A Rapid Method Using PCR-Based SCAR Markers for the Detection and Identification of *Phoma sclerotoides*: The Cause of Brown Root Rot Disease of Alfalfa

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ABSTRACT

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A rapid technique for identification and detection of *Phoma sclerotoides*, the causal agent of brown root rot of alfalfa, has been developed using polymerase chain reaction (PCR). Amplification products obtained from random amplified polymorphic DNA (RAPD) reactions were cloned and sequenced, and two extended primer sets were designed from the resulting data that were used to detect sequence-characterized DNA markers. A single 499-bp DNA amplification product was consistently obtained from primers PSB12₄₉₉ that was specific for 19 isolates of *P. sclerotoides* but was not produced from *Phoma medicaginis* or *Phoma betae*, or from other soilborne pathogens including *Aphanomyces euteiches*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium ultimum*, or *Phytophthora infestans*. A 499-bp amplification product was also produced from root tissue known to be infected with the fungus as verified by microscopic examination. A similar PCR product was obtained from soil samples collected from fields with an established infection of *P. sclerotoides* on alfalfa. This PCR-based assay enables detection of *P. sclerotoides* from alfalfa root tissue and in soil samples in a single day, including extraction of DNA, compared with standard methods that require up to 100 days for identification using agar media.

Additional keywords: pathogen detection, sequence-characterized amplified region

Phoma sclerotoides G. Preuss ex Sacc. has been associated with winter kill of alfalfa (*Medicago sativa* L.) and other legume crops in temperate regions with severe winters. The fungus was first reported on forage legumes in Canada in 1933 and identified as *Plenodomus meliloti* Dearn. & G.B. Sanford (12). The pathogen has also been found in Nova Scotia, Alaska, and the Yukon regions of Canada (12). *P. sclerotoides* has earned the com-

mon name brown root rot (BRR) of alfalfa because of the disease symptoms expressed in this host. BRR was first reported in the contiguous United States in 1996 near Farson, WY, after a severe winter resulted in a high mortality rate the following spring in established alfalfa fields (3,5). The pathogen has subsequently been isolated from alfalfa fields in eight counties in Wyoming (3) and was also reported recently from Montana and Idaho (F. A. Gray, *unpublished*). Hollingsworth suggested that BRR may be present in many of the alfalfa fields (ca. 100,000 ha) in Wyoming and other high-altitude growing areas in the Rocky Mountain region (1,4).

Aboveground symptoms on alfalfa caused by *P. sclerotoides* typically are found in plants at least 3 years old and include stunting and decline. Infected taproots develop dark brown lesions occasionally surrounded by a black border (2). The initially small root lesions coalesce, eventually causing a general rotting of the infected area. Crowns and shoots are often killed as a result of the root infection. Affected plants are generally slow to break dormancy in the early spring. Macroscopic

identification of BRR on alfalfa roots can be difficult, as the symptoms are readily confused with root rot symptoms caused by *Phytophthora medicaginis* (2). In addition, a complex of other soilborne pathogens including species of *Rhizoctonia*, *Aphanomyces*, and *Pythium* also infect alfalfa and may mask symptoms caused by *P. sclerotoides*.

Detection of fungal pathogens with molecular and polymerase chain reaction (PCR) techniques has been used successfully by others (6-9,13,14). Willits and Sherwood (14) were able to detect *Ustilago hordei* on barley leaves by PCR. Vandemark et al. (13) recently developed a PCR protocol using sequence characterized amplified regions (SCARs) (10) for identification of *Aphanomyces euteiches* in pea roots and *A. cochlidioides* in sugar beets. Because the isolation, growth, and subsequent identification of *P. sclerotoides* is time-consuming, requiring up to 100 days after transfer to agar media before conidia are released from pycnidia (12), the objective of this work was to develop a rapid and efficient method using SCARs to unambiguously detect the presence of *P. sclerotoides* in infected alfalfa tissue and in soil samples collected from alfalfa fields.

MATERIALS AND METHODS

DNA extraction. Eighteen isolates of *P. sclerotoides* collected from six regions in Wyoming (4), including Boulder, Farson, Eden, Laramie, Lyman, and Saratoga, and a Canadian isolate obtained from the American Type Culture Collection (ATCC 56515) were used in this study. Only slight morphological variability, primarily mycelial pigmentations, existed among each of the 18 Wyoming isolates and the Canadian isolate. The fungal cultures were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) in the dark and maintained in a growth chamber adjusted to $15.6 \pm 1.0^\circ\text{C}$ for 30 to 45 days. Mycelium was then separated from the agar substrate using a sterile spatula and desiccated overnight under vacuum in a SpeedVac SC100 system (Savant Instruments, Farmingdale,

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NY). Total nucleic acid was extracted from the dried mycelium using a modification of the method described by Vandemark et al. (13). Lyophilized mycelium (0.2 g) was macerated in liquid nitrogen using a mortar and pestle. The ground mycelium was hydrated for 10 min at room temperature in 3 ml of extraction buffer (200 mM Tris, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate [SDS]) and the DNA extracted with an equal volume of phenol/chloroform. The nucleic acid was precipitated with 0.1 vol of 0.3 M sodium acetate, pH 5.5, and 2.5 vol of ethanol. After centrifugation for 10 min at $10,000 \times g$, the pellet was washed with 70% ethanol and dried briefly under vacuum. The pellet was resuspended in 100 μ g of RNase H (Sigma, St. Louis, MO) per ml and incubated 15 min at 37°C to remove RNA. The suspension was extracted twice with equal volumes of phenol/chloroform, followed by a final wash in an equal volume of chloroform. The supernatant was precipitated in ethanol as described above, and the final DNA pellet was resuspended in Tris-EDTA (10 mM Tris, pH 8.0, and 1 mM EDTA). All DNA preparations were adjusted to 10 ng/ μ l using a spectrophotometer with ultraviolet absorbance at 260 nm. DNA was also extracted from other soilborne fungi including *Aphanomyces euteiches*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium ultimum*, and *Phytophthora infestans* as described above to provide experimental controls. In addition, DNA from two isolates of *Phoma medicaginis* and four isolates of *Phoma betae* was used to verify specificity of primers to *P. sclerotoides*.

Six-month-old alfalfa plants were inoculated with *P. sclerotoides* on infested barley grains as described by Hollingsworth (4). Briefly, two barley grains were placed against the taproots approximately 2.5 cm below the soil surface in December. Cotton was placed over the inocula and the soil replaced. Plants were placed outdoors for a total of 15 months winter exposure (January to May) at Laramie, WY, during a 3-year period. The average soil temperature for each of the consecutive 5-month winter periods was 0.6, 2.3, and 0.6°C, respectively. Plants were subsequently returned to the greenhouse and maintained at average air temperatures of 21°C day and 15°C night. Roots of inoculated plants were washed free of soil using tap water and blotted dry. Root tissue from noninoculated alfalfa roots was used as healthy controls. In addition, alfalfa plants naturally infected with *P. sclerotoides* were collected from fields in Farson, WY, during the 2001 growing season. Lesions from roots exhibiting symptoms of BRR were excised using a sterile scalpel. Total DNA was extracted from approximately 200 mg of root tissue per plant using the Fast DNA kit (Bio 101, Carlsbad, CA) according to the manufacturer's instructions. The final DNA preparation was diluted 1:100 using sterile distilled water and stored at -20°C.

Soil samples from greenhouse plants were collected near roots previously inoculated with *P. sclerotoides* as described above (4). Soil samples were also collected from a grower's fields near Farson, WY, where alfalfa plants exhibiting symptoms of BRR were observed. Soil samples used

as noninfested negative controls were collected, and 5- to 10-g aliquots were autoclaved at 121°C at 15 psi for 20 min. DNA was extracted from a 500-mg subsample of each soil sample using the Ultra-Clean DNA soil extraction kit (Mo-Bio, Solana Beach, CA) according to the manufacturer's protocol.

Screening for random amplified polymorphic DNA (RAPD) markers and detection of SCARs. DNA from each fungal isolate was screened for RAPD markers generated by random decamer primers (Operon, Inc., Alameda, CA) and amplified by PCR in 25- μ l reactions using the method described by Vandemark et al. (13). Reactions were resolved by agarose gel (1.4% wt/vol) electrophoresis, stained with ethidium bromide, visualized under ultraviolet light, and recorded with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA). Markers that were selectively amplified from all 19 isolates of *P. sclerotoides* but not from the other pathogens screened were excised from agarose gels. DNA was purified from the gel bands using GeneClean (Bio 101 Inc., Vista, CA) and cloned into the vector pCR2.1 Topo using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid DNA was purified from *Escherichia coli* using alkaline lysis (11), and clones containing the PCR product were identified by digestion with *Eco*R1 and visualized by agarose gel electrophoresis. The DNA products were sequenced using the dideoxy-chain termination method, and extended primers were subsequently designed based on the terminal sequences of the cloned markers for

Fig. 1. A, Agarose gel showing amplification products from polymerase chain reaction (PCR) of genomic DNA using the random amplified polymorphic DNA (RAPD) decamer primer OpC15. Marker indicates the 2.6-kb fragment of *Phoma sclerotoides* sequenced to design SCAR primers PSC15₂₆₀₀. **B,** Amplification products from PCR of genomic DNA of *P. sclerotoides* using the sequence-amplified characterized region (SCAR) primer pair OpC15₂₆₀₀. Lanes 1 to 7: isolates of *P. sclerotoides* from Farson, Lyman, Eden, Boulder, Saratoga, and Laramie regions in Wyoming; and Canada (ATCC), respectively. Lanes 8 and 9: *Phoma medicaginis*, lanes 10 to 13: *Phoma betae*, lane 14: *Aphanomyces euteiches*, lane 15: *Rhizoctonia solani*, lane 16: *Fusarium oxysporum*, lane 17: *Pythium ultimum*, lane 18: *Phytophthora infestans*, lane 19: water control.

conversion of the selected RAPD marker to a SCAR primer pair.

SCAR reactions. DNA (50 ng) extracted from pure mycelial cultures, root tissue, or soil samples was used in 25- μ l PCR reactions containing 15 mM Tris, pH 8.0, 2.5 mM MgCl₂, 100 mM each of dATP, dCTP, dGTP, and dTTP, 200 mM of each of the two respective SCAR primers, and 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Thermocycling parameters were optimized and a final profile was employed that consisted of a single cycle of 9 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 70°C, and 1 min at 72°C; and a final extension for 7 min at 72°C. SCAR reactions using DNA extracted from native field soil samples were carried out as described above except that the 35 cycle PCR procedure was increased to 40 cycles.

RESULTS

RAPD primer analysis. Thirty-nine RAPD decamer primers were evaluated in PCR reactions. Primers OpB1, OpB4, OpB12, OpC12, and OpC15 selectively amplified conserved DNA fragments from all isolates of *P. sclerotoides*. Products from two of the decamer primers that produced RAPD markers were selected as candidates for SCAR primers. Primer OpC15 produced an amplification product of approximately 2.6 kbp specific to each of the 19 isolates of *P. sclerotoides* (Fig. 1A). Primer OpB12 produced an amplification product consisting of 499 bases also unique to *P. sclerotoides* (Fig. 2A). The 2.6-kbp and 499-bp fragments were not

amplified from DNA of the other soilborne fungal pathogens *A. euteiches*, *R. solani*, *F. oxysporum*, *P. ultimum*, or *P. infestans*, or from the isolates of *P. betae* or *P. medicaginis*. Sequence data obtained from the cloned 2.6-kbp and 499-bp amplification products were used to design the SCAR primers used in all subsequent experiments and were designated PSC15₂₆₀₀ and PSB12₄₉₉, respectively. The extended SCAR sequences are illustrated below, and the underlined sequence represents the original primer used in RAPD reactions.

PSC15₂₆₀₀

PSC15-FS-30 5' GACGGATCAGCCCAC
GAAGGACCTTAACGC 3'

PSC15-RS-24 5' GACGGATCAGGTAGG
GATACCCGC 3'

PSB12₄₉₉

PSB12-FS-24 5' CCTTGACGCACAGAG
AATGATTGG 3'

PSB12-RS-26 5' CCTTGACGCATGTTA
GCATAAAATCC 3'

SCAR detection and analysis. The SCAR primer pair PSC15₂₆₀₀ amplified a single SCAR of 2.6 kbp from each of the 19 isolates of *P. sclerotoides* in all experiments (Fig. 1B). In addition, a SCAR identical in size was detected from isolates of *P. betae* and *P. medicaginis*. SCAR primer PSB12₄₉₉ produced a single amplification product from each of the *P. sclerotoides* isolates (Fig. 2B) but not from isolates of *P. betae* or *P. medicaginis*. Amplification products using SCAR primers PSC15₂₆₀₀ or PSB12₄₉₉ were not produced from DNA isolated from *A. euteiches*, *R. solani*, *F. oxysporum*, *P. ultimum*, or *P. infestans*. This suggests that PSB12₄₉₉ is

highly specific to *P. sclerotoides*, while PSC15₂₆₀₀ is specific to several different *Phoma* species.

Detection of *P. sclerotoides* from infected roots. Roots of alfalfa plants in the greenhouse exhibiting symptoms of BRR as well as roots from alfalfa plants naturally infected in the field were also evaluated by SCAR primers PSB12₄₉₉. The primers successfully amplified a single PCR product from all roots infected with *P. sclerotoides* that comigrated with the amplification product in the positive control. The PCR reactions did not produce products from root samples used as healthy controls (Fig. 3). Results of the PCR experiments were validated by subculturing root samples on PDA and subsequent identification of the fungal organism by microscopic examination. Fungal cultures were not obtained from asymptomatic alfalfa roots.

Detection of *P. sclerotoides* from soil samples. SCAR primers PSB12₄₉₉ amplified DNA products of the expected size from greenhouse soil samples artificially infested with *P. sclerotoides*. In addition, SCARs were produced from field soil samples collected from the rhizosphere of alfalfa plants exhibiting typical symptoms of BRR (Fig. 4). No amplification products were produced from autoclaved soil. In most cases, optimum results were obtained in PCR reactions when the DNA was diluted 1:10 with sterile water. Tissue excised from symptomatic roots of plants grown in the fields where positive soil samples were taken was microscopically examined and found to be infected with the pathogen.

Fig. 2. A, Agarose gel showing amplification products from polymerase chain reaction (PCR) of genomic DNA of *Phoma sclerotoides* using the random amplified polymorphic DNA (RAPD) decamer primer OpC12. Marker indicates the 499-bp fragment that was sequenced to design SCAR primer PSC12₄₉₉. **B,** Amplification products from PCR of genomic DNA of *P. sclerotoides* using the sequence-amplified characterized region (SCAR) primer pair PSC12₄₉₉. Lanes 1 to 7: isolates of *P. sclerotoides* from Farson, Lyman, Eden, Boulder, Saratoga, and Laramie regions in Wyoming; and Canada (ATCC), respectively. Lanes 8 and 9: *Phoma medicaginis*, lanes 10 to 13: *Phoma betae*, lane 14: *Aphanomyces euteiches*, lane 15: *Rhizoctonia solani*, lane 16: *Fusarium oxysporum*, lane 17: *Pythium ultimum*, lane 18: *Phytophthora infestans*, lane 19: water control.

DISCUSSION

P. sclerotioides is a difficult pathogen of alfalfa to detect rapidly using standard methods of culturing infected tissue on agar media, primarily due to the long growth periods necessary for production of fungal fruiting structures. Sixty to 100 days are typically required before characteristic fruiting structures are produced on agar media (12). We have designed SCAR primers that can detect the presence of *P. sclerotioides* in infected alfalfa roots or infested soil samples in a single day. The SCAR primer PSB12₄₉₉ produced a single diagnostic DNA amplification product consisting of 499 bp from all 18 isolates of *P. sclerotioides* collected from six regions in Wyoming and from one ATCC isolate of *P. sclerotioides* from Canada. The SCAR primer pair did not amplify this DNA product from the other *Phoma* species evaluated nor from other soilborne pathogens included in this study.

SCAR primers PSC15₂₆₀₀ amplified a single DNA product from three species of *Phoma*, including *P. sclerotioides*, *P. medicaginis*, and *P. betae*. It is possible that this SCAR may be diagnostic for other species of *Phoma*. The primers were not exhaustively tested on all available species of the organism, as such an evaluation was beyond the scope of this research. We found it interesting that the OpC15 RAPD primer produced the 2.6-kbp amplification product only in isolates of *P. sclerotioides*. However, with the design of PSC15₂₆₀₀, SCARs were consistently produced from all three *Phoma* species. Although the entire 2.6-kbp region was not sequenced in this study, it is surmised that an insufficient number of nucleotide bases in RAPD primer OpC15 were present to result in amplification of the 2.6-kbp product in the three species. Extension of the forward primer to 30 bases and the reverse primer to 24 bases in the design of PSC15₂₆₀₀ may have resulted in a sufficient number of base pair matches to produce the 2.6-kbp product from all three *Phoma* species tested.

The PSB12₄₉₉ SCAR primers produced a single amplification product from DNA isolated from infected alfalfa roots that were sampled from fields in Farson, WY, and from roots experimentally inoculated with *P. sclerotioides*. Isolation and identification of the fungal pathogen in experiments using agar media corroborated the results using SCARs. In some experiments (data not shown), *P. sclerotioides* was not recovered on PDA from symptomatic root tissue even though the PCR primers generated a 499-bp SCAR from the same root tissue. This suggests that the SCAR primer pairs provide the advantage of greater sensitivity over standard isolation techniques.

There is currently no efficient method available for isolating and concentrating pycnidia or pycnosclerotia of *P. sclerotioides* from soil, nor has a selective medium been developed for this pathogen.

Fig. 3. Detection of *Phoma sclerotioides* from alfalfa roots. Agarose gel showing amplification products produced by sequence-amplified characterized region (SCAR) primer pair PSC12₄₉₉ in polymerase chain reactions. Lane M: 100-bp ladder, lane 1: *P. sclerotioides* genomic DNA, lane 2: healthy alfalfa root, lanes 3 to 8: root samples from plants inoculated with *P. sclerotioides* and maintained under greenhouse conditions (see text), lanes 9 to 14: roots sampled randomly from alfalfa fields in Farson, WY, lane 15: water control.

Fig. 4. Detection of *Phoma sclerotioides* from soil samples. DNA was extracted from 500-mg aliquots. Agarose gel showing amplification products produced by sequence-amplified characterized region (SCAR) primer pair PSC12₄₉₉ in polymerase chain reactions. Lane M: 100-bp ladder, lane 1: *P. sclerotioides* genomic DNA, lane 2: noninfested soil, lanes 3 and 4: greenhouse soil samples from pots containing alfalfa plants infected with *P. sclerotioides*, lanes 5 to 8: random soil samples collected from alfalfa fields in Farson, WY, lane 9: water control.

The SCAR PSB12₄₉₉ primers detected *P. sclerotoides* in soil samples known to be infested with the pathogen based on isolation of the pathogen from alfalfa roots and the fungal thallus on agar media. Sufficient DNA of the pathogen was available in 0.5-g soil samples that the 499-bp SCAR product was routinely obtained. In several experiments, the final DNA suspension required additional dilutions before the SCAR product could be visualized adequately on agarose gels. Inhibitors of the PCR reaction may have been present in the samples after DNA extraction, or the final DNA suspension may have been so highly concentrated as to interfere with the reaction.

The detection system described in this study offers a rapid and accurate method for unambiguous identification of *P. sclerotoides* in infected alfalfa roots or in infested soil. The method is highly sensitive and can detect DNA of the pathogen at concentrations of 2 pg or less (data not shown). However, because we were interested in developing a rapid diagnostic test for detection of the pathogen, DNA concentrations of random samples used in our detection assays were not routinely quantified to provide optimum results. Dilution of the extracted DNA by 1:100 was sufficient in all cases for the primers to produce the 499-bp SCAR product from alfalfa roots that were infected with *P. sclerotoides*. This diagnostic assay may be used to detect the presence of *P. sclerotoides* in fields currently planted to alfalfa or in fields considered for planting with alfalfa in the future. Furthermore, this assay may be valuable for evaluating alfalfa germ plasm being screened for resistance to BRR, by greatly reducing the time required to assay large numbers of individual plants.

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