Fungicide Resistance in *Cercospora kikuchii*, a Soybean Pathogen

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Abstract

Price, P. P., III, Purvis, M. A., Cai, G., Padgett, G. B., Robertson, C. L., Schneider, R. W., and Albu, S. 2015. Fungicide resistance in *Cercospora kikuchii*, a soybean pathogen. Plant Dis. 99:1596-1603.

Isolates of *Cercospora kikuchii*, a soybean (*Glycine max*) pathogen causing Cercospora leaf blight and purple seed stain, were tested to determine baseline sensitivities ($n = 50$) to selected quinone outside inhibitor (QoI) fungicides by conducting radial growth assays on fungicide-amended media. Baseline effective fungicide concentration to inhibit 50% of fungal radial growth (EC_{50}) values were compared with EC_{50} values for isolates collected in 2011 ($n = 50$), 2012 ($n = 50$), and 2013 ($n = 36$) throughout soybean-producing areas in Louisiana. Median EC_{50} values for isolates subjected to QoI fungicides were significantly ($P = 0.05$) higher across all 3 years. Cross-resistance to QoI fungicides was observed in resistant isolates collected in 2011 to 2013. Discriminatory

doses were developed for QoI fungicides to distinguish between sensitive and resistant isolates. On average, 89% of all isolates screened in 2011 to 2013 were resistant to QoI fungicides. At a discriminatory dose of thiophanate methyl (TM), a methyl benzimidazole carbamate (MBC) fungicide, at 5 $\mu\text{g/ml}$, resistance was detected in the 2000, 2011, 2012, and 2013 collections at 23, 38, 29, and 36%, respectively. Isolates exhibiting multiple resistance to QoI fungicides and TM also were detected in 2011, 2012, and 2013 at frequencies of 34, 26, and 31%, respectively. Based on these results, Cercospora leaf blight management strategies in Louisiana using solo applications of QoI or MBC fungicides in soybean should be reconsidered.

Cercospora leaf blight (CLB), caused by *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M. W. Gardner, is a significant disease of soybean (*Glycine max* (L.) Merr.) in the United States, causing estimated average losses of 112,455 metric tons annually from 1996 to 2013 (Wrather and Koenning 2009). Applications of quinone outside inhibitor (QoI; Fungicide Resistance Action Committee [FRAC] group 11) and methyl benzimidazole carbamate (MBC; FRAC group 1) fungicides during the reproductive growth stages of soybean are used for managing CLB and other foliar diseases in Louisiana (Anonymous 2014). Fungicide use in Louisiana soybean increased from 1991 to 2006 from less than 5 to 38% of planted hectareage (USDA-NASS 2014). According to the United States Department of Agriculture–National Agricultural Statistics Service (USDA-NASS), the first use of QoI fungicides in Louisiana soybean occurred in 2000, covering approximately 5% of the planted hectareage (USDA-NASS 2014). Estimates from 2012 indicated that 42, 17, and 9% of the planted hectareage were treated at least once with the QoI fungicides azoxystrobin, pyraclostrobin, or trifloxystrobin, respectively (USDA-NASS 2014). Despite these preventative fungicide applications, Louisiana producers have struggled to manage CLB in recent years, and fungicide failures have become commonplace. These failures began around 2005 and, since that time, fungicide trials in Louisiana and Alabama have indicated marginal fungicide efficacy on CLB (Delaney et al. 2012; Lawrence et al. 2006; Padgett and Purvis 2006, 2007; Price and Padgett 2009; Price et al. 2013).

The 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 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 2014). QoI fungicides were introduced to the market in the mid-1990s, and resistance of fungal

pathogens to this chemistry class was documented in 1998 (FRAC 2013). Since then, approximately 60 species of fungi pathogenic to 20 important agronomic and horticultural crops have been identified as resistant to QoI fungicides (FRAC 2013). *Cercospora* spp. resistant to QoI fungicides in the United States include *C. beticola* (Kirk et al. 2012) and *C. sojina* (Zhang et al. 2012b).

Thiophanate methyl (TM) belongs to the MBC class of fungicides, which inhibit growth of fungi by interfering with microtubule assembly during mitosis (Howard and Aist 1977). FRAC also considers TM to be high risk for selecting resistant individuals in fungal plant pathogens (FRAC 2014). This resistance is due to a mutation in the β -tubulin gene leading to high levels of resistance in vitro and in vivo (Monma et al. 2003). Resistance to MBC fungicides was first documented in 1969, soon after their introduction; and, since this time, resistance to MBC fungicides of approximately 130 fungal pathogens of agronomic, fruit, horticultural, turf grass, and vegetable crops has been confirmed (FRAC 2013). Use of MBC fungicides in Louisiana was first documented in the early 1990s, with coverage of 3 to 4% of planted hectareage. Current estimates are unavailable from USDA-NASS; however, based on anecdotal evidence, approximately 15% of the planted hectareage in Louisiana is treated with MBC fungicides (personal observations). Resistance of *C. kikuchii* to TM was documented in isolates from soybean in Japan in the late 1980s (Imazaki et al. 2006; Sakai 1999). Field applications of MBC fungicides were determined to be highly selective for MBC-resistant isolates of fungal plant pathogens (Dovas et al. 1976; Imazaki et al. 2006; Ishii et al. 1985; Karaoglanidis et al. 2003; Ruppel et al. 1980). 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; Ishii et al. 1985; Karaoglanidis et al. 2003; Ruppel et al. 1980).

Cross-sensitivity or resistance is known to commonly occur with QoI fungicides across many species of plant-pathogenic fungi (Pasche et al. 2004; Wise et al. 2009). Additionally, MBC-resistant fungal isolates have exhibited cross-resistance to MBC fungicides in several documented cases (Cunha and Rizzo 2003; Keinath and Zitter 1998; Wong et al. 2008).

Many cases of multiple resistance to MBC and other fungicide modes of action in plant pathogens have previously been reported in situations where fungicides are extensively used, such as orchards, vegetable production, and greenhouse applications (Chapman et al.

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Accepted for publication 4 May 2015.

<http://dx.doi.org/10.1094/PDIS-07-14-0782-RE>
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2011; Myresiotis et al. 2007). Multiple resistance to Organo-tin, MBC, and triazole fungicides also was documented in *C. beticola*, a major pathogen of sugar beet (Bugbee 1995; Secor et al. 2010).

In order to determine whether fungicide failures observed for CLB in Louisiana soybean were due to resistance and to monitor for sensitivity, establishment of baseline sensitivities to QoI fungicides was necessary to determine whether sensitivity shifts are occurring or may occur in the future (Russell 2004). Therefore, the objectives of this study were to (1) establish baseline sensitivities of *C. kikuchii* in Louisiana to the QoI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin; (2) determine whether shifts in QoI sensitivity have occurred within the *C. kikuchii* population; and (3) determine *C. kikuchii* sensitivity to TM.

Materials and Methods

Isolate origins. In total, 166 isolates of *C. kikuchii* 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, and included 115 foliar and 30 seed isolates from the Macon Ridge Research Station near Winnsboro and 12 foliar and 16 seed isolates from the Dean Lee Research Station near Alexandria. Three isolates of unknown plant tissue origin were obtained from the Ben Hur Research Station near Baton Rouge (Table 1). Although these isolates were obtained from only three locations in Louisiana in 1 year, they were likely not exposed or had extremely limited exposure to QoI fungicides. Therefore, it was decided to use these isolates for baseline sensitivity assays because they were the only ones available for comparison in Louisiana.

In 2011, 2012, and 2013, isolates of *C. kikuchii* were obtained from symptomatic soybean leaves from commercial soybean fields throughout Louisiana. Because of the large scale of the project, it was not feasible to obtain prior fungicide use information for sampling locations. In all, 65 locations in 21 parishes were sampled in 2011, 36 locations in 25 parishes in 2012, and 7 locations in 5 parishes in 2013 (Table 2). 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 five symptomatic leaf sections, measuring 2 cm², were cut using scissors, then placed in sterile test tubes (20 by 125 mm). Sterile, distilled water (3 ml) 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 of *C. kikuchii* (Hartman et al. 1999; Matsumoto and Tomoyasu 1925; Murakishi 1951). Conidia were singly removed from the suspension with the aid of a glass needle fashioned from a micropipette. Because of the length and flexible nature of the conidia, single-spore isolation was 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 (approximately 22°C) for 5 to 7 days. Colonies exhibiting growth characteristics consistent with *C. kikuchii* were selected, transferred via hyphal tips to V8 agar (20% juice), and maintained at 25°C with a 12-h cycle of light and darkness.

Isolate identification. For DNA extraction, purification, and quantification, isolates from 2000, 2011, 2012, and 2013 were grown under ambient light at room temperature on 60-mm petri plates

containing potato dextrose agar (PDA) amended with chloramphenicol (75 µg/ml) for 1 week. A small amount of mycelium was scraped from the colony edge to a glass test tube containing 5 ml of Complete Medium (Jenns et al. 1989) and incubated in a rotary shaker at 150 rpm for 3 to 4 days. Genomic DNA was purified using a Promega Wizard (Promega Corp., Madison, WI) DNA Purification Kit as specified by the manufacturer's instructions. DNA concentrations were quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) and adjusted to 12.5 ng/µl. The internal transcribed spacer (ITS), including the 5.8S region, was amplified with the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White 1990) using the following conditions: 5 min at 95°C; followed by 35 cycles consisting of 30 s at 94°C, 45 s at 50°C, and 45 s at 72°C; with a subsequent final extension step of 7 min at 72°C. Polymerase chain reaction (PCR) products were direct sequenced using the same primers, edited using Sequencher 5.0 (GeneCodes, Ann Arbor, MI), and queried against a database of other *Cercospora* spp. using a BLASTn search in the National Center for Biotechnology Information database (National Center for Biotechnology Information 2014). BLASTn search results confirmed that all isolates were *Cercospora* spp. ITS sequences shared 99 to 100% sequence identity with several strains of *C. kikuchii*, including isolates

Table 2. Origin and status of *Cercospora kikuchii* isolates collected from 2011 to 2013 for quinone outside inhibitor (QoI) sensitivity in radial growth assays and in discriminatory dose assays for methyl benzimidazole carbamate resistance

Parish	Number of isolates	Number TM resistant ^z	Number of QoI tested	Number QoI resistant
Avoyelles	34	14	26	23
Caldwell	2	0	0	0
Cameron	1	0	1	1
Catahoula	24	7	22	20
Concordia	39	21	35	31
East Carroll	8	5	4	3
East Baton Rouge	2	0	2	0
Evangeline	7	1	5	5
Franklin	6	3	4	3
Grant	2	0	1	0
Jefferson	4	3	4	4
Davis				
LaSalle	12	0	9	5
Madison	2	2	2	2
Morehouse	5	3	1	1
Natchitoches	4	0	4	2
Ouachita	5	0	1	1
Pointe Coupee	16	11	11	10
Rapides	17	7	14	11
Richland	1	0	1	1
St. Landry	45	7	29	27
St. Martin	14	1	1	1
Tensas	13	7	13	11
Vermillion	9	4	9	8
West Carroll	2	2	0	0
West Baton Rouge	2	2	0	0

^z TM = thiophanate methyl.

Table 1. Origin and status of *Cercospora kikuchii* isolates from the year 2000 used in radial growth, baseline sensitivity assays to quinone outside inhibitor (QoI) fungicides, and discriminatory dose assays for methyl benzimidazole carbamate resistance

Parish	Number of isolates	Number TM resistant ^z	Number of QoI tested	Number QoI resistant
East Baton Rouge	3	3	3	0
Franklin	145	20	67	0
Rapides	28	18	24	0

^z TM = thiophanate methyl.

C35 (HM631728), CKBR 179 (AY633868), CBS 132633 (JX143619), and MUCC 590 (JX143620).

Resulting isolates from 2011 through 2013 received a numerical designation, and pools of 160 from 20 parishes, 82 from 18 parishes, and 36 from 7 parishes were available for analysis in 2011, 2012, and 2013, respectively. Unless otherwise indicated, cultures 21 to 35 days old were used in all assays and incubated on V8 agar, as previously described.

Determination of alternative respiration. 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 impacts 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* spp., and salicylhydroxamic acid (SHAM) may be used to inhibit this alternative pathway (Bradley and Pedersen 2011). Other studies have indicated that SHAM and propyl gallate (PG), another AOX inhibitor, may be toxic to fungal isolates in vitro (Seyran et al. 2010). In the current study, serial dilutions of SHAM were made in ethanol (95%), then added to sterilized, molten PDA (Cole-Parmer, Inc., Vernon Hills, IL) cooled to approximately 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 by 15 mm, approximately 20 ml/dish) and allowed to solidify. Twenty isolates were selected at random (RAND function; Microsoft Excel, Redmond, WA) for exposure to each AOX inhibitor. Mycelial discs (6 mm in diameter) were cut from stock cultures using a number 3 cork borer, inverted, transferred to the media, and placed in the incubator at 25°C with a 12-h cycle of light and darkness. Treatments were arranged in a completely randomized design with four replicates per isolate. The experiment was repeated once. After incubation for 5 (SHAM) or 7 (PG) days, colony diameter was measured using a circular inking template (Number 1304I; Pickett Industries, Santa Barbara, CA). Data were analyzed using mixed-model analysis (PROC MIXED; SAS Institute, Cary, NC), and mean colony diameters were compared with the Tukey-Kramer post hoc adjustment ($P = 0.05$).

Because radial growth of *C. kikuchii* was significantly reduced by SHAM, PG was included in assays to determine whether AOX occurred in these isolates of *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 10-fold from 0.001 to 10 µg/ml. A nonamended control was included, and PG (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 by 15 mm, approximately 20 ml/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 replicates per isolate and the experiment was repeated once. After 5 days, colony diameter was measured as previously described, and data were converted to percent inhibition by comparison with respective controls. The effective fungicide concentration to inhibit 50% of fungal radial growth (EC₅₀) was determined for each treatment by linear interpolation of a regression of log concentration by percent inhibition (GraphPad Prism 6.0, La Jolla, CA). The equation for the dose-response model is as follows: $Y = 0 + (100 - 0) / [1 + 10^{((\log EC_{50} - X) \times 1)}]$.

Mean EC₅₀ values for each isolate (with and without PG) were compared using a two-sample *t* test ($P = 0.05$) (PROC TTEST; SAS Institute).

In vitro sensitivity to QoI fungicides. Radial growth assays for assessing percent inhibition for QoI fungicides were utilized instead of spore germination assays as the method for determining fungicide sensitivity. Because of scant sporulation in culture, conidia were not available for comparing the two methods. Tenfold dilutions of

technical formulations (ChemService, Inc., West Chester, PA) of azoxystrobin (99.5%), pyraclostrobin (99.5%), and trifloxystrobin (99.3%) were performed in acetone, added (1.0 ml/liter) to sterilized, molten PDA (approximately 50°C), and aseptically dispensed into sterile petri dishes (15 by 100 mm, approximately 20 ml/dish). Acetone did not have a significant effect on fungal growth at this concentration (data not shown). Final concentrations for the QoI fungicides ranged from 0.001 to 10 µg/ml, with PDA amended with acetone (1.0 ml/liter) only included for comparison. Because of time and space constraints, 50 isolates were chosen at random (RAND function; Microsoft Excel) for each sensitivity profile. In some cases, fewer than 50 isolates were available for analysis. Isolates were transferred to amended PDA and incubated as previously described. Each experiment was conducted using a RCB design, with incubator shelves serving as blocks. There were four replicates per isolate, and all 50 isolates were included in one experimental "run". Each experiment was repeated once. Colony diameter was measured as previously described 5 days after transfer, and EC₅₀ values were calculated as previously described. Mean EC₅₀ values were determined for each isolate by combining data from the two experiments, and sensitivity profiles were constructed for each fungicide and year. Tests for normality were conducted using the Shapiro-Wilk method (GraphPad Prism 6.0). In most cases, even after attempts to normalize, data violated the assumption of normality; therefore, Kruskal-Wallis one-way analysis of variance was used to determine differences in distributions among years. Dunn's multiple comparison test was used post hoc to compare distributions between years (GraphPad Prism 6.0; $P = 0.05$).

Determination of QoI discriminatory doses. Obvious separation spans were evident in all QoI distributions from 2011, 2012, and 2013. That is, there was a large gap between low and high EC₅₀ values within the distributions. The midpoints of these separation spans were selected to distinguish between sensitive and resistant isolates. From the previously described in vitro sensitivity experiments, mean radial growth inhibition (%) in sensitive isolates was compared with that of resistant isolates to determine discriminatory doses for azoxystrobin, pyraclostrobin, and trifloxystrobin.

In vitro sensitivity to TM. In a preliminary experiment, isolates of *C. kikuchii* were transferred to PDA amended with a commercial formulation of TM at final concentrations of 0, 1.56, 6.25, 25, 100, 400, and 1,600 µg/ml (Imazaki et al. 2006). Sensitive isolates exhibited no growth at 1.56 µg/ml, while resistant isolates were uninhibited at 1,600 µg/ml (data not shown) (Imazaki et al. 2006). Simply, isolates were either highly sensitive or highly resistant. Based on these preliminary results and previous research with *C. kikuchii* and other *Cercospora* spp., a discriminatory dose of TM at 5 µg/ml was chosen to distinguish between TM-sensitive and -resistant isolates (Bugbee 1995; Campbell et al. 1998; Imazaki et al. 2006). Dilution of a technical formulation (ChemService, Inc.) of TM (99.1%) was performed in acetone, added (1.0 ml/liter) to sterilized, molten PDA (approximately 50°C), and aseptically dispensed into sterile petri dishes (15 by 100 mm, approximately 20 ml/dish). PDA amended with only acetone (1.0 ml/liter) was included for comparison. All isolates from 2000 ($n = 176$), 2011 ($n = 160$), 2012 ($n = 82$), and 2013 ($n = 36$) were tested for TM sensitivity. With the aid of a cork borer, 6-mm mycelial discs were cut from stock cultures, inverted, and aseptically transferred to PDA containing TM at 0 or 5 µg/ml. Each experimental run consisted of all of the isolates from a given year replicated three times in a RCB design, with incubator shelves serving as blocks. Each experiment for each year was repeated once. Cultures were incubated as previously described for 5 days, then observed for radial growth. Isolates were considered sensitive if no radial growth was observed and resistant if any growth was observed. The χ^2 test of independence was used to compare the frequencies of TM-sensitive, TM-resistant, QoI-sensitive, and QoI-resistant isolates from 2011, 2012, and 2013.

Results

Determination of alternative respiration. Results from this study indicate that SHAM was toxic to isolates of *C. kikuchii* from 2000 in vitro. At a concentration of 20 µg/ml, SHAM reduced colony

growth by approximately 80% (data not shown). Therefore, SHAM was not used in subsequent assays. An alternative AOX inhibitor, propyl gallate, did not significantly affect radial growth of *C. kikuchii* and was used in assays to inhibit AOX (data not shown). There were no significant differences when comparing individual isolate EC₅₀ values with and without the addition of propyl gallate; therefore, it was surmised that AOX did not occur in baseline isolates of *C. kikuchii*, and AOX inhibitors were not utilized in subsequent experiments (Table 3).

Baseline sensitivity to QoI fungicides. The baseline sensitivity of *C. kikuchii* to 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 fourfold span. The distribution was nonnormal ($W = 0.8454$, $P < 0.0001$), with outliers toward the less-sensitive end of the spectrum and a median of 0.08 µg/ml (Fig. 1). Sensitivity values for pyraclostrobin had a 1,000-fold range overall; however, two

isolates on either end of the spectrum explain this phenomenon. The remaining 48 isolates represented in the pyraclostrobin sensitivity profile had EC₅₀ values ranging from 0.003 to 0.02 µg/ml, a 17-fold range. The distribution also was nonnormal ($W = 0.7258$, $P < 0.0001$), with a median of 0.01 µg/ml (Fig. 1). 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 fourfold span. A similar, nonnormal ($W = 0.7475$, $P < 0.0001$) distribution pattern was observed for isolates exposed to trifloxystrobin, with a median of 0.01 µg/ml (Fig. 1).

Resistance to QoI fungicides. Overall azoxystrobin EC₅₀ values for 2011, 2012, and 2013, were 365-, 517-, and 299-fold higher, respectively, when compared with the baseline from 2000 (Fig. 2). Similarly, pyraclostrobin EC₅₀ values from 2011, 2012, and 2013 indicated QoI resistance with values of 635-, 782-, and 500-fold higher, respectively (Fig. 3), while trifloxystrobin sensitivity profiles from the same years had EC₅₀ values of 1,507-, 1,947-, and 1,650-fold higher, respectively (Fig. 4). All QoI-resistant isolates exposed to more than one QoI fungicide in this study exhibited cross resistance. Median EC₅₀ values for all three QoI fungicides used in the study were significantly higher for 2011 to 2013 when compared with the baseline distribution, as determined by Kruskal-Wallis one-way analysis of variance followed by Dunn's post hoc comparison ($P = 0.05$) (Table 4).

QoI discriminatory doses. 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. There were sensitivity distribution gaps of 20-, 10-, and 13-fold for azoxystrobin, pyraclostrobin, and trifloxystrobin, respectively. Isolates falling on the low and high sides of the midpoints of these points were considered sensitive or resistant, respectively. Mean radial growth inhibition for isolates sensitive to all three QoI fungicides was significantly higher than resistant isolates at 0.1, 1, and 10 µg/ml (Fig. 5).

TM and multiple resistance. In the 2000 population, 41 of 176 (23%) *C. kikuchii* isolates from three parishes were confirmed to be TM resistant. In 2011, 2012, and 2013, 38, 29, and 36% of isolates, respectively, were TM resistant (Table 3). Isolates of *C. kikuchii* resistant to both TM and QoI fungicides were not detected in the 2000 population. In 2011, 2012, and 2013, 35, 26, and 31% of all tested isolates, respectively, were multiple resistant. In this study, TM-resistant isolates of *C. kikuchii* had a tendency for QoI resistance, with 44 of 45 (98%) TM-resistant isolates in 2011 also resistant to QoI fungicides. For 2012 and 2013, 16 of 16 (100%) and 11 of 13

Table 3. Effect of propyl gallate (PG) on mean effective fungicide concentration to inhibit 50% of fungal radial growth (EC₅₀) values for 20 isolates of *Cercospora kikuchii* subjected to azoxystrobin concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml in radial growth assays

Isolate number	Mean EC ₅₀ without PG	Mean EC ₅₀ with PG	<i>t</i> Value	<i>P</i> value
3	0.035	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
78	0.215	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

Fig. 1. In vitro baseline sensitivity of *Cercospora kikuchii* to azoxystrobin, pyraclostrobin, and trifloxystrobin as determined by the effective concentration that inhibited 50% of radial growth (EC₅₀ values) of 50, 50, and 49 isolates, respectively.

Fig. 2. In vitro sensitivity profiles of *Cercospora kikuchii* to azoxystrobin for 50, 50, and 36 isolates from 2011, 2012, and 2013, respectively.

Fig. 3. In vitro sensitivity profiles of *Cercospora kikuchii* to pyraclostrobin for 49, 50, and 36 isolates from 2011, 2012, and 2013, respectively.

Fig. 4. In vitro sensitivity profiles of *Cercospora kikuchii* to trifloxystrobin for 49, 50, and 36 isolates from 2011, 2012, and 2013, respectively.

(85%) TM-resistant isolates, respectively, also exhibited QoI resistance. A χ^2 analysis of the 2011, 2012, and 2013 populations revealed that 96% of TM-resistant isolates also were resistant to QoI fungicides, and TM resistance was not independent of QoI resistance ($\chi^2 = 13.4274$, $P = 0.0002$) (Table 5).

Discussion

All baseline sensitivity distributions in this study were skewed and, according to FRAC, nonnormal baseline distributions could be a “clear warning of resistance” (Russell 2004). Previous research with other *Cercospora* spp. detailed similar baseline distributions to QoI fungicides (Bradley and Pedersen 2011; Zhang et al. 2012a). In some cases where these nonnormal distributions were observed, resistance was confirmed to QoI fungicides in subsequent years (Kirk et al. 2012; Wise et al. 2009; Zhang et al. 2012b). Although EC_{50} 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 sensitivity in fungal pathogens (Avila-Adame et al. 2003; Pasche et al. 2004; Wise et al. 2008; Zhang et al. 2012a). Other studies using conidial germination to determine baseline sensitivities to QoI fungicides in *Cercospora* spp. indicated ranges of 5- to 17-fold (Bradley and Pedersen 2011; Zhang et al. 2012a). In the current study, overall sensitivity to azoxystrobin in vitro was approximately sixfold less when compared with pyraclostrobin and trifloxystrobin, which could be an indication that the former was less effective on *C. kikuchii*. However, similar phenomena have been observed in other studies, with overall sensitivities of azoxystrobin ranging from 3- to 10-fold less when compared with pyraclostrobin and trifloxystrobin (Bradley and Pedersen 2011; Pasche et al. 2004; Wise et al. 2008). Two studies featuring *C. zea-maydis* and *C. sojae* indicated differences

of 46- to 180-fold in toxicity of azoxystrobin compared with pyraclostrobin or trifloxystrobin in vitro (Bradley and Pedersen 2011; Zhang et al. 2012a). However, the difference in toxicity in this study may not translate to field efficacy, with other studies indicating similar efficacy on CLB among all three QoI fungicides in vivo (Delaney et al. 2012; Padgett and Purvis 2007).

In 2011, 2012, and 2013, azoxystrobin, pyraclostrobin, and trifloxystrobin sensitivity profiles showed clear indications of resistance. Other research has indicated large differences in sensitivity 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). In this study, 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 (Avila-Adame et al. 2003; Myresiotis et al. 2007). Radial growth assays are tedious and 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 also may prove useful in rapidly identifying QoI-resistant isolates in field studies and further delineating QoI resistance in Louisiana and throughout the United States.

Three point mutations, or amino acid substitutions, on the cytochrome *b* gene have been attributed to QoI resistance in fungal pathogens: G143A, F129 L, and G137R (FRAC 2015). Resistance conferred by mutations at the F129 L and G137R locations has generally been characterized as partial, with resistance factors ranging from 5 to 15 (FRAC 2015). On the other hand, a change at the G143A location results in complete QoI immunity with resistance factors in the hundreds (FRAC 2015). Because of the large resistance factors (500- to 2,000-fold) of isolates in this study, it is likely that the G143A substitution has occurred; however, additional research is needed to confirm which genetic factors are associated with this QoI resistance.

Cross sensitivity or resistance to QoI fungicides was observed in all instances in this study. Other research has demonstrated similar phenomena in other fungal pathogens when exposed to QoI fungicides (Pasche et al. 2004; Wise et al. 2009), which is indicative of the identical mode of action among the QoI fungicides. Because of this similarity between QoI fungicides, it is not advisable to replace one QoI fungicide with another in a disease management situation, and rotation to a different mode of action is ideal.

Results indicate that TM resistance has existed in the CLB pathogen population since 2000. Resistance to MBC fungicides in other plant-pathogenic fungi has been documented since the late 1960s (FRAC 2013). Previous research indicated 100% cross-resistance in fungal

Table 4. Median effective fungicide concentration to inhibit 50% of fungal radial growth values for isolates of *C. kikuchii* from 2000, 2011, 2012, and 2013 exposed to azoxystrobin, pyraclostrobin, and trifloxystrobin^z

Year	Azoxystrobin	Pyraclostrobin	Trifloxystrobin
2000	0.1 a	0.01 a	0.01 a
2011	37.2 b	10.1 b	20.1 b
2012	57.6 c	12.2 b	29.1 b
2013	24.2 b	6.5 b	19.8 b
<i>P</i> value	<0.0001	<0.0001	<0.0001

^z Medians followed by the same letter do not significantly differ as determined by Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparison test.

Fig. 5. Mean ($\pm$ standard deviation) radial growth inhibition of isolates of *Cercospora kikuchii* from 2000, 2011, 2012, and 2013, sensitive and resistant to azoxystrobin, pyraclostrobin, and trifloxystrobin at a range of concentrations.

isolates to MBC fungicides (Cunha and Rizzo 2003; Keinath and Zitter 1998; Wong et al. 2008). In *C. beticola*, a pathogen of sugar beet, MBC resistance was reported in Greece and the United States using methods similar to the current study (Bugbee 1995; Karaoglanidis et al. 2003). In Japan, resistance of *C. kikuchii* to TM was first discovered in the 1980s (Sakai 1999), with later validating work in 2006 (Imazaki et al. 2006). The results of this study indicate that TM resistance in *C. kikuchii* is widespread throughout soybean-producing areas in Louisiana. These findings suggest that the use of this fungicide type imposed a selection pressure that led to the rise 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 was interpreted as maintenance of stability and competitiveness of resistant isolates (Ishii et al. 1985; Ruppel et al. 1980). Because of this confirmation of MBC resistance, widespread occurrence, high selection pressure of applications, and stability of fungicide resistance in the pathogen, MBC fungicides used alone for management of CLB or purple seed stain should be discouraged in areas where resistant isolates have been confirmed.

Multiple resistance to fungicides has been previously reported in other plant pathogens (Chapman et al. 2011; Myresiotis et al. 2007). Multiple resistance to Organo-tin, MBC, and triazole fungicides also was documented in *C. beticola* (Bugbee 1995; Secor et al. 2010). In this study, isolates that were resistant to TM were nearly certain to also be resistant to QoI fungicides. This finding was unexpected and, as others have 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 (Koller and Wilcox 2001). Further research is needed to elucidate this proclivity.

This is the first documentation of QoI and TM resistance in *C. kikuchii* in the United States. According to these results, QoI and TM resistance in *C. kikuchii* appears to be commonplace and widespread in soybean-producing areas throughout Louisiana. Although baseline isolates were confined to three locations, the locations were distributed among northern, central, and southern production systems and were the only isolates available from that time period representing the only option for baselines in Louisiana. Because differences between the baseline isolates and subsequent collections were well defined, the baseline isolates appeared to be adequate for Louisiana. Because pathogen populations and production practices are diverse, other areas may see differing results; however, this research details valid methods that can be utilized for fungicide sensitivity studies in other areas.

Because of the discovery of resistance, solo applications of QoI and MBC fungicides to soybean for management of CLB in areas in Louisiana where resistance is likely are not advisable at this time. Unfortunately, there are currently no fungicide products that provide consistent control of CLB in Louisiana. Recently, there has been interest in applying demethylation inhibitor (FRAC group 3) products

for CLB management, with inconsistent results. More research is needed to truly define the effectiveness of triazole compounds and, because producers are applying these products, the CLB pathogen population should be monitored for resistance development. Additionally, succinate dehydrogenase inhibitors (SDHI; FRAC group 7) have increasingly been used in Louisiana soybean production, also with inconsistent results on CLB. More research is needed to define the efficacy of SDHI compounds as well as the sensitivity of the CLB pathogen population to these fungicides.

Acknowledgments

We thank the Louisiana Soybean and Grain Research and Promotion Board for partially funding this project and the numerous student workers, associates, and colleagues who contributed to this project.

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Table 5. Test of independence (χ^2) of isolates of *Cercospora kikuchii* collected in 2011, 2012, and 2013 ($\chi^2 = 13.4274$, $P = 0.0002$) sensitive and resistant to thiophanate methyl (TM) and quinone outside inhibitors (QoI)

	QoI ^z		
TM	S	R	Total
Sensitive			
Frequency	27	84	111
Observed	14.59	45.41	60.00
Row percentage	24.32	75.68	...
Column percentage	90.00	54.19	...
Resistant			
Frequency	3	71	74
Observed	1.62	38.38	40.00
Row percentage	4.05	95.95	...
Column percentage	10.00	45.81	...
Total	30	155	185
	16.22	83.78	100.0

^z S = sensitive and R = resistant.

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