Rapid and Sensitive Detection of *Phytophthora sojae* in Soil and Infected Soybeans by Species-Specific Polymerase Chain Reaction Assays

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ABSTRACT

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Root and stem rot caused by *Phytophthora sojae* is one of the most destructive diseases of soybean (*Glycine max*) worldwide. *P. sojae* can survive as oospores in soil for many years. In order to develop a rapid and accurate method for the specific detection of *P. sojae* in soil, the internal transcribed spacer (ITS) regions of eight *P. sojae* isolates were amplified using polymerase chain reaction (PCR) with the universal primers DC6 and ITS4. The sequences of PCR products were aligned with published sequences of 50 other *Phytophthora* species, and a region specific to *P. sojae* was used to design the specific PCR primers, PS1 and PS2. More than 245 isolates representing 25 species of *Phytophthora* and at least 35

other species of pathogens were used to test the specificity of the primers. PCR amplification with PS primers resulted in the amplification of a product of approximately 330 bp, exclusively from isolates of *P. sojae*. Tests with *P. sojae* genomic DNA determined that the sensitivity of the PS primer set is approximately 1 fg. This PCR assay, combined with a simple soil screening method developed in this work, allowed the detection of *P. sojae* from soil within 6 h, with a detection sensitivity of two oospores in 20 g of soil. PCR with the PS primers could also be used to detect *P. sojae* from diseased soybean tissue and residues. Real-time fluorescent quantitative PCR assays were also developed to detect the pathogen directly in soil samples. The PS primer-based PCR assay provides a rapid and sensitive tool for the detection of *P. sojae* in soil and infected soybean tissue.

Additional keywords: disease management, molecular detection.

Root and stem rot caused by *Phytophthora sojae* is currently the most devastating disease of soybean (*Glycine max*), causing severe losses in soybeans worldwide (10). Areas that receive heavy rain may suffer plant mortality and yield losses of up to 100% in portions of affected fields. *P. sojae* was first reported around 1950 in Indiana and Ohio (17), and has since become widespread in many soybean-producing countries in the Americas (8,26,38). In the past few years, the disease has also caused serious soybean losses in northeastern China (29).

P. sojae is a homothallic species that produces large numbers of oospores in infected soybean tissues (10). The oospores can survive for many years in the soil after decomposition of the plant tissue (10). Following germination, the oospores produce numerous zoospores that swim through films of water to reach the roots that they subsequently infect, after which the pathogen grows into and among the root cells of the plant, causing serious root rot. Cropping of soybean in the same fields over successive years may increase the potential for damage by this disease (26). Given that increasing amounts of soybeans are being transported to and from different areas and countries, root rot caused by *P. sojae* constitutes a severe threat to worldwide soybean production. The development of a technique for the rapid detection of the pathogen in soybean production fields and in soil carried with transported soybeans will be very important for controlling the dispersion of *P. sojae* and maintaining "Phytophthora-free" soybean production.

The diagnosis of *Phytophthora* root rot is complex, since multiple species of soilborne pathogens infect soybean, including *Pythium* spp., *Fusarium* spp., and *Rhizoctonia* spp., and the symptoms caused by the different pathogens are very similar. Isolation of a pure culture is required for the diagnosis of this *Phytophthora* disease. The isolation and accurate identification of *P. sojae* are very difficult since this pathogen grows relatively slow. A rapid pathogen detection method and disease diagnostic assay would facilitate pathogen identification and lead to more effective management practices, such as determining the need for fungicide applications to limit disease incidence.

Polymerase chain reaction (PCR) offers advantages over the more traditional methods of pathogen detection and plant disease diagnosis since the organisms need not be cultured prior to detection by PCR and the technique is rapid and sensitive (2,9,18,27,36). In addition, though quantitative real-time PCR is being used increasingly in plant pathology for the accurate detection and quantification of plant pathogens (1,7,34,35,37), its application in the detection and quantification of pathogens from soil samples remains limited (5,6,11,20,40).

The objectives of this study were to develop a rapid and sensitive PCR-based method for the specific amplification of *P. sojae* from the soil carried with transported soybeans or in production fields, and to develop a rapid assay to detect the pathogen in infected soybean tissues. We also described the use of real-time PCR combined with fluorescent SYBR Green I dye to quantify *P. sojae* directly from soil.

MATERIALS AND METHODS

Source of isolates. *P. sojae* isolates were obtained from diseased soybean stems collected in Heilongjiang and Fujian provinces from 2002 to 2004. By using a leaf disk baiting method (22),

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color online.

additional isolates were baited from soybean residues and soil carried with soybeans imported from the United States, Canada, Brazil, and Argentina. The virulence and race type of these isolates have not been tested. Thirteen known races of *P. sojae* were provided by B. Tyler and J. Peng. The *P. sojae* isolates, as well as isolates of *Phytophthora* spp., *Pythium* spp., *Fusarium* spp., and various other pathogens, have been maintained in a collection at Nanjing Agricultural University, Department of Plant Pathology, and are listed in Tables 1 and 2.

Source of soil samples. A total of 118 soil samples were collected from the soybean production fields in Heilongjiang province of China in 2003 and 2004 where the soil type is phaeozem. In addition, 33 soil samples were collected from the soil carried with soybeans imported from the United States, Brazil, Argentina, and Canada. In commercial processing systems, soybeans are typically screened to remove contaminated soil and soybean residues. This soil and residue was collected for PCR assays and the samples evaluated are listed in Table 3.

Extraction of DNA from mycelium. *Phytophthora* isolates were cultured on tomato juice medium (39) (per liter, 200 ml of tomato juice, 0.1 g of CaCO₃, and 15 g of agar mixed with sterile distilled water, sterilized at 120°C for 20 min). Mycelia of each *Phytophthora* and *Pythium* isolate were obtained by growing the isolates in tomato juice broth (39) at 18 to 25°C (temperature-dependent isolates) for at least 5 days. Mycelia of the other fungi were grown in potato dextrose broth (10). The mycelia were harvested by filtration and frozen at -20°C. DNA was extracted from the mycelia according to a modified cetyltrimethylammonium bromide procedure (CTAB) (25). The DNA was dried at room temperature, resuspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0), and stored at -20°C.

Extraction of DNA from single oospores. Oospore suspensions were prepared and treated with KMnO₄ according to the method described by Chang and Ko (3). Single oospores were picked under a microscope and rinsed with 100 µl of sterile distilled H₂O in 1.5-ml Eppendorf tubes and then surface dried with tissue. Fifty microliters of 2% CTAB was added to the tube. The procedure for DNA extraction was identical to that described previously.

Extraction of *P. sojae* DNA from soil. A screening method was developed to collect oospores from soil collected above. Based on the size of *P. sojae* oospores, 200-, 300-, and 400-mesh screens of 20 cm in diameter were used to eliminate most of the soil, and a 600-mesh, 20-cm-diameter screen was used to collect the oospores. In addition, 20 g of soil was first macerated in 300 ml of water in a 500-ml beaker for 30 min, stirred with a glass rod for 10 min, and then added to a series of screens (200-, 300-, 400-, and 600-mesh from top to bottom). The screens were rinsed with copious amounts of water, and the material remaining on the 600-mesh screen was collected for DNA extraction and staining of oospores.

DNA was extracted directly from the soil samples according to a method modified from Heinz and Platt (14). Concentrated soil samples were ground to a fine powder in liquid nitrogen with a mortar and pestle, and approximately 0.4 g of the powder was transferred to a 1.5-ml centrifuge tube. The soil powder was first suspended in 0.5 ml of 0.4% skim milk powder solution by vigorous vortexing and then centrifuged (12,000 × g, 15 min) to remove the soil. The supernatant (approximately 0.4 ml) was transferred to a centrifuge tube into which 0.4 ml of proteinase K extraction buffer (50 mM Tris-HCl [pH 8.0], 25 mM EDTA, 100 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate, and proteinase K at 10 µg/ml) had been added, and the sample was incubated for 1 h at 55°C. One-half volume (0.4 ml) of 7.5 M ammonium acetate was added to the sample, and the precipitated cell debris was removed by centrifugation at 12,000 × g for 15 min. The nucleic acids in the supernatant were precipitated with

2 volumes of ethanol at -20°C for 30 min or overnight. The DNA was pelleted, rinsed with 70% ethanol, dried, dissolved in 5 µl of sterile water or 0.1× TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and used as a template for PCR amplification or stored at -20°C until use.

Discrimination of live and dead oospores. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) staining method was used to determine whether the oospores recovered from the soil were alive or dead (30). First, 0.05% MTT was added to the material gathered from the 600-mesh screen. The sample was incubated for 48 h at 35°C and then observed under a microscope. The oospores that germinated stained blue, and those in dormancy appeared pink, but dead oospores were either stained dark or not stained. Impurities such as pieces of soil remained unstained.

PCR. Each reaction for the DC6/internal transcribed spacer 4 (ITS4) protocol consisted of 2.5 µl of 10× PCR buffer, 1 µl of 2.5 mM dNTPs, 2.5 µl of 25 mM MgCl₂, 0.25 µl each of 20 µM primers, and 0.25 µl of *Taq* DNA polymerase (5 units/µl), to which were added template DNA and finally sterile distilled H₂O was added to a final volume of 25 µl. All reactions were performed in a PTC2000 PCR instrument (MJ Research, Watertown, MA). The thermal cycling settings used were an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. Negative controls lacking template DNA were performed in each experiment to test for contaminated reagents. All of the reagents used for the PCR amplification were purchased from Promega (Shanghai).

The products amplified from eight isolates of *P. sojae* by the universal primers DC6 and ITS4 (2) were purified according to Sambrook et al. (25) and fused into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. Samples of plasmids containing the PCR products were sent to Sangon in Shanghai for automated DNA sequencing on an ABI Prism System automated sequencer (Model 377, version 2.1.1, Perkin-Elmer Corp., Foster City, CA). Three clones from each isolate were sequenced. The sequences were aligned with published sequences from 50 other *Phytophthora* species (4) found in GenBank using ClustalW in BioEdit version 4.8.2 (13). A region specific to *P. sojae* was identified and used to design two PCR primers, PS1 and PS2, which were then synthesized by TaKaRa (Japan). The PCR conditions used with primers PS1 and PS2 were similar to those described above for ITS4 and DC6, except the annealing temperature was 66°C and 2.5 µl of bovine serum albumin (BSA) (0.1%) was added.

Quantitative detection of *P. sojae* in the soil samples. A real-time PCR method was developed to quantitatively detect *P. sojae* in soil. The reactions were carried out in a 50-µl volume that consisted of 0.5 µM of each primer (PS1/PS2); 5 µl of DNA solution extracted from soil samples; a 2.5-µl mixture containing 12.5 µM each dNTP; 5 µl of 10× PCR buffer; 2.5 mM Mg²⁺; 1.25 U of *Taq* DNA polymerase (Promega); 2.5 µl of 20× SYBR Green I (OPE Technology Development Co., Ltd., Shanghai, China); and sterile distilled H₂O to a final volume of 50 µl. The thermal cycling settings used were the same as those for the general PCR. Quantitative PCR experiments were performed in an ABI Prism 7000 Sequence Detection System (Perkin-Elmer). A standard curve was first established by plotting the log of a known concentration (10-fold dilution series from 100 fg to 10 ng in reaction volumes of 50 µl) of DNA from *P. sojae* against the threshold cycle (Ct) values and melting curve. Ct values resulting from assays of unknown samples were plotted onto this curve, and the inferred concentration of *P. sojae* was calculated. The DNA samples extracted from 20 g of soil were suspended in 10 µl of ultrapure water and used for PCR amplification. The amount of DNA per gram of soil was obtained from the standard curve. The concentrations of DNA were estimated based on six replicates.

TABLE 1. Isolates of *Phytophthora sojae* and other fungi used to screen the polymerase chain reaction (PCR)-specific primers (PS1/PS2)

Species ^a	Isolation/origin		No. isolates	PCR product ^c	
	Host/substrate	Source ^b		DC6/ITS4	PS1/PS2
<i>P. sojae</i>	<i>Glycine max</i>		138	+	+
<i>P. boehmeriae</i>	<i>Gossypium</i>	JS	2	+	–
	<i>Boehmeria niver</i>	JS	1	+	–
<i>P. nicotianae</i>	<i>Nicotiana tabacum</i>	YN	2	+	–
	<i>Citrus</i>	JS	2	+	–
	<i>Lycopersicon esculentum</i>	JS	1	+	–
	<i>Sophora chinensis</i>	JS	1	+	–
<i>P. cactorum</i>	<i>Malus pumila</i>	W. H. Ko	1	+	–
<i>P. drechsleri</i>	<i>Cucumis sativus</i>	JS	1	+	–
<i>P. palmivora</i>	<i>Citrus</i>	JS	1	+	–
<i>P. cinnamomi</i>	<i>Cedrus deodara</i>	JS	1	+	–
<i>P. capsici</i>	<i>Capsicum annuum</i>	JS	7	+	–
<i>P. tropicalis</i>	<i>Leucospermum</i>	W. H. Ko	1	+	–
<i>P. heveae</i>	Unknown	HN	1	+	–
<i>P. infestans</i>	<i>Solanum tuberosum</i>	FJ	2	+	–
	<i>Lycopersicon esculentum</i>	JS	3	+	–
<i>P. medicaginis</i>	<i>Medicago sativa</i>	ATCC	1	+	–
<i>P. cryptogea</i>	<i>Gerbera jamesonii</i>	JS	5	+	–
<i>P. colocasiae</i>	Unknown	ATCC	1	+	–
<i>P. megasperma</i>	<i>Matthiola incana</i>	CBS 305.36	1	+	–
<i>P. vignae</i>	<i>Vigna sinensis</i>	CBS 241.73	1	+	–
<i>P. syringae</i>	Unknown	CBS 132.23	1	+	–
<i>P. quercina</i>	<i>Quercus petraea</i>	CBS 781.95	1	+	–
<i>P. phaseoli</i>	Unknown	CBS 556.88	1	+	–
<i>P. lateralis</i>	<i>Chamaecyparis lawsoniana</i>	CBS 168.42	1	+	–
<i>P. idaei</i>	Raspberry	CBS 968.95	1	+	–
<i>P. hibernalis</i>	<i>Cirrus sinensis</i>	CBS 270.31	1	+	–
<i>P. fragariae</i> var. <i>rubi</i>	Raspberry	CBS 967.95	1	+	–
<i>P. erythroseptica</i>	<i>Solanum tuberosum</i>	CBS 129.23	1	+	–
<i>P. cambivora</i>	<i>Castanea sativa</i>	CBS 248.60	1	+	–
<i>Pythium ultimum</i>	Irrigation water	JS	1	+	–
<i>P. aphanidermatum</i>	<i>Cucumis sativus</i>	ZJ	2	+	–
	<i>Glycine max</i>	JS	1	+	–
<i>P. myriotylum</i>	Irrigation water	JS	1	+	–
<i>Pythium</i> sp.	Soil	JS	2	+	–
<i>Saprolegnia</i> sp.	Irrigation water	JS	1	–	–
<i>Chytridiomycetes</i> sp.	Irrigation water	JS	1	+	–
<i>Bremia lactucae</i>	<i>Lactuca L</i>	JS	1	+	–
<i>Peronospora</i>	<i>Gossypium</i>	JS	1	+	–
<i>Peronospora manshurica</i>	<i>Glycine max</i>	JS	3	+	–
<i>Fusarium solani</i>	<i>Gossypium</i>	JS	3	–	–
	<i>Glycine max</i>	JS	5	–	–
<i>F. oxysporum</i> f. sp. <i>vasinfectum</i>	<i>Gossypium</i>	JS	2	–	–
<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	<i>Cucumis sativus</i>	JS	3	–	–
<i>F. oxysporum</i> f. sp. <i>cubense</i>	<i>Musa sapientum</i>	FJ	1	–	–
<i>F. graminearum</i>	<i>Triticum aestivum</i>	JS	1	–	–
<i>F. equiseti</i>	Unknown	CGMCC	1	–	–
<i>F. avenaceum</i>	Unknown	CGMCC	1	–	–
<i>F. nivale</i>	Unknown	CGMCC	1	–	–
<i>F. sambucinum</i>	Unknown	CGMCC	1	–	–
<i>F. culmorum</i>	Unknown	CGMCC	1	–	–
<i>F. moniliforme</i>	<i>Oryza sativa</i>	L. F. Chen	1	–	–
	<i>Gossypium</i>	L. F. Chen	1	–	–
<i>Fusarium</i> sp.	<i>Gossypium</i>	JS	2	–	–
<i>Macrophoma kawatsukai</i>	<i>Malus pumila</i>	JS	1	–	–
<i>Alternaria alternata</i>	Soil	JS	1	–	–
<i>Alternaria</i> sp.	<i>Glycine max</i>	JS	1	–	–
<i>Botrytis cinerea</i>	<i>Cucumis sativus</i>	ZJ	1	–	–
<i>Endothia parasitica</i>	<i>Castanea mollissima</i>	JS	3	–	–
<i>Magnaporthe grisea</i>	<i>Oryza sativa</i>	YN	3	–	–
<i>Colletotrichum gossypii</i>	<i>Gossypium</i>	JS	1	–	–
<i>C. glycines</i>	<i>Glycine max</i>	JS	1	–	–
<i>Rhizoctonia solani</i>	<i>Gossypium</i>	JS	2	–	–
	<i>Glycine max</i>	SD	3	–	–
<i>Ascochyta gossypii</i>	<i>Gossypium</i>	JS	1	–	–
<i>Aspergillus</i> spp.	<i>Gossypium</i>	JS	1	–	–
<i>Verticillium dahliae</i>	<i>Gossypium</i>	JS	3	–	–
<i>V. albo-atrum</i>	<i>Medicago sativa</i>	JS	1	–	–
<i>Tilletia indica</i>	<i>Triticum aestivum</i>	JS	1	–	–

^a All *P. sojae* isolates and other *Phytophthora* species were maintained in the collection of Nanjing Agric. University. All other fungi genera were collected in this study.^b Isolates were isolated from the following sources: JS = Jiangsu province; SD = Shandong province; YN = Yunnan province; HN = Hainan province; ZJ = Zhejiang province; FJ = Fujian province; HLJ = Heilongjiang province; W. H. Ko from Hawaii University; L. F. Chen from Nanjing Agricultural University; and CGMCC = China General Microbiological Culture Collection Center in Beijing.^c PCR product: + = expected PCR product present; – = no PCR product band.

Specificity and sensitivity of primers PS1 and PS2. A total of 245 isolates representing 25 species of *Phytophthora* and 35 species of other pathogens (Tables 1 and 2) were used to determine the specificity of primers PS1 and PS2. Purified DNA and crude DNA extracted from zoospores or oospores of *P. sojae* were used for this purpose. The concentration of the purified *P. sojae* DNA was determined with a spectrophotometer (Beckman DU640; Beckman Coulter Inc., Fullerton, CA), and then a dilution series of the DNA, from 1.0 µg to 1 fg of DNA, was used to test the sensitivity of the detection procedure. To extract crude DNA from zoospores, 60 zoospores were suspended in 60 µl of double-distilled H₂O, 0.5 g of silica was added, and the mixture was vortexed for 1 min, after which

0.5, 1, 2, 3, to 9 µl of suspension solution was added to the PCR reactions. The amplified products were electrophoresed on 1% agarose gels containing ethidium bromide (0.5 µg/ml), which were run in 1× Tris-acetate-EDTA running buffer. A 100-bp DNA ladder (TaKaRa, Japan) was included on each gel as a molecular size standard. The experiments were repeated at least three times.

PCR amplification from diseased plant tissue. *P. sojae*-infected soybean tissues were collected from Heilongjiang province in 2003, and DNA was prepared from the diseased tissues by an NaOH lysis method for PCR, according to Wang et al. (33). Healthy soybean root tissue was used as a negative control. One microliter of this extract was used as the DNA template. The PCR conditions used were identical to those described above for amplification with the PS primers. The PCR amplifications were repeated at least three times per sample. In addition, to confirm that no PCR inhibitors were present in healthy soybean tissues, the primers ITS1 and ITS4, which amplify the ITS region of soybean, were used in PCR analyses as a positive control.

RESULTS

Sequence analysis. The sequences from the ITS regions of all eight *P. sojae* isolates from different sources were more than 99.5% identical. These sequences were aligned with previously published sequences from a corresponding region of the genomes of 50 other *Phytophthora* spp., including *P. sojae*, *P. drechsleri*, *P. medicaginis*, and *P. megasperma*. The variable sequences in the ITS1 and ITS2 regions were identified and used to design a pair of potential *P. sojae*-specific primers, PS1 (5'-CTGGATCATGAGCCCACT-3') and PS2 (5'-GCAGCCCGAAGGCCAC-3'). The entire ITS sequence from *P. sojae* isolate S317 was submitted to GenBank (accession no. AY742749).

Specificity. From all of the oomycete isolates, including *Phytophthora* spp., *Pythium* spp., and *Peronospora* spp., an approximate 1.3-kb product was amplified with primers DC6 and ITS4, with the exception of *Saprolegnia* sp., only one fungi isolate, *Chytridiomycetes* sp., yielded a product with the same size (Table 1). However, 138 isolates representing *P. sojae* exclusively yielded a product of approximately 330 bp (Tables 1 and 2; Fig. 1) with primers PS1 and PS2. Isolates representing all of the other *Phytophthora* species and the other genera tested yielded no amplification product with primers PS1 and PS2. However, when the annealing temperature decreased to less than 65°C, a specific 330-bp PCR product could be amplified from *P. drechsleri* (data not shown).

Sensitivity. PCR with primers PS1 and PS2 yielded positive results with 10 concentrations of purified DNA as the template, from 1 µg to 1 fg (Fig. 2A). In reactions containing crude DNA from zoospores, the DNA from 0.5 to 9 µl of zoospore DNA extract (60 zoospores) all allowed the amplification of the 330-bp DNA band (Fig. 2B). The DNA extracted from a single oospore was also sufficient for PCR detection (Fig. 2C).

Recovery of oospores from soil residues carried by soybeans. Twenty grams of soil was sieved by a series of screens and

TABLE 2. Isolates of *Phytophthora sojae* used to screen the polymerase chain reaction (PCR)-specific primers (PS1/PS2)

Isolate ^a	Isolation/origin		No. of isolates	Races	PCR product ^c	
	Host	Source ^b			DC6/ITS4	PS1/PS2
	<i>Glycine max</i>	HLJ	68	Unknown	+	+(68/68)
	<i>Glycine max</i>	FJ	28	Unknown	+	+
	<i>Glycine max</i>	USA	26	Unknown	+	+
	<i>Glycine max</i>	Brazil	2	Unknown	+	+
	<i>Glycine max</i>	Argentina	1	Unknown	+	+
P6497	<i>Glycine max</i>	B. Tyler	1	2	+	+
P7064	<i>Glycine max</i>	B. Tyler	1	7	+	+
P7074	<i>Glycine max</i>	B. Tyler	1	17	+	+
P7076	<i>Glycine max</i>	B. Tyler	1	19	+	+
R4	<i>Glycine max</i>	J. H. Peng	1	4	+	+
R5	<i>Glycine max</i>	J. H. Peng	1	5	+	+
R7	<i>Glycine max</i>	J. H. Peng	1	7	+	+
R8	<i>Glycine max</i>	J. H. Peng	1	8	+	+
R13	<i>Glycine max</i>	J. H. Peng	1	13	+	+
R17	<i>Glycine max</i>	J. H. Peng	1	17	+	+
R18	<i>Glycine max</i>	J. H. Peng	1	18	+	+
R19	<i>Glycine max</i>	J. H. Peng	1	19	+	+
R31	<i>Glycine max</i>	J. H. Peng	1	31	+	+

^a All isolates of *Phytophthora sojae* were maintained in the collection of Nanjing Agric. University.

^b Isolates were isolated from the following sources: FJ = Fujian province; HLJ = Heilongjiang province; B. Tyler from Virginia Polytechnic Institute and State University; and J. H. Peng from Dalin Quarantine Bureau of China.

^c PCR product: + = expected PCR product present; – = no PCR product band.

TABLE 3. Soil samples used for polymerase chain reaction (PCR) assay

Sources	Soil type	No. of soil samples	No. of PCR positive ^a	No. of leaf disk baiting positive ^b
Heilongjiang	Phaeozem	118	67	46
United States	Unknown	24	19	12
Canada	Unknown	6	4	3
Brazil	Unknown	2	1	1
Argentina	Unknown	1	1	1
Total		151	92	63

^a Number of soil samples that gave positive signals by PCR assay.

^b Number of soil samples that gave positive signals by leaf disk baiting assay.

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) products using the *Phytophthora sojae*-specific primers PS1 and PS2 designed in this study. PCR amplification products of 24 isolates of *P. sojae* (lanes 1 to 24) were collected from different sources as follows: Heilongjiang Province (lanes 1 to 12), United States (lanes 13 to 19), Brazil (lanes 20 to 22), Canada (lane 23), and Argentina (lane 24). Lane 25, no template DNA; M, 100-bp DNA ladder.

the oospores residing in 20 g of soil were collected into a soil sample of approximately 0.4 g per soil sample. The MTT staining method demonstrated that most of the oospores recovered from the soil were viable (Fig. 3). The DNA extracted from the concentrated soil samples was used for PCR amplification and produced a 330-bp band. In an artificial experiment, when only two oospores are present in 20 g of soil sample (soil type is phaeozem), all samples are PCR positive. This experiment was repeated more than 10 times with similar results.

The newly designed protocol described here was extensively applied on samples collected from quarantine bureaus of China. From 2004 to 2005, 33 soil samples collected from soybeans imported from the United States, Canada, Brazil, and Argentina were subjected to PCR assay, and 25 samples gave positive signals (Table 3; Fig. 4). Seventeen samples were further confirmed by the leaf disk baiting method, indicating the presence of the pathogen. This technique was also applied to 118 soil samples collected from the soybean production areas of Heilongjiang province from 2003 to 2004 and 67 of the samples gave positive signals, 46 of which were further confirmed by the leaf baiting method (Table 3). In addition, all soil samples positive in the leaf disk baiting method were confirmed by PCR.

PCR amplification from diseased plant tissue. After the preparation of template from the blighted soybean tissue by NaOH extraction followed by PCR amplification using primers PS1 and PS2, a single PCR product of approximately 330 bp was detected (Fig. 5). The presence of *P. sojae* in the diseased plants was confirmed by isolating the pathogen from the tissue to a pure culture. In contrast, no PCR product was amplified from healthy soybean tissues. Amplification carried out using the ITS universal

primer produced a 700-bp DNA band from healthy soybean tissue indicating the absence of PCR inhibitors in the healthy extract.

Quantitative detection of *P. sojae* in the soil samples. DNA extracted from two field soil samples collected in diseased soybean fields in Heilongjiang province and one other soil sample collected from imported U.S. soybeans were subjected to real-time fluorescent quantitative PCR using primers PS1 and PS2 (Fig. 6). The amount of DNA in each soil sample is shown in Table 4.

DISCUSSION

One objective of this work was to develop PCR primers for the rapid and specific amplification of *P. sojae* in the soil contaminating transported soybeans, infested soil in production fields, and diseased soybean tissues. PCR with primers PS1 and PS2 amplified a product of approximately 330 bp exclusively from all isolates of *P. sojae*. A simple mesh screen method to concentrate oospores from soil combined with the PCR assay allowed the detection of *P. sojae* from soil within 6 h. In addition, a quantitative real-time PCR was also developed to quantify *P. sojae* directly from soil. The PS primers were also able to be used to detect *P. sojae* in infected soybean tissues.

Fig. 3. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) staining of oospores recovered from the soil by the screen method. A, dormant oospore; B, germinating oospore; and C, dead oospore.

Fig. 2. Sensitivity of molecular detection using the *Phytophthora sojae*-specific primers PS1 and PS2 designed in this study. A, The purified DNA was used as template. No template DNA (lane 1). Lanes 2 to 11 were purified genomic DNA from 1 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg, respectively. M, 100-bp DNA ladder. B, Crude DNA from zoospores was used as template (60 zoospores were extracted to produce 60 µl of extract). No template DNA (lane 1). Lanes 2 to 11 were crude DNA extracted from zoospores using 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 9 µl, respectively. M, 100-bp DNA ladder. C, DNA from a single oospore was used as template. No template DNA (lane 1). Lanes 2 to 6 used DNA from a single oospore. M, 100-bp DNA ladder.

Fig. 4. PS primers amplification of *Phytophthora sojae* from the soil carried with the imported soybeans. Lane 1, no template DNA; lane 2, 100 pg of purified DNA of *P. sojae*; lanes 3 to 6, soil samples from different sources (United States [lanes 3 and 4], Brazil [lane 5], and Canada [lane 6]); and M, 100-bp DNA ladder.

Fig. 5. PS primers amplification of *Phytophthora sojae* from infected soybean tissues. Lane 1, no template DNA; lane 2, 100 pg of purified DNA of *P. sojae*; lane 3, DNA from infected soybean tissue; lane 4, DNA from healthy soybean tissue; lane 5, amplification of approximately 700-bp product from healthy soybean tissue with the universal primer set internal transcribed spacer 1 (ITS1)/ITS4; and M, 100-bp DNA ladder.

Sensitivity and specificity are very important for the detection of pathogens. The molecular detection sensitivity of one *P. nicotianae* assay is between 80 and 800 fg of DNA/μl (18), and another technique to detect *P. boehmeriae* has a detection sensitivity of approximately 10 to 100 fg (27). However, the PS primers provide a more sensitive technique for the detection of *P. sojae*, with only 1 fg of purified DNA required to detect the pathogen. Regarding the specificity, more than 245 isolates representing 25 species of *Phytophthora* and at least 35 other species of pathogens, many potentially associated with soybeans, did not generate a product with the PS primers, with one possible exception. The sequences of the PS primers vary from *P. drechsleri* sequences at only one nucleotide requiring the need to increase the annealing temperature to 66°C. *P. drechsleri* has a wide host range including various vegetable crops, lucerne, and a wide range of ornamentals and trees (28). However, this species does not infect soybeans (15) and is not likely to occur at detectable levels in soybean fields or soybean residue. False negatives could be avoided using the higher annealing temperature, through future work to develop more specific primers, or development of other techniques such as enzyme digestion and nested-PCR. In our assays, *P. drechsleri* was not encountered when isolates were obtained using the leaf baiting assay and culturing of the *Phytophthora* pathogens present.

Fig. 6. A, Standard curve obtained by plotting the log of a known DNA concentration of *Phytophthora sojae* (10 pg to 100 ng) against the threshold cycle (Ct) values obtained from real-time quantitative polymerase chain reaction (PCR) assays. B, Melting curve profile for real-time PCR amplification of *P. sojae* pure genomic DNA. The negative derivative of fluorescence with respect to temperature is plotted as dF/dT versus temperature to obtain a graphical representation of the melting peaks.

The PCR assay allows the rapid detection of the pathogen in soil. Although *P. sojae* is a quarantine pathogen in China, more than 20 million tons of soybeans are imported into China annually. In addition, because most of the soybeans used as seeds in China are transported from Heilongjiang province, where *P. sojae* has been isolated, rapid determination of whether *P. sojae* exists in the soil carried with the transported soybeans is not only important for the soybean trade between China and other countries, but also for controlling the spread of *P. sojae* within China. The PS primer-based PCR assay requires only 6 h for the detection of *P. sojae* in soil. Compared with the extensively used soybean leaf disk baiting method which needs at least 15 to 20 days for pathogen detection (22), the newly designed PCR assay provides a much more efficient method, which has been extensively applied in some quarantine bureaus of China during the past several years. Given its speed and efficiency, the PCR method is specially fit as a tool in quarantine measures.

The thick-walled oospores of *P. sojae* may remain dormant and persist in soybean residues and soil for many years in the fields (10). Sensitive detection of the pathogen from soil will lead to more effective disease management practices. However, the quantity of oospores in soil is limited and the extraction of target DNA directly from soil is difficult. To concentrate the soilborne oospores before DNA extraction, a method to collect oospores through a series of screens was developed, by means of which all of the oospores in 20 g of soil could be collected in a final 0.4-g volume of soil. The screening procedure, combined with the PCR assay, allowed the detection of as few as two oospores in a 20-g soil sample. This procedure constitutes a very efficient method for the detection of *P. sojae* from the soil directly. To our knowledge, this is the first report of a method to recover *P. sojae* oospores from soil, and most oospores were demonstrated to be viable by the MTT staining method.

Many PCR-based molecular tools have been developed to detect *Phytophthora* species and diagnose associated diseases over the past several years (9,12,18,19,21,23,24,27). Recently, Judelson and Tooley (16), Tooley et al. (31), and Trout et al. (32) reported the development of PCR primers to detect *P. infestans* from infected potato. Kong et al. (18) developed PCR primers to detect *P. nicotianae* in irrigation water. To our knowledge, there has been no previous report of the molecular detection of *P. sojae*. In general, *Phytophthora* spp. are difficult to isolate into pure culture, and baiting and culturing processes are time-consuming. The technique described here circumvents these traditional protocols. To detect *P. sojae* in infected soybean tissues, we adopted a quick lysis assay for infected plant material that eliminates the need for time-consuming extraction of pathogen DNA prior to PCR (33). These techniques should allow the rapid, accurate detection of *P. sojae* from soil and infected plants.

Although this is the first report about the molecular detection of *P. sojae*, which has been used extensively in China during the past several years (Table 3), there are still some limitations in the application of this protocol to monitor *P. sojae* in soybean production fields. Issues associated with spatial distribution of inoculum would require multiple soil samples to be evaluated, and the soil sieving by screens could result in the cross contamination among different soil samples. In general, in such cases, the leaf baiting

TABLE 4. Results of quantitative polymerase chain reaction assay

Samples ^a	HLJ1	HLJ2	US1
Amount ^b	3.29 ± 1.50 fg	16.13 ± 9.59 fg	54.86 ± 22.27 fg

^a HLJ1 and HLJ2 represent the soil samples collected from Huanan County and Jiamusi city of Heilongjiang province, respectively. US1 represents a soil sample collected from soybeans imported from the United States in 2004.

^b The amount of DNA per gram of soil, the concentrations of DNA were estimated after six replicates.

method was applied first, and after the water-soaked lesion appeared, the leaf disk was subjected to PCR detection.

Though there are still some limitations to the PCR assay reported here, the sensitivity, specificity, efficiency, and speed make the PCR assay a highly useful technique for detecting *P. sojae* from the soil carried with transported soybeans. Because this PCR assay also has significant practical applications in the detection of *P. sojae* in infected soybean tissues and infested field soils, it constitutes a powerful tool for controlling the dispersion of *P. sojae* and developing disease control strategies.

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