Using PCR to Distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* from Other Soybean Fungal Pathogens and to Detect Them in Soybean Tissues

A. W. Zhang, Department of Crop Sciences; G. L. Hartman, USDA/ARS and Department of Crop Sciences, 70 EASB, 1101 W. Peabody; L. Riccioni, Istituto Sperimentale per la Patologia Vegetale, Rome, Italy; W. D. Chen, Illinois Natural History Survey; R. Z. Ma, Department of Animal Sciences; and W. L. Pedersen, Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana 61801-4723

ABSTRACT

Zhang, A. W., Hartman, G. L., Riccioni, L., Chen, W. D., Ma, R. Z., and Pedersen, W. L. 1997. Using PCR to distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* from other soybean fungal pathogens and to detect them in soybean tissues. Plant Dis. 81:1143-1149.

Restriction fragment length polymorphism analyses of polymerase chain reaction (PCR) amplified DNA were used to distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* isolates from other soybean fungal pathogens. Primers made to the conserved sequences of nuclear ribosomal DNA amplified the internal transcribed spacer (ITS) regions of *D. phaseolorum* var. *meridionalis* and *P. longicolla*. The PCR products were cloned and then sequenced. Specific primers, Phom.I and Phom.II, were designed from the polymorphic regions of *D. phaseolorum* and *P. longicolla* isolates from soybean to distinguish them from other soybean fungal pathogens. These ITS-derived primers amplified a 337-bp-specific DNA fragment from *P. longicolla*, *D. phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *sojae*, and *Phomopsis* spp. from 20 different hosts. No amplified product was observed using DNA of seven other soybean fungal pathogens or soybean DNA. The detection limit of PCR using primers Phom.I and Phom.II was 2.5×10^{-7} dilution of fungal DNA extracted from samples of 10 pooled seeds and as low as a 1:15 (*Phomopsis*:soybean) ratio when using 10 ng of DNA per μ l from each *P. longicolla* and soybean. PCR did not produce products using primers Phom.I and Phom.II with DNA extracted from noninfected seeds, but specific bands were observed from samples of 10 pooled seeds and from individually infected seeds. A specific band was observed as well from DNA extracts of tissue samples from symptomless plants inoculated with *P. longicolla* and *D. phaseolorum* var. *sojae*.

Phomopsis seed decay of soybean (*Glycine max* (L.) Merr.) is a major cause of poor quality soybean seeds in the United States and other soybean-growing areas of the world (30). The disease is caused primarily by *Phomopsis longicolla* T.W. Hobbs (teleomorph unknown), but *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. (anamorph *Phomopsis phaseoli* (Desmaz.) Sacc.), which has three varieties, *D. phaseolorum* var. *sojae* (Lehman) Wehm, *D. phaseolorum* var. *caulivora* Athrow & Caldwell, and *D. phaseolorum* var. *meridionalis* Fernandez (14,19,24), also has been reported to be seedborne in soybeans

(1,30). Soybean seed mortality has been correlated with the incidence of Phomopsis seed decay fungi in seed lots (16), with a reduction in seed germination of up to 90% (21). Planting seed lots with infection levels of more than 40% has been reported to cause significant yield losses (33). Latent (21) and systemic infections (20) by *D. phaseolorum* and *P. longicolla* make it difficult to distinguish between healthy and infected plants and/or seeds. Infected seeds also may introduce the pathogen into new areas (15), and seed detection is important to avoid yield losses (9). *P. longicolla* also is the most important organism causing off-color soybean seeds, which is one of the primary quality-rating factors that negatively influences the market grade of soybeans (18,29).

Because of the economic impact of Phomopsis seed decay, some seed-testing laboratories in the United States test soybean seed lots for the causal fungi. One commonly used procedure is the agar plate bioassay (29), which is rather labor-intensive and time-consuming. Serological methods, such as enzyme-linked immunosorbent assays (ELISA), also have been used for detecting *D. phaseolorum* and *P. longicolla* in plants and seeds (6,17,32), but they are not used routinely in seed-testing laboratories. Molecular methods,

such as polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) analysis, are now widely used in fungal taxonomic and phylogenetic studies (7,8,11,34), and PCR-based diagnostic methods recently have been used to detect fungal pathogens in plant tissues (3,27).

Molecular genetic differences among *Phomopsis* spp. from different hosts have been studied by sequencing the amplified internal transcribed spacer (ITS) (25). Random amplified polymorphic DNA (RAPD) analysis was used to differentiate *D. phaseolorum* var. *meridionalis* isolates from *Macrophomina phaseolina* and *Phoma* sp. (5) and confirm the existence of at least three different subspecific taxa of *D. phaseolorum* that infect soybeans (13). There has been little work done, however, using molecular techniques to detect *D. phaseolorum* and *P. longicolla* in plant tissues. A molecular method that could detect the fungus in seeds may provide information needed, for example, by the European Community (EC), which requires a phytosanitary certification for soybean seed imports and does not allow importation of seeds with more than 15% seed infection by fungi causing Phomopsis seed decay (EC Directive 9.92).

The present study is a step toward the development of a PCR-based diagnostic method to detect *D. phaseolorum* and *P. longicolla* in soybean plant tissues and in soybean seeds. The objectives were to (i) use RFLP analyses of PCR-amplified DNA within the ITS regions of nuclear ribosomal DNA (rDNA) to distinguish soybean isolates of *D. phaseolorum* and *P. longicolla* from other soybean pathogens, and (ii) develop *D. phaseolorum* and *P. longicolla* specific primers based on sequence analysis of the ITS region to detect these fungi in soybean plants and seeds.

MATERIALS AND METHODS

Origin of isolates and DNA extraction. *D. phaseolorum* and *P. longicolla* isolates were obtained from a wide geographic area from various contributors, while other fungal genera were isolated from soybean in Illinois (Table 1). *D. phaseolorum* and *P. longicolla* isolates were obtained from infected soybean seeds or stems and were single-spored. Identification was con-

Corresponding author: G. L. Hartman
Email: ghartman@uiuc.edu

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Accepted for publication 17 June 1997.

Publication no. D-1997-0804-05R

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1997.

firmed on the basis of mycological descriptions (19,24,30).

The fungi were grown on potato dextrose agar (PDA; Difco, Detroit, MI). Pieces of agar from the margins of actively growing colonies were used to inoculate potato dextrose broth (PDB), and cultures were grown at 24°C for 48 to 168 h. Mycelia were collected following centrifugation at 12,000 rpm for 10 min. Total genomic DNA of mycelia and 1 g of healthy soybean plant tissues (stem and leaf) were extracted using a previously described protocol (3).

RFLP analyses of PCR-amplified DNA. ITS4 and ITS5 primers (34) were used to amplify DNA fragments from seven *P. longicolla* isolates, two *D. phaseolorum* var. *meridionalis* isolates, a 1:1 mixture of a *P. longicolla* isolate and a *D.*

phaseolorum var. *meridionalis* isolate, seven other soybean pathogens (Table 1), and healthy soybean tissue. PCR was performed with a Perkin-Elmer DNA Thermal Cycler (Applied Biosystems, Perkin-Elmer Cetus, Emeryville, CA) using 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris-HCl at pH 8.3, 0.2 mM each of dTTP, dATP, dGTP, dCTP, 50 pmol of the primers, 2.5 units *Taq* DNA polymerase, and 25 ng of genomic DNA in a final volume of 50 µl. Reactions were run for 1 cycle at 96°C for 5 min, 35 cycles, each at 94°C, for 45 s, 53°C for 30 s, and 72°C for 1.5 min. The amplification efficacy was checked by agarose gel electrophoresis using 5 µl of PCR products.

Twenty restriction enzymes, *Afl*III, *Alu*I, *Cla*I, *Dde*I, *Dra*I, *Eco*RI, *Eco*RV, *Hga*I, *Hgi*CI, *Hind*III, *Hinf*I, *Mae*III, *Nae*I,

*Nsp*BII, *Rsa*I, *Sau*II, *Taq*2, *Xba*I, *Xho*I, and *Xho*II, were used to generate RFLP patterns. From each sample, 5 µl of PCR product from the ITS region was digested for 4 h with 5 to 10 units of restriction enzyme at 37°C. Aliquots (20 µl) were loaded on a 2% horizontal agarose gel before electrophoretic separation.

DNA sequencing and sequence analysis. The ITS regions of *P. longicolla* isolates 3113 and 3114, and *D. phaseolorum* var. *meridionalis* isolates STC-2 and STC-5, were amplified by primers ITS4 and ITS5. PCR products were size-separated by 1% agarose gel electrophoresis in Tris-acetate-EDTA buffer. DNA was purified from agarose blocks using the Gel Purification Kit 50 (Qiagen Inc., Chatsworth, CA). The purified DNA was ligated into the pCR 2.1 vector and transformed into Shot competent *Escherichia coli* cells following the instructions of the "Original TA-Cloning Kit", Version E (Invitron Corp., San Diego, CA). Four colorless colonies were randomly selected from Luria-Bertani agar plates. After the insertion size was confirmed, selected colonies were cultured in a medium containing 4% circlegrow (Gibco BRL, Gaithersburg, MD) in water at 37°C for 12 h before extracting plasmid DNA. The purified DNA was sequenced on an Applied Biosystems 373 A DNA Sequencer using DiDeoxy Dye Terminator (Applied Biosystems Division, Perkin-Elmer Cetus, Emeryville, CA).

Design of *D. phaseolorum*- and *P. longicolla*-specific primers. The DNA sequences were edited by SeqEd version 1.0.3 (Applied Biosystems Division) and analyzed by the Sequencing Analysis Package (GCG, University of Wisconsin, Madison). Based on the full-length sequences and compared with other sequence information from *Phomopsis* spp. (25), *D. phaseolorum*- and *P. longicolla*-specific primers, Phom.I and Phom.II, were designed for each selected clone with the aid of the computer program Primer Designer (version 2.0, Scientific and Educational Software, Stateline, PA). Specific primers were synthesized by the Biotechnology Center, University of Illinois at Urbana-Champaign.

Specificity of primers Phom.I and Phom.II. DNA extracted from seven other soybean fungal pathogens, three *P. longicolla* isolates, two *D. phaseolorum* var. *meridionalis* isolates, two *D. phaseolorum* var. *caulivora* isolates, two *D. phaseolorum* var. *sojae* isolates, 20 isolates of *Phomopsis* spp. from different hosts, and from healthy soybean tissue, were tested for their amplification with primers Phom.I and Phom.II.

P. longicolla DNA was used at a concentration of 10 ng/µl or diluted with soybean DNA 15 times from 1:1 to 1:15, and 1:30. DNA was extracted from a sample of 10 pooled seeds from a seed lot known to

Table 1. List of fungal species, isolates, their host affiliation, location of collection, and contributor used in this study

Fungus	Host	Location	Contributor ^a
<i>Cercospora sojae</i>	<i>Glycine max</i>	Illinois	G. L. Hartman
<i>Rhizoctonia solani</i>	<i>G. max</i>	Illinois	G. L. Hartman
<i>Macrophomina phaseolina</i> I	<i>G. max</i>	Illinois	G. L. Hartman
<i>M. phaseolina</i> II	<i>G. max</i>	Illinois	G. L. Hartman
<i>Fusarium solani</i>	<i>G. max</i>	Illinois	G. L. Hartman
<i>Sclerotinia sclerotiorum</i>	<i>G. max</i>	Illinois	G. L. Hartman
<i>Phialophora gregata</i>	<i>G. max</i>	Illinois	G. L. Hartman
<i>Septoria glycines</i>	<i>G. max</i>	Illinois	G. L. Hartman
<i>Diaporthe phaseolorum</i> var. <i>meridionalis</i> STC-5	<i>G. max</i>	Arkansas	J. Rupe
STC-2	<i>G. max</i>	Arkansas	J. Rupe
624	<i>G. max</i>	Florida	S. A. Rehner
<i>D. phaseolorum</i> var. <i>caulivora</i> 713	<i>G. max</i>	Italy	A. Porta-Puglia
720	<i>G. max</i>	Italy	A. Porta-Puglia
<i>D. phaseolorum</i> var. <i>sojae</i> AK25A	<i>G. max</i>	Arkansas	J. Rupe
SW93-10	<i>G. max</i>	Korea	E. W. Park
<i>Phomopsis longicolla</i> r1258	<i>G. max</i>	Illinois	G. L. Hartman
MO151	<i>G. max</i>	Missouri	G. L. Hartman
AKI	<i>G. max</i>	Arkansas	J. Rupe
VS8	<i>G. max</i>	Illinois	G. L. Hartman
MO123	<i>G. max</i>	Missouri	G. L. Hartman
3113	<i>G. max</i>	Illinois	G. L. Hartman
3114	<i>G. max</i>	Illinois	G. L. Hartman
<i>Phomopsis</i> spp. 570	<i>Oxydendrum</i>	Virginia	S. A. Rehner
438	<i>Actinidia</i>	California	S. A. Rehner
532	<i>Chamaecyparis</i>	New Jersey	S. A. Rehner
537	<i>Kalmia</i>	New Jersey	S. A. Rehner
533	<i>Epigaea</i>	New Jersey	S. A. Rehner
597	<i>Solanum</i>	Dominican Rep.	S. A. Rehner
441	<i>Lindera</i>	Maryland	S. A. Rehner
514	<i>Picea</i>	New Jersey	S. A. Rehner
512	<i>Juniperus</i>	Virginia	S. A. Rehner
629	<i>Chamaedorea</i>	Mexico	S. A. Rehner
510	<i>Carpinus</i>	New Jersey	S. A. Rehner
487BR	<i>Paulownia</i>	Maryland	S. A. Rehner
487	<i>Paulownia</i>	Maryland	S. A. Rehner
445	<i>Vaccinium</i>	Massachusetts	S. A. Rehner
476	<i>Vaccinium</i>	New Jersey	S. A. Rehner
495	<i>Vaccinium</i>	Washington	S. A. Rehner
522	<i>Sassafras</i>	New Jersey	S. A. Rehner
526	<i>Sassafras</i>	West Virginia	S. A. Rehner
499	<i>Asparagus</i>	New Jersey	S. A. Rehner
577	<i>Asparagus</i>	Taiwan	S. A. Rehner

^a G. L. Hartman, USDA/ARS, Urbana, IL; A. Porta-Puglia, Istituto Sperimentale per la Patologia Vegetale, Rome, Italy; J. Rupe, University of Arkansas; S. A. Rehner, USDA/ARS, Beltsville, MD (25).

have 25% of the seed infected with *D. phaseolorum* and/or *P. longicolla* based on plating 200 seeds on PDA. Extracted DNA was suspended in 250 μ l of dd (double distilled) H₂O and then 10-fold diluted with ddH₂O to 5×10^{-6} . DNA from all dilutions was amplified with primers Phom.I and Phom.II under the PCR conditions of 1 cycle at 96°C for 5 min, 45 cycles, each at 95°C, for 30 s, 60°C for 30 s, and 72°C for 1 min. The composition of each PCR reaction was the same as the PCR reactions previously mentioned, except that 2 μ l of extract of DNA was used per reaction for each dilution. This experiment was repeated once.

Detection of *D. phaseolorum* and *P. longicolla* from soybean seed lots and plants. Ten seed lots of cv. Williams obtained from the University of Illinois research farm in 1994 were tested by PDA plate method (29) for *D. phaseolorum* and *P. longicolla* infection. Seeds were treated with 95% ethanol for 30 s, 0.5% NaOCl for 1 min, 2.5% paraquat (Gramoxone, Zeneca Ag Products, Wilmington, DE) for 3 min, rinsed three times with ddH₂O, and then incubated in PDB for 12 to 18 h. A microextraction technique (12) was used to extract DNA from 10 to 30 single seeds per 10 seed lots. Two μ l of the extracted DNA was amplified with primers Phom.I and Phom.II. For the samples of 10 pooled seeds, the DNA was extracted using a previously described protocol (3).

In the greenhouse, 2-week-old soybean seedlings (cv. Williams) were sprayed with a mycelial suspension (10^7 to 10^8 hyphal fragments per ml obtained from 3-day-old cultures grown in PDB and blended for 2 min) of either *P. longicolla* isolates 3113 and 3114, *D. phaseolorum* var. *meridionalis* isolates STC-2 and STC-5, or *D. phaseolorum* var. *sojae* isolate AK25A. Nine plants (three per replicate) were sprayed with each isolate or water. Fifteen days after inoculation, three symptomless plants were randomly sampled per isolate and the water control. Stems and petioles from each plant were cut separately into 1-cm lengths and leaf tissue into 1×0.5 cm sections. These were surface-disinfected in 0.5% NaOCl for 1 min and rinsed three times in ddH₂O. Five randomly selected pieces from each plant were placed on a PDA plate. DNA was extracted from randomly selected 0.2 g of cut fresh tissue samples using a microextraction technique (12). The DNA extracts were used in PCR assays with primers Phom.I and Phom.II (2 μ l of DNA for each reaction). DNA extracts from healthy soybean tissue were used as a control. The remaining plants that were not used for fresh tissue samples were sprayed with a 2.5% paraquat solution. Presence or absence of fungal fruiting structures on stems was recorded 10 days later. Stem sections without fungal fruiting bodies were transferred to PDA.

Fig. 1. Gel electrophoresis of amplified products by polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region with (A) primers ITS4 and ITS5 of different soybean fungal pathogens and soybean tissue, and banding patterns after digestion with restriction enzymes (B) *AluI*, (C) *DdeI*, and (D) *RsaI*. Lanes 1 to 6: isolates of *Phomopsis longicolla* (MO151, AKI, VS8, MO123, 3113, and 3114); lanes 7 and 9: *Diaporthe phaseolorum* var. *meridionalis* (624 and STC-2, respectively); lane 8: mixed DNA of *D. phaseolorum* var. *meridionalis* (STC-5) and *P. longicolla* (3113); lanes 10 to 19: DNA of soybean tissue, *Septoria glycines*, *Phialophora gregata*, *Sclerotinia sclerotiorum*, *P. longicolla* (r1258), *Fusarium solani*, *Macrophomina phaseolina* I, *M. phaseolina* II, *Rhizoctonia solani*, and *Cercospora sojae*, respectively; and lane M: molecular weight markers (Gibco-BRL) 1-kb DNA ladder in A and 123-bp DNA ladder in B to D.

In order to reduce nonspecific amplification and the effect of inhibitors in plant tissues, the hot start technique (4) was used in the PCR analysis. Two master stocks: M1 with DNA, primers, PCR buffer, and water; and M2 with dNTP, *Taq* polymerase, and water were added sequentially.

RESULTS

RFLP analyses of PCR-amplified DNA. PCR with primers ITS4 and ITS5 amplified fragments from 550 to 850 bp for the nine soybean fungal pathogens. *D.*

phaseolorum and *P. longicolla* isolates had a unique fragment of 605 bp (Fig. 1A). The *Phomopsis* isolates were distinguished from the *Diaporthe* isolates after digestion only with *AluI* and *RsaI* (Fig. 1B and D). Isolates within *P. longicolla* and those within *D. phaseolorum* var. *meridionalis* each had distinct banding patterns. *AluI*, *RsaI*, and *DdeI* produced a complete distinguishable RFLP banding pattern that distinguished *D. phaseolorum* and *P. longicolla* from other fungi (Fig. 1B to D).

DNA sequencing and design of *D. phaseolorum*- and *P. longicolla*-specific

primers. After multiple sequence alignment analysis of the ITS1 and ITS2 regions, the *D. phaseolorum*- and *P. longicolla*-specific primers were designed as: Phom.I (5'-GAGCTCGCCACTAGATTTCAGGG-3') and Phom.II (5'-GGCGGC-CAACCAAACCTCTTGT-3'). The primers had a GC content of 56 to 57% and were located in ITS1 and ITS2 regions, respectively, of rDNA. Their complete amplified fragment was 337 bp (Fig. 2).

Specificity of primers Phom.I and Phom.II. A 337-bp DNA fragment was amplified from all *P. longicolla* and *D. phaseolorum* isolates, but not from other soybean pathogens or soybean DNA using PCR with primers Phom.I and Phom.II (Fig. 3). Twenty *Phomopsis* isolates from different hosts also were amplified by these primers.

The detection limit of PCR using primers Phom.I and Phom.II was 2.5×10^{-7} dilution of fungal DNA extracted from samples of 10 pooled seeds and as low as a 1:15 (*Phomopsis*:soybean) ratio when using 10 ng of DNA per μ l from each *P. longicolla* and soybean (Fig. 4).

Detection of *D. phaseolorum* and *P. longicolla* from soybean seed lots and plants. Both DNA extraction methods produced DNA from infected soybean plants and seeds that was suitable for PCR. PCR did not produce products using primers Phom.I and Phom.II with DNA extracted from 10 pooled seeds with 0% infection (Table 2). Seed lots with 5 to 40% infection had specific bands (Fig. 5). A specific band from individually sampled infected seeds was detected with Phom.I and Phom.II. In seed lots 7 and 8, the infection percentages from single-seed PCR detections were higher than from the PDA plating (Table 2).

Soybean plants infected with isolates of *P. longicolla* and *D. phaseolorum* var. *sojae* isolate AK25A produced pycnidia on some plants 15 days after inoculation. After applying paraquat, pycnidia were observed on the stems of all inoculated plants; no pycnidia were observed on non-inoculated plants. *D. phaseolorum* var. *meridionalis* did not infect plants, and neither pycnidia nor perithecia were observed on desiccated stems. The fungus was not reisolated from stem samples incubated on PDA.

PCR amplified a specific band with *D. phaseolorum*- and *P. longicolla*-specific primers using tissue samples from symptomless plants inoculated with *P. longicolla* and *D. phaseolorum* var. *sojae*, but not from plant samples inoculated with *D. phaseolorum* var. *meridionalis* (Table 3 and Fig. 6).

DISCUSSION

RFLP analyses of PCR-amplified DNA have been used to differentiate fungal isolates within the same species (8,11). In our study, analysis distinguished *P. longicolla*

Fig. 2. Schematic locations of the *Diaporthe phaseolorum*- and *Phomopsis longicolla*-specific polymerase chain reaction primers, Phom.I and Phom.II, which amplified a 337-bp fragment (inclusive of the primer) and the "universal" internal transcribed spacer (ITS) primers ITS4 and ITS5, which amplified a 605-bp fragment of *D. phaseolorum* and *P. longicolla* rDNA.

Fig. 3. Gel electrophoresis of products amplified by the polymerase chain reaction (PCR) using *Diaporthe phaseolorum*- and *Phomopsis longicolla*-specific primers Phom.I and Phom.II, and DNA from different soybean fungal pathogens. Lane M: 1-kb DNA ladder; lanes 1 to 10: *Cercospora sojina*, *Rhizoctonia solani*, *Macrophomina phaseolina* I, *M. phaseolina* II, *Fusarium solani*, *Septoria glycines*, *Sclerotinia sclerotiorum*, *Phialophora gregata*, *S. glycines*, and DNA of soybean tissue; lanes 11 to 13: *P. longicolla* (MO151, AK1, MO123); lanes 14 and 15: *Diaporthe phaseolorum* var. *sojae* (AK25A, SW93-10); lanes 16 and 17: *D. phaseolorum* var. *caulivora* (713, 720); and lanes 18 and 19: *D. phaseolorum* var. *meridionalis* (624, STC-2).

Fig. 4. Polymerase chain reaction amplification with specific primers Phom.I and Phom.II using different ratios of *Phomopsis* DNA with soybean DNA. Lanes 1 to 13: ratios of *Phomopsis* DNA with soybean DNA from 1:30 and in decreasing increments of 1:15 to 1:0; lane 14: soybean DNA; lanes 15 to 21: 5×10^{-6} to 5×10^{-1} in $10 \times$ increments of DNA extracted from 10 pooled seeds of a seed lot with 25% of *Diaporthe phaseolorum* and/or *Phomopsis longicolla* infection; and lane M: 1-kb DNA ladder.

and *D. phaseolorum* var. *meridionalis* from other soybean pathogens; however, based on the restriction enzymes we used, there were no differences within the isolates of *P. longicolla* and *D. phaseolorum* var. *meridionalis*.

The rDNA of related organisms generally are highly conserved, while the ITS region is less conserved (34). The more extensive sequence divergence in the ITS region has been the basis for the development of PCR assays used to detect pathogens from infected plants (3,27), including soybeans (10). Using the homologous regions on both sides of the 5.8S rDNA of *D. phaseolorum* and *P. longicolla* isolates, we developed specific primers that amplified a 337-bp fragment from the ITS1 and ITS2 regions from 147 bp to 440 bp on the rDNA. These primers also amplified this fragment for 20 *Phomopsis* spp. isolates originating from different hosts, while no amplification was observed using DNA from other soybean fungal pathogens. Compared with RFLPs, these primers produced only one specific band and allowed for the detection of *D. phaseolorum* and *P. longicolla* in plant tissues.

The *D. phaseolorum*– and *P. longicolla*–specific primers have two major applications, including the detection of *D. phaseolorum* and *P. longicolla* latent infection in plants and in soybean seeds. The *D. phaseolorum*– and *P. longicolla*–specific primers detected *D. phaseolorum* and *P. longicolla* infections in symptomless tissues and potentially could be used to monitor latent infections and colonization of plants in soybean fields. This may have some predictive value in terms of evaluating infection early in the season before signs of the fungus are present. The other application could be useful for seed testing laboratories in which molecular detection may be more reliable and faster than the traditional plating of seeds on agar.

Seedborne bacterial and viral pathogens have been detected using PCR, which has been shown to be faster and more sensitive than other detection techniques (2,22,23,26,28). Detection of seedborne fungal pathogens by PCR has not been extensively reported, but PCR has the potential to be routinely used in seed testing laboratories for important seedborne pathogens like *D. phaseolorum* and *P. longicolla*. Using specific primers instead of serological assays theoretically has an advantage in reducing the number of false positive reactions that may occur due to cross-reactivity of antibodies with antigens derived from the plant or other microbes. In addition, since small amounts of DNA are required for PCR assays, the problems associated with low antigen titer with ELISA can be resolved (3). Compared with plating seeds on PDA, which sometimes can mask the isolation of certain fungi when seeds have multiple infections, PCR-specific primers have high selectivity

(10,27) and should be very useful for detecting target organisms on seeds with multiple infections.

The use of PCR to detect pathogens in plant tissues will become even more effective once inhibitors of PCR contained in seeds and/or plant tissues are removed (10,13,27). The immunocapture method for virus detection and preincubated cultures for bacteria coupled with PCR have been effective in overcoming inhibitors (26,28). In our study, seeds were soaked for 18 h

before DNA extraction. This probably increased the amount of living fungal DNA, reduced the background of dead cell DNA, and removed PCR inhibitors by diffusion into the liquid medium (26). When coupled with the hot-start method (5), nonspecific amplification from PCR was minimized. The detection limit of PCR using primers Phom.I and Phom.II was 2.5×10^{-7} dilution of fungal DNA extracted from samples of 10 pooled seeds.

Table 2. Detection of *Diaporthe phaseolorum* and/or *Phomopsis longicolla* in soybean seeds (cv. Williams) by potato dextrose agar (PDA) plate method and polymerase chain reaction (PCR) with specific primers Phom.I and Phom.II for 10 individual seeds and pooled samples of 10 seeds

Seed lot	PDA plate (%) ^a	Single seed sample by PCR (%) ^b	Pooled sample of 10 seeds by PCR ^c
1	0	0	–
2	0	0	–
3	20	20	+
4	0	0	–
5	5	5	+
6	0	0	–
7	25	30	+
8	10	20	+
9	0	0	–
10	40	40	+

^a Percentage of seed infection detected by plating 200 seeds per seed lot.

^b Percentage based on 10 individual seeds per seed lot except in lot 5 = 20 seeds and lot 8 = 30 seeds.

^c (–) absence or (+) presence of specific 337-bp amplified fragments after PCR with primers Phom.I and Phom.II (Fig. 6).

Table 3. Detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* in soybean plants (cv. Williams) inoculated with *D. phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *sojae*, and *P. longicolla* by polymerase chain reaction (PCR) with specific primers Phom.I and Phom.II

Fungus	Isolate	Infection ^a			PCR detection		
		Plant 1	Plant 2	Plant 3	Plant 1	Plant 2	Plant 3
<i>D. phaseolorum</i> var. <i>sojae</i>	AK 25A	+	+	+	+	+	+
<i>D. phaseolorum</i> var. <i>meridionalis</i>	STC-2	–	–	–	–	–	–
	STC-5	–	–	–	–	–	–
<i>P. longicolla</i>	3113	+	+	+	+	+	+
	3114	+	+	+	+	+	+
Control		–	–	–	–	–	–

^a Plant infection was determined by plating tissues on potato dextrose agar and confirming the presence of *D. phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *sojae*, and *P. longicolla*.

Fig. 5. Polymerase chain reaction amplification with specific primers Phom.I and Phom.II from 10 pooled seeds of seed lots (cv. Williams) with different *Diaporthe phaseolorum* and *Phomopsis longicolla* infection rates. Lane M: 1-kb ladder; lanes 1 to 10: seed lots with 0, 0, 20, 0, 5, 0, 25, 10, 0, and 40% infection rate, respectively.

Fig. 6. Polymerase chain reaction amplification with specific primers Phom.I and Phom.II from symptomless soybean plants (cv. Williams) 15 days after inoculation with *Diaporthe phaseolorum* and *Phomopsis longicolla* isolates. The *D. phaseolorum* var. *meridionalis* inoculation was negative (Table 3). Lanes 1 to 3: water-sprayed control; lanes 4 to 6: plants inoculated with *P. longicolla* (3114); lanes 7 to 9: plants inoculated with *P. longicolla* (3113); lanes 10 to 12: plants inoculated with *D. phaseolorum* var. *meridionalis* (STC-5); lanes 13 to 15: plants inoculated with *D. phaseolorum* var. *meridionalis* (STC-2); lanes 16 to 18: plants inoculated with *D. phaseolorum* var. *sojae* (AK25A); and lane M: 1-kb DNA ladder.

The extraction of DNA also is a critical step for the use of PCR in seed testing. Besides the microorganisms for which 0% tolerance is required, it is necessary to purify DNA from 100 to 200 single seeds from each seed lot, which is considered a representative sample in most seed testing laboratories. The DNA micro-extraction proved to be an effective alternative, and although DNA extraction from 100 seeds was time-consuming, it may be improved by using other methods, such as a DNA fast kit, which could increase the efficiency of PCR detection by reducing PCR inhibitors while reducing the time for DNA extraction (4). In order to make PCR detection of *D. phaseolorum*- and *P. longicolla*-infected seeds more efficient, we are focusing our efforts on improving DNA extraction from soybean tissues, especially from seeds, and on the potential use of gel-free PCR (28), which could simplify the PCR detection assay.

In terms of soybean seed quality, discolored seeds are presently evaluated equally in the application of discount schedules of the market grade, even though not all discoloration may reduce seed quality. Seed infected by *D. phaseolorum* and *P. longicolla* is the number one cause of soybean seed discoloration and also is known to reduce seed quality (18,31). The use of a rapid and sensitive technique to estimate the presence of *D. phaseolorum* and *P. longicolla* in seeds could present an economical advantage to producers if discount schedules for fungal-damaged soybean seeds are reevaluated.

ACKNOWLEDGMENTS

We thank Harris Lewin for the use of the sequencer, Ron Warsaw for his assistance in inoculation of plants, and J. Rupe, S. A. Rehner, E. W. Park, and A. Port-Puglia for isolates. The participation of Luca Riccioni was supported by the Ministero per le Risorse Agricole, Alimentari e Forestali of Italy (Biotechnology Program, Project No. 451, Area 10).

LITERATURE CITED

- Anderson, T. R. 1985. Seed moulds of soybean in Ontario and the influence of production area on the incidence of *Diaporthe phaseolorum* var. *caulivora* and *Phomopsis* sp. Can. J. Plant Pathol. 7:74-78.
- Audy, P., Braat, C. E., Saindon, G., Huang, H. C., and Laroche, A. 1996. A rapid and sensitive PCR-based assay for concurrent detection of bacteria causing common and halo blight in bean seed. Phytopathology 86:361-366.
- Beck, J. J., and Ligon, J. M. 1995. Polymerase chain reaction assays for the detection of *Stagonospora nodorum* and *Septoria tritici* in wheat. Phytopathology 85:319-324.
- Beever, J. E., Da, Y., Ron, M., and Lewin, H. A. 1994. A genetic map of nine loci on bovine chromosome 2. Mammal Genome 5:542-545.
- Blakemore, E. J. A., Jaccoud Filho, D. S., and Reeves, J. C. 1994. PCR for the detection of *Pyrenophora* species, *Fusarium moniliforme*, *Stenocarpella maydis*, and *Phomopsis/Diaporthe* complex. Pages 205-213 in: Modern Assays for Plant Pathogenic Fungi. A. Schots, F. M. Dewey, and R. P. Oliver, ed. CAB International, Wallingford, UK.
- Brill, L. M., McClary, R. D., and Sinclair, J. B. 1994. Analysis of two ELISA formats and antigen preparations using polyclonal antibodies against *Phomopsis longicolla*. Phytopathology 84:173-179.
- Bruns, T. D., White, T. J., and Taylor, J. W. 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. 22:525-564.
- Buscot, F., Wipf, D., Di Battista, C., Munch, J. C., Botton, B., and Martin, F. 1996. DNA polymorphism in morels: PCR/RFLP analysis of the ribosomal DNA spacer and microsatellite-primed PCR. Mycol. Res. 100:63-71.
- Chamberlain, D. W., and Gray, L. E. 1974. Germination, seed treatment, and microorganisms in soybean seed produced in Illinois. Plant Dis. Rep. 58:50-54.
- Chen, W., Gray, L. E., and Grau, C. R. 1996. Molecular differentiation of fungi associated with brown stem rot and detection of *Phialophora gregata* in resistant and susceptible soybean cultivars. Phytopathology 86:1140-1148.
- Chen, W. D., and Hoy, J. W. 1993. Molecular and morphological comparison of *Pythium arrhenomanes* and *P. graminicola*. Mycol. Res. 97:1371-1378.
- Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA miniprep: Version 2. Plant Mol. Biol. Rep. 1:19-22.
- Demeke, T., and Adams, R. P. 1992. The effects of plant polysaccharides and buffer additives of PCR. Biotechniques 12:332-334.
- Fernandez, F. A., and Hanlin, R. T. 1996. Morphological and RAPD analysis of *Diaporthe phaseolorum* from soybean. Mycologia 88:425-440.
- Garzonio, D. M., and McGee, D. C. 1983. Comparison of seed and crop residues as sources of inoculum for pod and stem blight of soybeans. Plant Dis. 67:1374-1376.
- Gleason, M. L., and Ferriss, R. S. 1985. Influence of soil water potential on performance of soybean seeds infected by *Phomopsis* sp. Phytopathology 75:1236-1241.
- Gleason, M. L., Ghabrial, S. A., and Ferriss, R. S. 1987. Serological detection of *Phomopsis longicolla* in soybean seeds. Phytopathology 77:371-375.
- Hepperly, P. R., and Sinclair, J. B. 1978. Quality losses in *Phomopsis*-infected soybean seed. Phytopathology 68:1684-1687.
- Hobbs, T. W., Schmitthenner, A. F., and Kutter, G. A. 1985. A new *Phomopsis* species from soybean. Mycologia 77:535-544.
- Kilpatrick, R. A. 1957. Fungi associated with flowers, pods, and seeds of soybeans. Phytopathology 47:131-135.
- Kmetz, K. T., Schmitthenner, A. F., and Ellett, C. W. 1978. Soybean seed decay: Prevalence of infection and symptom expression caused by *Phomopsis* sp., *Diaporthe phaseolorum* var. *caulivora*. Phytopathology 68:836-840.
- Leite, R. P., Jr., Jones, J. B., Somodi, G. C., Minsavage, G. V., and Stall, R. E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. Plant Dis. 79:917-922.
- Maes, M., Garbeva, P., and Kamoen, O. 1996. Recognition and detection in seed of the *Xanthomonas* pathogens that cause cereal leaf streak using rDNA spacer sequences and polymerase chain reaction. Phytopathology 86:63-69.
- Morgan-Jones, G. 1989. The *Diaporthe/Phomopsis* complex: Taxonomic considerations. Pages 1699-1706 in A. J. Pascale, ed. Proc. World Soybean Res. Conf. IV. Orientacion Grafica Editora, Buenos Aires, Argentina.
- Rehner, S. A., and Uecker, F. A. 1994. Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis*. Can. J. Bot. 72:1666-1674.
- Schaad, N. W., Cheong, S. S., Tamaki, S., Hatziloukas, E., and Panopoulos, N. J. 1995. A combined biological and enzymatic amplification (Bio-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. Phytopathology 85:243-248.
- Schilling, A. G., Moller, E. M., and Geiger, H. H. 1996. Polymerase chain reaction-based assays for the detection for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86:515-522.
- Schoen, C. D., Knorr, D., and Leone, G. 1996. Detection of potato leafroll virus in dormant potato tubers by immunocapture and a fluorogenic 5' nuclease RT-PCR assay. Phytopathology 86:993-999.
- Sinclair, J. B. 1982. Seed testing: Detection of pathogens. Pages 112-115 in: Soybean Seed Quality and Stand Establishment. J. B. Sinclair and J. A. Jacobs, eds. INTSOY Ser. No. 22. University of Illinois, Urbana-Champaign.
- Sinclair, J. B. 1995. Reevaluation of grading standards and discounts for fungus-damaged soybean seeds. J. Am. Oil Chem. Soc. 72:1415-1419.
- Sinclair, J. B., and Backman, P. A. 1989. Compendium of Soybean Diseases. 3rd ed.

- American Phytopathological Society, St. Paul, MN.
32. Velicheti, R. K., Lamison, C., Brill, L. M., and Sinclair, J. B. 1993. Immunodetection of *Phomopsis* species in asymptomatic soybean plants. *Plant Dis.* 77:70-73.
33. Wallen, V. R., and Seaman, W. L. 1963. Seed infection of soybean by *Diaporthe phaseolorum* and its influence on host development. *Can J. Bot.* 41:13-21.
34. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols, A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.