Screening a Diverse Soybean Germplasm Collection for Reaction to Purple Seed Stain Caused by *Cercospora kikuchii*

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Abstract

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Purple seed stain (PSS), caused by *Cercospora kikuchii*, is a prevalent soybean disease that causes latent seed infection, seed decay, purple seed discoloration, and overall quality deterioration. The objective of this research was to screen soybean accessions from the United States Department of Agriculture germplasm collection for resistance to PSS. In total, 123 plant introductions (PI) from 28 different countries, representing maturity groups (MG) III, IV, and V, were screened. Incidence of *Cercospora* leaf blight (% CLB), visual PSS (% PSS), and seed infected by *C. kikuchii*

(% *C. kikuchii*) in harvested seed were determined. In 2007, % *C. kikuchii* was 2 to 51% for MG III, 2 to 35% for MG IV, and 0 to 33% for MG V. In 2008, % *C. kikuchii* was 0 to 45% for MG III, 1 to 71% for MG IV, and 0 to 15% for MG V. In total, four and ten PI from MG III and IV, respectively, were identified as resistant to PSS in both years. Highly positive correlations were found for inoculated versus noninoculated treatments and for % PSS versus % *C. kikuchii* infection. The PSS-resistant PI identified in this study will be valuable to breeders in developing resistant cultivars.

Cercospora kikuchii (Tak. Matsumoto & Tomoy.) M. W. Gardner) is a widespread soybean pathogen that causes *Cercospora* blight, leaf spot, and purple seed stain (PSS; 6,22). PSS is present in every region of the world where soybean crops are grown (17). PSS was first reported in 1924 in Indiana (4,22), and occurs in almost every soybean-growing region in the United States (6).

Although PSS has not been reported to cause significant yield reduction in soybean (22), it can cause a significant reduction in overall seed quality. Latent seed infection by *C. kikuchii* results in seed decay with reduced vigor, purple seed discoloration, and overall seed deterioration, which can lead to a reduction of seed-lot grade and value when brought to commodity and food-grade markets (15,22,23,27,29,32).

Seed infection and disease development are favored by high temperature and humidity during the early reproductive stages of soybean (21). Leaf and pod infection occurs at temperatures of 15 to 32°C (11,20), although optimum infection occurs at 25°C (19). In addition, 18 to 24 h of dew is favorable for optimum leaf and pod infection (11,20). However, infection can occur as long as humidity is high enough for conidia to germinate and infect soybean, even during dry weather conditions that usually occur later in the growing season (27). Symptoms of *C. kikuchii* manifest as discolorations on leaves, petioles, stems, pods, and seed. Leaf and pod discoloration is characterized by reddish-to-purple areas with a leathery, bronzing appearance. Symptoms on soybean seed manifest as pale to dark-purple discolorations that range from covering small portions to the whole surface of the seed (22).

Strategies to control the disease include tillage, crop rotation (1), fungicide applications at pod-filling stages (25), and the use of genetic resistance (7,8,16,24,31). Differences in resistance, ranging from susceptible to resistant, were found in a screening of 17 soybean cultivars for foliar, seed, and asymptomatic infection by *C. kikuchii* (14). Resistance to *C. kikuchii* was found in plant introduction (PI) 80837, with heritability estimations of 0.91 in the F_2 and 0.51 in

the F_3 generation (16,31), and was governed by a single, dominant gene (8). The use of *C. kikuchii*-resistant genotypes to control the disease is economical and environmentally friendly. With this in mind, soybean accessions are excellent sources of genetic diversity, playing a role as gene banks for the improvement of soybean germplasm. However, a comprehensive screening of the United States Department of Agriculture (USDA) Soybean Germplasm Collection has not been conducted to identify new sources of resistance to PSS.

The objective of this study was to characterize the reaction of soybean accessions from a selection of USDA germplasm by inoculation with a field isolate of *C. kikuchii*. In this article, we describe the reaction of 123 PI from 28 different countries representing maturity groups (MG) III, IV, and V, which were screened in the field during 2007 and 2008 at Kibler, AR.

Materials and Methods

***C. kikuchii* isolates.** Seed with purple discoloration from soybean grown in Kibler, AR in 2006 were used to obtain local isolates of *C. kikuchii*. Seed were disinfested with 0.5% NaOCl for 90 s and rinsed twice with distilled water. Disinfested seed were then placed on Difco potato dextrose agar (PDA; Becton, Dickinson, and Company, Franklin Lakes, NJ) medium amended with rifampicin (100 mg/liter), ampicillin (0.25 g/liter), and fenpropathrin ([RS]-a-cyano-3-phenoxybenzyl-2,2,3,3-tetramethyl-cyclopropanecarboxylate) (0.14 mg/liter). Once colonies were formed, they were transferred to a fresh petri dish with PDA for purification and isolation of multispore isolates. Isolates were visually classified, based on morphology, as *C. kikuchii*, and stored at 4°C. Pathogenicity of the multispore isolates was evaluated and transfers from selected isolates with proven pathogenicity were used to generate colonies on V8 media (14). The V8 medium for inoculum preparation was made by autoclaving a mixture of 800 ml of distilled water, 200 ml of V8, 3 g of CaCO₃, and 20 g of Difco agar.

Inoculum preparation. Sporulation of *C. kikuchii* was induced as described by El-Gholl et al. (2) and Schuh (19). Colonies from PDA-grown isolates of *C. kikuchii* were macerated with the agar under a laminar-flow hood in an autoclaved Waring blender jar. The slurry was then spread onto V8 media in petri dishes that were subjected to 12 h of darkness and 12 h of white fluorescent light at 25°C for 15 days to induce conidial production (14,19).

Inoculum was prepared as described by Orth and Schuh (14), with some modifications. Briefly, sporulating cultures were mixed with

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Table 1. List of soybean accessions used for field screening of *Cercospora* leaf blight in Arkansas

Line	MG ^a	Origin
PI 62202	III	China
PI 68609	III	China
PI 79797	III	China
PI 88486	III	China
PI 88490	III	China
PI 88492	III	China
PI 92659	III	China
PI 104708	III	Poland
PI 153243	III	Belgium
PI 153292	III	France
PI 153309	III	France
PI 189891	III	France
PI 189920	III	France
PI 189969	III	France
PI 229738	III	Algeria
PI 235339	III	Uruguay
PI 283331	III	Morocco
PI 283332	III	Morocco
PI 398620	III	South Korea
PI 398697	III	South Korea
PI 398726	III	South Korea
PI 398752	III	South Korea
PI 404154	III	Georgia
PI 404160	III	Georgia
PI 407903	III	South Korea
PI 407907	III	South Korea
PI 408026	III	South Korea
PI 408321	III	South Korea
PI 416988	III	Japan
PI 417015	III	Japan
PI 417094	III	China
PI 417361	III	Japan
PI 417559	III	Poland
PI 424155	III	South Korea
PI 424159	III	South Korea
PI 424247	III	South Korea
PI 424444	III	South Korea
PI 424550	III	South Korea
PI 437124	III	Georgia
PI 437338	III	Russian Federation
PI 437357	III	Russian Federation
PI 437482	III	Russian Federation
PI 437710	III	China
PI 438310	III	North Korea
PI 438312	III	Algeria
PI 438335	III	Algeria
PI 438341	III	Algeria
PI 438357	III	Bulgaria
PI 438409	III	Germany
PI 438427	III	India
PI 438435	III	Morocco
PI 438501	III	United States
PI 458111	III	South Korea
PI 458123	III	South Korea
PI 464920	III	China
PI 476922	III	Vietnam
PI 479722	III	China
PI 504481	III	Taiwan
PI 504487	III	Taiwan
PI 504488	III	Taiwan
PI 504504	III	Taiwan
PI 506528	III	Japan
PI 506592	III	Japan
PI 506766	III	Japan
PI 506769	III	Japan

(continued in next column)^a Maturity group.**Table 1.** *(continued from preceding column)*

Line	MG ^a	Origin
PI 506883	III	Japan
PI 506894	III	Japan
PI 507016	III	Japan
PI 507189	III	Japan
PI 507239	III	Japan
PI 507268	III	Japan
PI 507439	III	Japan
PI 507530	III	Japan
PI 509044	III	United States
PI 518287	III	Taiwan
PI 518674	III	United States
PI 522215	III	Russian Federation
PI 547812	III	United States
PI 547827	III	United States
PI 547840	III	United States
PI 547851	III	United States
PI 547884	III	United States
PI 547891	III	United States
PI 547892	III	United States
PI 548262	III	United States
PI 548298	III	Canada
PI 548304	III	United States
PI 548340	III	Canada
PI 548383	III	United States
PI 548388	III	United States
PI 548587	III	United States
PI 567528	III	China
PI 567541	III	China
PI 567560	III	China
PI 567574	III	China
PI 578368	III	China
PI 578486	III	India
PI 603167	III	North Korea
PI 603395	III	China
PI 603497	III	China
PI 19986	IV	United States
PI 59849	IV	Japan
PI 62199	IV	China
PI 64747	IV	Japan
PI 69507	IV	China
PI 70013	IV	China
PI 80466	IV	Japan
PI 80473	IV	Japan
PI 80479	IV	Japan
PI 80488	IV	Japan
PI 80498	IV	Japan
PI 80828	IV	Japan
PI 80834	IV	Japan
PI 82210	IV	South Korea
PI 84594	IV	South Korea
PI 84946	IV	South Korea
PI 85424	IV	South Korea
PI 85658	IV	South Korea
PI 87074	IV	South Korea
PI 89074	IV	China
PI 89772	IV	China
PI 90402	IV	China
PI 90406	IV	China
PI 91082	IV	South Korea
PI 91153	IV	China
PI 91346	IV	China
PI 92463	IV	Russian Federation
PI 92651	IV	China
PI 92713	IV	China
PI 96118	IV	South Korea
PI 96169	IV	South Korea
PI 97038	IV	South Korea

(continued on next page)

Table 1. (continued from preceding page)

Line	MG ^a	Origin
PI 97235	IV	South Korea
PI 105579	IV	France
PI 153321	IV	France
PI 157395	IV	South Korea
PI 157434	IV	South Korea
PI 157435	IV	South Korea
PI 157445	IV	South Korea
PI 158765	IV	China
PI 159923	IV	Peru
PI 167277	IV	Turkey
PI 170380	IV	Turkey
PI 171652	IV	Turkey
PI 172901	IV	Turkey
PI 189926	IV	France
PI 235335	IV	Uruguay
PI 235340	IV	Uruguay
PI 235344	IV	Uruguay
PI 235346	IV	Uruguay
PI 253665	IV	China
PI 264555	IV	Argentina
PI 274421	IV	Japan
PI 283330	IV	Taiwan
PI 304218	IV	Taiwan
PI 319532	IV	China
PI 323555	IV	India
PI 323556	IV	India
PI 346307	IV	India
PI 346308	IV	India
PI 355067	IV	United States
PI 355068	IV	United States
PI 355069	IV	United States
PI 355070	IV	United States
PI 361103	IV	Romania
PI 371611	IV	Pakistan
PI 378682	IV	Japan
PI 398293	IV	South Korea
PI 398303	IV	South Korea
PI 404153	IV	Georgia
PI 404159	IV	Georgia
PI 404161	IV	Georgia
PI 404163	IV	China
PI 404164	IV	China
PI 404165	IV	China
PI 404167	IV	China
PI 404170	IV	China
PI 404172	IV	China
PI 404173	IV	China
PI 404185	IV	China
PI 407733	IV	China
PI 416779	IV	Japan
PI 416984	IV	Japan
PI 416987	IV	Japan
PI 417005	IV	Japan
PI 417071	IV	Japan
PI 417135	IV	Japan
PI 417243	IV	China
PI 417339	IV	Japan
PI 417345	IV	China
PI 417380	IV	China
PI 417434	IV	Japan
PI 417457	IV	Japan
PI 417472	IV	Japan
PI 417578	IV	United States
PI 423929	IV	Japan
PI 79832	V	China
PI 89061	V	China
PI 103079	V	China

(continued in next column)

Table 1. (continued from preceding column)

Line	MG ^a	Origin
PI 159319	V	South Africa
PI 170895	V	South Africa
PI 170896	V	South Africa
PI 172902	V	Turkey
PI 179825	V	China
PI 283327	V	Taiwan
PI 322693	V	Angola
PI 346306	V	India
PI 346309	V	India
PI 355067	V	United States
PI 381659	V	Uganda
PI 381668	V	Uganda
PI 407749	V	China
PI 407752	V	China
PI 407758	V	China
PI 407763	V	China
PI 407765	V	China
PI 416807	V	Japan
PI 417034	V	Japan
PI 417098	V	Japan
PI 417329	V	Japan
PI 417335	V	Japan
PI 417356	V	Japan
PI 417366	V	Japan
PI 417387	V	Japan
PI 417420	V	Japan
PI 417423	V	Japan
PI 417567	V	Taiwan
PI 417582	V	United States
PI 436563	V	China
PI 471931	V	Nepal
PI 471938	V	Nepal
PI 471942	V	Nepal
PI 476889	V	Vietnam
PI 476890	V	Vietnam
PI 476905	V	China
PI 476909	V	Vietnam
PI 476917	V	China
PI 476920	V	Vietnam
PI 476931	V	Vietnam
PI 483466	V	China
PI 483468	V	China
PI 504510	V	Thailand
PI 506791	V	Japan
PI 506844	V	Japan
PI 506934	V	Japan
PI 507056	V	Japan
PI 507121	V	Japan
PI 507126	V	Japan
PI 507159	V	Japan
PI 507212	V	Japan
PI 507305	V	Japan
PI 507485	V	Japan
PI 507690	V	Russian Federation
PI 508268	V	United States
PI 518729	V	China
PI 543795	V	United States
PI 544354	V	United States
PI 548422	V	United States
PI 548460	V	United States
PI 548464	V	United States
PI 548466	V	United States
PI 548653	V	United States
PI 548667	V	United States
PI 548974	V	United States
PI 548977	V	United States
PI 548991	V	United States

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15 ml of distilled water and 0.5% polysorbate 80 (sorbitan mono-9-octadecenoate poly [oxy-1,2-ethanediyl] derivatives). The surface of the colonies was rubbed to separate the conidia from the mycelia. The resulting suspension was filtered through four layers of cheesecloth to eliminate mycelia fragments, then diluted to a concentration of 135,000 conidia/ml in 2007, as determined by a hemacytometer. In 2008, the final concentration of the inoculum was adjusted to 80,000 conidia/ml.

Pathogenicity test. Among the 394 isolates obtained, two were selected (PSS1 and PSS2) based on amount of spores produced. Pathogenicity of the two isolates was evaluated to select the most aggressive one to be used for the field experiment. Four soybean genotypes with known field susceptibilities were grown in the greenhouse: two susceptible genotypes (IA3001 and Vinton 81) and two resistant genotypes (UA 4805 and V97-3000). The experiment was arranged in a randomized complete block design. Each genotype was grown in 12 pots (three sets of four) and each pot contained two plants. Two sets of pots were inoculated at R1, R5, and R7 stage (3), each set with a different isolate (PSS1 or PSS2). One set of pots was not inoculated and used as a control. The experimental unit was the pot (rep) within an isolate or control treatment. Inoculation was performed using a manual sprayer, applying the inoculum with a conidial concentration of 135,000 and 80,000 conidia/ml in 2007 and 2008, respectively. Inoculation was directed to wet the leaves and pods of the whole plants, and inoculated plants were then placed in a dew chamber at 25°C for 24 h. Once past the incubation period, plants were moved to the greenhouse, where foliar symptoms of the disease were observed. Plants were harvested at maturity and the percentage of *C. kikuchii* infection was calculated through the observation of colonies recovered from the seed, which was determined by placing symptomatic seed on PDA (10 seeds from each treatment per plate), isolating the pathogen, and calculating percentage of infection (% seed infected per plate).

Plant materials. In all, 274 soybean accessions of MG III, IV, and V (Table 1) were randomly selected from the USDA germplasm collection (National Soybean Research Center, Urbana, IL). These lines were acquired from 28 different countries and consisted of important and diverse centers of origin or areas of important commercial

production of soybean. The lines were sent to winter nurseries in Argentina and Costa Rica for seed increase during winter 2006. Based on amount and quality of seed obtained, 123 accessions, 41 each from MG III, IV, and V, were selected for field screening in 2007. Four cultivars with known PSS field reactions were also included in each MG experiment as checks. Based on results from the 2007 study, 78 PI were chosen for the 2008 study. A seed sample was collected from the most susceptible and most resistant PI selected, which included 26 PI each from MG III, IV, and V for the 2008 screening experiments, with the same four commercial checks in each MG.

Field screening. The field experiment was conducted at the University of Arkansas Vegetable Research Station at Kibler. The soybean plants were planted on a Dardanelle silt loam (fine-silty, mixed, superactive, thermic Typic Argiudolls) with 1.5% organic matter and with soil test results (spring of 2007) of pH 6.9, EC 80 $\mu\text{mhos cm}^{-1}$, P at 53.2 mg kg⁻¹, and K at 236 mg kg⁻¹. In 2007, 123 soybean genotypes were grouped by maturity into three separate experiments. Each experiment was arranged in a strip-plot design with three replications (blocks). The strip was inoculation treatment (inoculated, noninoculated) and the subplot was genotype (41 PI and four checks in each MG experiment). In 2008, when only the most susceptible and most resistant PI were used, each experiment consisted of 26 selected PI and four checks. In both years, each subplot consisted of a single row, 3 m long, planted with 100 seeds with 1.1-m row spacing. Plants were inoculated in the field as described by Roy and Abney (17) at the R5 growth stage (GS) (3) using a CO₂ pressurized backpack sprayer (R & D Sprayers, Inc. Opelousas, LA), which was equipped with Teejet, low-pressure, flat, spray-tip nozzles (8003 LP-SS) and calibrated to 20 psi. The fields received 2.0 to 2.5 cm of water weekly by overhead irrigation. At 15 days after inoculation, foliar symptoms and disease incidence were assessed as the percentage of the plot infected by *Cercospora* leaf blight (% CLB).

During fall 2007 and 2008, each plot was harvested into a bulk sample, a subsample of 100 seeds was taken from each bulk sample, and the percentage of seed with PSS (% PSS) was determined visually. Additionally, percentage of seed infected with *C. kikuchii* (% *C. kikuchii*) was assessed by surface disinfecting 50 seeds from each bulk sample in 0.5% NaOCl for 90 s and placing them on PDA at 10 seeds/plate in five plates. The PDA was amended with rifampicin (100 mg/liter), ampicillin (0.25 g/liter), and ([RS]-a-cyano-3-phenoxybenzyl-2,2,3,3-tetramethyl-cyclopropanecarboxylate) (0.14 mg/liter). Seed were incubated for 7 days at 24 to 25°C, with a 14-h photoperiod, under fluorescent white light to induce PSS (7).

Analysis of variance (ANOVA) was conducted using JMP 8 (SAS, Cary, NC) for both variables to compare treatment and genotypic differences using GLM procedure. Correlations among % CLB, % PSS, and % *C. kikuchii* were also performed. Data were analyzed separately for 2007 and 2008 due to the different number of soybean genotypes tested and different environmental conditions in both years. Resistant and susceptible responses were separated based on the LSMeans (± 2 standard deviations [SD]) of the most resistant and the

Table 1. (continued from preceding page)

Line	MG ^a	Origin
PI 549027	V	China
PI 549045	V	China
PI 553038	V	United States
PI 553044	V	United States
PI 553049	V	United States
PI 553052	V	United States
PI 559934	V	United States
PI 561358	V	China

Table 2. Incidence of purple seed stain (PSS), *Cercospora kikuchii*, and *Cercospora* leaf blight (CLB) in genotypes grouped by maturity and year

MG ^a , treatment	% PSS				% <i>C. kikuchii</i>				% CLB	
	2007		2008		2007		2008		2007	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
MG III										
Inoculated	22.4	0.0–51.0	5.7	0.0–20.0	24.6	2.0–51.0	11.5	0.0–45.0	4.2	0.0–15.0
Noninoculated	15.6	0.1–47.0	5.4	0.3–20.0	22.8	1.3–49.0	10.5	0.0–40.0	3.1	0.0–12.0
MG IV										
Inoculated	11.8	0.0–33.0	7.3	0.0–42.0	17.9	2.0–35.0	13.0	1.3–71.0	12.9	0.0–38.0
Noninoculated	9.7	0.0–41.0	4.3	0.0–21.0	15.4	0.0–42.0	8.4	0.7–34.0	10.2	1.7–30.0
MG V										
Inoculated	2.3	0.0–16.3	1.9	0.0–13.0	5.0	0.0–33.0	4.9	0.0–15.0	13.3	0.0–36.7
Noninoculated	1.3	0.0–15.0	1.0	0.0–5.0	3.8	0.0–29.0	4.7	0.7–11.3	8.2	0.0–22.0

^a MG = maturity group.

most susceptible checks for MG III and IV and based upon Fisher's protected least significant difference (LSD) for MG V because there were no checks to produce thresholds for MG V. In order to be accurate for genotypic classification, asymptomatic infected seed was taken into consideration by using % *C. kikuchii*, which is the percentage of *C. kikuchii* colonies recovered from a sample of soybean seed.

Table 3. Threshold for classification of reactions of maturity group (MG) III and IV plant introductions to *Cercospora kikuchii* infection, using the parameter LSMean $\pm$ 2 standard deviations (SD) of resistant and susceptible checks

Year	MG	Checks ^a	LSMean	SD	Lower limit	Upper limit
2007	III	AG4403 R	2.7	2.3	7.3	33.6
		IA3001 S	48.0	7.2		
	IV	UA4805 R	4.7	1.2	7.0	43.0
2008	III	AP350 S	51.3	4.2		
		AG4403 R	7.3	4.6	16.6	22.7
	IV	IA3001 S	8.7	7.0		
		UA4805 R	2.0	2.0	6.0	42.7
		AP350 S	70.7	14.1		

^a R = resistant and S = susceptible.

Table 4. Resistant and susceptible varieties as classified using the two standard deviation from the mean of the check cultivar in each group

Year, variety	MG ^a	Tolerance levels and genotypes	<i>C. kikuchii</i> infection (%)	
			Mean	Range
2007	III			
	Resistant	PI 438312, PI 417361, PI 235339, PI 548340, and PI 476922	4.4	2.0–7.3
	Susceptible	PI 153309, PI 438357, PI 548298, PI 438435, PI 283331, PI 603167, PI 283332, PI 504481, PI 88490, PI 504487, PI 547840, and PI 404160	41.2	35.3–51.3
	IV			
	Resistant	PI 159923, PI 172901, PI 235340, PI 153321, PI 167277, PI 264555, PI 404159, PI 84594, PI 171652, PI 80488, and PI 404173	4.0	2.0–6.7
	Susceptible	No genotypes identified as susceptible	...	...
2008	III			
	Resistant	PI 408321, PI 235339, PI 603167, PI 417559, PI 417361, PI 548340, PI 438409, PI 104708, PI 79726, PI 416988, PI 438335, PI 438310, PI 438427, PI 476922, PI 437124, and PI 404160	3.8	0.0–10.7
	Susceptible	PI 88490, PI 283332, PI 438357 and PI 547840	34.2	24.0–45.3
	IV			
	Resistant	PI 172901, PI 264555, PI 167277, PI 84594, PI 80488, PI 355070, PI 235340, PI 87074, PI 171652, PI 361103, PI 404173, PI 416779, PI 159923, and PI 404159	3.5	1.3–6.0
	Susceptible	No genotypes identified as susceptible	...	...

^a Maturity group.

Results

Pathogenicity test. Results from the pathogenicity test revealed that isolate PSS1 produced greater percentages (6.1%) of *C. kikuchii* infection than PSS2 (1.2%) in susceptible cultivars. No symptoms were observed on the noninoculated checks. Symptoms on susceptible cultivars were characterized by extensive purple discoloration on leaves, petioles, pods, and stems. Purple discoloration areas were also observed on seed of susceptible cultivars, which had high percentages of recovery of *C. kikuchii* when plated on PDA.

Field experiment: ANOVA and means table. ANOVA revealed significant treatment–entry interaction effect on % PSS in 2007 ($P < 0.0001$) and both % PSS and % *C. kikuchii* in 2008 ($P = 0.007$ and < 0.0001 , respectively) in MG IV, and % CLB ($P < 0.05$) in 2007 in MG V (data not shown). No significant interaction was observed among these variables in MG III (Table 2). The length of the growth period and the fact that development stages occurred under different environmental conditions may explain the responses observed in each MG. Significant differences among entries were observed for all variables and all year–treatment combinations, which suggested that genotypic effects offset the effects of the treatment applied and environmental conditions. However, and unlike MG III and IV, MG V had significant differences among treatments in four of the five response–year combinations, which suggested parallel responses of entries between treatments.

Variety classification and selection of stable genotypes. We classified 5 and 16 varieties of MG III as resistant and 12 and 4 varieties as susceptible in 2007 and 2008, respectively. In both resistant and susceptible varieties, % *C. kikuchii* was similar between years. Using the most susceptible check (IA3001) LSMean – 2SD (22.7%) (Table 3) for % *C. kikuchii* from the inoculated treatment, we observed that PI 88490, PI 283332, PI 438357, and PI 547840 were susceptible, whereas almost all other genotypes did not express many disease symptoms under the 2008 environmental conditions when compared with the most resistant check (AG4403) LSMean + 2SD (16.6%) (Table 4).

We classified 11 and 14 varieties of MG IV as resistant in 2007 and 2008, respectively, but no susceptible varieties were identified in MG IV. Using the most susceptible check (AP350) LSMean – 2SD (42.66%) (Table 3) from percentage of *C. kikuchii* infection in the inoculated treatment, no susceptible genotypes were identified. Using the most resistant check (UA 4805) LSMean + 2SD (6%) in the classification (Table 3), more than half of the genotypes developed only low incidence of infection. In 2008, weather conditions were not conducive for disease development, and there was no significant difference between resistant and susceptible PI. However, the resistant genotypes selected in 2007 were also resistant in 2008, except for PI 153321, which had a resistant response in 2007 but was not tested in 2008 (Table 4).

Correlations for each MG between % PSS and % *C. kikuchii* were moderate to high, positive, and significant (Table 5), suggesting that % PSS could also be a good and easy parameter to measure the presence of the disease. The % PSS has also been used to classify cultivars in other studies (14). Poor or slightly negative correlations between % CLB versus % PSS and % *C. kikuchii* were observed in our study (Table 6), similar to the results reported in previous studies (14,22,27), suggesting that resistance to CLB and PSS may be governed by different genes.

Resistant and susceptible genotypes for MG V were separated using the LSD for % *C. kikuchii* from the inoculated treatments. This was done in order to be accurate for genotypic classification by taking into consideration the asymptomatic, infected seed and recovery of the pathogen. We were not able to use the best resistant or worst susceptible LSMean $\pm$ 2SD, because the checks had different reactions than we expected. Due to the unfavorable weather conditions resulting in delayed disease development, most genotypes developed very low % *C. kikuchii* infections. Therefore, resistant genotypes could not be identified for MG V. PI 507690 was the only genotype to be identified as susceptible based on the % *C. kikuchii* infection in the inoculated treatments.

The genotypes selected in both years had consistent reactions, which increased confidence in their reaction. These PI should be further evaluated in other environments to assess their use in breeding programs as donors of resistant genes. In MG III, PI 417361, PI

235339, PI 548340, and PI 476922 and, in MG IV, PI 159923, PI 172901, PI 235340, PI 167277, PI 264555, PI 404159, PI 84594, PI 171652, PI 80488, and PI 404173 were selected for consistency in resistance in both years.

Discussion

Weather conditions favorable for *C. kikuchii* sporulation are generally present in early production systems in Arkansas, where MG III and IV soybean cultivars are planted in late April to early May. Seed development for these MG generally occurs during environmental conditions highly favorable for seed infection with *C. kikuchii* and other fungi (16,30) giving rise to poor seed quality (12). In our study, we confirmed previous research findings that reported greater % PSS and % *C. kikuchii* in MG III and IV genotypes (14) than in MG V genotypes. These genotypes matured later in the season, when temperatures were lower and conditions were less favorable for disease development (27).

Additionally, year-to-year differences in temperature and precipitation during inoculation with *C. kikuchii* affected disease occurrence in our experiments. Higher temperatures and drier conditions in 2007 (mean maximum and minimum temperatures of 35.6 and 23.3°C, respectively, for August and 30.2 and 18.5°C for September; 170.9 mm of rain accumulation for both months) occurred and was the likely cause of higher % PSS and % *C. kikuchii* infection. In contrast, lower infection rates occurred in 2008 due to cooler temperatures with higher humidity (mean maximum and minimum temperatures of 32 and 21.8°C, respectively, for August and 28 and 17.2°C for September; 394.5 mm of rain accumulation for both months) (13). This is consistent with previous studies in Arkansas where exceptionally dry years with enough humidity for conidial germination of *C. kikuchii* resulted in greater % PSS infection rates than years with cooler temperatures and greater humidity, particularly for early-maturing cultivars (27).

Inoculum used in our experiment was prepared as described by Orth and Schuh (14), with a final spore concentration of 1.35×10^5 to increase the pressure of the pathogen in the field. It is worth noting that sporulation of *C. kikuchii* isolates, particularly after 1 year

of storage and multiplication in vitro, is difficult. We observed that isolate PSS1 had reduced sporulation in 2008, although conditions used to induce sporulation were similar to those used in 2007. Therefore, we reduced the final inoculum concentration to 8×10^4 . This decline in spore production when isolates were older in vitro was also reported by El-Gholl et al. (2). The reduced sporulation of isolate PSS1 likely contributed to the generally lower infection rates in 2008 compared with 2007. We found that maintenance of pathogenic isolates in vitro is challenging; therefore, natural infection serves as an alternative in screening response to *C. kikuchii* infection. However, there is a risk for lack of disease expression when weather conditions are nonconducive for disease development. Another alternative to consider would be the maintenance of the isolate with periodic inoculations in the greenhouse. However, the drawback would be that possible genetic mutations in the isolates could occur during the multiple infection events.

Many different GS have been chosen to inoculate soybean plants in previous studies. Orth and Schuh (14) inoculated soybean in the field at the R4 GS (full pod before seed initiation). Schuh (20) inoculated plants at four different stages of reproductive development (full bloom, post bloom, small pod, and large pod) and studies conducted by Ploper et al. (16) inoculated at the R2 GS (full bloom). Our protocol to inoculate plants was to cover developed pods with inoculum suspension at the R5 GS (beginning seed) and evaluate the effect of inoculation at later GS. In previous studies, inoculations at advanced GS produced lower incidence of seed infection (18), and higher incidence of infection were observed for inoculations at flowering periods (10,17). Therefore, earlier inoculation could have helped to produce higher levels of disease in our study. Inoculation was delayed in 2008 to GS R5.5 (3) due to problems in getting the pathogen to sporulate and due to high levels of precipitation before and after inoculation (13). These problems should be taken into consideration for future field screening studies with artificial inoculations.

With exception of MG IV for % PSS in 2007 and 2008 and % *C. kikuchii* in 2008, the treatment–entry interaction effect was not significant for % PSS and % *C. kikuchii*, suggesting that genotypes

Table 5. LSMeans correlations between inoculated (Inoc) and noninoculated (Noninoc) treatments for percentage of purple seed stain (% PSS) and percentage of *Cercospora kikuchii* infection (% *C. kikuchii*), and LSMeans correlation between % PSS and % *C. kikuchii*^a

Correlations	Year	MG III	n	P	MG IV	n	P	MG V	n	P
Inoc vs Noninoc										
% PSS	2007	0.87	45	<0.0001	0.80	45	<0.0001	0.78	45	<0.0001
	2008	0.76	30	<0.0001	0.67	30	<0.0001	0.75	30	<0.0001
% <i>C. kikuchii</i>	2007	0.88	45	<0.0001	0.86	45	<0.0001	0.88	45	<0.0001
	2008	0.84	30	<0.0001	0.86	30	<0.0001	0.78	30	<0.0001
% PSS vs % <i>C. kikuchii</i>										
Noninoculated	2007	0.76	45	<0.0001	0.85	45	<0.0001	0.90	45	<0.0001
	2008	0.72	30	<0.0001	0.83	30	<0.0001	0.63	30	<0.0001
Inoculated	2007	0.81	45	<0.0001	0.80	45	<0.0001	0.78	45	<0.0001
	2008	0.75	30	<0.0001	0.97	30	<0.0001	0.72	30	<0.0001
Overall	2007	0.77	90	<0.0001	0.82	90	<0.0001	0.83	90	<0.0001
	2008	0.73	60	<0.0001	0.94	60	<0.0001	0.67	60	<0.0001

^a MG = maturity group and n = number of LSMeans from genotypes evaluated.

Table 6. Correlations between *Cercospora* leaf blight (CLB) and percentage of purple seed stain (% PSS) or percentage of *Cercospora kikuchii* infection (% *C. kikuchii*) in maturity groups (MG) III, IV, and V in 2007^a

Correlations	MG III	n	P	MG IV	n	P	MG V	n	P
% PSS vs CLB									
Noninoculated	0.26	128	0.0029	−0.04	119	0.6861	−0.03	126	0.7012
Inoculated	0.27	131	0.0008	−0.04	115	0.6895	0.01	125	0.9078
Overall	0.28	259	<0.0001	−0.02	234	0.7726	0.03	251	0.5892
% <i>C. kikuchii</i> vs CLB									
Noninoculated	0.18	128	0.0399	−0.05	119	0.5734	−0.12	126	0.1784
Inoculated	0.29	131	0.0008	−0.07	115	0.4486	−0.05	125	0.6064
Overall	0.25	259	<0.0001	−0.04	234	0.5003	−0.05	251	0.4704

^a n = number of data points used in correlation calculations.

exhibited consistent responses and similar rankings when they were exposed to both treatments. Generally, the reactions of genotypes in the three experiments with different maturities were stable among blocks, especially for 2007 experiments. In general, higher % PSS and % *C. kikuchii* rates were found for inoculated treatment than for noninoculated treatment. However, the differences were not always significant, suggesting that natural infection could be a viable option for evaluation of PSS and *C. kikuchii* infection.

With few exceptions, no significant differences were observed between inoculated and noninoculated treatments, suggesting that natural infection was as effective as artificial inoculation, especially for conducive years like 2007. Artificial inoculation is helpful for assessing incidence of PSS but not necessarily for evaluation of reaction to CLB or *C. kikuchii* infection. The range of LSMeans for inoculated and noninoculated treatments was similar, in general, for all three variables evaluated. The range of LSMeans was greater for the inoculated than noninoculated treatment, suggesting that artificial inoculation could be helpful in the screening for the disease, particularly when the weather conditions are nonconductive. A moderate to high correlation coefficient (0.67 to 0.88) between inoculated and noninoculated treatments also supports the conclusion that natural infection and artificial inoculation are both effective in ranking the genotypes. However, artificial inoculation would ensure a high pressure of inoculum, especially in nonfavorable years for disease expression.

In the disease cycle, colonization of soybean plants by *C. kikuchii* occurs through stomata (22), resulting in foliar blight. No hypersensitive reaction (HR) was found among the genotypes tested in all three MG from either inoculated or noninoculated plots. HR is characterized by rapid death of cells attacked by the pathogen or adjacent to the infection point as a necrotic response to invasion of the organism (5). A wide variety of responses associated with different levels of % CLB, % PSS, and % *C. kikuchii* suggested that resistance may be governed by major and minor genes, and that there exists vertical resistance for the extreme low or high response values and horizontal resistance for the intermediate response values (26) in reaction to PSS and *C. kikuchii* infection.

We observed, as in previous studies (14,28), that high incidence of CLB did not represent high incidence of PSS. A good example is that the MG IV resistant check, PI 80837, carries a resistance gene for PSS (7,9) but presented high incidence of CLB. The difference among % PSS, % *C. kikuchii*, and % CLB shows the complexity of this disease. However, % PSS and % *C. kikuchii* are positively correlated and may be controlled by the same resistance mechanism, whereas CLB appears to be conditioned by a different resistance mechanism at a different developmental stage.

Field screening is a simple and effective way to identify resistant responses to PSS among different soybean genotypes, especially for early maturing groups such as III and IV. Screening for MG V is challenging, because pod filling and seed maturation in the late growing season does not generally coincide with favorable weather conditions for epidemics of PSS. Artificial inoculation is helpful and should be considered to increase the disease pressure but it is very difficult to achieve the best results in the field due to weather conditions, differences in maturity, and technical challenges. With few exceptions, a nonsignificant treatment–entry interaction effect for all variables tested suggested consistent behaviors among the genotypes across replications and when subjected to inoculated and noninoculated treatments. High correlations between natural infection and artificial inoculation are supportive evidence that natural infection can be a good alternative for field screening. Concomitantly, strong positive correlations between % PSS and % *C. kikuchii* implies that visual rating is an acceptable way to score the presence of the disease, especially when recovery of the pathogen is not an objective. The sources of resistance identified in this study may be used in future genetic studies or in the development of new varieties with improved resistance to PSS.

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Literature Cited

- Almeida, A. M. R., Saraiva, O. F., Farias, J. R. B., Gaudencio, C. A., and Torres, E. 2001. Survival of pathogens on soybean debris under no-tillage and conventional tillage systems. *Pesq. Agropecu. Bras.* 36:1231-1238.
- El-Gholl, N. E., Alfieri, S. A., Ridings, W. H., and Schouties, C. L. 1982. Growth and sporulation *in vitro* of *Cercospora apii*, *Cercospora arachidicola*, *Cercospora kikuchii*, and other species of *Cercospora*. *Can. J. Bot.* 60:862-868.
- Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. *Crop Sci.* 11:929-931.
- Gardner, M. W. 1926. Indiana plant diseases, 1925. *Proc. Indiana Acad. Sci.* 36:231-247.
- Gaumann, E. 1946. Type of defensive reactions in plants. *Phytopathol.* 36:624-633.
- Grau, C. R., Dorrance, A. E., Bond, J., and Russin, J. S. 2004. Fungal diseases. Pages 679-763 in: *Soybeans: Improvement, Production, and Uses*, 3rd ed. Agron. Monogr. 16. H. R. Boerma and J. F. Specht, eds. ASA, CSSA, and SSSA, Madison, WI.
- Jackson, E. W. 2004. Resistance to *Phomopsis* seed decay and purple seed stain in soybean and virulence differences among *Phomopsis* spp. causing seed decay. Doctoral dissertation, University of Arkansas, Fayetteville.
- Jackson, E. W., Fenn, P., and Chen, P. 2006. Inheritance of resistance to purple seed stain caused by *Cercospora kikuchii* in PI 80837 soybean. *Crop Sci.* 46:1462-1466.
- Jackson, E. W., Feng, C., Fenn, P., and Chen, P. 2008. Genetic mapping of resistance to purple seed stain in PI 80837 soybean. *J. Hered.* 99:319-322.
- Lavolette, F. A., and Athow, K. L. 1972. *Cercospora kikuchii* infection of soybean as affected by stage of plant development. (Abstr.) *Phytopathology* 62:771.
- Martin, K. F., and Walters, H. J. 1982. Infection of soybean by *Cercospora kikuchii* as affected by dew temperature and duration of dew periods. (Abstr.) *Phytopathology* 72:974.
- Mayhew, W. L., and Caviness, C. E. 1994. Seed quality and yield of early-planted, short-season soybean genotypes. *Agron. J.* 86:16-19.
- NOAA Satellite and Information Service. Online publication. <http://www.ncdc.noaa.gov/oa/mpp/freedata.html>
- Orth, C. E., and Schuh, W. 1994. Resistance of 17 soybean cultivars to foliar, latent, and seed infection by *Cercospora kikuchii*. *Plant Dis.* 78:661-664.
- Pathan, M. A., Sinclair, J. B., and McClary, R. D. 1989. Effects of *Cercospora kikuchii* on soybean seed germination and quality. *Plant Dis.* 73:720-723.
- Ploper, L. D., Abney, T. S., and Roy, K. W. 1992. Influence of soybean genotype on rate of seed maturation and its impact on seedborne fungi. *Plant Dis.* 76:287-292.
- Roy, K. W., and Abney, T. S. 1976. Purple seed stain of soybeans. *Phytopathology* 66:1045-1049.
- Roy, K. W., and Abney, T. S. 1997. Antagonism between *Cercospora kikuchii* and other seedborne fungi of soybeans. *Phytopathology* 67:1062-1066.
- Schuh, W. 1991. Influence of temperature and leaf wetness period on conidial germination *in vitro* and infection of *Cercospora kikuchii* on soybean. *Phytopathology* 81:1315-1318.
- Schuh, W. 1992. Effect of pod development stage, temperature, and pod wetness duration on the incidence of purple seed stain of soybeans. *Phytopathology* 82:446-451.
- Schuh, W. 1993. Influence of interrupted dew periods, relative humidity, and light on disease severity and latent infections caused by *Cercospora kikuchii* on soybean. *Phytopathology* 83:109-113.
- Schuh, W. 1999. *Cercospora* blight, leaf spot, and purple seed stain. Pages 17-18 in: *Compendium of Soybean Diseases*, 4th ed., G. L. Hartman, J. B. Sinclair, and J. C. Rupe, eds. American Phytopathological Society, St. Paul, MN.
- Sinclair, J. B., and Backman, P. A. 1989. Part IV. Soybean disease management strategies. Pages 94-100 in: *Compendium of Soybean Diseases*, 3rd ed. J. B. Sinclair and P. A. Backman, eds. American Phytopathological Society, St. Paul, MN.
- Srisombun, S., and Supapornhem, P. 1993. Inheritance of soybean resistance to purple seed stain. *Soybean Genet. Newsl.* 20:92-93.
- TeKrony, D. M., Egli, D. B., Stucky, R. E., and Loeffler, T. M. 1985. Effect of benomyl applications on soybean seedborne fungi, seed germination, and yield. *Plant Dis.* 69:763-765.
- Van der Plank, J. E. 1963. Plant disease in biological warfare. Pages 212-222 in: *Plant Diseases: Epidemics and Control*. Academic Press, New York.
- Walters, J. A. 1980. Soybean leaf blight caused by *Cercospora kikuchii*. *Plant Dis.* 64:961-962.
- Walters, H. J. 1985. Purple seed stain and *Cercospora* leaf blight. Pages 503-506 in: *Proc. 3rd World Soybean Res. Conf.* Ames, IA. R. Shibles, ed. Westview Press, Boulder, CO.
- Wilcox, J. R., and Abney, T. S. 1973. Effects of *Cercospora kikuchii* on soybeans. *Phytopathology* 63:796-797.
- Wilcox, J. R., Abney, T. S., and Frankenberger, E. M. 1985. Relationships between seedborne soybean fungi and altered photoperiod. *Phytopathology* 75:797-800.
- Wilcox, J. R., Lavolette, F. A., and Martin, R. J. 1975. Heritability of purple seed stain resistance in soybeans. *Crop Sci.* 15:525-526.
- Yeh, C. C., and Sinclair, J. B. 1982. Effect of *Cercospora kikuchii* on soybean seed germination and its interaction with *Phomopsis* sp. *Phytopathol. Z.* 105:265-270.