Molecular detection of *Phytophthora sojae* in *Glycine max* through conventional and real-time PCR

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INTRODUCTION

Phytophthora rot, caused by *Phytophthora sojae* Kaufmann & Gerdemann, is one of the most damaging diseases of soybean (*Glycine max*) in the U.S. (4). In 2005, *P. sojae* caused an estimated yield loss of 1.1 million tons in the U.S. (7). Field diagnosis of Phytophthora rot may be difficult due to symptoms resembling those caused by other pathogens such as *Pythium* or *Diaporthe* species. Symptoms include root rot and stem rot (Fig. 1). Methods to identify *P. sojae* involve direct isolation on semi-selective media. The development of a PCR-based assay for the rapid and sensitive detection of *P. sojae* could facilitate pathogen identification and lead to more effective disease management.

PCR assays for rapid and specific detection of *P. sojae* using two sets of primers have been reported by other researchers, but they have limitations or have not been validated. PS1-PS2 were developed in China and PSOJF1-PSOJR1 were developed at Michigan State University (1,6). *P. sojae* is not the only soybean-infecting *Phytophthora* species in the Midwestern U.S. A previously undescribed *Phytophthora* species has been found associated with soybean in Illinois and Indiana (2,3). The two *Phytophthora* species can be distinguished based on morphology, but culturing is time consuming, laborious, and often very difficult (Fig. 2). Therefore, a PCR assay must not only detect *P. sojae*, but must also distinguish *P. sojae* from other *Phytophthora* species that could potentially be associated with soybean. The objective of this study was to evaluate PCR methods for rapid and specific detection of *P. sojae*.

Fig. 1. Soybean plant with a stem lesion caused by *P. sojae* infection.

Fig. 2. Cultures showing differences in morphology of *P. sojae* and the undescribed *Phytophthora* species after 6 days of growth on potato dextrose agar (PDA). Growth of *P. sojae* (left) was limited, while the undetermined *Phytophthora* (right) grew abundantly and formed a rosette pattern.

MATERIALS AND METHODS

Isolates and DNA extraction

Genomic DNA was extracted from mycelium of 92 isolates of fungi and oomycetes, consisting of 10 genera and 30 species (Table 1). All isolates were grown in nutrient broth media for at least 5 days at 22° C. The mycelia and spores were harvested by filtration, homogenized in a FastPrep® instrument (QBiogene, Irvine, CA), and DNA was extracted using a FastPrep® FastDNA kit. DNA was stored at -20° C.

Testing PS1-PS2 primers for specificity

PCR primers PS1-PS2 were tested to determine specificity to *P. sojae* using conventional PCR (sPCR). Reactions were carried out using the protocol described by Wang *et al.* (6). Initial reactions did not produce similar results so the protocol was modified by using annealing temperatures ranging from 55 to 66° C. Reactions were carried out in a PTC-100 PCR instrument (MJ Research, Watertown, MA) and an Eppendorf Mastercycler (Eppendorf, Waterbury, NY).

Testing PSOJF1-PSOJR1 primers for specificity with real-time PCR

The PSOJ primers were evaluated with quantitative, real-time PCR (qPCR) using SYBR Green technology. The PCR reactions were conducted in a volume of 25 µl that contained 5 µl of DNA solution extracted from mycelia, 12.5 µl TaqMan universal PCR master mix (ABI), 2.5 µl 1× SYBR Green I, 450 nM each of the forward and reverse primers, and 2.75 µl of molecular grade water. Thermal cycling parameters consisted of 2 min at 50° C and 10 min at 95° C, followed by 40 cycles of 15 s at 95° C and 1 min at 60° C. Cycle threshold values were calculated using ABI software. Reactions were run in duplicate and analyzed based on absolute quantification using ABI software. Analysis of samples was repeated twice. DNase/RNase treated water controls were included in all reactions.

Testing PSOJF1-PSOJR1 primers for specificity with conventional PCR

The PSOJ primers were also evaluated with sPCR. The PCR reactions were conducted in a volume of 25 µl that contained 2.5 µl of DNA solution extracted from mycelia, 12.5 µl of GoTaq master mix (Promega), 0.25 µl each of 20 µM forward and reverse primer, and 9.5 µl of molecular grade water. Reactions were performed in a PTC-100 PCR instrument and an Eppendorf Mastercycler gradient instrument. The thermal cycling settings were 94° C for 5 min, followed by 35 cycles of 94° C for 30 s, 66° C for 30 s and 72° C for 30 s, followed by 72° C for 10 min. Annealing temperatures ranging from 55 to 66° C were tested. Negative controls were used that lacked template DNA.

Plant inoculations and sampling

A modified inoculum layer method using cultivar 'Sloan' seedlings was used for plant inoculations (5). The roots of inoculated and noninoculated plants were washed 14 days after planting and all plants were assessed for rot symptoms and examined microscopically for the presence of oospores. Segments of symptomatic root tissue were surface disinfested, rinsed with sterile water, and embedded in modified PARP medium to recover *P. sojae* (5). Putative *P. sojae* colonies were grown in broth media and DNA was extracted as described above. DNA was extracted from the remaining root tissue with a FastPrep® FastDNA kit.

Detection of *P. sojae* in soil

Soil samples were collected from three soybean fields in Minnesota where plants exhibited symptoms of Phytophthora rot, as well as from one field where plants lacked visible symptoms. An artificially infested soil sample was also prepared by autoclaving one field soil sample and adding a mycelial slurry of *P. sojae*. DNA was isolated from soil samples with the MoBio PowerSoil™ kit and used for sPCR and qPCR analysis.

Table 1. Isolates of *Phytophthora sojae* and other pathogens used to test specific primers PSOJF1-PSOJR1 and results from the testing with conventional PCR (sPCR) and real-time PCR (qPCR)

Genus and species	Number of isolates	sPCR[a]	qPCR[b,c]
Aphanomyces euteiches	1	-	ND
Diaporthe phaseolorum var. *caulivora*	2	-	ND
Fusarium acuminatum	2	-	ND
Fusarium equiseti	2	-	ND
Fusarium graminearum	2	-	ND
Fusarium oxysporum	4	-	ND
Fusarium proliferatum	2	-	ND
Fusarium redolens	2	-	ND
Fusarium solani	4	-	ND
Fusarium sporotrichioides	2	-	ND
Fusarium subglutinans	2	-	ND
Fusarium virguliforme	1	-	ND
Macrophomina phaseolina	2	-	ND
Phialophora gregata	2	-	ND
Phomopsis longicolla	2	-	ND
Phytophthora cactorum	2	-	ND
Phytophthora cinnamomi	2	-	ND
Phytophthora cryptogea	2	-	ND
Phytophthora drechsleri	1	-	ND
Phytophthora infestans	1	-	ND
Phytophthora medicaginis	1	-	ND
Phytophthora nicotianae	2	-	ND
Phytophthora sansomeana	2	-	ND
Phytophthora sojae	32	+	17.8
Phytophthora taxon 'Walnut'	1	-	ND
Phytophthora sp. (Illinois)	7	-	ND
Pythium irregulare	1	-	ND
Pythium oligandrum	1	-	ND
Rhizoctonia solani	2	-	ND
Sclerotinia sclerotiorum	3	-	ND

[a] sPCR product: + = expected PCR product band present; - = no PCR product band detected.
[b] Ct values are averages from duplicate reactions that were repeated using qPCR.
[c] Samples with Ct values greater than 35 were considered to have nondetectable (ND) DNA.

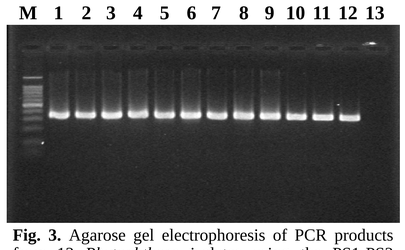

Fig. 3. Agarose gel electrophoresis of PCR products from 12 *Phytophthora* isolates using the PS1-PS2 primer set with an annealing temperature of 55° C. *P. sojae* (lanes 1 to 7), undescribed *Phytophthora* (lanes 8 and 9), *P. nicotianae* (lane 10), *P. cinnamomi* (lane 11), and *P. cactorum* (lane 12); No template DNA (Lane 13); M, 100-bp DNA ladder.

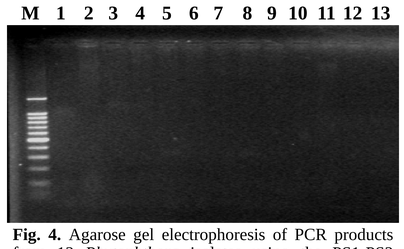

Fig. 4. Agarose gel electrophoresis of PCR products from 12 *Phytophthora* isolates using the PS1-PS2 primer set with an annealing temperature of 66° C. *P. sojae* (lanes 1 to 7), undescribed *Phytophthora* (lanes 8 and 9), *P. nicotianae* (lane 10), *P. cinnamomi* (lane 11), and *P. cactorum* (lane 12); No template DNA (Lane 13); M, 100-bp DNA ladder.

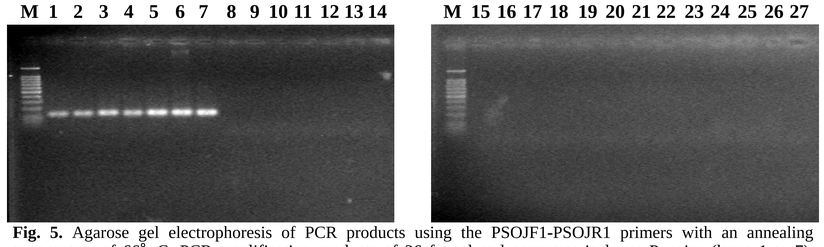

Fig. 5. Agarose gel electrophoresis of PCR products using the PSOJF1-PSOJR1 primers with an annealing temperature of 66° C. PCR amplification products of 26 fungal and oomycete isolates: *P. sojae* (lanes 1 to 7), undescribed *Phytophthora* (lanes 8 to 14), *P. nicotianae* (lane 15), *P. cinnamomi* (lanes 16 & 17), *P. cactorum* (lane 18), *P. medicaginis* (lane 19), *Pythium* (lanes 20 and 21), *A. euteiches* (lane 22), *F. virguliforme* (lane 23), *P. gregata* (lanes 24 and 25), and *R. solani* (lane 26); Lane 27, no template DNA; M, 100-bp DNA ladder.

Table 2. Comparison of culture isolation and conventional polymerase chain reaction (sPCR) for detection of *Phytophthora sojae* in plant tissue

Plant sample	Culture plating detection	PSOJF1-PSOJR1 sPCR detection
Greenhouse plants, noninoculated	-	-
Greenhouse plants inoculated with *P. sojae*	+	+

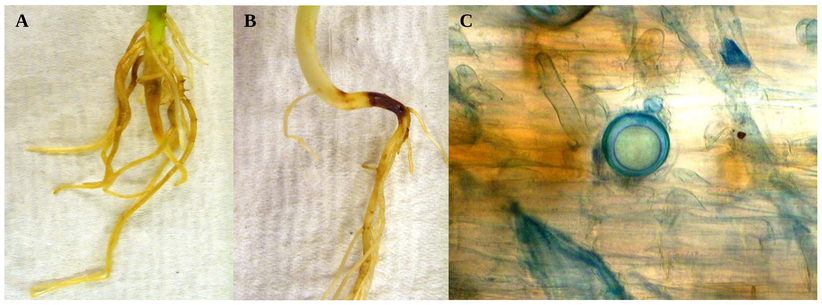

Fig. 6. Symptoms and signs of *P. sojae* infection in inoculated soybean seedlings. **A.** Soybean seedling with taproot necrosis. **B.** Soybean seedling with a necrotic lesion on the hypocotyl. **C.** Oospore visualized in an inoculated soybean seedling root.

Table 3. Results from the use of conventional PCR (sPCR) and real-time PCR (qPCR) assays based on the PSOJ primer set for detection of *Phytophthora sojae* in soil samples

Soil sample	sPCR detection[a]	qPCR detection[b,c]
SROC-212A	+	22.6
RM-KST	+	24.7
BM-RCo.	+	24.1
Sterilized field soil infested with *P. sojae*	+	27.5
Field soil from a location without *P. sojae*	-	-

[a] sPCR product: + = expected PCR product band present; - = no PCR product band detected.
[b] Ct values are averages from duplicate reactions that were repeated using qPCR.
[c] Samples with Ct values greater than 35 were considered to have nondetectable DNA.

Results and Discussion

- Our results indicate the PS1-PS2 primer set was not specific for *P. sojae*. DNA was amplified from five *Phytophthora* species tested when using annealing temperatures between 55 and 65° C (Fig. 3). Wang *et al*. reported specificity by increasing the annealing temperature to 66° C. When we increased the annealing temperature to 66° C there was no amplification of DNA from any of the *Phytophthora* species tested (Fig. 4).
- When the PSOJF1-PSOJR1 primer set was used for both qPCR and sPCR, *P. sojae* DNA was detected, but DNA from 60 other fungal and oomycete isolates tested was not (Table 1). This indicates that these primers are specific for detecting *P. sojae*.
- Greenhouse plants inoculated with *P. sojae* exhibited root rot and oospores were visualized in roots (Fig. 6A to C). Inoculated plants also yielded putative *P. sojae* cultures used to test the PSOJ primers and gave positive results with sPCR (Table 2). Control plants were asymptomatic and oospores were not detected in their roots. DNA extracted from plants inoculated with *P. sojae* tested positive with sPCR, whereas DNA from control plants was negative (Table 2). The PSOJ primers have also been used to detect *P. sojae* from symptomatic field-grown plants (data not shown).
- *P. sojae* was detected in soil using sPCR and qPCR. The pathogen was detected in soil from three fields containing plants with symptoms of Phytophthora rot, but not from a field lacking symptomatic plants (Table 3). *P. sojae* was also detected in an artificially infested soil sample using both sPCR and qPCR (Table 3).

Conclusions

- The PSOJF1-PSOJR1 primer set was species-specific for detecting *P. sojae* with both qPCR and sPCR.
- The PSOJF1-PSOJR1 primer set allowed the rapid detection of *P. sojae* from infected plant material and from soil. Therefore, these primers can be used as a diagnostic and research tool to improve management of Phytophthora rot.
- The PS1-PS2 primer set was not specific for detecting *P. sojae*. Caution should be used if these primers are utilized for detecting *P. sojae* and diagnosing Phytophthora rot.

Acknowledgements

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References

1. Hart, P. 2004. Application of real time PCR for detection and identification of soybean pests in Michigan. online: http://fieldcrop.msu.edu/documents/ID%20soybean%20pests%20GR03-004.pdf.
2. Malvick, D. K. and Grunden, E. 2004. Traits of soybean-infecting *Phytophthora* populations from Illinois agricultural fields. Plant Disease 88:1139-1145.
3. Reeser, P. W., Scott, D. H., and Ruhl, G.E. 1991. Recovery of race non-classifiable *Phytophthora megasperma* f. sp. *glycinea* from soybean roots in Indiana in 1990. Phytopathology 81:1201 (Abstract).
4. Schmitthenner, A. F. 2000. Phytophthora rot of soybean. Online. Plant Health Progress doi:10.1094/PHP-2000-0601-1001-HM.
5. Schmitthenner, A.F. and Bhat, R.G. 1994. Useful methods for studying *Phytophthora* in the laboratory. Ohio Agricultural Research and Development Center Special Circular 143. Ohio State University, Department of Plant Pathology, Wooster.
6. Wang, Y., Zhang, W., Wang, Y., and Zheng, X. 2006. Rapid and sensitive detection of *Phytophthora sojae* in soil and infected soybeans by species-specific polymerase chain reaction assays. Phytopathology 96:1315-1321.
7. Wrather, J.A. and Koenning, S. R. 2006. Estimates of disease effects on soybean yields in the United States 2003-2005. Journal of Nematology 38:173-180.