methyl Benzimidazole Carbamate-Resistant Isolates of *Cercospora kikuchii* to be Quinone Outside Inhibitor-Resistant. In the 2000 population, no MBC isolates were detected that were also QoI-resistant. Chi-square analysis of the 2011 and 2012 populations revealed that 98% of MBC-resistant isolates also were resistant to QoI fungicides (Table 5.5). Populations from 2000, 2011 and 2012 had incidences of MBC-resistant isolates also resistant to QoI fungicides of 0, 98, and 100%, respectively (Figure 5.10). Estimated probabilities of MBC-resistant isolates also resistant to QoI fungicides were 0.05, 0.98, and 0.93 for 2000, 2011, and 2012, respectively (Figure 5.10). When comparing the population from 2000 to the 2011 and 2012 populations, respective MBC-resistant isolates were 20 and 19 times more likely to exhibit QoI

resistance in 2011 and 2012 (Figure 5.11). In other words, MBC-resistant isolates of *C. kikuchii* were nearly certain to also exhibit QoI-resistance. There was no significant difference in the relative risk of MBC resistant isolates exhibiting QoI resistance between the 2011 and 2012 populations (Figure 5.11).

Table 5.4. Detailed parish information indicating number of tested and confirmed quinone outside inhibitor-resistant isolates of *Cercospora kikuchii* collected in 2000, 2011, and 2012.

Parish	2000		2011		2012	
	No. Tested	QOI-R*	No. Tested	QOI-R	No. Tested	QOI-R
Avoyelles	0	0	12	12	2	2
Caldwell	0	0	0	0	1	1
Cameron	0	0	0	0	1	1
Catahoula	0	0	14	13	8	7
Concordia	0	0	29	27	6	4
East Carroll	0	0	0	0	4	3
East Baton	3	0	2	0	0	0
Evangeline	0	0	3	3	5	4
Franklin	67	0	2	1	1	1
Jefferson Davis	0	0	0	0	1	1
LaSalle	0	0	9	5	0	0
Madison	0	0	0	0	2	2
Morehouse	0	0	1	1	0	0
Ouachita	0	0	0	0	1	1
Pointe Coupee	0	0	7	6	4	4
Rapides	28	0	11	8	3	3
Richland	0	0	1	1	0	0
Saint Landry	0	0	4	3	13	11
Saint Martin	0	0	0	0	1	1
Tensas	0	0	0	0	3	1
Vermilion	0	0	5	4	4	4

*Quinone outside inhibitor resistant.

In the 2000 population, 0 out of 98 isolates from three parishes were multiple resistant to MBC and QoI fungicides. In 2011, 44 of 45 MBC-resistant isolates from 10 parishes were determined to be resistant to QoI fungicides. For 2012, 16 of 16 MBC-resistant isolates from 12 parishes exhibited QoI resistance as well (Tables 5.3 and 5.4).

Discussion

Resistance to MBC fungicides in other plant pathogenic fungi has been documented since the late 1960s (Schroeder and Provvidenti 1969, Ruppel et al 1975, 1980, Ishii et al 1985, Dovas et al 1976, Henry and Trivellas 1989, Elad et al 1992, Moorman and Lease 1992, Raposo et al 1996, Campbell et al 1998, Kenaith and

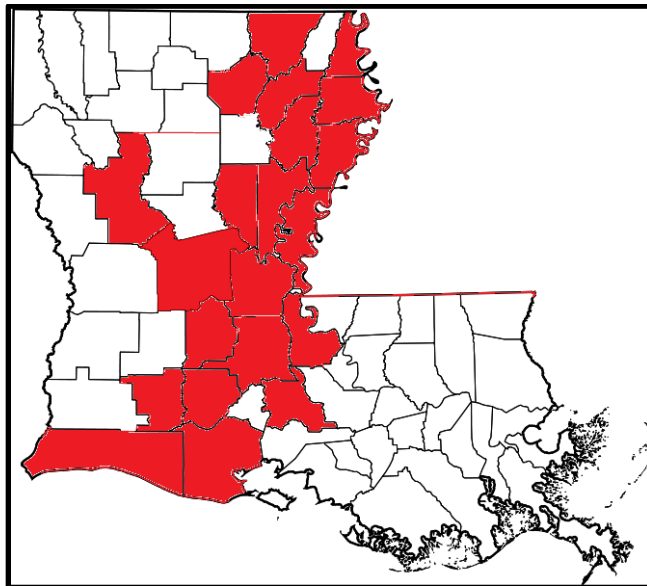


Figure 5.7. Louisiana parishes with confirmed quinone outside inhibitor resistance in isolates of *Cercospora kikuchii* collected in 2011 and 2012.

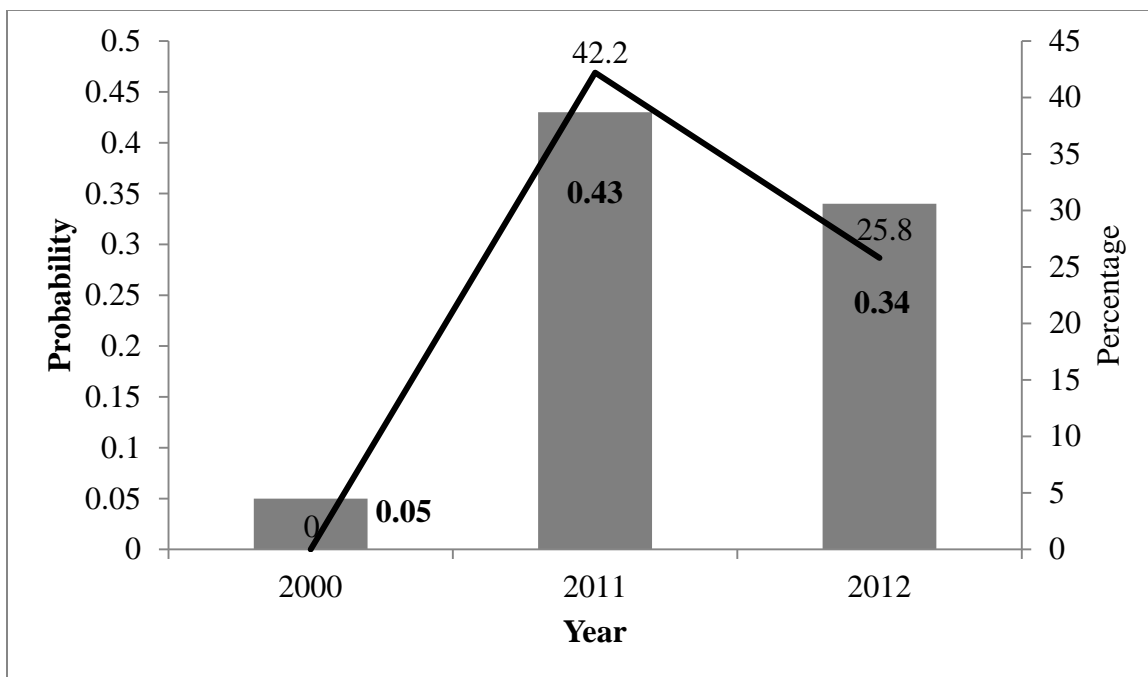


Figure 5.8. Probabilities and observed percentages of multiple resistance to methyl benzimidazole carbamate and quinone outside inhibitor fungicides in isolates of *Cercospora kikuchii* collected in 2000, 2011, and 2012.

Zitter 1998, Weiland and Halloin 2001, Culbreath et al 2002, Karaoglanidis et al 2003, Yoshimura et al 2003, Imazaki et al 2006, Karaoglanidis and Bardas 2006, Myresiotis et al 2007, Wong et al 2008, Koch et al 2009, Secor et al 2010, Quello et al 2010, Sun et al 2010, Weber 2011, Chapman et al 2011). Previous research

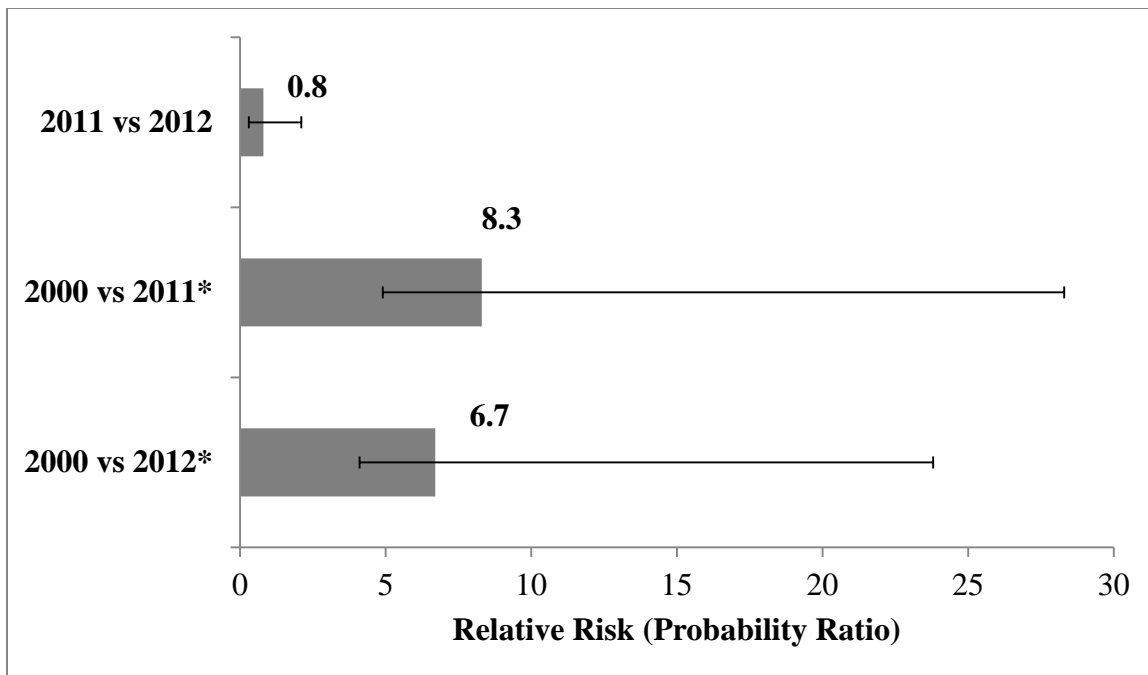


Figure 5.9. Relative risk and 95% confidence limits of multiple resistance to methyl benzimidazole carbamate and quinone outside inhibitor fungicides in isolates of *Cercospora kikuchii* collected in 2000, 2011, and 2012.

*Denotes statistical significance (PROC LOGISTIC, n = 262, Wald $X^2 = 27.952$, $P < 0.0001$).

indicated cross-resistance in fungal isolates to MBC fungicides (Kenaith and Zitter 1998, Cunha and Rizzo 2003, Wong et al 2008). Thus, isolates resistant to thiophanate-methyl in this study were assumed resistant to all MBC fungicides. In *C. beticola*, a closely-related pathogen of sugar beet, MBC resistance was reported in Greece and the United States using methods similar to the current study (Secor et al 2010, Karaoglanidis et al 2003). Additionally, MBC resistance was reported in *C. arachidiola* and *Cercosporidium personatum*, closely related pathogens of peanut (Culbreath et al 2002). In Japan, resistance of *C. kikuchii* to MBC fungicides was first discovered in the 1980s (Sakai 1999) with later validating work in 2006 (Imazaki et al). Results from the current study provide the first evidence of MBC resistance in *C. kikuchii* in the United States. MBC resistance in *C. kikuchii* is widespread throughout soybean producing areas in Louisiana. These findings suggest that use of this fungicide imposed a selection pressure that led to the development of resistant strains. This scenario has been repeated in the past where MBC applications selected for resistant strains, which slowly declined after discontinuation of use, and this was interpreted as maintenance of stability and competitiveness of resistant isolates (Ruppel et al 1980, Ishii et al 1985, Karaoglanidis and Bardas 2006, Secor et al 2010).

Table 5.5. Chi-Square analysis of methyl benzimidazole carbamate and quinone outside inhibitor-sensitive and -resistant isolates of *Cercospora kikuchii* collected in 2011 and 2012.

Chi Square Analysis of Proportions of MBC and QoI Sensitive and Resistant Isolates from 2011 and 2012				
		QOI		
MBC		S ¹	R ²	Total
Frequency	S	26	62	88
Percent		17.45	41.61	59.06
Row %		29.55	70.45	
Col %		96.30	50.82	
Frequency	R	1	60	61
Percent		0.67	40.27	40.94
Row %		1.64	98.36*	
Col %		3.70	49.18	
Total		27	122	149
		18.12	81.88	100.00

¹Sensitive. ²Resistant.

*Percentage of MBC-resistant isolates also resistant to QoI fungicides ($X^2 = 18.909$, $P < 0.0001$).

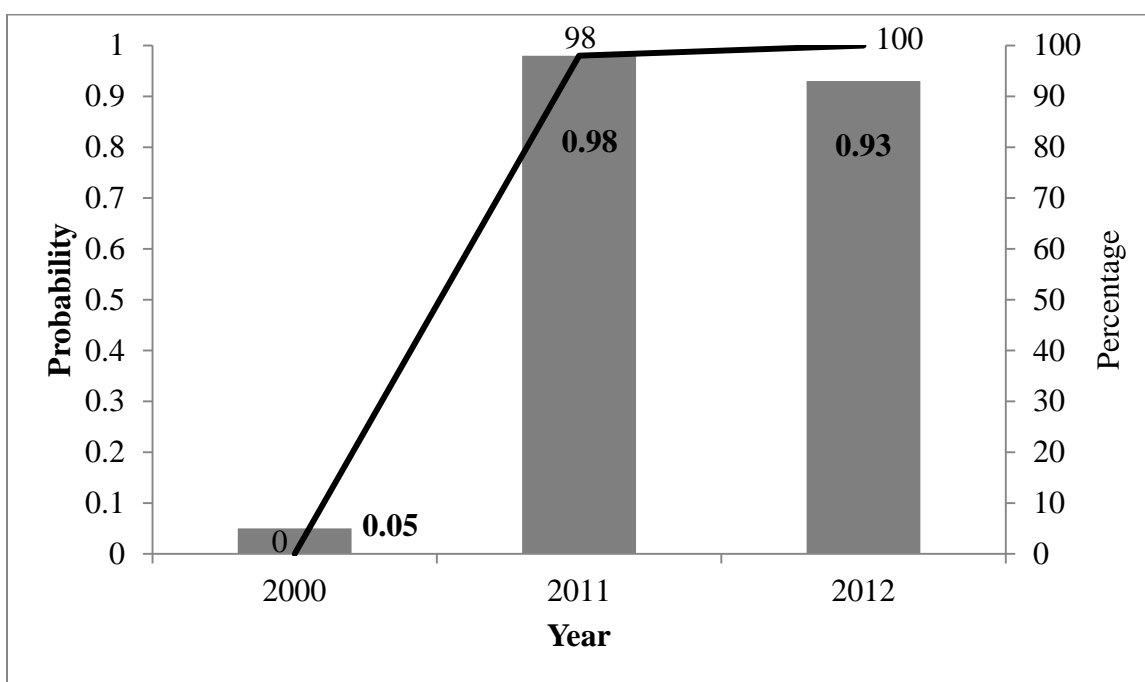


Figure 5.10. Probabilities and observed percentages of methyl benzimidazole carbamate-resistant isolates of *Cercospora kikuchii* collected in 2000, 2011, and 2012 also resistant to quinone outside inhibitor fungicides.

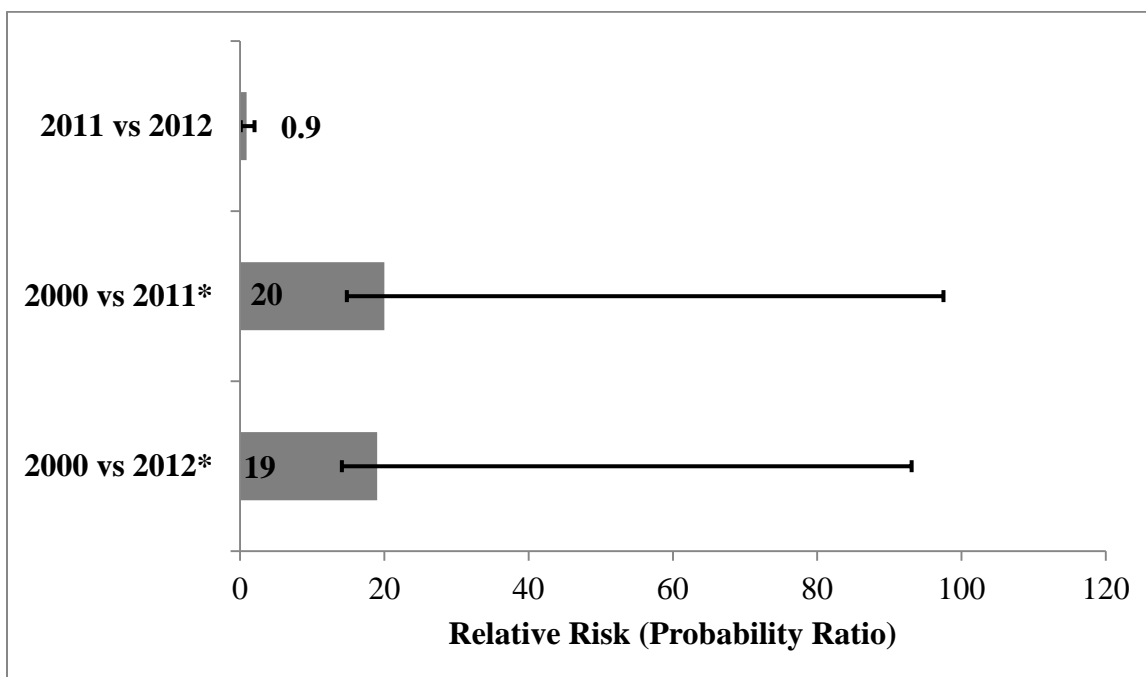


Figure 5.11. Relative risk and 95% confidence limits of quinone outside inhibitor resistance in methyl benzimidazole carbamate-resistant isolates of *Cercospora kikuchii* from 2000, 2011, and 2012.

*Denotes statistical significance (PROC LOGISTIC, n = 100, Wald $X^2 = 36.848$, $P < 0.0001$).

Because of this confirmation of MBC resistance, widespread occurrence, high selection pressure of applications, and stability of fungicide resistance in the pathogen, MBC fungicide use for management of CLB/PSS should be discontinued in areas where resistant isolates have been confirmed.

Many cases of resistance to QoI fungicides in plant pathogens were previously documented (Avila-Adame et al 2003, Pasche 2004, Wise et al 2009, Secor et al 2010, Kirk et al 2012, Zhang et al 2012b). *Cercospora* species confirmed resistant to QoI fungicides include *C. beticola* and *C. sojina* (Bolton et al 2012, Zhang et al 2012b, respectively). Resistance of *C. kikuchii* to QoI fungicides was determined and shown to be widespread in Louisiana (See Chapter 3). In the current study, relative risk of QoI resistance in *C. kikuchii* was determined to be higher than that of MBC resistance. Additionally, the probability of QoI resistance was significantly higher in the 2011 and 2012 populations when compared to the 2000 population, which is logical because the latter population likely was not exposed to QoI fungicides. The likelihood of QoI resistance in 2011 and 2012 populations was 82 and 84%, respectively, indicating the vast majority of the pathogen

population is comprised of resistant individuals. With QoI resistance confirmation, high probability, and widespread occurrence, QoI applications also should not be recommended for management of CLB/PSS in areas where resistance has been confirmed in Louisiana.

Multiple resistance to fungicides has been previously reported in other plant pathogens (Elad et al 1992, Moorman and Lease 1992, Bugbee 1995, Raposo et al 1996, Koller and Wilcox 2001, Myresiotis et al 2007, Sun et al 2010, Weber 2011, Chapman et al 2011, May-De Mio et al 2011). Multiple resistance to MBC, QoI, and DMI fungicides in the related *C. beticola* were previously reported in Greece and the United States (Karaoglanidis et al 2003, Secor et al 2010). Results from the current study provide the first evidence of multiple resistance to MBC and QoI fungicides in *C. kikuchii* in the United States. Isolates from 2011 and 2012 were more likely to develop multiple resistance when compared to the 2000 population. Additionally, MBC-resistant isolates of *C. kikuchii* had a tendency for QoI resistance, with probabilities of QoI resistance of approximately 98 and 93% for 2011 and 2012, respectively. This finding was unexpected, and as Koller and Wilcox (2001) hypothesized, may be an indication of a relationship between resistance mechanisms of the fungus to the two fungicide types or possibly higher mutation rates in MBC-resistant isolates. Further research is needed to elucidate this proclivity. The occurrence of multiple resistance could also be an indication that alternating or tank-mixing MBC and QoI fungicides would be an ineffective management practice in mitigating resistance. Finally, the discovery of multiple resistance to MBC and QoI fungicides and proclivity of MBC-resistant isolates of *C. kikuchii* to also be QoI-resistant further reinforces that MBC and QoI applications should not be recommended for management of CLB/PSS.

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CHAPTER 6 SUMMARY AND CONCLUSIONS

In 1921, purple seed stain (PSS), purple “speck”, or “Shihan”, of soybean was reported in Korea. Matsumoto and Tomoyasu first described the pathogen infecting seeds, seedlings, stems, pods, and leaves. The disease was first observed in the United States in 1924, and the causal agent is currently identified as *Cercospora kikuchii*. *Cercospora* leaf blight/purple seed stain caused average losses of 140,500 metric tons in soybean annually from 1996 to 2012, and disease incidence and severity have markedly increased over the past 10-15 years in Louisiana.

Fungicide applications during the reproductive stage of development of soybean are recommended by the Louisiana State University Agricultural (LSU AgCenter) for management of CLB/PSS in Louisiana. Historically, a methyl benzimidazole carbamate (MBC) fungicide, thiophanate-methyl (Topsin™) and two quinone outside inhibitor (QoI) fungicides: azoxystrobin (Quadris™) and pyraclostrobin (Headline™) have been recommended by the LSU AgCenter for CLB/PSS management. Since 2012, trifloxystrobin (Gem™) has been recommended alone for CLB/PSS management, along with two demethylation inhibitor (DMI) fungicides: flutriafol (Topguard™) and tetraconazole (Domark™). Propiconazole and other DMI fungicides are recommended in mixtures with QoI fungicides by the LSU AgCenter. Fungicide use statistics are limited for Louisiana, but current application estimates range from 40 to 75% of planted hectares. Additionally, in recent years, fungicide use has been encouraged by industry and soybean prices have increased, which have likely increased the number of fungicide applications.

QoI fungicides interfere with mitochondrial respiration by blocking electron transport at the quinol-oxidizing site of the cytochrome bc_1 complex (III), which subsequently affects spore germination and hyphal growth. This mode of action is highly specific and can be overcome by a single-step mutation resulting in a high risk of resistance development in plant pathogens. Since QoI introduction in 1996, 56 species of fungi

pathogenic to 20 horticultural and agronomic crops have been identified as resistant to QoI fungicides. QoI fungicides are commonly used in Louisiana, and efficacy on CLB/PSS appears to have decreased since their inception.

Fortunately, a collection of *C. kikuchii* isolates from the year 2000 that were likely not exposed to QoI fungicides were available for baseline sensitivity assays to azoxystrobin, pyraclostrobin, and trifloxystrobin. Alternative respiration, a phenomenon by which fungi bypass the effects of QoI fungicides, did not occur in these isolates, so testing proceeded without the addition of alternative respiration inhibitors. Radial growth assays provided baseline sensitivities with similar characteristics to those generated by others with conidial germination assays. Overall mean baseline sensitivities (based on EC₅₀ values) of *C. kikuchii* to azoxystrobin, pyraclostrobin, and trifloxystrobin were 0.102, 0.017, and 0.015 µg/ml, respectively. Intrinsic activity of pyraclostrobin and trifloxystrobin was greater than azoxystrobin, which has been a common observation in numerous other studies and usually does not translate to differences in disease control in the field. Additionally, results indicated cross-sensitivity between isolates exposed to azoxystrobin and trifloxystrobin, which is in agreement with other research with QoI fungicide sensitivities. All three baseline sensitivities to QoI fungicides in this study were non-normal with outliers towards the less-sensitive ends of the spectra. Previous research dictates that this type of distribution could indicate a propensity towards QoI resistance development. Therefore, it was concluded that populations of *C. kikuchii* should be monitored over time for possible resistance development.

Isolates of *C. kikuchii* representing the soybean production areas in Louisiana, a broad geographical range, were collected for QoI sensitivity analysis in 2011 and 2012. For isolates from 2011, overall mean sensitivities of *C. kikuchii* to azoxystrobin, pyraclostrobin, and trifloxystrobin were 32.6, 10.8, and 22.6 µg/ml, respectively. Similar results were observed in 2012 with respective overall sensitivities of 52.7, 13.3, and 29.2 µg/ml. When compared with baselines that were established with the 2000 collection, isolates were significantly less-sensitive and had much higher EC₅₀ values, a clear indication of QoI resistance. This is the first confirmation of QoI resistance in *C. kikuchii*. Additionally, results indicated that QoI resistance is

widespread in Louisiana with resistant isolates confirmed in 21 parishes. From this research, discriminatory dose values were established for azoxystrobin, pyraclostrobin, and trifloxystrobin, and these values were used to determine that Louisiana populations of *C. kikuchii* are comprised of approximately 83% resistant individuals. Additionally, discriminatory doses were used to illustrate that QoI applications have no effect on resistant isolates of *C. kikuchii* in the field. Furthermore, sensitivities of individual isolates to azoxystrobin, pyraclostrobin, and trifloxystrobin were significantly and positively correlated, indicating cross-resistance to QoI fungicides in *C. kikuchii*. Based on estimates of QoI-resistant individuals, widespread occurrence, and likelihood of cross-resistance, QoI fungicides should be discouraged for management of CLB/PSS in Louisiana.

Demethylation inhibitor (DMI) fungicides were commercially introduced around 1975. These fungicides interact with cytochrome P450s at the site of the 14 α -demethylase (CYP51) and C-22 desaturase (CYP61) blocking 14 α -demethylation resulting in an accumulation of 5-hydroxy sterol, which is the toxic component of the fungicide. Several changes throughout the sterol biosynthesis pathway must occur in plant pathogenic fungi for disease control problems to occur with DMI fungicides, and this results in a medium risk of resistance development. Since their introduction, 29 plant pathogenic species infecting approximately 20 horticultural and agronomic crops have been confirmed resistant to DMI fungicides. DMI fungicides were not recommended for CLB/PSS management until 2012 in Louisiana, but it is likely that applications of DMI fungicides were made prior to this time for management of CLB/PSS and other diseases.

As previously-mentioned, a collection of isolates from 2000 was available to determine baseline sensitivities to the DMI fungicides: flutriafol, propiconazole, and tetraconazole. It is unknown if these isolates were previously exposed to DMI fungicides in the field. Overall mean *in vitro* baseline sensitivities to flutriafol, propiconazole, and tetraconazole were 0.452, 0.182, and 1.667 $\mu\text{g/ml}$, respectively. Baselines for all three DMI fungicides were non-normal with outliers towards the less-sensitive ends of the spectra indicating a possible propensity for resistance development. Overall *in vitro* toxicity of tetraconazole was less than flutriafol and propiconazole, and previous research indicates that it is not uncommon to observe differences between *in vitro* toxicities between DMI fungicides. Additionally, sensitivities of individual isolates to flutriafol and

propiconazole were correlated, indicating cross-sensitivity to DMI fungicides in *C. kikuchii*. After baselines were established, it was concluded that monitoring *C. kikuchii* populations over time was prudent; particularly with the recent recommendation changes and increased use of DMI fungicides.

Isolates of *C. kikuchii* representing the soybean production areas in Louisiana, a broad geographical range, were collected for DMI sensitivity analysis in 2011 and 2012. For isolates from 2011, overall mean sensitivities of *C. kikuchii* to flutriafol, propiconazole, and tetraconazole were 0.398, 0.411, and 0.960 $\mu\text{g/ml}$, respectively. In 2012, respective overall sensitivities were 0.826, 0.446, and 1.22 $\mu\text{g/ml}$. Statistically significant differences towards less sensitivity were detected for flutriafol and propiconazole distributions when compared to baselines, which could be indicative of a sensitivity shift. Interestingly, a shift towards more sensitivity to tetraconazole was detected when compared to the baseline, which indicated that the baseline isolates had been previously exposed to tetraconazole. Unfortunately, previous field fungicide exposure of the baseline isolates was not determinable. Furthermore, isolates outlying towards the less-sensitive end of the spectrum in 2012 for all three DMI fungicides could be an indication of a shift towards resistance. Additionally, sensitivities to flutriafol, propiconazole, and tetraconazole were significantly and positively correlated, which are indications of cross-sensitivity to DMI fungicides in *C. kikuchii*. Based on this research, there were indications of possible resistance development, so further monitoring of DMI sensitivities should continue in *C. kikuchii*.

Thiophanate-methyl belongs to the MBC class of fungicides, which inhibit growth of fungi by interfering with microtubule assembly during mitosis. This fungicide class is considered to be “high-risk” for inducing resistance in fungal plant pathogens, which arises from a mutation in the β -tubulin gene. Resistance to MBC fungicides was first documented soon after their introduction, and since has occurred in nearly 130 fungal pathogens of fruit, vegetable, turf grass, horticultural, and agronomic crops. Additionally, resistance of *C. kikuchii* to thiophanate-methyl has been documented in Japan since the late 1980s. Fungicide use records

and estimates are scarce, but the USDA-NASS indicates that MBC fungicides have been used in Louisiana soybean since the early 1990s. Finally, thiophanate-methyl is currently recommended for CLB/PSS management in Louisiana.

Preliminary research indicated that isolates were either highly-sensitive or highly-resistant to thiophanate-methyl, which has been observed in other laboratory bioassays with MBC fungicides. Other studies indicated nearly 100% cross-resistance among all MBC fungicides. Therefore, *C. kikuchii* isolates resistant to thiophanate-methyl should also be considered resistant to all MBC fungicides. Isolates of *C. kikuchii* from 2000, 2011, and 2012 populations were screened for resistance to thiophanate-methyl using a discriminatory dose of 5 µg/ml. Respective percentages of resistant isolates for each year were 23, 45, and 36, with similar respective estimated probabilities of 0.23, 0.39, and 0.30. Therefore, it was concluded that in a given year, approximately 31% of individuals in a given population of *C. kikuchii* will be MBC-resistant. Results also indicated that MBC resistance in *C. kikuchii* is widespread across soybean production areas in Louisiana. This is the first documentation of MBC resistance in *C. kikuchii* in the United States. Other research indicated that MBC applications are highly-selective for resistance, and that resistant individuals are stable, competitive, and remain in populations for long periods of time. For these reasons, MBC applications for management of CLB/PSS should be discouraged in Louisiana.

Discovery of multiple-resistance in *C. kikuchii* to QoI and MBC fungicides resulted from these studies. There were no actual multiple resistant isolates observed in the 2000 population likely because of no exposure to QoI fungicides. However, 5% multiple-resistant isolates were hypothetically added to the baseline population for statistical comparisons. Probabilities of multiple resistance to MBC and QoI fungicides for populations from 2000, 2011, and 2012 were 0.05, 0.43, and 0.34, respectively, and relative risk of multiple resistance were significantly higher in 2011 and 2012 populations when compared to the 2000 population. Additionally, when considering only MBC-resistant isolates, probabilities of multiple resistance to QoI fungicides were 0.05, 0.98, and 0.93, for the 2000, 2011, and 2012 populations, respectively, and MBC-resistant isolates from 2011 and 2012 were more likely to be QoI-resistant. These results indicate a proclivity of MBC-resistant isolates to also

express QoI resistance. Further research is needed to elucidate this tendency. To our knowledge, this is the first documentation of multiple fungicide resistance in *C. kikuchii*, and this discovery should further discourage the use of these two chemistry types for CLB/PSS management in Louisiana.

This project confirmed MBC and QoI resistance in *C. kikuchii* and provides a partial explanation as to why management of CLB/PSS has become challenging in recent years. However, many other research opportunities have come to light as a result of this research and literature reviews:

1. Monitoring of populations of *C. kikuchii* for sensitivity to MBC, QoI, and DMI fungicides should continue to detect less or more sensitivity depending on fungicide use in a given area at a given time.
2. Although there were not large differences in DMI sensitivities between the baseline and subsequent populations in this study, this does not necessarily translate to field efficacy of DMI fungicides. More research is needed to determine if DMI fungicides are efficacious on CLB/PSS.
3. Discriminatory doses developed in this project may be useful in studying the effects of fungicide applications (rate, timing, multiple applications, and mixed active ingredients) on field populations of *C. kikuchii*. Additionally, discriminatory doses could provide a more efficient means of population monitoring statewide or prove useful in studying field implications of multiple resistance to MBC and QoI fungicides in *C. kikuchii*.
4. Results from this project provide a means by which to monitor *C. kikuchii* sensitivity to novel fungicides in the future.
5. Results from this research suggest many opportunities for determining the molecular mechanisms of fungicide resistance (particularly QoI and multiple-resistance) in *C. kikuchii*.
6. Isolates used in this study could also be used in molecular diversity studies of *C. kikuchii* in soybean.
7. Results from this research provide a basis and indicate a need for reformed fungicide recommendations in Louisiana.

VITA

Paul Patrick Price, III (Trey), son of Paul Patrick Price, Jr. and Deborah Lynn Brouillette Kincaid was born in 1976, in Winnsboro, Louisiana. He is a lifelong resident of Louisiana, and graduated from Winnsboro High School in May 1994. He earned a Bachelor of Science degree in Agronomy from Louisiana Tech University in Ruston, Louisiana in March 1999, and immediately began graduate studies under the direction of Dr. H. Lynn Walker studying Plant Pathology. Trey earned his Masters of Science in Biology from Louisiana Tech University in August 2000. After a two-year stint in Environmental Consulting with PPM Consultants, Inc. of Monroe, Louisiana, he began work as an Extension Associate with LSU AgCenter in May 2003. After two growing seasons, Trey transferred to a Research Associate position with Dr. B. Rogers Leonard at the Macon Ridge Research Station (MRRS) where he remained for five growing seasons conducting research in entomology. In 2008, he was accepted into the Plant Pathology graduate program at Louisiana State University under the direction of Drs. Raymond W. Schneider and G. Boyd Padgett while continuing to work full-time as a Research Associate in Entomology. In 2010, Trey transferred to a Research Associate position in Plant Pathology at MRRS under the direction of Dr. G. Boyd Padgett where he is currently employed. He currently resides in Winnsboro, Louisiana, is married to Beth and has a daughter and a son, Camryn and Keegan. Trey is currently a doctoral candidate in the Department of Plant Pathology and Crop Physiology at Louisiana State University.