Development of a Quantitative Polymerase Chain Reaction Detection Protocol for *Cercospora kikuchii* in Soybean Leaves and Its Use for Documenting Latent Infection as Affected by Fungicide Applications

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ABSTRACT

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Cercospora leaf blight (CLB) of soybean, caused by *Cercospora kikuchii*, is a serious disease in the southern United States. A sensitive TaqMan probe-based real-time quantitative polymerase chain reaction (qPCR) assay was developed to specifically detect and quantify *C. kikuchii* in naturally infected soybean plants. The sensitivity was 1 pg of genomic DNA, which was equivalent to about 34 copies of genome of *C. kikuchii*. Using this qPCR assay, we documented a very long latent infection period for *C. kikuchii* in soybean leaves beginning at the V3

growth stage (as early as 22 days after planting). The levels of biomass of *C. kikuchii* remained low until R1, and a rapid increase was detected from the R2/R3 to R4/R5 growth stages shortly before the appearance of symptoms at R6. The efficacy of various fungicide regimens under field conditions also was evaluated over a 3-year period using this qPCR method. Our results showed that multiple fungicide applications beginning at R1 until late reproductive stages suppressed the development of *C. kikuchii* in leaves and delayed symptom expression. Different fungicide chemistries also had differential effects on the amount of latent infection and symptom expression during late reproductive growth stages.

Additional keywords: cercosporin, *CTB6*, disease management, *Glycine max*.

Cercospora kikuchii (T. Matsumoto & Tomoy.) M.W. Gardner is the causal agent of *Cercospora* leaf blight (CLB) and purple seed stain (PSS) of soybean (24). This pathogen can affect all aerial parts of the plant including leaves, petioles, stems, pods, and seeds (21,34,36). PSS is widely distributed in all soybean growing regions of the world and primarily affects seed germination leading to reduced stands and poor seed quality. Symptoms of CLB include brick red lesions on petioles and the development of purplish or bronze colored leaves in the upper canopy during late reproductive growth stages (mid-R5 to R6) (12). CLB has been a minor disease in the southern United States, but it has become a serious concern to soybean producers in recent years. The disease has been reported in other major soybean producing states such as Iowa. CLB is capable of causing substantial yield losses, and it is associated with a disorder known as green stem in which stems remain green while pods and seeds progress to maturity (12,18,29,38,39).

Several attempts have been made to identify soybean cultivars with resistance to CLB. Moderate levels of resistance were found in some soybean lines, but there was no relationship between resistance to CLB and PSS because soybean cultivars resistant to CLB were susceptible to PSS and vice versa (24,36). Development of CLB symptoms depends upon environmental conditions with hot dry conditions at the end of the season leading to severe blight symptoms (*personal observations*). Because most commer-

cial cultivars lack resistance to *C. kikuchii*, many growers have resorted to early planting with short season maturity groups to avoid favorable conditions for development of CLB later in the season (22). In addition, the pathogen is genetically diverse and soybean cultivar disease response varies by location and time (3–5).

Another alternative in CLB management is the use of fungicides. However, results from extensive field evaluations, in which fungicides were applied to foliage at the mid-reproductive growth stage (R3) but before symptom expression, have not been effective (27,30).

Latent infection is defined as the state in which a host is infected with a pathogen but does not show symptoms (1). Earlier studies showed that *C. kikuchii* and other fungi often cause latent infections in soybeans (14,32). As a result, disease symptoms only become visible long after initial infection and usually at late growth stages when plants are under stress or there is a sudden change in nutritional or environmental conditions (6,14). We hypothesized that fungicide efficacy would be increased if the chemicals were applied during the latent infection stage which may occur earlier than R3 or R5. However, chemicals for disease management are normally applied between R3 and R5 for late season diseases. In order to time fungicide application at the latent infection period, it is necessary to establish when latent infection occurs. The advent of traditional polymerase chain reaction (PCR) made it possible to detect plant pathogens based on primers designed for a specific part of their genomic sequence. However, traditional PCR, which involves separation of reaction products on an agarose gel for visualization and qualitative analysis, is not suitable for automation when there is a need to screen a large number of samples. The development of high throughput real-time PCR technology, which uses primers and a fluorogenic

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probe to amplify a specific target sequence, allows monitoring the PCR amplification process in real time and the quantification of target sequence by measuring fluorescence signal without the need for post-PCR gel analysis. After the first successful application of real-time quantitative PCR (qPCR) for detecting *Phytophthora* strains in their host plants, it has been successfully employed in detecting several plant pathogens including fungi (2,20, 23,26), bacteria (8,35), and viruses (15,33). It also has been used to quantify the progression of pathogens based on absolute quantification of pathogen DNA (17).

In this study, we report the (i) development of a qPCR assay for quantifying genomic DNA of *C. kikuchii*, (ii) quantitative assessments of *C. kikuchii* using this qPCR protocol from infected soybean leaves during different plant growth stages before the onset of symptoms, and (iii) comparisons of DNA content of *C. kikuchii* in infected plants subjected to different fungicide treatments as an example of how this technology can be used in evaluating fungicide efficacy.

MATERIALS AND METHODS

Cercospora species. The *Cercospora* species and isolates used in this study are listed in Table 1. Isolates of *C. kikuchii* were recovered from infected soybean plants collected from fields in different locations in Louisiana in 2001 (3). The *C. sojina* isolate RWS1, causal agent of frog eye leaf spot in soybean, was recovered from a soybean plant near Baton Rouge, LA in 2007. All isolates were single-spored and stored on slants of potato dextrose agar at 5°C in the dark. Samples of genomic DNA for *C. sojina* isolate CS223, *C. zeae-maydis*, *C. zeina*, and *C. beticola* were provided by B. Bluhm (University of Arkansas, Fayetteville, AR).

Development of specific qPCR primers and probe for *C. kikuchii*. Because of limited genome sequence information available for *C. kikuchii*, 14 sets of PCR primers (Supplemental Table 1) specific for eight cercosporin toxin biosynthesis (CTB) pathway genes and four adjacent open reading frames (ORFs) in *C. nicotianae* were designed based upon known sequences (7). These primers were used to amplify their corresponding genes and ORFs from *C. kikuchii*. PCR was performed on genomic DNA from *C. kikuchii* and *C. sojina*. The CNCTB6 primer pair, which targeted the NADPH-dependent oxidoreductase gene (CTB6) from *C. nicotianae* (GenBank accession DQ991508), produced a specific amplification product from *C. kikuchii* but not from *C. sojina*. The PCR fragment amplified from *C. kikuchii* was confirmed to be the CTB6 gene by direct sequencing (data not shown). The qPCR primers (CKCTB6-2F: 5'-CACCATGCTA GATGTGACGACA-3', CKCTB6-2R: 5'-GGTCCTGGAGGCAG CCA-3') and the fluorescent probe (CKCTB6-PRB: 5'-FAM-CTCGTCGCACAGTCCCGCTTCG-TAMRA-3'), which was specific to the *C. kikuchii* CTB6 gene, were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) based on the region of CTB6 DNA sequences of *C. kikuchii* that are different

from *C. nicotianae*, and they were custom synthesized by IDT DNA, Inc. (Coralville, IA).

qPCR assay and quantification of DNA of *C. kikuchii* in field samples. qPCR was performed with MicroAmp optical 8-tube strips and optical 8-cap strips (Applied Biosystems, Foster City, CA) in the ABI 7000 sequence detection system (Applied Biosystems) under standard conditions. Each reaction contained 7.5 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl of 10 µM for each primer (final concentration 666 nM each primer), 0.6 µl of 10 µM (final concentration of 400 nM) probe, and 1 µl of 10 ng/µl or 100 ng/µl template DNA. HPLC grade water was added to adjust the final reaction volume to 15 µl. For standard curves, triplicates of 1 µl of serial dilutions of DNA of *C. kikuchii* (1 pg/µl to 1,000 pg/µl) in 10 ng/µl of healthy soybean DNA in order to simulate field samples were used as template. DNA from healthy soybean leaves was obtained from greenhouse-grown plants. In addition, each qPCR run of field samples also included *C. kikuchii* standards (1,000 pg, 100 pg, 10 pg, and 1 pg) in triplicate as references and DNA from *C. sojina* isolate RWS1 as a negative control to ensure specificity. DNA concentrations of *C. kikuchii* in each field sample were calculated as picogram of *C. kikuchii* DNA per nanogram of soybean DNA. For soybean samples with less than 1 pg of DNA of *C. kikuchii* in 10 ng of soybean DNA (Ct value > 37.75), a 100 ng DNA template was used in a second qPCR reaction and the resulting Ct value was converted to picogram of pathogen DNA per nanogram of soybean DNA. The absolute quantity of DNA of *C. kikuchii* in field samples, which was used to determine the level of infection by *C. kikuchii*, was calculated by using the Ct value of each sample and the regression equation obtained with DNA standards.

Plant materials, sampling details, and fungicide sprays. Field studies to determine when soybean plants became infected with *C. kikuchii* using qPCR were conducted in 2007 (first planting [22 May], second planting [5 June], and third planting [27 June]), 2009, and 2010. Field experiments in each year were arranged in randomized complete blocks. Each treatment (field plot) in the experiments described in Table 2 was replicated three times with each plot consisting of four rows on 76 cm centers by 12 m long. Plants were maintained according to standard practices (19). The following soybean cultivars, all of which were susceptible to CLB in previous evaluations, were included in these experiments: RC 5892 (Croplan Genetics, St. Paul, MN), DP 5634 RR and DP 5808 RR (Asgrow Seed Co., Kalamazoo, MI), and Pioneer 95Y20 (Pioneer Hi-Bred International, Johnston, IA). The following fungicides (rates of application), which are representative of the triazole and strobilurin chemistries that are commonly used in soybean production, were used: flutriafol (512 ml ai/ha) (Topguard, Cheminova, Research Triangle Park, NC); metconazole (548 ml ai/ha) (Caramba) plus pyraclostrobin (322 ml ai/ha) (Headline) (BASF Corp., Research Triangle Park, NC); and pyraclostrobin alone (877 ml ai/ha). Fungicides were

TABLE 1. *Cercospora* species tested for specificity of the quantitative polymerase chain reaction (qPCR) primers and probe developed in this study for *C. kikuchii*

<i>Cercospora</i> species	Isolate	Host	Source	Detection ^a
<i>C. kikuchii</i>	MRL 6020-2B ^b	Soybean	Leaf	Yes
<i>C. kikuchii</i>	DLL 6013-1B ^b	Soybean	Leaf	Yes
<i>C. kikuchii</i>	DLL 50908-1B ^b	Soybean	Leaf	Yes
<i>C. kikuchii</i>	MRS 6020-2B ^b	Soybean	Seed	Yes
<i>C. kikuchii</i>	MRS 5012-1A ^b	Soybean	Seed	Yes
<i>C. sojina</i>	RWS1	Soybean	Leaf	No
<i>C. sojina</i>	CS223 ^c	Soybean	Leaf	No
<i>C. zeae-maydis</i>	SCOH1-5 ^c	Maize	Leaf	No
<i>C. zeina</i>	DYPA ^c	Maize	Leaf	No
<i>C. beticola</i>	S1 ^c	Sugarbeet	Leaf	No

^a Yes indicates significant amplification in qPCR and No indicates no amplification.

^b See Cai and Schneider (3).

^c DNA obtained from B. Bluhm, University of Arkansas.

applied with a tractor mounted, four-row spray boom fitted with flat spray nozzles on 38 cm centers in a total volume of 175 liters/ha at 275 kPa. Leaf samples were collected from plants in each plot for each treatment. If plants were scheduled to be sprayed with fungicides on a sampling date, samples were collected immediately before spraying. Each sample consisted of 30 randomly collected uppermost fully expanded leaflets from the center two rows in each plot. Uppermost leaflets were used because CLB symptoms first appear on upper leaflets that are exposed to sunlight (12). Leaf samples were frozen in liquid nitrogen and stored at -80°C until further processing.

CLB disease severity ratings. CLB does not lend itself to quantitative estimates of disease severity, e.g., percent leaf area affected, because symptoms begin as small brick red necrotic lesions on petioles and progress ultimately to the classic leathery purple discoloration of leaves exposed to direct sunlight. Therefore, the following semiquantitative scale, which has been used in previous studies for assessing disease severity, was used: 1 = no symptoms; 2 = few symptoms on petioles (up to 10% of petioles exhibit symptoms); 3 = all symptoms of category 2 present plus up to 5% of leaves affected including leaf veins; 4 = all symptoms of category 3 present plus up to 10% of leaves affected; 5 = all symptoms of category 4 present plus approximately 50% petioles and up to 30% of leaves with symptoms; 6 = up to 100% of petioles affected but leaves do not have reddish cast and are not chlorotic; 7 = up to 20% of upper leaves becoming reddened or chlorotic; 8 = moderate chlorosis, reddening and necrosis on up to 50% of upper leaves; and 9 = symptoms as in category 8 and symptoms are severe plus defoliation has occurred (27,31). This scale (1 to 9) was converted to quantitative increments (0 to 100%) (1 = 0%; 2 = 5%; 3 = 10%; 4 = 15%; 4.5 = 20%; 5 = 30%; 5.5 = 45%; 6 = 60%; 7 = 80%; 7.5 = 90%; 8 = 95%; and 9 = 100%) by assessing percent incidence and severity of petiole symptoms (31). These assessments were used in all statistical analyses. At least 20 plants in the center two rows were examined, and a mean value was assigned to each plot. Disease incidence was 100% when plants were rated during all 3 years. CLB ratings were done at R6 during all years except for the third planting in 2007 for which we could not obtain CLB ratings because of the development of green stem syndrome.

DNA extraction. All 30 leaflets from each sample were ground to a fine powder in liquid nitrogen with a prechilled mortar and pestle. Three subsamples of 100 mg each were transferred to 2.0-ml microcentrifuge tubes, and DNA was extracted with a GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. DNA from *C. kikuchii* and *C. sojina* was extracted with the same kit using 100 mg of lyophilized ground mycelial powder. Mycelia were collected by filtration from 5-day-old cultures grown in potato dextrose broth on rotary shakers in the dark. Mycelia were

washed three times with sterile distilled water before being lyophilized, ground with a mortar and pestle, and stored at -80°C until further processing. All DNA samples were diluted to 10 ng/ μl based on DNA quantification by measuring absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). The uniformity of diluted genomic DNA was verified using qPCR assays with primers specific for plant 18S rRNA (Zm18s1F: 5'-GAGAAACGGC TACCACATCCA-3' and Zm18s1R: 5'-ACGCGCCCGGTATT GTTAT-3') and SYBR Green dye chemistry on eight randomly chosen leaf samples, all of which yielded similar Ct values with a mean of 14.95 ± 0.2 standard deviations.

Data analysis. Analysis of variance using the mixed procedure (PROC MIXED) of SAS (version 9.1, SAS Institute, Cary, NC) was performed on data from the fungicide-sprayed and non-sprayed plants. Means were compared by Tukey's honestly significant difference at $P < 0.05$ with Kramer's adjustment for unbalanced design (16).

RESULTS

Development of qPCR primers specific for *C. kikuchii*. CNCTB6F and CNCTB6R primers (Supplemental Table 1) amplified a 1.14-kb PCR product only in the presence of genomic DNA from *C. kikuchii* but not from *C. sojina* (Supplemental Figure 1). When field samples were tested, these primers produced a specific product from soybean leaves either with CLB symptoms or seedlings grown from purple stained seeds but not from healthy leaves collected from greenhouse-grown plants. These results indicated that the amplification of *CTB6* gene by CNCTB6F and CNCTB6R primers can be used to differentiate between *C. kikuchii* and *C. sojina* and that the primers can detect *C. kikuchii*. Homology analysis of the sequenced PCR product (GenBank accession JF830016, this study) showed that the *CTB6* gene shared 97% sequence identity between *C. kikuchii* and *C. nicotianae* (data not shown). The DNA region containing the most sequence differences between *C. kikuchii* and *C. nicotianae* was used to design qPCR primers and probe (Fig. 1). The specificity of qPCR primers CKCTB6-2F and CKCTB6-2R and fluorescent

Fig. 1. Quantitative polymerase chain reaction (qPCR) target region of *CTB6* from *Cercospora kikuchii* (CK) (GenBank accession JF830016) and *C. nicotianae* (CN) (GenBank accession DQ991508). Open boxes indicate variable nucleotides between CK and CN. Arrows indicate location of qPCR primers (CKCTB6-2F and CKCTB6-2R) and probe (CKCTB6-PRB) developed in this study.

TABLE 2. Experimental details for field studies related to *Cercospora* leaf blight of soybean conducted over a 3-year period

Experiment	Year	Planting date	Cultivar	Growth stage at sampling ^a	Sampling date (DAP) ^b	Fungicide application (DAP)	Fungicide treatment ^c
A	2007	22 May	RC 5892 DP 5634 RR	R2 – R6	58, 78, 85, 107	62, 78	Pyraclostrobin plus metconazole
B	2007	5 June	RC 5892 DP 5634 RR	V8 – R6	44, 64, 71, 93	64	Pyraclostrobin plus metconazole
C	2007	27 June	RC 5892 DP 5634 RR	V3 – R5	22, 42, 57, 71	42	Pyraclostrobin plus metconazole
D	2009	15 May	DP 5808 RR	V4 – R7	35, 52, 59, 70, 76, 85, 97, 109, 116, 124	48, 55, 74, 90, 103	Pyraclostrobin
E	2010	26 May	95Y20	V5 – R5	33, 44, 58, 100	30, 51, 77, 99, 113	Flutriafol

^a See Fehr et al. (9) for description of soybean growth stages.

^b DAP, days after planting.

^c Text provides foliar application details.

probe CKCTB6-PRB was tested by performing qPCR with genomic DNA from *C. kikuchii*, *C. sojae*, *C. zeae-maydis*, *C. zeina*, *C. beticola*, greenhouse-grown healthy soybean leaves (negative control), and the no-template control (NTC). Amplification was detected only in the presence of *C. kikuchii* genomic DNA as assessed by changes in fluorescence intensity. No significant fluorescence was detected in qPCR containing genomic DNA from the other *Cercospora* species, healthy soybean, or NTC.

Standard curves and sensitivity of qPCR assays. Quantification of serially diluted DNA of *C. kikuchii* showed a linear relationship (Fig. 2A) between the amount of genomic DNA (x) and Ct values (y) ($y = -1.434\ln(x) + 37.913$) with an R^2 value of 0.9984 and a slope of -3.3 , indicating a nearly 100% amplification efficiency (Fig. 2B). Mean Ct values for *C. kikuchii* DNA ranged from 27.87 for 1,000 pg to 37.75 for 1 pg, which was reliably detected in all samples. Therefore, the lowest detection limit of our assay for DNA of *C. kikuchii* was 1 pg in 10 or 100 ng of soybean DNA. The addition of as much as 1 ng of DNA from *C. sojae* per 10 ng of soybean DNA still produced a negative result ($Ct \geq 40$), which confirmed the high specificity of this assay.

Visual rating of disease severity and quantification of *C. kikuchii* DNA in soybean leaf samples using qPCR. For the first and second plantings in 2007 (experiments A and B, Table 2), CLB symptoms were more severe in the nonsprayed DP 5634 RR plants compared with sprayed plants ($P = 0.01$) (Fig. 3A), whereas there was no difference in disease severity between fungicide-sprayed and nonsprayed RC 5892 plants. No symptoms were observed for leaf samples of either cultivar collected at the last sampling (R5 stage) from the third planting (experiment C, Table 2). DNA of *C. kikuchii* was detected in all samples of all three plantings from V3 through R6. In general, the fungal DNA levels were lower in plants sprayed with fungicides compared to nonsprayed controls. In the first planting, the amount of DNA of *C. kikuchii* in soybean leaves collected from nonsprayed plants from R2 to R4 significantly increased from 0.02 to 1.32 pg and 0.11 to 1.86 pg in cultivars RC 5892 and DP 5634 RR ($P < 0.0001$), respectively, while it remained at low levels in sprayed plants until R4 in both cultivars (Fig. 3B). Fungal DNA levels did not change from R4 to R6 in nonsprayed or sprayed plants in both cultivars, but had significantly lower levels in sprayed plants compared to nonsprayed plants. Soybean plants receiving two fungicide sprays at 62 days after planting (DAP) (R2) and 78 DAP (R4) had very low levels of fungal DNA compared to nonsprayed plants (Fig. 3B). In the second planting (experiment B, Table 2), the amount of DNA of *C. kikuchii* detected in nonsprayed soybean plants from V8 to V9 to R6 increased from 0.03 to 1.35 pg in RC 5892 ($P < 0.0001$) and from 0.1 pg at R2 to 3.12 pg at R6 in DP 5634 RR ($P < 0.0001$) (Fig. 3C). However, for the fungicide-treated plants, pathogen DNA increased from R5 to R6 in RC 5892 but was unchanged in DP 5634 RR. Cultivar DP 5634 RR had consistently higher levels of fungal DNA compared to RC 5892 in nonsprayed and sprayed plants at every growth stage (Fig. 3C). For the third planting (experiment C, Table 2), DNA of *C. kikuchii* was detected as early as 22 DAP at V4. Fungal DNA remained low until R3 in both cultivars whether sprayed or nonsprayed but increased from R3 to R5 (Fig. 3D). The levels of *C. kikuchii* detected at R5 in the third planting were lower than those samples collected at R5 from the first and second plantings. For RC 5892, the levels of *C. kikuchii* detected in nonsprayed plants was higher than plants sprayed at R5, but there was no significant difference between these two treatments for DP 5634 RR.

In 2009 (experiment D, Table 2), there was no significant difference in CLB severity ($P = 0.37$) between sprayed and nonsprayed plants for DP 5808 RR (Fig. 4A). There was no significant change in fungal DNA from V4 to R4 in nonsprayed plants and from V4 to R5 in sprayed plants (Fig. 4B). However, fungal

DNA increased from 1.15 pg at late R4 to 20.4 pg at R7 in nonsprayed plants ($P < 0.0001$) and increased from 2.41 pg at R6 to 11.67 pg at R7 in sprayed plants ($P < 0.0001$). Nonsprayed plants were about 7 to 10 days ahead of sprayed plants with regard to fungal DNA level. There was almost a twofold increase in fungal DNA in nonsprayed plants compared to sprayed plants at R7 (Fig. 4B).

In 2010, CLB symptoms were more severe in nonsprayed plants and plants with one spray (1S) compared with plants with three (3S) and five (5S) sprays ($P = 0.0001$) (experiment E, Table 2) (Fig. 5A). There was no significant change in fungal DNA from V5 to R2 in any treatment. However, at R5, nonsprayed plants had the highest content of DNA of *C. kikuchii* (4.99 pg), followed by plants with one and three sprays (Fig. 5B). The least amount of fungal DNA was detected in plants with five sprays (Fig. 5B). There was a significant difference in the amount of fungal DNA between sprayed and nonsprayed plants at R5. In all sprayed plants, there was a more than 10-fold increase in *C. kikuchii* DNA from R2 to R5. However, the rate of increase in the nonsprayed plants was much higher (Fig. 5B).

DISCUSSION

CLB has become a serious concern for soybean producers in the southern United States in recent years because most soybean cultivars are susceptible (29) and estimated yield loss caused by *C. kikuchii* in the United States in 2007 was 2.7 million bushels (39). Given the lack of disease resistance, the use of fungicides

Fig. 2. Quantitative polymerase chain reaction (qPCR) amplification plots and standard curves for serial dilutions of DNA of *Cercospora kikuchii*. **A**, Representative qPCR fluorescent amplification curves for *C. kikuchii* DNA concentrations from left to right are 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 0.1 pg in triplicate. Each standard was spiked with 10 ng of soybean DNA. **B**, Data represent mean cycle threshold (Ct) values of at least six replicates of each dilution and error bars indicate standard deviation. Ct values were plotted against known DNA concentrations of *C. kikuchii* and the linear regression equation for calculating quantity of DNA of *C. kikuchii* in soybean leaf sample is shown inside the graph.

has become the primary means by which this disease is managed. However, management of CLB with fungicides has been less than satisfactory even though *C. kikuchii* is sensitive to commonly used fungicides in in vitro tests (28). We hypothesized that this apparent inconsistency may be attributed to a long latent period during which the pathogen may become established within leaf tissues in sites where fungicide concentration may be too low to suppress subsequent symptom expression. Thus, fungicides would have to be applied during or prior to initial infection, and there would have to be sufficient residual activity or additional applications to suppress pathogen development during reproductive stages of plant growth. Cerkaskas and Sinclair (6) first observed the development of *C. kikuchii* lesions on stems and pods of soybean plants that had been dipped in a solution of the herbicide paraquat 2 weeks earlier compared with nondipped tissues. They suggested that the pathogen exists in a latent state in soybean tissues until the inducement of plant senescence by the herbicide (6). Klingelfuss and Yorinori (14) also observed latent infection of soybeans at the R5 stage and they went on to show that symptoms developed even after three applications of diphenconazole during the pod filling stage of development (R5.2 to R5.5). However, as they did not sample at earlier vegetative or reproductive stages, the leaves could have been infected before the R5.2 stage of development (14). In this study, we documented an extended latent period and showed that fungicides had a greater impact on pathogen development within the host as application occurred early. Our findings suggest that it is essential to apply fungicides during the latent period even though symptoms may not become ap-

parent for several weeks. Fungicide applications at R1 before the rapid increase in pathogen biomass (R2/R3 to R4/R5), likely would result in the highest level of disease suppression. This study quantified the fungal biomass present over time using qPCR and assessed the effects of fungicide applications on the increase in fungal biomass. Furthermore, we showed that cultivar may also be a factor in pathogen development. To our knowledge this is the first report of using qPCR for detection and quantification of *C. kikuchii* in soybean plants.

The qPCR primer set (CKCTB6-2F and CKCTB6-2R) and probe (CKCTB6-PRB), which were based on the *CTB6* gene sequence from *C. kikuchii*, produced a specific amplicon using genomic DNA from *C. kikuchii* but not from other *Cercospora* species tested in this study. The detection limit was 1 pg of *C. kikuchii* DNA in the presence of 10 to 100 ng of soybean DNA. This was equivalent to 34 copies of the genomes, which is based upon an estimate of the genome size of *C. kikuchii* of 28.4 Mb (or equivalent of 0.03 pg) (13). The sensitivity was similar in the absence of soybean DNA with the detection limit of 1 pg of *C. kikuchii* DNA. However, in order to simulate soybean leaf samples, the standard curve was constructed with serial dilutions of *C. kikuchii* genomic DNA in the presence of 10 ng of soybean genomic DNA. A similar approach was used in quantifying *Fusarium virguliforme* in artificially inoculated soybean roots (20). The qPCR protocol developed in this study was quantitative over four orders of magnitude (1,000 pg to 1 pg) of fungal genomic DNA; these results were similar to those obtained by Guo et al. (11). The relatively higher detection limit compared with other studies with

Fig. 3. *Cercospora* leaf blight severity ratings and quantification of DNA of *Cercospora kikuchii* in leaves of soybean cultivars RC 5892 and DP 5634 RR in 2007 in nonsprayed (U) and sprayed (S) plants. Arrows indicate the days after planting (DAP) when fungicides were applied. **A**, Bars represent mean disease severity of three plots within each treatment, and error bars indicate standard deviation. Groups of means with the same letters were not significantly different according to Tukey's honestly significant difference test at $P < 0.05$. **B**, First planting. Soybean leaf samples were collected at R2 (58 DAP), R4 (78 DAP), R5 (85 DAP), and R6 (107 DAP) growth stages. In sprayed treatments, a mixture of pyraclostrobin and metconazole was applied at R2 (62 DAP) and R4 (78 DAP) growth stages. Error bars indicate standard deviation. **C**, Second planting. Soybean leaf samples were collected at V8-V9 for RC 5892 and R2 for DP 5634 RR (44 DAP), R3-R4 (64 DAP), R5 (71 DAP), and R6 (93 DAP) growth stages. In sprayed treatments, a mixture of pyraclostrobin and metconazole was applied at R3 to R4 (64 DAP) growth stage. Error bars indicate standard deviation. **D**, Third planting. Soybean leaf samples were collected at the following growth stages: V3 for RC 5892 and V3 to V4 for DP 5634 RR (22 DAP), R1 to R2 for RC 5892 and R2 for DP 5634 RR (42 DAP), R2 (49 DAP), R3 (57 DAP), and R5 (71 DAP). In sprayed treatments, a mixture of pyraclostrobin and metconazole was applied at R1 to R2 (42 DAP).

femtogram sensitivity may be attributed to the use of internal transcribed spacer (ITS) sequences used in other studies (25) that have far more copies than structural genes, such as the toxin biosynthetic pathway gene (*CTB6*) that we used in this study. Also, using the cercosporin pathway gene was important in differentiating *C. kikuchii* from *C. sojae* because both are capable of infecting soybean, but only *C. kikuchii* is known to produce cercosporin (10). In addition, a BLAST search done at NCBI revealed that the ITS1 and ITS2 sequences of *C. kikuchii* share 97 and 100% similarity with *C. sojae*, respectively, and 100% similarity with other species of *Cercospora*, such as *C. beticola*, *C. apii*, *C. capsici*, and *C. rodmanii*. These similarities render ITS sequences of *C. kikuchii* of little use for developing species-specific qPCR primers.

C. kikuchii was detected in soybean leaf samples during the V3 to V4 stage, long before the development of visible symptoms at the R6 stage. We documented a general trend of increasing amount of fungal DNA in nonsprayed soybean plants from R2/R3 to R4/R5 during 2007 and 2010 and post-R4 during 2009. These findings suggest that latent infection progresses until plants begin reproductive growth, at which time there is a steady increase in fungal biomass leading ultimately to symptom development. We believe that this increase in fungal biomass is not caused by secondary infection because we have not observed any sporulation by the pathogen on surfaces of purple leaves, which is the classic symptom of CLB and first appears during early R6. We have observed sporadic sporulation on blighted leaves during late

R6 that may cause secondary infections. In addition to the results shown here, time-of-application experiments clearly showed that fungicide applications during late vegetative or early reproductive growth stages were most effective in managing CLB (28).

Results from this study indicated that latent infection responded differentially to different fungicide combinations, which may be a reflection of the greater systemic activity of triazole fungicides used in this study. While systemicity is not the only determinant of fungicide efficacy, this trait would be essential for suppressing infection if a single application was made at R1. In addition, residual activity would be required if the sudden increase in fungal biomass occurred at R3 or later. In 2007, the cultivars sprayed with a mixture of pyraclostrobin and metconazole for the first two plantings had significantly lower levels of fungal DNA at later stages of soybean growth compared with nonsprayed controls. Whereas in 2009, multiple applications of pyraclostrobin alone only delayed the onset of rapid pathogen development by one growth stage compared with nonsprayed controls. However, in 2010, a single flutriafol application at V5 was enough to limit development of *C. kikuchii* as there was no significant difference in fungal DNA content between the treatment receiving the single V5 spray and the treatment receiving three sprays with the last one being applied at R3. The treatment receiving five sprays, with the last one occurring at R6, had the lowest fungal DNA content at the end of the season. However, three and five fungicide applications are impractical commercial practices for managing CLB. Clearly, fungicides must be applied during early latent stages in

Fig. 4. *Cercospora* leaf blight disease severity ratings and quantification of DNA of *Cercospora kikuchii* in leaves of soybean cultivar DP 5808 RR in 2009. Arrows indicate days after planting (DAP) when fungicides were applied. **A**, Bars represent mean disease severity of three plots within each treatment, and error bars indicate standard deviation. Letters represent groups of means that were determined according to Tukey's honestly significant difference test. Different letters indicate significant differences at $P < 0.05$. **B**, Soybean leaf samples were collected at the following growth stages: V4 (35 DAP), early R2 (52 DAP), late R2 (59 DAP), R3 (70 DAP), R4 (76 DAP), late R4 (85 DAP), R5 (97 DAP), R6 (109 DAP), late R6 (116 DAP), and R7 (124 DAP). In sprayed treatments, pyraclostrobin was applied at the following growth stages: R1 (48 DAP), R2 (55 DAP), R3 (74 DAP), R4 (90 DAP), and R5 (103 DAP). Error bars indicate standard deviation.

Fig. 5. *Cercospora* leaf blight disease severity ratings and quantification of DNA of *Cercospora kikuchii* in leaves of soybean cultivar Pioneer 95Y20 in 2010. Arrows indicate days after planting (DAP) when fungicides were applied. **A**, Bars represent mean disease severity of four plots within each treatment, and error bars indicate standard deviation. Different letters indicate significant differences ($P < 0.05$) according to Tukey's honestly significant difference test. **B**, Soybean leaf samples were collected at the following growth stages: V5 (33 DAP), R1 (44 DAP), R2 (58 DAP), and R5 (100 DAP). Flutriafol was sprayed at V5 (30 DAP) in the treatment with one spray (1S); at V5 (30 DAP), R1 to R2 (51 DAP), and R3 (77 DAP) in the treatment with three sprays (3S); and at V5 (30 DAP), R1 to R2 (51 DAP), R3 (77 DAP), R5 (99 DAP), and R6 (113 DAP) in the treatment with five sprays (5S). Error bars indicate standard deviation.

order to suppress pathogen development. This conclusion was recently bolstered by the findings of Schneider et al. (31) who demonstrated that a single application of flutriafol during late vegetative stages or at first flower (R1) was more efficacious than multiple applications beginning at R3 or later. We recognize that these results were obtained in different years and from different cultivars; however, similar trends were observed across years and cultivars.

It is likely that fungicidal control of other diseases with protracted latent infection periods would benefit from similar studies. For example, the soybean rust pathogen, *Phakopsora pachyrhizi*, also has an extended latent period (up to 60 days), and fungicide applications are most efficacious when applied shortly before the rapid increase in fungal biomass, which occurs about 2 weeks before pustules become evident (37).

With the exception of the cultivar DP 5634 RR in 2007, there was not a clear relationship between DNA content of *C. kikuchii* and disease severity. This may be attributed to the effects of environment on symptom expression. For example, CLB is generally most severe when plants are exposed to hot, dry conditions late in the season. It is possible that the pathogen is induced to produce more cercosporin in the plant or the plant may be more sensitive to the toxin under these conditions even though fungal biomass may not be affected. In addition, cultivars may respond differently to equivalent amounts of endophytic fungal biomass within their tissues. These relationships warrant further study.

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