Evaluation of Commercial Soybean Cultivars for Reaction to Phomopsis Seed Decay

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Abstract

Phomopsis seed decay (PSD), caused by *Phomopsis longicolla* (syn. *Diaporthe longicolla*), is an economically important soybean disease causing poor seed quality. Planting resistant cultivars is one of the most effective means to control PSD. In this study, 16 commercially available maturity groups IV and V soybean cultivars, including two previously identified PSD-resistant and two PSD-susceptible checks, were evaluated for seed infection by *P. longicolla* in inoculated and noninoculated plots, and harvested promptly or with a 2-week delay in harvest. The test was conducted at Stoneville, Mississippi, in 2012 and 2013. Seed

infection by *P. longicolla* ranged from 0.5 to 76%, and seed germination ranged from 18 to 97%. One MG IV cultivar (Morsoy R2 491) and five MG V cultivars (Progeny 5650, Progeny 5706, Asgrow 5606, Asgrow 5831, and Dyna-Gro33C59) had significantly ($P \leq 0.05$) lower percent seed infected by *P. longicolla* than their respective susceptible checks and other cultivars in the same tests. Information obtained from this study will be useful for soybean growers and breeders for selection of cultivars for planting or breeding and future genetic studies in the development of cultivars with improved resistance to PSD.

Phomopsis seed decay (PSD), caused primarily by a seed-borne fungus, *Phomopsis longicolla* (syn. *Diaporthe longicolla* (Hobbs) Santos), is one of the most economically important diseases of soybean, *Glycine max* (L.) Merrill (Hobbs et al. 1985; Li 2011; Li et al. 2015a; Santos et al. 2011; Sinclair 1993). This disease causes poor seed quality and occurs in most soybean production areas worldwide, especially in the mid-southern region of the United States (Li et al. 2011, 2015c; Sinclair 1993). In addition to *P. longicolla*, there are other important fungal pathogens causing soybean diseases, such as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. (anamorph *P. phaseoli* (Desm.) Sacc.) including *D. phaseolorum* var. *caulivora* K. L. Athow & R. M. Caldwell, and *D. aspalathi* E. Jansen, Castl. and Crous (syn. *D. phaseolorum* var. *meridionalis* F. A. Fernández), and *D. phaseolorum* var. *sojae* (S. G. Lehman) in the *Diaporthe-Phomopsis* complex. Although these pathogens may be associated with PSD (Hartman et al. 2015), research has demonstrated that soybean seeds, instead of stems and other soybean tissues, are more susceptible to *P. longicolla* than to other *Diaporthe* species (Xue et al. 2007). Differences in aggressiveness on soybean among *P. longicolla* isolates have been found, and isolates from nonlegume hosts infect soybean aggressively (Li et al. 2001, 2010).

Soybean seed infected by *P. longicolla* can be discolored with shriveled and elongated shapes or have cracked seed coats appearing chalky white. However, in some cases, infected soybean seed do not exhibit visible symptoms or signs (Kulik and Sinclair 1999; Sinclair 1993). Symptomless soybean seed with Phomopsis infections could have low seed germination and seedling vigor, and poor stands as seeds with symptoms (Gleason and Ferriss 1985; Sinclair 1993). *P. longicolla* can alter seed composition, decrease oil quality, and cause high frequency of moldy and/or split beans (Hepperly and Sinclair 1978), resulting in poor seed quality. Significant yield loss

of soybean caused by PSD has been reported, but varied by year and environment (Baird et al. 2001; Wrather et al. 2003; Wrather and Koenning 2009). In 2009, warm and humid environments favored disease development (Balducchi and McGee 1987; Rupe 1990; Rupe and Ferriss 1986; TeKrony et al. 1983) during the late growing season in the southern United States, and PSD caused soybean yield losses over 0.33 million metric tons in 16 southern states (Koenning 2010). However, in 2010 and 2011, there was little infection by *P. longicolla* in Arkansas, Mississippi, and Missouri because of hot and dry conditions during seed maturation (Li et al. 2015c). High incidence of seed decay has also occurred when plants were deficient in potassium or when infected by *Soybean mosaic virus* (Koning et al. 2001, 2003).

Several control strategies have been used to manage PSD, including crop rotation with non-soybean host crops, conventional tillage, foliar fungicide treatments (Cross et al. 2012; Jardine 1991; TeKrony et al. 1985; Wrather et al. 2004; Xue et al. 2007), harvesting mature seeds on time, and use of host genetic resistance (Jackson et al. 2005; Pathan et al. 2009; Roy et al. 1994). Use of PSD-resistant soybean cultivars is the most economical and environmentally friendly means to control PSD (Jackson et al. 2005, 2009; Li and Chen 2013; Pathan et al. 2009; Roy et al. 1994; Zimmerman and Minor 1993).

Comprehensive screening of plant introductions from the USDA Germplasm Collection (<https://www.ars-grin.gov/npgs/>) for resistance to PSD has been reported (Brown et al. 1987; Li et al. 2011, 2015c). However, information about the reaction of commercial soybean cultivars to *P. longicolla* is lacking. We hypothesized that PSD-resistant gene(s) may exist in commercial soybean cultivars, and cultivars with different levels of resistance to PSD could be identified. The objective of this study was to identify soybean cultivars with resistance to PSD and high seed quality by evaluating reactions to *P. longicolla* infection following inoculation and either prompt or delayed harvest at maturity or with a 2-week delay in harvest post-maturity.

Materials and Methods

Soybean cultivars. Sixteen commercially available soybean cultivars from maturity groups IV and V were selected from the “Cross-Reference Guide for Common Soybean Varieties-2011” that were recommended by the Mississippi Soybean Promotion Board (<http://mssoy.org>) based on yield performance in Mississippi Soybean Variety Trials at multiple locations. Information about the reaction of those cultivars to Phomopsis seed decay was lacking. Two previously identified PSD resistant checks, SS93-6181 (Pathan et al. 2009) and PI 424324B (Li et al.

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2011), and two susceptible checks, AP 350 (Jackson et al. 2005) and Osage, were also included in this study (Table 1). Osage is a conventional cultivar developed and commonly grown in Arkansas (Chen et al. 2007). Osage also was reported to be resistant to several important diseases in the mid-southern U.S.A., including southern stem canker, sudden death syndrome, *Soybean mosaic virus*, and frogeye leaf spot (Chen et al. 2007), but appeared to be susceptible to PSD in Mississippi in a preliminary test (Li, unpublished). All soybean seeds were obtained from Mississippi State University, Delta Research and Extension Center.

Field experiments. Field experiments were conducted at Stoneville, Mississippi, in a Sharkey clay soil (very fine, smectitic, thermic Chromic Epiaquert) in 2012 and 2013. This field has been used to screen soybean plant introductions for resistance to PSD since 2006. For all experiments, seeds were planted at a rate of 33 seeds/m of row, in 2.74 m-long double-row plots with a 0.91-m row spacing. The plot size was 1.82 m × 2.74 m. Each plot of each cultivar was a single replication.

There were separate experiments for each maturity group (MG IV and MG V). Experimental design for each experiment was a split-split plot with inoculation treatment (inoculated and noninoculated) as the main plot and harvest date (prompt harvest or a 2-week delay in harvest) as subplot. Cultivars were randomly arranged with four replications as paired plots, one for prompt harvest and one for delayed harvest. All plants (approximately 80) in each plot were manually harvested. Pre-emergence herbicides of Gramoxone at 2.33 liters/ha and Dual at 1.8 liters/ha (Syngenta Crop Protection, Greensboro, NC) were applied on the second day after planting. Since that, there was no chemical treatment to the field because experiments involved pathogen inoculation. Weed removal was conducted manually. Planting dates in each year are listed in Table 2.

Inoculations were performed at the R5 growth stage (Fehr and Caviness 1997) each year (Table 2). To promote infection, plants were irrigated two to three times a day following inoculation if there

was no rain. Approximately 250 liters of water were applied for each irrigation. Irrigation was conducted with overhead Rain Bird Brass Impact Sprinklers (Model #25P JDA-C). Sprinklers were connected to a nurse wagon equipped with a Honda engine/pacer poly pump (Bell Inc., Inverness, MS). In the following 6 to 7 weeks (from R5 to R8 growth stages) after inoculation, plots were irrigated two to three times a week if needed.

Inoculum preparation and application. Isolate MSPL 10-6 of *P. longicolla* was used for inoculation in the experiments. This isolate was originally from field-grown soybean plants in Stoneville, MS, and its genome sequence is publicly available (Li et al. 2015b). Isolate MSPL 10-6 has also been used in previous studies to identify new sources of resistance to PSD (Li et al. 2011, 2015c). To prepare the inoculum, the isolate was grown at 24°C on potato dextrose agar (Difco Laboratories, Detroit, MI) adjusted to pH 4.8 with 25% lactic acid (APDA) after autoclaving. Inoculum was prepared as previously reported (Li et al. 2011, 2015c). Briefly, cultures were induced to sporulate under a fluorescent light output of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a 12-h photoperiod for 30 to 45 days. Sporulating cultures were flooded with sterile deionized water three times, agitated to dislodge conidiospores, and filtered with four layers of sterile cheesecloth to eliminate the agar. Conidiospore concentrations were adjusted to approximately $1.5 \times 10^5/\text{ml}$ with a hemacytometer (Hausser Scientific, Blue Bell, PA). The quality of the inoculum was determined by the colony-forming unit (CFU) of diluted conidiospore suspension containing 100 conidiospores on APDA. Field inoculations were conducted using a battery-operated backpack sprayer Solo Model 416 (Flojet Co., Santa Ana, CA) with a hand-held boom containing a single nozzle with an adjustable orifice at 207 kPa. Conidiospore suspension was sprayed directly on the pods and then evenly across the foliage in each plot. Plots were sprayed to runoff (Li et al. 2011, 2015c). Approximately 500 ml of conidiospore suspension was applied to each plot.

Seed assays. Prior to planting, 30 seeds of each line provided by Mississippi State University, Delta Research and Extension Center, were assayed for incidence of *P. longicolla*. After manual harvest, 50 randomly chosen seeds (13% moisture) from each plot (200 seeds

Table 1. A list of soybean cultivars evaluated for resistance to *Phomopsis longicolla*

Cultivar	Maturity group	Seed coat color	Seed source	Country
Armor ARX 1482	IV	Yellow	Armor Seed Co.	U.S.A.
Morsoy R2 491	IV	Yellow	Missouri Farmers Association	U.S.A.
Morsoy R2S 480	IV	Yellow	Missouri Farmers Association	U.S.A.
Pioneer 94Y90	IV	Yellow	Pioneer Hibred Seed Co.	U.S.A.
SS93-6181 ^y	IV	Yellow	University of Missouri	U.S.A.
AP 350 ^z	IV	Yellow	Agripro Seed Co.	U.S.A.
Asgrow 5606	V	Yellow	Monsanto	U.S.A.
Asgrow 5831	V	Yellow	Monsanto	U.S.A.
Dyna-Gro 37RY52	V	Yellow	Crop Production Services	U.S.A.
Dyna-Gro 33C59	V	Yellow	Crop Production Services	U.S.A.
Progeny 4949	V	Yellow	Erwin Keith	U.S.A.
Progeny 5650	V	Yellow	Monsanto	U.S.A.
Progeny 5706	V	Yellow	Monsanto	U.S.A.
Terral 59R16	V	Yellow	Monsanto	U.S.A.
PI424324B ^y	V	Green	USDA Germplasm Collection	South Korea
Osage ^z	V	Yellow	University of Arkansas	U.S.A.

^y Resistant check.

^z Susceptible check.

Table 2. Planting, inoculation, and harvest dates for evaluating soybean cultivars for response to *Phomopsis longicolla* in Stoneville, MS, in 2012 and 2013

Year	Planting date	Harvest dates ^z					
		Inoculation dates ^y		Maturity group IV		Maturity group V	
		Maturity group IV	Maturity group V	Prompt	Delayed	Prompt	Delayed
2012	25 April	19 July, 31 July	25 July, 8 Aug.	14 Sep. – 21 Sep.	28 Sep. – 5 Oct.	27 Sep. – 10 Oct.	12 Oct. – 25 Oct.
2013	30 April	16 July, 30 July	30 July, 13 Aug.	16 Sep. – 27 Sep.	30 Sep. – 10 Oct.	18 Oct.	30 Oct.

^y Two treatments. Soybean plants were either inoculated with spore suspension of *P. longicolla* (1 to $1.5 \times 10^5/\text{ml}$) at the R5 growth stage (inoculated treatment) or sprayed with distilled water (noninoculated treatment).

^z Harvests were conducted manually: Prompt (at R8 growth stages) versus delayed harvest (at R8 + 2 weeks).

for each soybean cultivar) were assayed to determine the percent seed infection by *P. longicolla*, percent standard seed germination, and visual seed quality with the methods as previously reported (Li et al. 2015c). Seeds were surface-disinfected in 0.5% sodium hypochlorite for 3 min, rinsed in sterile distilled water, and then placed on APDA (Li et al. 2011, 2015c). Five seeds were placed on APDA in each 100 mm × 15 mm Petri dish where one seed was placed in the center and the others were placed equidistant around the outside of the dish, approximately 10 mm from the side with approximately 30 mm between seeds. All seed plates were incubated for 4 days at 24°C. Putative *P. longicolla* colonies and the type strain TWH P74 (ATCC 60325) were transferred to new APDA plates. Two isolates from each cultivar were single-spore purified for verification. *P. longicolla* was initially identified with a comparison of the type strain using morphological characteristics including conidial type, sporulation, and stromal pattern according to Hobbs et al. (1985). Further identification was confirmed by analysis of the internal transcribed spacer (ITS) regions of rDNA amplified by PCR with primers ITS1, 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4, 5'-TCCTCCGCTTATTGATATGC-3' (Li et al. 2015b; White et al. 1990), and the translation elongation factor 1-α gene (TEF-1 α) with primers EF1-728F, 5'-CATCGAGAAGTTCGAGAAGG-3',

and EF1-986R, 5'-TACTTGAAGGAACCTTACC-3' (Carbone and Kohn 1999; Li and Hartman 2003). All sequences were analyzed using BLASTn at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. Other soybean seed pathogens, such as *Alternaria* spp., *Cercospora* spp., and *Fusarium* spp., have different culture morphology from *P. longicolla* and were identified based on the description in Hartman et al. (2015).

The number of seeds infected with *P. longicolla* was recorded and calculated as percent seed infection. Seed germination of 100 arbitrarily selected seeds from each plot was determined using a standard soybean seed germination protocol (Association of Official Seed Analysts 2001). Visual scoring of seed quality was determined with a scale of 1 to 5 as previously reported (Li et al. 2015c), in which 1 = excellent (no discolored seed); 2 = good (less than 10% discolored seed); 3 = fair (11 to 30% discolored seed); 4 = poor (31 to 50% discolored seed); and 5 = very poor (more than 50% discolored seed). Seed wrinkling, molding, mottling, and discoloration were the factors in estimating seed quality (Li et al. 2015c).

Weather data of total precipitation, number of rainy days, average maximum temperatures, and maximum relative humidity during the soybean growing season were obtained from the Stoneville, MS, weather station (<http://ext.msstate.edu/anr/drec/stations.cgi?defstation=Stoneville>).

Table 3. Analysis of variance of *Phomopsis* seed infection, germination, and visual seed quality of 16 cultivars from inoculated field trials with two harvest times in Stoneville, MS, in 2012 and 2013^r

Source of variance	Num DF ^s	PSD ^t		Germination ^u		Visual score ^v	
		F	P ≥ F	F	P ≥ F	F	P ≥ F
Fixed effects							
Maturity group IV							
INOC ^w	1	25.77	<0.0001	41.76	<0.0001	0.00	0.9857
CV ^x	5	3.96	0.0022	7.57	<0.0001	1.53	0.1844
INOC × CV	5	0.63	0.6743	2.28	0.0503	2.27	0.0511
HAR ^y	1	164.08	<0.0001	41.15	<0.0001	3.97	0.0485
INOC × HAR	1	0.04	0.8384	10.03	0.0019	0.00	0.9971
CV × HAR	5	4.24	0.0013	2.98	0.014	1.53	0.1848
INOC × CV × HAR	5	0.17	0.9716	0.15	0.9791	2.27	0.0514
YR ^z	1	133.49	<0.0001	9.02	0.011	0.00	0.9986
INOC × YR	1	10.04	0.0019	27.68	0.0002	3.81	0.0742
CV × YR	5	2.33	0.046	3.12	0.0107	2.28	0.0507
INOC × CV × YR	5	0.07	0.9968	0.52	0.764	1.54	0.1825
HAR × YR	1	73.59	<0.0001	2.96	0.0878	0.00	0.9991
INOC × HAR × YR	1	0.06	0.8146	11.15	0.0011	3.89	0.0507
CV × HAR × YR	5	2.74	0.0218	1.86	0.1056	2.28	0.0507
INOC × CV × HAR × YR	5	1.51	0.1892	0.70	0.6249	1.53	0.1836
Maturity group V							
INOC	1	22.47	<0.0001	3.36	0.0680	1.31	0.2629
CV	9	11.76	<0.0001	2.76	0.0043	1.96	0.0612
INOC × CV	9	0.93	0.5043	1.75	0.0778	1.97	0.0603
HAR	1	13.67	0.0003	2.76	0.0979	0.00	0.9994
INOC × HAR	1	10.01	0.0018	3.06	0.0813	0.00	0.9935
CV × HAR	9	7.5	<0.0001	1.80	0.0692	0.00	1.0000
INOC × CV × HAR	9	1.64	0.1058	1.96	0.0442	0.00	1.0000
YR	1	372.88	<0.0001	0.27	0.6030	2.36	0.1587
INOC × YR	1	6.01	0.0152	2.22	0.1376	2.40	0.1556
CV × YR	9	5.6	<0.0001	1.82	0.0654	5.65	<0.0001
INOC × CV × YR	9	0.85	0.5699	1.85	0.0602	5.66	<0.0001
HAR × YR	1	18.72	<0.0001	2.76	0.0979	0.00	0.9994
INOC × HAR × YR	1	0.7	0.4036	3.06	0.0813	0.00	0.9935
CV × HAR × YR	9	5.9	<0.0001	1.80	0.0692	0.00	1.0000
INOC × CV × HAR × YR	9	0.67	0.7340	1.96	0.0442	0.00	1.0000

^r Analysis of variance (ANOVA) was performed using generalized linear mixed model (PROC GLMMIX) of SAS.

^s The numerator degree of freedom.

^t *Phomopsis* seed infection was calculated as percent seed infection by *Phomopsis longicolla* from the seed plating assays.

^u Percent seed germination based on tests of 100 seeds from each of four replications.

^v Visual quality of seeds were assessed using a scale of 1 to 5.

^w Soybean plants were inoculated with a spore suspension of *P. longicolla* (1 to 1.5 × 10⁵/ml) at the R5 growth stage. Noninoculated plants were sprayed with distilled water.

^x Cultivar.

^y Two harvest times: prompt harvest (R8 growth stage) versus delayed harvest (R8+2 weeks).

^z Year.

Data analyses. Statistical analysis of data were performed using the generalized linear mixed procedure (PROC GLMMIX) of SAS (version 9.4, SAS Institute, Cary, NC) with a negative binomial distribution and a log link function specified for *Phomopsis* seed

infection (Littell et al. 2006). Seed germination and seed quality scores were calculated as the mean percentage of each cultivar. Data were combined across the two years to test if there were interactions between cultivar and year when “year” was the fixed effect, and to

Table 4. Percent seed infection by *Phomopsis longicolla* of six maturity group IV soybean cultivars in replicated, inoculated field tests with prompt and delayed harvests in Stoneville, MS, in 2012 and 2013

Cultivar	Year	Maturity group	Phomopsis (%) ^s			
			Prompt harvest ^t		Delayed harvest ^u	
			Non ^v	Inoc ^w	Non	Inoc
AP 350 ^x	2012	IV	15.0 ab ^y	26.0 a	48.0 ab	43.0 abc
Armor ARX 1482	2012	IV	19.0 ab	18.0 abc	42.0 abc	49.0 ab
Morsoy R2 491	2012	IV	11.0 b	12.0 bc	28.0 c	31.0 bc
Morsoy R2s 480	2012	IV	11.0 b	10.0 c	60.0 a	64.0 a
Pioneer 94Y90	2012	IV	20.0 a	23.0 ab	33.0 bc	45.0 ab
SS93-6181 ^z	2012	IV	12.0 b	13.0 bc	44.0 abc	41.0 bc
Mean			14.7	17.0	42.5	45.5
AP 350	2013	IV	1.6 a	2.4 ab	0.8 a	8.0 a
Armor ARX 1482	2013	IV	1.5 a	3.2 ab	1.2 a	3.7 b
Morsoy R2 491	2013	IV	1.4 a	2.1 ab	0.5 a	4.0 b
Morsoy R2s 480	2013	IV	1.4 a	1.8 b	1.8 a	4.2 b
Pioneer 94Y90	2013	IV	1.6 a	5.2 a	1.8 a	4.2 b
SS93-6181	2013	IV	1.4 a	1.9 b	1.4 a	4.3 b
Mean			1.5	2.8	1.3	4.7

^s Percent seed infection by *P. longicolla* from plating assays analyzed with a negative binomial distribution and log link function.

^t Harvested promptly at R8 growth stage.

^u Delayed harvest 2 weeks after R8.

^v Noninoculated treatment.

^w Inoculated treatment.

^x Susceptible check.

^y Means followed by the same letter within a column are not significantly different as determined by the least significant difference test at $P \leq 0.05$.

^z Resistant check.

Table 5. Percent seed infection by *Phomopsis longicolla* of 10 maturity group V soybean cultivars in replicated, inoculated field tests with prompt and delayed harvests in Stoneville, MS, in 2012 and 2013

Cultivar	Year	Maturity group	Phomopsis (%) ^s			
			Prompt harvest ^t		Delayed harvest ^u	
			Non ^v	Inoc ^w	Non	Inoc
Asgrow 5606	2012	V	6.0 cd ^x	6.0 c	14.7 bc	20.0 b
Asgrow 5831	2012	V	6.0 cd	19.0 b	10.0 cd	14.0 bc
Dyna-Gro 37RY52	2012	V	11.0 ab	12.0 b	34.0 a	59.0 a
Dyna-Gro 33C59	2012	V	9.0 bc	18.0 b	11.0 bcd	23.0 b
Progeny 4949	2012	V	11.0 ab	20.0 ab	25.0 ab	47.0 ab
Progeny 5650	2012	V	6.0 d	7.0 bc	6.0 d	13.0 bc
Progeny 5706	2012	V	5.0 d	9.0 bc	14.0 bc	14.0 bc
Terral 59R16	2012	V	22.0 a	22.0 a	24.0 abc	53.0 a
PI 424324B ^y	2012	V	6.0 d	6.0 c	6.0 d	5.0 c
Osage ^z	2012	V	12.0 ab	13.0 b	36.0 a	76.0 a
Mean			10.3	13.7	17.2	31.9
Asgrow 5606	2013	V	2.6 bcd	4.0 abc	2.6 a	1.8 d
Asgrow 5831	2013	V	2.2 bcd	5.0 ab	0.5 bc	6.5 ab
Dyna-Gro 37RY52	2013	V	2.2 bcd	2.2.bcd	3.3 a	5.2 abc
Dyna-Gro 33C59	2013	V	4.6 ab	6.8 a	1.0 abc	4.8 abc
Progeny 4949	2013	V	5.4 a	3.2 bcd	2.2 ab	7.0 a
Progeny 5650	2013	V	1.4 cde	1.0 de	1.8 abc	1.6 de
Progeny 5706	2013	V	0.8 de	2.0 cde	1.0 abc	2.7 cd
Terral 59R16	2013	V	3.2 abc	3.2 bcd	2.0 abc	3.3 bcd
PI 424324B	2013	V	0.0 e	0.6 e	0.0 c	0.0 e
Osage	2013	V	6.6 a	3.4 abc	2.6 ab	6.6 ab
Mean			2.9	3.2	1.7	4.0

^s Percent seed infection by *P. longicolla* from plating assays analyzed with a negative binomial distribution and log link function.

^t Harvested promptly at R8 growth stage.

^u Delayed harvest 2 weeks after R8.

^v Noninoculated treatment.

^w Inoculated treatment.

^x Means followed by the same letter within a column are not significantly different as determined by the least significant difference test at $P \leq 0.05$.

^y Resistant check.

^z Susceptible check.

compare cultivars within a year, in which year was the random effect and the cultivar was the fixed effect. The cultivars were compared with Fisher's least significant difference (LSD) at $P \leq 0.05$. The PROC CORR procedure of SAS was used to compute Pearson's correlation coefficients between percent seed infected by *P. longicolla* and germination rate, and between percent seed infection and visual seed quality.

Results

Maximum air temperatures during the soybean growing season in 2012 averaged 29.8°C and ranged from 21.5 to 33.9°C. In 2013, air temperatures averaged 28.8°C and ranged from 22.3 to 33.8°C. In August, most of plants were at the R5 or R6 growth stage. The average daily maximum air temperature for August in both 2012 and 2013 was similar at 33.9 and 33.8°C, respectively, which was higher than 24°C, the optimal temperature for PSD. In 2012, total precipitation during June, July, and August was 162, 116, and 169 mm, respectively, while in 2013, total precipitation during those months was 93, 49, and 51 mm, respectively.

Results from seed assays prior to planting indicated that soybean cultivars selected for planting were generally of good quality. Seeds obtained for our tests were free of *P. longicolla*. However, the PSD-causing pathogen was found in seeds harvested from the field experiments. All putative *P. longicolla* isolates obtained from seed plating assays had typical morphology similar to the type strain TWH P74 (ATCC 60325), although there were slight differences in stromal patterns. DNA sequences of 16 selected isolates were identical at the ITS region (accession MF134860) and the *TEF-1α* gene (MF189565). These had 99 to 100% identity with many reported sequences of *P. longicolla* strains in GenBank, such as isolate FAU657 (KJ590727) and type strain TWH P74 (NR_144924) at the ITS regions, and isolate FAU657 (590766), isolate 600 (AF398896), and ER 1678 (HQ445912) in *TEF-1α* gene. However, they were only 84% similar with *P. sojae* (syn. *D. phaseolorum* var. *sojae*) isolates, such as FAU604 (KJ590759), PS/KR (JF461471), and FAU499 (KJ590760). Other seed-borne pathogens, such as *Cercospora* spp., which produces purple coloration on seed coat and APDA, and *Alternaria* spp., which has an inverted club shape conidia, were easily identified. Isolation frequency of *Cercospora* spp. ranged from 0 to 24%, *Fusarium* spp. were from 0 to 30%, while *Alternaria* spp. were rarely isolated.

Analysis of variance of *Phomopsis* seed infection of the experiments involving genotypes of MG IV and MG V indicated that there were significant differences ($P \leq 0.05$) in cultivars (CV), inoculation (INOC) treatments, harvest time (HAR), and years (YR). There also were interactions of CV × HAR, INOC × YR, CV × YR, HAR × YR, and CV × HAR × YR (Table 3). Mean percentage infected seeds for maturity groups (MGs) IV and V cultivars are shown in Table 4 and Table 5, respectively. Cultivars Morsoy R2S 480 (MG IV) and Progeny 4949 (MG V) had low *Phomopsis* seed infection when harvested on time but were susceptible to PSD with delayed harvest (Tables 4 and 5). The MG IV cultivar Morsoy R2 491 had significantly ($P \leq 0.05$) lower percent seed infected by *P. longicolla* than the susceptible check and all other cultivars. Although none of the MG V cultivars had significantly lower percent seed infected by *P. longicolla* than their respective resistant check PI 424324B, five cultivars (Progeny 5650, Progeny 5706, Asgrow 5606, Asgrow 5831, and Dyna-Gro33C59) had significantly lower *Phomopsis* seed infection than the susceptible group V cultivar Osage.

Effects of harvest time and year on *P. longicolla* seed infection with the combination of inoculation treatments are shown in Table 6. In most cases, *P. longicolla* infection was higher in plots with delayed harvest than those with prompt harvest. For example, susceptible cultivar AP 350 had 20.5% infection if harvested on time and 45.5% infection with delayed harvest in 2012. However, resistant PI 424324B had the same low percentages of *Phomopsis* seed infection in both harvest schemes in 2012 (Table 6).

There were differences in seed germination and visual scores of seed quality among cultivars (Table 7). For example, in 2012, PSD-resistant cultivar Morsoy R2 491 had higher seed germination of 96 and 87% in the noninoculated and inoculated treatments, respectively, when harvested on time. However, susceptible cultivar AP 350 had seed germination of 79 and 69% in the noninoculated and inoculated treatments, respectively. In general, inoculation treatments reduced seed germination in most cultivars. For example, in 2013, cultivar Armor ARX 1482 had 91% seed germination in the noninoculated trial but had only 76% seed germination in the inoculated trial when harvested on time. In 2013, Asgrow 5606 had 80% germination in the noninoculated trial but had only 59% seed germination in the inoculated trial with prompt harvest (Table 7). However, inoculation treatment did not reduce the percentage of seed germination in some cultivars, such as Dyna-

Table 6. Effect of harvest time and year in replicated field tests in Stoneville, MS, in 2012 and 2013 on percent seed infection by *Phomopsis longicolla*^t

Cultivar	Maturity group	2012 harvest			2013 harvest		
		Prompt ^u	Delayed ^v	<i>P</i> ^w	Prompt	Delayed	<i>P</i>
AP 350 ^x	IV	20.5 ab ^y	45.5 b	0.0055	2.0 ab	4.4 a	0.0007
Armor ADX 1482	IV	19.5 abc	45.5 b	0.0049	2.4 ab	2.5 b	0.1340
Morsoy R2 491	IV	11.5 bc	29.5 c	0.0264	1.8 b	2.3 b	0.1151
Morsoy R2S 480	IV	10.5 c	62.0 a	<0.0001	1.6 b	3.0 ab	0.0036
Pioneer 94Y90	IV	21.5 a	39.0 bc	0.0310	3.4 a	3.0 ab	0.2229
SS93-6181 ^z	IV	12.5 bc	42.5 b	0.0004	1.7 ab	2.9 ab	0.0117
Mean		15.8	44.0		2.1	3.0	
Asgrow 5606	V	6.0 d	17.4 c	0.0062	2.2 b	3.3 bcd	0.3343
Asgrow 5831	V	12.5 c	12.0 c	0.8550	3.5 ab	3.6 bc	0.5467
Dyna-Gro37RY52	V	11.5 c	46.5 ab	<0.0001	4.2 a	2.2 cdef	0.0532
Dyna-Gro 33C59	V	13.5 c	17.0 c	0.3736	2.9 ab	5.7 a	0.0125
Progeny 4949	V	17.0 b	36.0 b	0.0022	4.6 a	4.3 ab	0.9830
Progeny 5650	V	6.5 cd	9.5.0 c	0.4083	1.7 bc	1.2 ef	0.4085
Progeny 5706	V	7.0 cd	14.0 c	0.1338	1.8 bc	1.4 def	0.6305
Terral 59R16	V	22.0 b	38.5 b	0.0434	2.6 ab	3.2 bcde	0.5289
PI 424324B ^z	V	6.0 d	5.5 c	0.9750	0.0 c	0.3 f	0.5845
Osage ^x	V	12.5 c	56.0 a	<0.0001	4.6 a	5.0 ab	0.5371
Mean		12.0	24.5		2.8	3.0	

^t Percent seed infection by *P. longicolla* from plating assays analyzed with a negative binomial distribution and log link function.

^u Harvested promptly at R8 growth stage.

^v Delayed harvest 2 weeks after R8.

^w Probability value for test of significance.

^x Susceptible check.

^y Means followed by the same letter within a column are not significantly different by the least significant difference test at $P \leq 0.05$.

^z Resistant check.

Gro37RY52 and Dyna-Gro 33C59 in the on-time harvest trials in 2012 (Table 7). In general, percent seed infected by *P. longicolla* was negatively correlated with germination rate, but positively correlated with visual seed quality in delayed harvest of MGVI with inoculated treatment and overall harvest time in the inoculated trial of MG V (Table 8). None of the noninoculated trials had significant correlations between percent seed infected by *P. longicolla* and visual seed quality scores.

Discussion

Environmental conditions have been reported to play an important role in the development of PSD (Gleason and Ferriss 1985; Rupe

1990; Spilker et al. 1981). The favorable environments for PSD, such as warm and humid conditions during the late season from beginning pod fill through harvest, promote the colonization of *P. longicolla*, causing high levels of seed infection.

In the southern United States, the “early soybean production system (ESPS)” is very popular (Heatherly 1999), where early maturing cultivars are planted in late March through April, and mature in August to early September. This system allows the crop to take advantage of plentiful spring and early summer rains to increase chances to avoiding late-season drought, leading to increased yields (Heatherly 1999). However, environments in August are usually hot and humid, which could cause severe PSD and poor seed germination in the

Table 7. Percent seed germination of soybean cultivars and visual seed quality in replicated field tests in Stoneville, MS, in 2012 and 2013

		Harvest							
		Prompt ^r				Delayed ^s			
		GM ^t		VQ ^u		GM		VQ	
Cultivar	Year	Non ^v	Inoc ^w	Non	Inoc	Non	Inoc	Non	Inoc
Maturity group IV									
Armor ARX 1482	2012	78.8	73.0	2.1	2.4	75.5	54.3	2.4	2.5
Morsoy R2 491	2012	95.5	86.8	2.1	2.0	86.5	77.8	2.3	2.3
Morsoy R2s 480	2012	87.3	82.8	2.1	2.0	61.0	30.0	2.1	2.4
Pioneer 94Y90	2012	83.3	63.5	2.3	2.1	75.0	61.8	3.0	3.0
SS93-6181 ^x	2012	83.0	84.8	2.0	1.8	81.8	63.0	2.8	2.8
AP 350 ^y	2012	79.0	61.3	2.5	2.1	52.3	40.3	3.1	3.5
Mean		84.5	75.3	2.2	2.1	72.0	54.5	2.6	2.7
LSD ^z		8.5	24.5	0.4	0.4	21.1	13.9	0.7	0.7
Armor ARX 1482	2013	90.7	76.3	1.6	1.9	50.7	44.8	2.6	3.6
Morsoy R2 491	2013	87.3	81.4	1.9	2.0	62.4	50.3	2.3	2.9
Morsoy R2s 480	2013	91.3	83.3	1.6	2.0	34.5	28.7	2.3	3.0
Pioneer 94Y90	2013	82.0	79.0	1.6	2.0	33.2	46.8	2.6	2.8
SS93-6181	2013	82.7	70.5	1.3	2.1	41.8	18.3	3.0	3.6
AP 350	2013	85.2	69.0	1.4	2.0	49.3	18.0	2.4	3.4
Mean		86.5	76.6	1.6	2.0	45.3	34.5	2.5	3.2
LSD		22.3	29.0	0.7	0.7	30.5	25.1	0.7	0.9
Maturity group V									
Asgrow 5606	2012	94.3	92.8	2.1	1.5	96.8	87.3	2.5	1.6
Asgrow 5831	2012	95.3	94.0	2.0	1.5	94.8	95.0	2.3	1.8
Dyna-Gro 37RY52	2012	94.0	96.0	1.8	1.3	76.5	74.5	2.0	1.9
Dyna-Gro 33C59	2012	93.3	94.8	1.9	1.5	94.0	92.5	1.9	1.8
Progeny 4949	2012	92.3	86.8	2.6	2.1	71.8	58.8	3.8	2.9
Progeny 5650	2012	93.5	89.8	1.8	2.3	92.3	87.8	2.1	2.0
Progeny 5706	2012	96.0	91.0	2.1	1.9	94.0	95.0	2.1	2.1
Terral 59R16	2012	91.0	87.3	2.4	1.8	77.3	70.8	2.6	2.4
PI 424324B ^x	2012	97.0	92.8	1.3	1.6	96.5	97.8	1.5	1.5
Osage ^y	2012	93.3	95.0	1.6	1.6	80.8	75.5	1.8	2.0
Mean		94.0	92.0	2.0	1.7	87.5	83.5	2.3	2.0
LSD		5.7	10.8	0.6	0.6	12.5	13.4	0.7	0.6
Asgrow 5606	2013	80.3	59.0	2.1	2.3	59.0	77.0	1.8	2.0
Asgrow 5831	2013	70.0	60.5	1.8	2.5	60.5	74.8	1.6	2.8
Dyna-Gro 37RY52	2013	67.4	48.2	1.8	1.6	48.2	48.3	1.8	2.1
Dyna-Gro 33C59	2013	62.5	40.3	1.8	2.3	40.3	65.3	1.5	2.5
Progeny 4949	2013	50.2	42.6	2.3	2.3	42.6	17.3	2.4	3.4
Progeny 5650	2013	87.3	66.8	1.6	2.3	66.8	55.8	1.9	2.1
Progeny 5706	2013	90.3	87.8	2.0	2.4	87.8	85.8	1.6	2.4
Terral 59R16	2013	57.0	55.0	2.6	2.6	55.0	55.1	2.3	3.5
PI 424324B	2013	95.5	89.5	1.8	1.3	89.5	92.2	1.0	1.1
Osage	2013	73.0	42.1	2.0	2.1	42.1	40.9	1.9	2.0
Mean		73.3	59.2	2.0	2.2	59.2	61.2	1.8	2.4
LSD		24.5	23.3	0.7	0.7	23.3	24.3	0.4	0.7

^r Harvested promptly at R8 growth stage.

^s Delayed harvest 2 weeks after R8.

^t Percent seed germination based on tests of 100 seed from each of four replications.

^u Visual seed quality were assessed using a scale of 1 to 5.

^v Noninoculated control sprayed with distilled water.

^w Plants were inoculated with spore suspension of *Phomopsis longicolla* (2×10^5 /ml) at the R5 stage.

^x Resistant check.

^y Susceptible check.

^z Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

south (Mayhew and Caviness 1994). In this study, we analyzed the effect of harvest times (prompt versus delayed harvest) on the incidence of PSD. Our research has demonstrated that seed infected by *P. longicolla* was more severe with delayed harvest compared with prompt harvest at maturity. Cultivars that had low Phomopsis seed infection when harvest was prompt could be susceptible to *P. longicolla* with delayed harvest. An example is Morsoy R2S 480, which had the lowest Phomopsis seed infection in the on-time harvest trial, but had the highest seed infection in the delayed harvest trial in 2012. Therefore, testing cultivars under a delayed harvest regime or under the conditions that favor PSD disease development is a useful method for identification of PSD-resistant cultivars.

Wrather et al. (2003) reported that yield losses caused by PSD varied by years. Our results are in agreement with this finding. In our study, there were significant ($P \leq 0.01$) differences of percent seed infection by *P. longicolla* in 2012 and 2013. Overall, the incidence of PSD was higher in 2012 than that in 2013. This difference could be due to, but not limited to, the weather conditions in each year. Although the maximum air temperatures were similar during the soybean growing season in 2012 and 2013, total precipitation during August in 2012 (169 mm) was three times higher than in 2013 (51 mm), when most plants were at R5 growth stages when beans began to develop (Fehr and Caviness 1997). The difference in August rainfall between the two years was likely the main cause of difference in PSD incidence between the two years.

Pathogen infection is often erratic under field environments due to uneven distribution of the pathogen. Inoculation treatments have been used to increase disease pressure in field and greenhouse experiments for identification and confirmation of disease resistance in soybean (de Farias Neto et al. 2006; Hartman et al. 1997; Li et al. 2011; Mueller et al. 2003; Shakiba et al. 2012). Artificial inoculation under controlled conditions can reduce the incidence of disease escape by providing a more uniform environment for pathogen growth and disease development. In this study, regardless of maturity group harvest scheme, seeds from inoculated trials had higher incidence of PSD than noninoculated trials. However, if the weather conditions are non-conducive for PSD development, inoculation treatment may not differentiate resistant from susceptible lines. For example, in a previously published experiment in 2011, extremely hot and dry weather during late season resulted in almost no seed infection in inoculated and noninoculated trials in Arkansas, Missouri, and Mississippi (Li et al. 2015c). Moreover, if the weather conditions

are wet and humid, there was little or no significant difference between inoculated and noninoculated treatments (Li et al. 2011). The quality and quantity of the pathogen inoculum must be taken into account for field screening experiments with inoculation treatments. Inoculation experiments can fail if poor quality inoculum (the pathogen is unviable or has low virulence or was not properly maintained) is used. In screening diverse soybean germplasm lines for reaction to purple seed stain (PSS) caused by *Cercospora kikuchii*, Alloatti et al. (2015) found that an old isolate of *C. kikuchii* with reduced sporulation likely contributed to the lower infection rate. In one of our previous field tests (unpublished data), inoculation with a spore suspension of less than 1.5×10^4 /ml did not produce good seed infection, especially when the weather was not favorable for PSD. The use of natural infection could be an alternative approach for screening resistance to soybean diseases in the field, but the best results will only be obtained when the environmental conditions are conducive for disease development. There is often a high risk for plants escaping pathogen infection if the pathogen is not evenly distributed in the field, leading to false conclusions in identifying resistance.

Seed quality is very important for the soybean growers and processors. PSD is a soybean seed disease causing poor seed quality and reducing seed germination. In this study, susceptible cultivar AP 350 had poor germination, especially in inoculated trials with delayed harvest, while resistant genotypes, such as Morsoy R2 491, PI 424324B, and Progeny 5706 had high germination. We also found that percentages of Phomopsis seed infection were significantly ($P \leq 0.01$) and negatively correlated with seed germination in either noninoculated or inoculated treatments with delayed harvest. However, the negative correlation between Phomopsis seed infection and germination rate was not significant in the prompt harvest trials. This is likely due to the lower seed infection rates compared with delayed harvest.

The visual seed scoring method was developed to evaluate seed quality (Li et al. 2015c). Since there are different seed-borne pathogens in soybean (Baird et al. 2001; Hartman et al. 2015), seed with poor appearance could be coinfecting by other pathogens besides *P. longicolla*. In this study, a number of different fungal pathogens, such as *Alternaria*, *Cercospora*, and *Fusarium* spp. were identified during the seed plating assays. Therefore, identification of sources of resistance to multiple seed-borne pathogens can greatly improve seed quality of soybean.

The use of PSD-resistant cultivars to protect soybean crops from the disease is economical and environmentally prudent, especially when using the popular ESPS in southern states. In this study, significant differences in seed infection among cultivars in response to *P. longicolla* attack were observed. Differences among cultivars were also reflected in seed germination and visual seed quality. Six cultivars, including one MG IV (Morsoy R2 491) and five MG V (Progeny 5650, Progeny 5706, Asgrow 5606, Asgrow 5831, and Dyna-Gro33C59), had significantly ($P \leq 0.05$) lower seed infection than the susceptible checks and other cultivars in the same tests at both harvest regimes. The fact that group IV cultivars had more infection to Phomopsis than group V cultivars may be related to relative maturity. Information obtained from this study will be useful for soybean growers and breeders for selection of cultivars for planting or breeding and future genetic studies in the development of cultivars with improved resistance to PSD.

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Table 8. Pearson correlation coefficients between percent seed infection with *Phomopsis longicolla* and seed germination, and visual seed quality in replicated field tests in Stoneville, MS, in 2012 and 2013

Maturity group	Treatment ^u	Harvest ^v	Germination ^w		Visual quality ^x	
			r ^y	p ^z	r	p
IV	Non	Prompt	-0.6324	0.1779	0.1900	0.7184
IV	Inoc	Prompt	0.5273	0.2824	0.0847	0.8732
IV	Non	Delayed	0.7835	0.0026	0.3499	0.2649
IV	Inoc	Delayed	-0.6111	0.0071	0.4500	0.0610
IV	Non	All times	-0.8784	<0.001	0.0332	0.8960
IV	Inoc	All times	-0.8094	0.0014	0.1548	0.6309
V	Non	Prompt	-0.6149	0.0585	0.1010	0.7814
V	Inoc	Prompt	-0.5037	0.1378	0.5420	0.1056
V	Non	Delayed	-0.6533	<0.0001	0.1558	0.3371
V	Inoc	Delayed	-0.7977	0.0057	0.4381	0.2054
V	Non	All times	-0.7479	0.0001	0.0386	0.8717
V	Inoc	All times	-0.6255	0.0032	0.4439	0.0500

^u Inoculation treatments. Non = noninoculated control sprayed with distilled water; Inoc = inoculated with spore suspension of *P. longicolla* (2×10^5 /ml) at the R5 growth stage.

^v Prompt: harvested at R8 growth stages, delayed: harvested 2 weeks after R8.

^w Percent seed germination.

^x Visual seed quality were assessed using a scale of 1 to 5.

^y Pearson correlation coefficients.

^z Probability.

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