

## Isolates of *Diaporthe–Phomopsis* from weeds and their effect on soybean

A. Mengistu, L.A. Castlebury, J.R. Smith, A.Y. Rossman, and K.N. Reddy

**Abstract:** Greenhouse and laboratory studies were conducted to determine the identity and pathogenicity of *Diaporthe–Phomopsis* species complex recovered from eight weed species. The identifications of the eight isolates representing four taxa, including two apparently undescribed species of *Phomopsis*, were based on colony features in pure culture, morphology of alpha or beta-conidia, and internal transcribed spacer sequences. Of the eight isolates, *Phomopsis* sp. A, from *Eclipta prostrata* (eclipta), *Phomopsis longicolla* isolates from both *Ipomoea lacunosa* (pitted morning-glory) and *Chamaesyce nutans* (nodding spurge), and *Diaporthe phaseolorum* from *Desmanthus illinoensis* (Illinois bundle-flower) caused significant levels of infection on soybean hypocotyls, pods, and seeds. These four isolates from weed species also caused systemic infection of seed similar to the soybean isolate of *P. longicolla*. *Diaporthe phaseolorum* isolated from *Caperonia palustris* (Texasweed) and *Aster exilis* (slender aster), *Phomopsis* sp. B, from *Sida spinosa* (prickly sida), and *Phomopsis* sp. A from *Polygonum aviculare* (prostrate knotweed) were not pathogenic to soybean. This is the first demonstration that *Phomopsis* sp. A, *P. longicolla*, and *D. phaseolorum* isolated from eclipta, pitted morning-glory, nodding spurge, and Illinois bundle-flower cause seed infection of soybean.

**Key words:** *Phomopsis longicolla*, nuclear DNA ITS, seed quality, soybean, weeds.

**Résumé :** Des études en serre et en laboratoire furent effectuées dans le but de déterminer l'identité et le pouvoir pathogène d'espèces de *Diaporthe–Phomopsis* isolées de huit espèces d'adventices. Les identifications des huit isolats, qui représentaient quatre taxons, y compris deux espèces de *Phomopsis* apparemment non décrites, furent basées sur les caractéristiques des colonies en culture pure, la morphologie des conidies alpha ou bêta et les séquences des espaceurs transcrits internes. Des huit isolats, le *Phomopsis* sp. A, isolé de l'*Eclipta prostrata* (Mahakanni), les isolats de *P. longicolla* isolés à la fois de l'*Ipomoea lacunosa* (volubilis) et du *Chamaesyce nutans* (euphorbe penchée), et le *Diaporthe phaseolorum* du *Desmanthus illinoensis* (mimosa de l'Illinois) causèrent d'importantes infections sur les hypocotyles, les gousses et les graines de soja. Ces quatre isolats obtenus d'adventices causèrent aussi une infection systémique des graines similaire à celle causée par l'isolat du soja du *P. longicolla*. Le *Diaporthe phaseolorum* isolé du *Caperonia palustris* et de l'*Aster exilis*, le *Phomopsis* sp. B, isolé du *Sida spinosa*, et le *Phomopsis* sp. A isolé du *Polygonum aviculare* (renouée des oiseaux) ne furent pas pathogènes pour le soja. Ces résultats constituent la première démonstration que le *Phomopsis* sp. A, le *P. longicolla* et le *D. phaseolorum* isolé du Mahakanni, du volubilis, de l'euphorbe penchée, et du mimosa de l'Illinois causent l'infection des graines de soja.

**Mots-clés :** *Phomopsis longicolla*, ITS d'ADN nucléaire, qualité des graines, soja, adventices.

### Introduction

Infection of soybean (*Glycine max* (L.) Merrill) seed is one of several ubiquitous problems caused by members of the *Diaporthe–Phomopsis* complex (Hepperly et al. 1980; Roy 1997; Roy et al. 1994) and is commonly attributed to *Phomopsis longicolla* T.W. Hobbs (Hobbs et al. 1985). Even though infested crop debris and soil are the major

sources of primary inoculum, diseased seeds are an important factor in the long-range dissemination of the pathogen (Hartman et al. 1999). Severely infected seeds are shriveled, elongated, and cracked and appear white and chalky. However, seeds may be infected and not show symptoms. Generally, infected seeds do not germinate or are slow to germinate. Soybean pods can become infected at any time after they are formed, but significant seed infections do not

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occur before physiological maturity. Seed infection tends to be more severe when harvest is delayed. Progressive spread in the plant is caused by infection from conidia dispersed by splashed water.

Besides its effect on soybean seed, *P. longicolla* has been documented to infect some weed species (Li et al. 2001; Roy 1997; Roy et al. 1994; Uecker 1989). Weeds can serve as alternate hosts for fungal pathogens that affect crop plants (Borromeo et al. 1993; McLean and Roy 1991; Roy et al. 1994; White et al. 1990), including soybean plants and seed (Black et al. 1996; Hepperly et al. 1980). However, there have been questions about whether strains isolated from weeds in soybean fields might actually cause infection in soybeans. The association between weeds and *P. longicolla* has been established previously (Roy 1997; Roy et al. 1994; Uecker 1989). However, the morphological identification of *Diaporthe-Phomopsis* isolates recovered from weeds has been difficult because of overlapping morphological characters (Hobbs et al. 1985; Morgan-Jones 1989). The weed species used in this experiment were selected because they represent the most commonly found weeds in soybean fields in Stoneville, Mississippi. However, these weed species are also distributed extensively throughout the lower Mississippi Delta region and beyond.

Previous studies have used wound inoculation techniques to determine the pathogenicity of *P. longicolla* on soybean seedlings (Mengistu and Reddy 2005; Li et al. 2001; Roy 1997; Roy et al. 1994). However, although wound inoculation techniques produce definitive results, they may not provide an accurate assessment of the pathogenicity of *P. longicolla* on soybean seed. Methods other than hypocotyl inoculation, such as the inoculation of vegetative plant parts (mainly leaves) and mature pods and seeds, are needed to establish the association with seed infection. The objectives of this research were (i) to identify *Diaporthe-Phomopsis* species recovered from weeds using morphological characteristics and internal transcribed spacer (ITS) sequences and (ii) to establish whether infection of vegetative and reproductive plant parts by these weed isolates can result in soybean seed infection.

## Materials and methods

### Morphological identification of the weed isolates

A single isolate of *Diaporthe-Phomopsis* from each of the eight weed species, eclipta (*Eclipta prostrata* L.), prickly sida (*Sida spinosa* L.), Illinois bundle-flower (*Desmanthus illinoensis* (Michx.) Macmill. ex B.L. Robins.), Texasweed (*Caperonia palustris* (L.) St. Hil.), prostrate knotweed (*Polygonum aviculare* L.), pitted morning-glory (*Ipomoea lacunosa* L.), nodding spurge (*Chamaesyce nutans* (Lag.) Small), slender aster (*Aster exilis* Ell.), and one isolate from soybean recovered from seed in Stoneville Mississippi were plated on acidified potato dextrose agar (APDA, pH 5.6; Difco Laboratories, Detroit, Mich.)<sup>2</sup> From each sporulating isolate, 30 monoconidial *Phomopsis*-like isolates were transferred and from each nonsporulating isolate, 30 hyphal tip transfers were made and examined for morphological

and cultural characteristics. Each isolate was examined for sporulation, conidial dimensions, pattern of stroma, and presence or absence of beta-conidia and perithecia when cultured on APDA. Because the 30 monoconidial isolates and hyphal-tip cultures were morphologically similar within each of the eight original isolates, a single conidial isolate from each sporulating weed and soybean as well as a single hyphal tip from each nonsporulating isolate was transferred to new plates of APDA and grown for molecular identification and for additional morphological and cultural characterization. Cultures of the eight isolates from weeds and one isolate from soybean were deposited in the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands) and dried cultures were deposited in the US National Fungus Collections (BPI). These accession numbers and the GenBank accession numbers for DNA sequences are given in Table 1.

### DNA sequencing

DNA sequences from each weed and soybean isolate were compared with reference sequences from the type strain of *P. longicolla* (ATCC 60325 = FAU 600) and from a well-characterized strain of *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. (ATCC 64802 = FAU 458) (White et al. 1990). The PureGene tissue kit (Gentra Systems, Minneapolis, Minn.) was used to extract DNA from approximately 50 mg of mycelia scraped from the surface of 3- to 5-day-old cultures growing on Difco potato dextrose agar (PDA). Primers ITS 5 and ITS 4 (White et al. 1990) were utilized for the ITS regions 1 and 2, including the 5.8S rDNA.

The ITS gene regions were amplified in 50 µL reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, Calif.) under the following reaction conditions: 10–15 ng of genomic DNA, 200 µmol/L of each dNTP, 2.5 units AmpliTaq Gold (Applied Biosystems, Foster City, Calif.), 25 pmol of each primer, and 10 µL of the supplied 10 × PCR buffer with 15 mmol/L MgCl<sub>2</sub>. The thermal cycler program was as follows: 10 min at 95 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; and a final extension period of 10 min at 72 °C. After amplification, the PCR products were purified with QIAquick columns (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions. Amplified products were sequenced with the BigDye version 3.1 ready reaction kit (Applied Biosystems, Foster City, Calif.) on an ABI 3100 automated DNA sequencer. Raw sequences were edited into contigs using Sequencher version 4.5 for Windows (Gene Codes Corporation, Ann Arbor, Mich.) and were deposited in GenBank as AY745016–AY7455024 (Table 1).

### Test for pathogenicity

Two greenhouse experiments (experiments 1 and 2) were set up for testing the pathogenicity of the weed and one soybean isolates. These two experiments used a randomized complete block design with four replications. The experimental unit was a pot of 15 L capacity that was filled with soil, sand, and Jiffy-mix (1:1:1 ratio) and seeded with four plants per pot.

<sup>2</sup>Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

**Table 1.** Identification of *Diaporthe-Phomopsis* isolates, culture deposits (CBS and BPI), and the GenBank accession for isolates recovered from weeds and soybean at Stoneville, Mississippi.

Isolate name	Taxon	Culture No.*	Dried specimen*	GenBank accession No.	Host common name	Host scientific name
STAM 26 <sup>†</sup>	<i>Phomopsis</i> sp. A	CBS 116016	BPI 877411	AY745022	Eclipta	<i>Eclipta prostrata</i>
STAM 27 <sup>†</sup>	<i>P. longicolla</i>	CBS 121120	BPI 877412	AY745023	Pitted morning-glory	<i>Ipomoea lacunosa</i>
STAM 28 <sup>†</sup>	<i>P. longicolla</i>	CBS 116017	BPI 877413	AY745016	Nodding spurge	<i>Chamaesyce nutans</i>
STAM 29 <sup>†</sup>	<i>D. phaseolorum</i>	CBS 116018	BPI 877414	AY745017	Illinois bundle-flower	<i>Desmanthus illinoensis</i>
STAM 30	<i>D. phaseolorum</i>	CBS 116019	BPI 877415	AY745024	Texasweed	<i>Caperonia palustris</i>
STAM 31	<i>D. phaseolorum</i>	CBS 116020	BPI 877416	AY745018	Slender aster	<i>Aster exilis</i>
STAM 32	<i>Phomopsis</i> sp. B	CBS 116021	BPI 877417	AY745019	Prickly sida	<i>Sida spinosa</i>
STAM 33	<i>Phomopsis</i> sp. B	CBS 116022	BPI 877418	AY745020	Prostrate knotweed	<i>Polygonum aviculare</i>
STAM 35 <sup>†</sup>	<i>P. longicolla</i>	CBS 116023	BPI 877419	AY745021	Soybean	<i>Glycine max</i>

\*CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; BPI, US National Fungus Collections, Beltsville, Md.

<sup>†</sup>Isolates that produced alpha-conidia.

Four inoculation methods were used: hypocotyl, leaf, pod, and seed inoculations. Four plants per replication with a total of 16 plants were used to determine infections on hypocotyls, leaf, pod, and seed.

#### Hypocotyl inoculation

Hypocotyl inoculation was performed by wound inoculation at 3 mm below the cotyledonary node at the V1 growth stage (Fehr et al. 1971) with a 2 mm × 2 mm plug of a 7-day-old fungal culture. Inoculated plants were incubated at 30 °C with high humidity (95%–100%) for 24 h and then placed on a greenhouse bench. After 10 days, sixteen 5 mm stem pieces were removed from the inoculated plants and disinfected with 0.25% NaOCl for 60 s, blotted dry, and plated onto APDA. The percentages of dead seedlings and *Phomopsis* spp. recovered from inoculated hypocotyls were recorded. Control plants were wounded but not inoculated.

#### Leaf inoculation

Leaf inoculation was performed at the R5 stage (Fehr et al. 1971) using a sporulating 18-day-old culture. Spores at a concentration of  $1 \times 10^6$ /mL were sprayed over the entire plant, covering it until runoff occurred. The plants were then incubated at 30 °C with high humidity (95%–100%) for 24 h and then placed on a greenhouse bench. After 72 h, sixteen 5 mm leaf disks per soybean line were removed from the leaf at the uppermost node and disinfected with 0.25% NaOCl for 60 s, blotted dry, and plated onto APDA. In addition, pods and seeds harvested from leaf inoculation at R5 were assayed at the R7 growth stage (Fehr et al. 1971) (experiments 1 and 2). Control plants were sprayed with sterile distilled water at R5.

#### Pod inoculation

Pod inoculation was performed at the R7 growth stage by excising 16 noninoculated pods and disinfesting them with 0.25% NaOCl for 60 s. Disinfested pods were soaked in a spore suspension of  $1 \times 10^6$  spores·mL<sup>-1</sup> for 5 min and incubated in sealed plastic containers for 24 h at 30 °C. Pods were then removed from the incubator, air-dried, placed in sterile coin envelopes for 7 days, disinfested, and plated on APDA. The seeds within pods and shelled pods were assayed separately (experiments 1 and 2). Control pods were sprayed with sterile distilled water at R7.

#### Seed inoculation

Seed inoculation was performed as follows: 100 seeds were taken from noninoculated plants at the R7 growth stage (experiments 1 and 2), disinfested, and soaked in sterile distilled water for 24 h at room temperature (24 °C). Seeds were strained, blotted dry, and then resoaked in a  $1 \times 10^6$  spores·mL<sup>-1</sup> spore suspension for 5 min. Inoculated seeds were then incubated in a sealed plastic container for 24 h at 30 °C. Seeds were removed from the incubator, air-dried, and placed in sterile coin envelopes for 7 days. Seeds were then disinfested with 0.25% NaOCl for 60 s, blotted dry, and placed on APDA. Culture plates were incubated at 24 °C for 4 days, and the recovered cultures were identified and recorded. Control seeds were sprayed with sterile distilled water at R7.

#### Experiment 1

This experiment was to test the pathogenicity of the eight weed isolates (STAM 26 to STAM 33) and an isolate from soybean (STAM 35) using the four inoculation methods described above on ‘Maverick’ soybeans (Sleper et al. 1998). Assays were performed on inoculated hypocotyls at V1; assays on leaf discs, pods, and seeds were performed on plants inoculated at R5 stage. Assays were also performed on pods and seeds on plants inoculated at the R7 stage. All samples were plated separately on APDA and incubated at 24 °C for 4 days. Four plants per replication for a total of 16 plants were used to determine infections on hypocotyls, leaf, pod, and seed. Assays were performed by surface disinfecting plant samples from each inoculation type with 0.25% NaOCl solution for 60 s and then blotting dry.

#### Experiment 2

This experiment was set up to inoculate four soybean lines and one soybean cultivar with known resistance and susceptibility to *P. longicolla*, using a single isolate from eclipta (STAM 26). The STAM 26 isolate was selected based on its morphological similarity to a *P. longicolla* isolate from soybean (STAM 35). The soybean lines used were PI 93055, PI 417479, PI 80837, and PI 592947. The soybean cultivar used was ‘Maverick’. PI 417479 (Brown et al. 1987) and PI 80837 (Jackson et al. 2005) were reported to be resistant, whereas PI 93055, PI 592947, and ‘Maverick’ (A. Mengistu, unpublished data) are susceptible. Four inoc-

ulation methods similar to experiment 1 were used. Assays from experiment 2 were performed on hypocotyls and leaves that were inoculated at R5, whereas pods and seeds were assayed from plants that were inoculated at R5 and R7 stages similar to experiment 1.

For nonsporulating isolates, an 18 day-old culture grown on APDA broth from each isolate was ground in a Waring blender for 1 min. The mycelia were passed through a 28-mesh screen (600  $\mu\text{m}$  openings) to remove large debris and standardize the mycelial length. The mycelia were then resuspended in 10 mL of sterile distilled water. The concentration of mycelial fragments was then adjusted to  $1 \times 10^6$  fragments  $\text{mL}^{-1}$ . Inoculation and assays were performed similar to the sporulating isolates used in experiment 1.

Data were analyzed using SAS MIXED procedure (SAS Institute Inc. 2005) with analysis of variance of means separated by Fisher's protected least significant difference.

## Results

### Morphological identification of *Diaporthe* or *Phomopsis* isolates

Four of the eight weed isolates (STAM 26, STAM 27, STAM 28, and STAM 29; Table 1) produced colonies morphologically similar to those produced by soybean isolates of *P. longicolla* (STAM 35). These isolates produced alpha-conidia and stromata, formed a concentric pattern, and produced no perithecia. Alpha-conidia were hyaline, ellipsoidal to fusiform, and guttulate. The size of alpha-conidia fell within the range of 5–9  $\mu\text{m}$   $\times$  1.5–3.5  $\mu\text{m}$ . Beta-conidia and perithecia did not develop for any isolate when grown on APDA or sterile soybean stems in potato broth. Isolates from pitted morning-glory (STAM 27) and nodding spurge (STAM 28) were identified as *P. longicolla* based on the morphological description reported by Hobbs et al. (1985). The other four weed isolates (STAM 30, STAM 31, STAM 32, and STAM 33) had similar morphological characteristics to STAM 26 to STAM 29 except that no alpha-conidia were produced.

### Identification of *Diaporthe* or *Phomopsis* isolates with DNA sequences

The ITS sequences of the isolates from pitted morning-glory (STAM 27), nodding spurge (STAM 28), and soybean (STAM 35) were identical to the ITS sequence from the type strain of *P. longicolla* (GenBank accessions U11411, U11357, as *Phomopsis* sp.; Rehner and Uecker 1994).

The isolate from Illinois bundle-flower (STAM 29) was identified as *D. phaseolorum* (reference strain ATCC 64802 = FAU 458; GenBank accessions U11323 and U11373 as *Phomopsis* sp.; Rehner and Uecker 1994) based on identical ITS sequences but produced both alpha- and beta-conidia. In the current study, the taxonomic concept of *D. phaseolorum*, as outlined by Uecker (1989), includes both varieties *sojae* and *phaseolorum* but excludes the *D. phaseolorum* var. *caulivora* Athow & Caldwell, the causal agent of northern stem canker, and *Diaporthe asplathi* van Rensburg, Castleb., & Crous (*D. phaseolorum* var. *meridionalis* F.A. Fernandez), the causal agent of southern stem canker.

Isolates from Texasweed (STAM 30) and slender aster (STAM 31) did not produce conidia, but their ITS sequences were identical to the *D. phaseolorum* isolates from Illinois bundle-flower (STAM 29). Only the isolate from Illinois bundle-flower was moderately pathogenic on soybean. The *D. phaseolorum* isolates differed from *P. longicolla* by 3.8% (19/495 total differences). The isolate from prickly sida (*Phomopsis* sp. B, STAM 32) was not identical to any known *Diaporthe*–*Phomopsis* ITS sequence available in GenBank and differed from the other three ITS sequence groups sampled in this study by 3.8%–5.8%. The closest matches in GenBank were sequences from *P. longicolla* (AF000207), *Phomopsis* sp. from *Vaccinium* (AF317586), and *D. melonis* var. *brevistylispora* (AB105147); all differed by approximately 3.5%–4.0%.

The isolates from eclipta (STAM 26), and prostrate knotweed (STAM 33) had identical sequences to one another and differed from the type strain of *P. longicolla* by 4.0% (20/494 total differences) and from *D. phaseolorum* by 3.0% (15/494 total differences).

### Test for pathogenicity

#### Experiment 1

The pathogenic form of the *Phomopsis* sp. is presented in this result as *Phomopsis* sp. A to separate it from the non-pathogenic form of *Phomopsis* sp. B. Isolates from eclipta (STAM 26), pitted morning-glory (STAM 27), nodding spurge (STAM 28), and Illinois bundle-flower (STAM 29) caused infections of the hypocotyls, leaves, pods, and seeds (Table 2). Infections on hypocotyl, leaf, pod, and seed ranged from 25% to 100%, from 7% to 10%, from 55% to 90%, and from 1% to 5%, respectively, for the above isolates inoculated at R5. These isolates had lower infection on hypocotyl and leaf compared with infection on similar tissues from the soybean isolate (STAM 35) that had 100%, 100%, 90%, and 15% on hypocotyl, leaf, pod and seed, respectively (Table 2).

Pods inoculated at the R7 stage using STAM 26, STAM 27, STAM 28, STAM 29, and STAM 35 had infection of 100%, 100%, 98%, 45%, and 100%, respectively (Table 2). However, infection of seeds within pods was lower with 50%, 25%, 30%, and 10% for STAM 26, STAM 27, STAM 28, and STAM 29, respectively. STAM 35, the soybean isolate caused seed infection of 80% (Table 2).

Infection from seed inoculation at the R7 stage was significant (100%) for STAM 26, STAM 27, STAM 28, and STAM 35. However, infection from STAM 29 on seed was moderate (55%).

The nonsporulating isolates, STAM 30, STAM 31, STAM 32, and STAM 33 inoculated with mycelium did not cause infection on the susceptible soybean cultivar 'Maverick'. No *Phomopsis* sp. A or B, *D. phaseolorum*, or *P. longicolla* were recovered from noninoculated control plants.

#### Experiment 2

Results from this experiment were similar to those from experiment 1 (Table 3). Of the four inoculation methods used on the four soybean lines and one soybean cultivar, hypocotyl inoculation using STAM 26 caused 100% of the plants to be killed. Inoculations made at the R5 growth

**Table 2.** (A) Mean percent infection of hypocotyl, leaf, pod, and seed on soybean ‘Maverick’ when inoculated with eight weed and one soybean isolate in experiment 1 and (B) results of the ANOVA.

Isolate name	Taxon	Host	Hypocotyl*	Leaf <sup>†</sup>		Pod <sup>‡</sup>		Seed <sup>§</sup>	
				Leaf disc	Pod	Shelled pod	Seed	Seed	Seed <sup>§</sup>
STAM 26 <sup>  </sup>	<i>Phomopsis</i> sp. A	Eclipta	100 a	10 f	80 b	5 f	100 a	50 d	100 a
STAM 27 <sup>  </sup>	<i>P. longicolla</i>	Pitted morning-glory	100 a	7 f	65 c	1 f	100 a	25 e	100 a
STAM 28 <sup>  </sup>	<i>P. longicolla</i>	Nodding spurge	99 a	8 f	90 ab	1 f	98 a	30 e	100 a
STAM 29 <sup>  </sup>	<i>D. phaseolorum</i>	Illinois bundle-flower	25 e	10 f	55 cd	2 f	45 d	10 f	55 cd
STAM 30	<i>D. phaseolorum</i>	Texasweed	0 f	0 f	0 f	0 f	0 f	0 f	0 f
STAM 31	<i>D. phaseolorum</i>	Slender aster	0 f	0 f	0 f	0 f	0 f	0 f	0 f
STAM 32	<i>Phomopsis</i> sp. B	Prickly sida	0 f	0 f	0 f	0 f	0 f	0 f	0 f
STAM 33	<i>Phomopsis</i> sp. B	Prostrate knotweed	0 f	0 f	0 f	0 f	0 f	0 f	0 f
STAM 35 <sup>  </sup>	<i>P. longicolla</i>	Soybean	100 a	100 a	90 ab	15 ef	100 a	80 b	100 a
		Control inoculation	0 f	0 f	0 f	0 f	0 f	0 f	0 f

  

(B) ANOVA results.			<i>P</i> > <i>F</i>
Source of variation	df	<i>F</i>	
Inoculation	3	250.44	<0.0001
Isolates	8	185.22	<0.0001
Inoculation × isolates	24	2.52	0.0003
LSD ( <i>P</i> ≤ 0.05)	12.5		

**Note:** Mean infection values with the same letter are not significantly different (*P* ≤ 0.05).

\*Hypocotyl inoculation and assay were performed at the V1 stage.

<sup>†</sup>Leaf inoculation was done at the R5 stage. The leaf disc assay was performed at R5, and pod and seed assays were performed at the R7 stage.

<sup>‡</sup>Pod excised and inoculated with spores at the R7 stage. The shelled pod and seeds within the pod were assayed from inoculated pods at the R7 stage.

<sup>§</sup>Seed inoculation and assay was performed at the R7 stage.

<sup>||</sup>Isolates that produced alpha-comidia.

**Table 3.** (A) Mean percent infection on four soybean lines and one soybean cultivar inoculated with *Eclipta prostrata* isolate (STAM 26, *Phomopsis* sp. A) on the hypocotyl, leaf, pod, and seed for experiment 2 and (B) results of the ANOVA.

(A) Percent infection.							
Soybean line/cultivar	Hypocotyl*	Leaf <sup>†</sup>			Pod <sup>‡</sup>		
		Leaf disc	Pod	Seed	Shelled pod	Seed	Seed <sup>§</sup>
PI 93055	100 a	2 f	82 b	2 f	99 a	44 d	99 a
PI 417479	100 a	8 f	91 a	1 f	90 a	17e	98 a
PI 80837	100 a	4 f	100 a	1 f	99 a	78 bc	99 a
PI 592947	100 a	9 f	85 b	2 f	89 ab	29e	96 a
'Maverick'	100 a	2 f	90 a	5 f	98 a	66	94 a
Control	0 f	0 f	0 f	0 f	0 f	0 f	0 f

  

(B) ANOVA results.			
Source of variation	df	F	P > F
Inoculation	3	280.47	<0.0001
Isolates	4	4.12	0.0039
Inoculation × isolates	12	2.77	0.0002
LSD ( $P \leq 0.05$ )	11.9		

**Note:** Mean infection values with the same letter are not significantly different ( $P \leq 0.05$ ).

\*Hypocotyl inoculation at the V1 stage.

<sup>†</sup>Leaf inoculation was done at the R5 stage. Leaf disc assay was performed at R5, and pod and seed assays were performed at the R7 stage.

<sup>‡</sup>Pods were excised and inoculated with spores at the R7 stage. Shelled pod and seeds within pod were assayed at the R7 stage.

<sup>§</sup>Seed inoculation and assay were performed at the R7 stage.

stage produced infections on leaves, pods, and seed ranging from 2% to 9%, from 82% to 100%, and from 1% to 5%, respectively.

Inoculations of pod at R7 had seed infection within those pods that ranged from 89% to 99% and from 17% to 78%, respectively. When inoculation was done directly on seed at R7, the infection was 94%–99%. Seed infection from pods inoculated at the R7 stage was lower for PI 417479 (17%) than for PI 93055 (44%) and PI 80837 (78%) (Table 3). No *Phomopsis* species were recovered from noninoculated control plants.

## Discussion

Using ITS sequences, weed isolates were identified as belonging to *P. longicolla*, *Diaporthe phaseolorum*, and two unidentified *Phomopsis* species (*Phomopsis* sp. A and *Phomopsis* sp. B). Of the isolates identified morphologically as *P. longicolla*, the isolates from *eclipta* (STAM 26) and prostrate knotweed (STAM 33) differed from the type strain by 4.0%, indicating that these isolates represent a species distinct from *P. longicolla*. The isolate from prickly sida (STAM 32) represents another *Phomopsis* species distinct from *P. longicolla*. Results of DNA sequencing also indicate that morphological identifications of these closely related species of *Diaporthe*–*Phomopsis* are not sufficient to differentiate species and that comparison with DNA sequences of well-characterized and (or) type strains is required for accurate identification. Additional genes and isolates will be required to determine if these isolates truly represent genetically distinct lineages and to definitively identify *Phomopsis* spp. A and B.

*Phomopsis* sp. A, *P. longicolla*, and *D. phaseolorum* isolates recovered from *eclipta*, pitted morning-glory, nodding

spurge, and Illinois bundle-flower caused significant levels of infection on hypocotyls, leaves, pods, and seed of soybean. Among these, only the isolates from pitted morning-glory and nodding spurge were *P. longicolla*. All sporulating isolates caused significant infection, whereas the non-sporulating isolates produced no infection. Even though *D. phaseolorum* from Texasweed and slender aster, *Phomopsis* sp. B from prickly sida, and *Phomopsis* sp. A from prostrate knotweed were nonpathogenic on soybean, it is premature to make a generalization that these weeds may not support pathogenic forms. These weeds may have the potential to be sources of inoculum for *P. longicolla* or other pathogenic species that may infect and reduce soybean seed quality throughout soybean growing regions of North America. Texasweed is distributed across the southeastern United States, mostly in the Gulf States. Slender aster is found from Maine to Florida and west across the lower south to the Pacific. Others are distributed throughout the southeastern United States, lower Midwest, and eastern United States (e.g., *eclipta*, nodding spurge, and prickly sida). Prostrate knotweed is distributed throughout the United States and Canada. Further tests are needed to include more weed species from different geographical locations to fully establish the ecology and epidemiology of *Diaporthe*–*Phomopsis* isolates from weeds and their ability to infect soybean seed and impact seed quality.

Soybean plants that were inoculated at the R5 growth stage had infected leaves, pods, and seed, when assayed later at the R7 growth stage, indicating that the pathogenic forms of *Phomopsis* sp. A, *P. longicolla*, and *D. phaseolorum* were capable of causing systemic infection.

Pods (unshelled) inoculated at the R7 stage were severely infected, as were the seed within those pods. However, infection from shelled pods by these isolates exceeded that of

infection of seeds within the pods, suggesting that there may be physical or physiological barriers slowing the movement of the pathogen from the pod to the seed. The inoculation techniques and the environmental conditions that were used in this test may simulate some of the conditions occurring in nature, where alternate periods of wet and dry conditions were used to favour pod infection at maturity (Hartman et al. 1999).

Genotypic differences in infection levels on seed from R7 pod inoculation among the four soybean lines and one soybean cultivar indicate a potential use of this technique for the evaluation of germplasm for resistance. However, the method may require a refinement. The fact that seeds from inoculated pods of PI 80837 had a high percent recovery (78%) indicates either that the inoculum level used in this experiment may have been excessive or that STAM 26 may be particularly aggressive. It may also mean that PI 80837 may not be as resistant as previously thought (Jackson et al. 2005). Before drawing any conclusions about the resistance of these soybean lines and soybean cultivar, more experiments with different spore concentrations need to be conducted. Spore concentration and exposure time of seeds and pods to inoculum may alter the relative responses of the soybean lines and soybean cultivar.

This study demonstrated that weed isolates were identified and characterized using DNA sequencing. These results also provided new information that *Phomopsis* sp. A, *P. longicolla*, and *D. phaseolorum* isolated from eclipta, pitted morning-glory, nodding spurge, and Illinois bundle-flower can cause seed infection of soybean.

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