Distribution of *Phoma sclerotoides* on Alfalfa and Winter Wheat Crops in the North Central United States

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ABSTRACT

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Brown root rot of alfalfa (*Medicago sativa*), caused by *Phoma sclerotoides*, has been reported in several states in the northern United States and in western Canada. A survey was conducted to determine the distribution of the fungus in Minnesota and Wisconsin. Isolates of the pathogen were recovered from roots of alfalfa, winter wheat, and perennial ryegrass plants. The internal transcribed spacer (ITS) 1, 5.8S, and ITS2 of the rDNA of the isolates from alfalfa and wheat were identical and matched the sequences of a *P. sclerotoides* isolate from Wyoming. The fungus was found to be widespread in both states and was detected in roots of alfalfa plants from 17 counties in Minnesota and 14 counties in Wisconsin using polymerase chain reaction (PCR)-based assays. A real-time PCR assay was developed that increased sensitivity of detecting the pathogen from plant tissues and soil. The isolates from alfalfa caused disease on inoculated winter wheat plants. Although the fungus was previously found associated with roots of diseased cereal and turfgrass plants, this is the first demonstration of pathogenicity of *P. sclerotoides* on wheat.

Additional keywords: *Lolium perenne*, *Triticum aestivum*, winterkill

Brown root rot (BRR) of alfalfa (*Medicago sativa* L.) is caused by *Phoma sclerotoides* G. Preuss ex Sacc. (formerly *Plenodomus meliloti* Dearness & Sanford). The fungus is a slow-growing plant pathogen and saprophyte endemic to the prairie soils of Canada (1,17,18). The fungus was first studied on forage legumes during the mid-1920s (1). Later, it was identified from diseased plants in the Northwest Territories, Nova Scotia, Alaska, the Yukon, as well as British Columbia, Alberta, Saskatchewan, and Manitoba (1,19). In the 1980s, the fungus was found causing widespread root rot of alfalfa in Alberta (1). Only recently, *P. sclerotoides* was identified from diseased alfalfa in the contiguous United States. During the spring of 1996, *P. sclerotoides* was found to be the cause of widespread winterkill of alfalfa in

southwestern Wyoming (3), and was detected on a diseased alfalfa sample by the Montana Plant Disease Clinic (15). The pathogen was later isolated from diseased roots of alfalfa located in eight Wyoming counties (4,5,9) and one Idaho county (8). In the spring of 2003, *P. sclerotoides* was first identified from diseased alfalfa plants in Minnesota and Wisconsin (11,16). The prevalence of the disease and distribution of *P. sclerotoides* throughout the remainder of the United States, particularly the upper-tier states, is largely unknown. Because optimal growth of the fungus occurs at 10 to 15°C (5,8), the disease is suspected to contribute to winterkill in regions where winters are extended. Brown root rot is responsible for winter injury and stand decline of a number of forage legumes including alfalfa, red clover (*Trifolium pratense* L.), bird's foot-trefoil (*Lotus corniculatus* L.), alsike clover (*Trifolium hybridum* L.), sweet clover (*Melilotus* spp.), and common sainfoin (*Onobrychis viciifolia* Scop.) (1).

Symptoms of BRR on alfalfa include brown, circular-shaped lesions on taproots and lesions girdling, or banding, smaller lateral or feeder roots. Over time, lesions can coalesce and eventually cause plant death (1). Rot of the upper taproot and

lower crown interferes with carbohydrate and protein storage and increases susceptibility of alfalfa plants to winterkill (10).

Hard red winter wheat (*Triticum aestivum* L.) commonly exhibits widespread winterkill in Minnesota (21). Stand losses from adapted cultivars have been attributed exclusively to weather-related plant stresses. While the potential of *P. sclerotoides* to cause disease on winter wheat is currently unknown, it is a recognized pathogen on over-wintering turfgrass species (18). Although winter wheat acreage continues to increase in Minnesota, many growers avoid planting the crop because of its risk for winterkill. Winter wheat may be grown in rotation with alfalfa, a known host of *P. sclerotoides*, so it is critical to determine if it is a host for the fungus so that effective disease management strategies can be implemented.

Polymerase chain reaction (PCR)-based techniques have become widely used for the detection of plant pathogens (2,13,14). Because PCR is specific, sensitive, and rapid, it is ideal for identifying a slow-growing fungus such as *P. sclerotoides* (12). Real-time PCR is even more sensitive, more efficient, and more effectively quantitative than conventional endpoint PCR (2,14). Recent development of affordable, real-time PCR techniques makes real-time PCR particularly suitable for high throughput detection and quantification of pathogens in plant tissues and soil.

The objectives of this work were to (i) determine the distribution of *P. sclerotoides* in the Upper Midwestern United States by surveying for the pathogen in stands of alfalfa and winter wheat; (ii) determine if *P. sclerotoides* causes root rot of hard red winter wheat; and (iii) develop a sensitive and quantitative real-time PCR-based assay to detect and quantify the density of *P. sclerotoides* in roots of alfalfa, cereal crops, and soil.

MATERIALS AND METHODS

Isolation of *P. sclerotoides* from alfalfa and winter wheat roots. Between 2003 and 2005, 12 alfalfa plants were collected at random from a total of seven commercial production fields located in Marshall, Pennington, Red Lake, and Polk

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counties in northwest Minnesota and in Otter Tail County in west central Minnesota (84 total plant samples). Stand health and age ranged from excellent (approximately 1-year-old) to severely declining (3-years-old). Collected plants were placed into self-sealing plastic bags and stored at 4°C until processed. Roots were rinsed with tap water to remove soil, and tissue isolations were made from discolored roots of plants with symptoms and from randomly selected roots of symptomless plants. Root pieces were disinfected for 3 min in a 10% sodium hypochlorite solution, rinsed for 3 min in sterile distilled water, and air-dried for 3 min on filter paper. The root pieces were then placed on water agar and maintained in a dark incubator at 5°C for 4 months. If beaked pycnidia characteristic of *P. sclerotioides* were observed in at least one petri dish, the collection site was considered positive for *P. sclerotioides*. Pure cultures of *P. sclerotioides* were obtained by transferring disinfected pycnidia to potato dextrose agar (PDA) plates.

During the spring of 2004, at least 12 winter wheat plants were collected at random from 22 commercial production fields (264 plants) located in Kittson (eight fields), Roseau (five fields), Marshall (one field), Pennington (one field), Polk (five fields), and Norman (one field) counties located in northwestern Minnesota and Redwood County (one field) located in southwestern Minnesota. In addition, plants from one perennial ryegrass (*Lolium perenne* L.) seed production field in Roseau County were collected during 2004. Plants were placed into self-sealing plastic bags, stored under refrigeration, and processed as previously described. Disinfested seminal and nodal roots were placed in petri dishes containing sterile water agar. Dishes were maintained in a dark incubator at 5°C for at least 2 months. If beaked pycnidia characteristics of *P. sclerotioides* were observed, a pure culture of the fungus was obtained as before. In addition, DNA was extracted from winter wheat and perennial ryegrass samples from each location for detection of *P. sclerotioides* by a PCR-based assay.

Survey for *P. sclerotioides* in alfalfa fields. A survey for *P. sclerotioides* was conducted in alfalfa stands of the Upper Midwest beginning in the fall of 2003 and continuing to fall of 2005. Overall, plants from 265 fields were evaluated. The majority of plants were from Minnesota (113 fields in a total of 38 counties) and Wisconsin (102 fields in 19 counties), with a small number of samples from Iowa (four fields), Illinois (five fields), Idaho (three fields), and Wyoming (four fields), as well as from Ontario (five fields) and Manitoba (27 fields). Plants were collected by extension educators, university researchers, producers, and others from commercial production fields and experimental plots.

Plants were collected from healthy and declining stands. From five to 20 plants were removed randomly across fields. At least five plants were assayed by PCR from each location.

In preparation for the PCR assay, DNA was isolated from alfalfa, winter wheat, and perennial ryegrass roots using the Fast DNA kit and Fast Prep instrument (Q-BIOgene Solon, OH). Approximately 200 mg of root tissue was homogenized in 500 µl of buffer CLS-Y, 400 µl of buffer CLS-VF, and 100 µl of buffer PPS. Samples were agitated for 30 s on setting 5. DNA of *P. sclerotioides* was detected using a modified version of a previously established endpoint PCR-based assay specific for *P. sclerotioides* (12). PCR reactions were performed in 25-µl volumes containing 50 ng template DNA, 2.5 µl 10× reaction buffer (Promega Corp., Madison, WI), 2.5 µl dNTP mixture (2.5 mM each dNTP; TaKaRa Bio Inc., Otsu, Shiga, Japan), 1 µM SCAR primers, and 0.625 units *Taq* DNA polymerase. Reaction conditions were 2 min at 95°C for one cycle, 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C for 40 cycles, followed by 7 min at 72°C for one cycle. The 499-bp amplicon was detected by agarose gel electrophoresis.

DNA sequence of the rDNA internal transcribed spacers (ITS) and 5.8S rDNA. Isolates of *P. sclerotioides* were grown on PDA plates at 15°C for 5 weeks. Isolates of *P. medicaginis* and *P. pinodella* were grown on PDA plates at 25°C for 3 weeks. DNA was extracted from mycelium and spores scraped from the surface of plates using the Q-BIOgene FastDNA kit as per the manufacturer's protocol for fungal DNA. The ITS regions of all samples were PCR amplified with the universal primer pair ITS1 5'-TCCGTAGGT GAACCTGCGG-3' and ITS4 5'-TCC TCCGCTATTGATATGC-3' (20). PCR reactions were performed in 50-µl reactions containing 100 ng of template DNA, 5 µl 10× ThermoPol PCR buffer (New England Biolabs, Beverly, MA), 5 µl dNTP mixture (2.5 mM each dNTP; TaKaRa Bio), 50 ng each oligonucleotide primer, and 5 units *Taq* DNA polymerase. Thermocycling conditions consisted of an initial denaturing step at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 7 min. Verification of amplification was done by UV illumination in a 1% agarose gel after electrophoresis in the presence of ethidium bromide.

For cloning, PCR products were purified from the gel using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA). The fragments were ligated into vector pGEM-T II (Promega Corp.) and transformed into competent cells of *E. coli* according to the manufacturer's instructions. Plasmids were isolated using the Qiaprep Spin Miniprep Kit (Qiagen).

Samples were sequenced by the Advanced Genetic Analysis Center (University of Minnesota) with primers ITS1 or ITS4. Sequences were aligned using ClustalW (1.83) Multiple Sequence Alignment.

Inoculation of winter wheat with *P. sclerotioides*. On 4 October 2004, cultivar Jerry hard red winter wheat seed were planted into Deepot Cells (Stuewe and Sons, Inc., Corvallis, OR) containing a pasteurized native soil:sand mixture (1:1) at a depth of 2.54 cm. Plants were maintained in the greenhouse at 10°C (±5°C) under natural lighting for 7 days. A total of 125 potted test plants were situated in Deepot support trays (Stuewe and Sons).

Inoculum was prepared using a modified protocol (7) from four *P. sclerotioides* isolates obtained from diseased alfalfa roots from four locations in Minnesota during 2004 (Thief River Falls [TRF], Red Lake Falls [RLF], Holt, and Fergus Falls [FF]). Sterile barley grains were placed into five self-sealing plastic bags and inoculated with one each *P. sclerotioides* isolate. One bag of noninoculated sterile grain was used as the nontreated control treatment. Sealed bags were maintained in a dark growth chamber at 5°C where they were massaged periodically to increase colonization of the fungus on the substrate.

Plants were placed outside on 11 October 2004, and pots were situated above the soil surface at the University of Minnesota Northwest Research and Outreach Center, Crookston, MN. The lower portion (sides and bottom) of each group of 25 pots was isolated from other pots by placing a plastic 189-liter bag around pots. The top edge of each bag was pulled upward through pot support openings in trays before pots were inserted. Pots were positioned snugly into perimeter tray support openings, securing the plastic barrier in place. This primarily was done to reduce wind penetration of cold air and prevent possible cross-contamination between *P. sclerotioides* isolates. The test was then surrounded snugly by a wheat straw bale perimeter, and loose straw was forced between racks and inserted into openings to reduce air circulation during the winter. The test was watered during the fall as needed to promote plant establishment and growth.

On 26 October, when plants were 20 days old, three holes approximately 2.54 cm deep were made in the soil in each pot. Holes were placed midway between the seedling and the pot wall (approximately 1 cm from the seedling). One barley grain was dropped into each hole and the soil was pinched closed. In total, soil surrounding each test plant contained either three sterile barley grains or three grains infested with one of the four isolates of *P. sclerotioides* (total of 25 pots each).

After more than 6 months outside, trays of potted plants were returned to the greenhouse on 29 April 2005. Plants were maintained in the greenhouse for 2 weeks

until roots could be examined microscopically for pathogen signs and disease symptoms. Root tissues of live plants, regardless of symptom expression, were processed 17 May 2005 to complete Koch's postulates. Tissues were disinfected as described above and placed into petri dishes containing sterile water agar. Dishes were maintained in a dark incubator at 5°C for more than 4 months. Dishes were examined on 27 September for beaked pycnidial production. If present, pycnidia were surface-disinfected for 30 s in a 10% sodium hypochlorite solution, rinsed for 30 s in sterile distilled water, and placed in dishes containing PDA. Pure cultures were examined and compared with original cultures of *P. sclerotoides*.

Real-time PCR assay. PCR reactions were carried out using SYBR Green Supermixes with ROX (Bio-Rad Laboratories, Inc., Hercules, CA). Primers for real-time PCR (forward primer 5'-CCTGAA GCCGTCCAAGTT-3', reverse primer 5'-GCTCACCGAACACGACGCTTT-3') were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and were selected within the sequence of the target gene amplicon (499 bp) for the conventional endpoint PCR assay (12). The real-time amplicon was designed to be 130 bp (from bp 253 to bp 372 of the 499-bp amplicon). Each PCR reaction was 25 µl total volume with primers at a concentration of 50 nM. Real-time PCR detection was conducted using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction was incubated at 50°C for 2 min and 95°C for 3 min followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting curve analysis was conducted according to the ABI software. Standard curves were constructed using dilutions of gel-purified PCR amplicon (499 bp) generated by the endpoint PCR reaction. Copy number for the target gene was determined based on DNA concentration of the gel-purified PCR amplicon. A dilution series of standard DNA was included to generate a standard curve for each experimental plate. Cycle threshold (Ct) values for PCR standards were not affected when the standards were measured in a background of up to 35 ng alfalfa root DNA. Each sample was amplified in triplicate.

Standard curves were also constructed using 10-fold dilutions of purified *P. sclerotoides* DNA. A linear relationship existed between Ct values and the logarithm of starting DNA concentration for a range of concentrations between 1.5 pg and 1.5 ng of purified *P. sclerotoides* DNA. Ct values generated by real-time PCR analysis of 100 to 100,000 starting copies of the conventional PCR amplicon roughly corresponded to Ct values generated by 1.5 pg to 1.5 ng of purified *P. sclerotoides* DNA, respectively. As the gel-purified DNA amplicon provided a greater range of linear

values than the purified *P. sclerotoides* DNA, the gel-purified PCR amplicon was used to develop the real-time PCR assay.

Soil DNA extraction. Spores from *P. sclerotoides* cultures grown on PDA plates were suspended in sterile distilled water and counted with a hemacytometer. Ten-fold dilutions were prepared and added to 250-mg samples of uninfested soil collected from fields at the University of Minnesota Experiment Station, St. Paul, MN. Total DNA was prepared from soil using the Power Soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA).

RESULTS

Distribution of *P. sclerotoides* on alfalfa in Minnesota and Wisconsin. Pure cultures of *P. sclerotoides* were obtained from alfalfa plants from five locations (Table 1). They originated from production fields in Pennington County (TRF), Red Lake County (RLF), Otter Tail County (FF), Polk County (Bratvold), and Marshall County (Holt). In vitro, the cultures had beaked pycnidia characteristic of *P. sclerotoides* and produced single-celled hyaline elliptical conidia.

The DNA sequence from the ITS1, 5.8S, and ITS2 of the nuclear rDNA of isolates from alfalfa plants from Minnesota were compared with the sequence from *P. sclerotoides* isolates originating from Canada (ATCC 56515) and Wyoming (ATCC MYA295) as well as to type cultures of *P. medicaginis* (ATCC 52798), which causes spring black stem and leaf spot of alfalfa, and *P. pinodella* (ATCC 32162), the causal agent of Ascochyta foot rot and black stem of pea. Sequences from all isolates were deposited in GenBank (Table 1). The length of the sequences from *P. sclerotoides* ATCC 56515, *P. medicaginis* ATCC 52798, and *P. pinodella* ATCC 32162 were 436, 430, and 431 bp, respectively. The *P. sclerotoides* isolate from

Wyoming and the isolates from Minnesota were identical in sequence and differed from isolate ATCC 56515 from Canada by three base changes, all in the ITS2 sequence. In contrast, six indels and 59 base changes were observed between the sequence from *P. sclerotoides* ATCC 56515 and *P. medicaginis* ATCC 52798, and nine indels and 63 base changes between *P. sclerotoides* ATCC 56515 and *P. pinodella* ATCC 32162. A BLASTn search of GenBank with the *P. sclerotoides* sequence found the closest matching sequence (95% identity) was to the corresponding rDNA from *Leptosphaeria doliolium* (anamorph *Phoma hoehnelii*).

To rapidly detect *P. sclerotoides* in plant samples, we used a modified version of a conventional endpoint PCR-based assay previously shown to be specific for the fungus (12). Amplicons of the expected size (499 bp) were generated from the DNA of numerous alfalfa roots that were sampled. Test amplicons co-migrated with amplicons generated by PCR of purified *P. sclerotoides* DNA. The fungus was identified in alfalfa samples taken from sites in Minnesota, Wisconsin, Idaho, and Wyoming, but not in alfalfa samples taken from fields in Iowa, Illinois, Ontario, or Manitoba. In Minnesota and Wisconsin, the fungus was found to be relatively widespread. About one-third (21 out of 57) of the counties surveyed had at least one field in which plants tested positive for the fungus by the endpoint PCR assay (Table 2). In Minnesota and Wisconsin, the fungus appeared concentrated in three main regions: the Red River Valley in northwestern Minnesota, the St. Croix River Valley in Minnesota and Wisconsin, and west of Green Bay in northeastern Wisconsin (Fig. 1). The fungus was detected in alfalfa roots with brown necrotic lesions and also from alfalfa roots asymptomatic for BRR. Plants positive for the fungus originated from

Table 1. Cultures of *Phoma* species, host, place of origin, and GenBank accession numbers of DNA sequence for the ITS1, 5.8S, and ITS2 regions of the rDNA

Isolate designation ^a	Source	<i>Phoma</i> species	Host	Origin	GenBank accession number
56515	ATCC ^b	<i>P. sclerotoides</i>	Alfalfa	Canada	DQ525733
MYA295	ATCC	<i>P. sclerotoides</i>	Alfalfa	Wyoming	DQ530443
52798	ATCC	<i>P. medicaginis</i>	Alfalfa	South Dakota	DQ525735
32162	ATCC	<i>P. pinodella</i>	Pea	Netherlands	DQ525734
TRF	Field	<i>P. sclerotoides</i>	Alfalfa	Minnesota	DQ530444
Bratvold	Field	<i>P. sclerotoides</i>	Alfalfa	Minnesota	DQ530449
RLF	Field	<i>P. sclerotoides</i>	Alfalfa	Minnesota	DQ530450
FF	Field	<i>P. sclerotoides</i>	Alfalfa	Minnesota	DQ530451
Holt	Field	<i>P. sclerotoides</i>	Alfalfa	Minnesota	DQ530452
Roosevelt	Field	<i>P. sclerotoides</i>	Perennial ryegrass	Minnesota	DQ530444
Roseau	Field	<i>P. sclerotoides</i>	Winter wheat	Minnesota	DQ530446
Hallock	Field	<i>P. sclerotoides</i>	Winter wheat	Minnesota	DQ530447
Oslo	Field	<i>P. sclerotoides</i>	Winter wheat	Minnesota	DQ530448

^a TRF, isolate from near Thief River Falls, MN; Bratvold, isolate from near Crookston, MN; RLF, isolate from near Red Lake Falls, MN; FF, isolate from near Fergus Falls, MN; Roosevelt, isolate from near Roosevelt, MN; Roseau, isolate from near Roseau, MN; Hallock, isolate from near Hallock, MN; Oslo, isolate from near Oslo, MN.

^b American Type Culture Collection.

both younger (2-year-old) and older (3- to 11-year-old) stands. In some, but not all, cases the presence of the fungus was associated with winter injury and/or stand decline.

Identification of *P. sclerotiioides* from winter wheat and perennial ryegrass. Winter wheat plants from 22 locations in Minnesota were assayed for *P. sclerotiioides* with the endpoint PCR test and root tissue isolations made. Plants from a site in Kittson County and Marshall County were positive by PCR. Pure cultures of *P. sclerotiioides* with characteristic beaked pycnidia were obtained from plants from Marshall County (Oslo), Kittson County (Hallock), and Roseau County (Roseau) (Table 1). In addition, an isolate was obtained from perennial ryegrass from Roseau County (Roosevelt). DNA sequencing of the ITS1, 5.8S, and ITS2 of the rDNA was performed for the wheat and perennial ryegrass isolates. The sequences were identical to the sequences obtained

from alfalfa in Minnesota except for a single base deletion in the Roosevelt perennial ryegrass isolate in the ITS2 region.

Real-time PCR-based assay. To further assess the presence of *P. sclerotiioides* in plant roots and surrounding soil, we developed a more sensitive, quantitative assay based on real-time PCR. Primers for the real-time PCR assay were selected from the DNA sequence amplified in the conventional endpoint PCR assay (12). A standard curve was constructed by plotting average threshold cycles (Ct values) for 10-fold dilutions of the conventional PCR amplicon versus the logarithm of the starting copy number for each dilution of the conventional endpoint PCR amplicon (Fig. 2). The standard curve showed a linear relationship between Ct values and the logarithm of the starting template copy number. The assay was quantitative and reproducible over a wide range of concentrations, greater than five orders of magnitude. The assay readily detected as few as

10 copies of the *P. sclerotiioides* target gene. For all experiments, the linear coefficient of the standard curve was $R^2 = 0.97$ to 0.99 and the slope of the curve was in a range corresponding to 90 to 100% efficiency for the PCR reaction. Nonspecific amplification was not observed by either gel electrophoresis of the PCR product or melting curve analysis, confirming the specificity of the assay design.

Alfalfa DNA samples from the survey conducted in 2005 were re-analyzed using the real-time PCR assay. The amount of *P. sclerotiioides* DNA in each sample was quantified by comparing the average Ct values for the alfalfa sample with Ct values on the standard curve. Samples identified positive by the conventional endpoint PCR assay were confirmed positive by the real-time PCR assay. As for the conventional assay, the real-time PCR assay did not detect *P. sclerotiioides* in the DNA of healthy, uninfected alfalfa plants propagated in the greenhouse (data not shown).

Table 2. Total survey of alfalfa fields in the United States and Canada for *Phoma sclerotiioides* on alfalfa roots using endpoint polymerase chain reaction (PCR)^a

State	County	Number of fields sampled	Number of positive fields ^b	State/province	County	Number of fields sampled	Number of positive fields
MN	Aitkin	4	0	WI	Chippewa	6	1
MN	Benton	5	1	WI	Columbia	3	1
MN	Big Stone	1	0	WI	Dunn	1	1
MN	Carver	4	0	WI	Eau Claire	4	0
MN	Cass	2	1	WI	Fond du Lac	4	0
MN	Cottonwood	1	0	WI	Grant	3	0
MN	Dakota	4	0	WI	Juneau	1	0
MN	Dodge	3	0	WI	Lafayette	1	0
MN	Fillmore	3	0	WI	Manitowoc	3	0
MN	Freeborn	1	0	WI	Marinette	1	1
MN	Goodhue	1	1	WI	Monroe	8	0
MN	Hennepin	1	0	WI	Oconto	1	1
MN	Isanti	1	0	WI	Pierce	3	1
MN	Itasca	5	0	WI	Polk	3	0
MN	Kandiyohi	1	0	WI	Rock	2	0
MN	Marshall	3	1	WI	Shawano	19	1
MN	Martin	1	0	WI	St. Croix	28	6
MN	McLeod	1	0	WI	Washburn	5	0
MN	Meeker	2	0	WI	Waupaca	6	0
MN	Morrisson	3	0		Total	102	13
MN	Mower	2	0	ID	Fairfield	3	2
MN	Murray	3	0	WY	Goshen	2	1
MN	Nobles	3	0	WY	Natrona	2	2
MN	Olmsted	1	0		Total	4	3
MN	Otter Tail	6	1	IL	Champaign	2	0
MN	Pennington	3	2	IL	Carroll	1	0
MN	Pipestone	3	0	IL	Kane	1	0
MN	Polk	1	1	IL	Bureau	1	0
MN	Red Lake	10	2		Total	5	0
MN	Rock	1	1	IA	Polk	1	0
MN	Sherburne	2	1	IA	Not identified	4	0
MN	Sibley	2	0	IA	Boone	1	0
MN	Stearns	3	0		Total	6	0
MN	Steele	3	0	ONT		5	0
MN	Wabasha	5	1	MB		27	0
MN	Washington	3	1		Grand total	265	34
MN	Winona	13	2				
MN	Wright	2	0				
Total		113	16				

^a Five to 20 plants were collected randomly from each field. At least five plants were assayed from each field.

^b A field was considered positive for *P. sclerotiioides* if at least one root DNA sample from collected plants yielded a 499-bp product by the endpoint PCR assay.

However, the more sensitive real-time assay identified *P. sclerotioides* DNA in alfalfa samples that were negative by the conventional endpoint PCR assay. In the plants sampled in 2005, six out of 53 fields (in four out of 20 counties) tested positive using conventional endpoint PCR, whereas 51 out of 53 fields (in 18 out of 20 counties) tested positive using real-time PCR (Table 3). The real-time PCR assay also detected low levels of *P. sclerotioides* in plants from 22 out of 27 sites in Manitoba, just north of the Red River Valley, which had tested negative by the endpoint PCR assay (data not shown). The relative amount of *P. sclerotioides* in alfalfa roots determined by real-time PCR was evaluated according to the calculated starting copy number of the target gene for each sample. Root DNA samples with 10 to 100 calculated copies of the target gene were considered to have very low amounts of the fungus, whereas >100 to 1,000 copies was considered a low level, >1,000 to 10,000 copies was considered a moderate level, and >10,000 copies was considered a high level. Generally, only root DNA samples with high amounts of the fungus as measured by the real-time PCR assay were identified as positive by conventional endpoint PCR and by isolation of the fungus from roots.

DNA was prepared from soil samples spiked with conidia from *P. sclerotioides* cultures. Figure 3A shows a linear relationship between the number of spores in the spiked samples and the number of

copies of the target gene determined by real-time PCR over a wide range of starting concentrations. The real-time assay was able to reproducibly detect as few as 25 copies of the target gene per sample, or the equivalent of 1,000 copies per 100 mg of soil. DNA of *P. sclerotioides* was effectively detected in a background of total DNA extracted from the soil, and the calculated spore number based on real-time PCR was close to the predicted spore number for 100% DNA recovery and amplification (Fig. 3B). Preliminary studies with the real-time PCR assay identified *P.*

sclerotioides in the soil of alfalfa fields in Otter Tail and Stearns counties of Minnesota, where the fungus was identified in the roots of alfalfa plants by conventional endpoint PCR (data not shown).

Alfalfa isolates of *P. sclerotioides* cause root rot on winter wheat. Daily weather information was collected manually less than 1 km from the test site. Monthly air temperature lows for the 2004–2005 winter exposure period were: –3.3°C, Oct.; –15.5°C, Nov.; –26.6°C, Dec.; –37.7°C, Jan.; –26.1°C, Feb.; –20.5°C, Mar.; and –4.4°C, April. During January, low daily

Fig. 2. Real-time polymerase chain reaction (PCR) standard curve for *Phoma sclerotioides*. Three replicate PCR reactions were performed for each quantity (1.4×10^1 to 1.4×10^5 copies) of target DNA. Error bars represent one standard deviation.

Fig. 1. Counties in Minnesota (left) and Wisconsin (right) in which *Phoma sclerotioides* was identified in alfalfa plants. Counties in which positive plants were identified by real-time polymerase chain reaction (PCR) (gray) and by both real-time PCR and endpoint PCR (black).

air temperatures were bitter cold for 17 nonconsecutive days, ranging between -23.3°C and -38°C . January high daily air temperatures remained in the negative digits for 29 of 31 days. Thirty-eight percent (47 plants) of test plants died during the winter exposure period from environmental stresses unrelated to disease (Table 4). These plants did not have symptoms of root or crown rot disease. In addition, seven plants were not recoverable. It is likely that missing plants were eaten by rabbits or migrating geese. Fifty plants (40%) were killed during the winter and had pycnidia characteristic of *P. sclerotoides* attached to roots that were brown and rotted. A total of 21 plants were alive in May of 2005, nine from the noninoculated control treatment and 12 from inoculated treatments. Of the 100 inoculated plants, four had neither signs of *P. sclerotoides* nor root rot symptoms. The remaining eight live plants from inoculated treatments had pycnidia embedded or superficially attached to partially rotted root systems. All plants were either killed or missing from the Holt isolate treatment, while as many as five plants remained alive but colonized by the RLF isolate (Table 4). *P. sclerotoides* was identified from pure cultures using colony, pycnidia, and spore morphologies.

DISCUSSION

A 3-year survey for *P. sclerotoides*, primarily in Minnesota and Wisconsin, found the fungus to be widespread in both states on alfalfa roots. Although the fungus

has been recognized as the cause of BRR of alfalfa for many years (1), diagnosis of BRR was difficult due to the slow growth of the pathogen in culture. Development of a PCR assay specific for *P. sclerotoides* (12) made diagnosis more rapid and has facilitated testing of plants from numerous locations. The high frequency of identification of the fungus in several areas, such as the Red River Valley of Minnesota, suggests that certain locations may be more favorable for pathogen survival and plant infection. It is possible that the fungus is widespread in the northern tier states of the United States and the Prairie Provinces of Canada (3). Surveys in additional locations are needed to determine the complete range of the fungus. The survey showed that in some but not all cases, the fungus was associated with alfalfa plants suffering stand decline or winter injury. Due to the slow growth of the fungus, symptoms may take three or more winter seasons to become apparent (17), although under conducive environmental conditions disease can occur after one winter (6,7). Both environmental and biotic factors may contribute to winter injury and winterkill of alfalfa. At locations in which the fungus is present and in years when the environment supports disease development, BRR is likely a primary cause of plant death. However, the disease may be overlooked much of the time.

P. sclerotoides was identified on roots of both winter wheat and perennial ryegrass. Previously, the fungus was identified in association with monocots (18); how-

ever, pathogenicity had not been demonstrated. In this study, isolates of *P. sclerotoides* from alfalfa were used to inoculate roots of hard red winter wheat plants in the fall and disease was assessed the following spring. Although there was a high frequency of mortality in the non-inoculated plants, mortality was higher in inoculated plants, and a large proportion of these plants had pycnidia of *P. sclerotoides* in rotted roots. From this evidence we conclude that the fungus is a pathogen of winter wheat and causes root rot, which likely contributes to winterkill of plants in the field. These results support the current recommendations for management of BRR, which include rotation to annual crops. Additional studies are needed to validate these preliminary findings.

The isolates causing disease on alfalfa in Minnesota were confirmed to be *P. sclerotoides* by sequencing the ITS1, 5.8S, and ITS2 of the nuclear rDNA. The sequences from the Minnesota isolates were identical to the isolate from Wyoming. In addition, isolates from winter wheat had the same sequence as the alfalfa isolates. Interestingly, the isolate from perennial ryegrass had a single base deletion in the ITS2 region. Additional isolates from ryegrass are needed to determine the incidence of this variant. The alfalfa and wheat isolates differ from the *P. sclerotoides* isolate from Canada by three base changes in the ITS2 region. This suggests that there may be regionally distinct populations of the fungus.

Table 3. Comparing the identification of *Phoma sclerotoides* in alfalfa roots from plants collected in 2005 using real-time polymerase chain reaction (PCR) versus endpoint PCR^a

State	County	Number of fields sampled	Number of positive fields by endpoint PCR ^b	Number of positive fields by real-time PCR ^c	Relative level of <i>P. sclerotoides</i> assessed by real-time PCR ^d
MN	Benton	3	0	3	Low-moderate
MN	Carver	1	1	1	Moderate
MN	Fillmore	1	0	1	Low-moderate
MN	Goodhue	1	1	1	Moderate
MN	Hennepin	1	0	0	Not detected
MN	Isanti	1	0	1	Moderate
MN	Otter Tail	1	0	1	Moderate
MN	Sherburne	1	0	1	Very low
MN	Stearns	4	0	4	Low-moderate
MN	Winona	8	0	7	Very low-moderate
Total		22	2	20	
WI	Dunn	1	1	1	High
WI	Eau Claire	1	0	1	Moderate
WI	Fond du Lac	4	0	3	Very low-moderate
WI	Juneau	1	0	1	Low
WI	Manitowoc	3	0	3	Very low
WI	Pierce	1	0	1	Low
WI	Shawano	1	0	0	Not detected
WI	St. Croix	13	3	13	Low-moderate
WI	Washburn	5	0	5	Very low-low
WI	Waupaca	1	0	1	Very low
Total		31	4	29	

^a Five to 20 plants were collected randomly from each field. At least five plants were assayed from each field. DNA samples from roots of collected plants were assayed by the endpoint and real-time PCR assays.

^b A field was considered positive for *P. sclerotoides* if at least one root DNA sample from collected plants yielded a 499-bp product by the endpoint PCR assay.

^c A field was considered positive if at least one root DNA sample from collected plants had >100 calculated copies of the target gene.

^d Very low = 10 to 100 calculated copies of the target gene; low = >100 to 1,000 copies; moderate = >1,000 to 10,000 copies; high = >10,000 copies.

A real-time PCR assay for *P. sclerotoides* was developed to increase the sensitivity of detection and to be able to quantify fungal material in plant and soil samples. A sensitive assay is needed for detection of the pathogen in soil, as the

amount of the fungus in individual samples can be low. Although the real-time PCR assay can detect as few as 10 copies of the target gene, approximately 100 copies are reproducibly detected in DNA extracted from alfalfa roots. Reproducible detection

by the endpoint assay is approximately 10,000 copies, making the real-time assay more sensitive by several orders of magnitude. Low to moderate amounts of the fungus were detected in roots of plants surveyed in 2005 that lacked typical BRR symptoms. This is further evidence that the fungus can be detected in alfalfa roots before disease symptoms occur. The real-time assay identified nine additional counties with alfalfa plants harboring low to moderate amounts of the fungus. The real-time PCR assay will be useful in future studies to assess population density of the fungus in soil to establish a threshold level of the fungus for causing disease, for identifying environmental conditions conducive to pathogen survival and increase, and to determine potential rotational crops that reduce pathogen density in soil.

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Fig. 3. Real-time polymerase chain reaction (PCR) quantitation of *Phoma sclerotoides* in soil samples. Soil samples (250 mg) were spiked with known quantities (2.5×10^3 to 2.5×10^6) of *P. sclerotoides* conidia. **A**, Target gene copy number increases in a linear fashion with increasing numbers of spores added to soil. Efficiency of PCR amplification = 90%, $R^2 = 0.98$. **B**, Open bars represent calculated spore number based on real-time PCR, whereas solid bars represent predicted spore number for 100% DNA recovery and 100% efficiency of PCR. Error bars represent standard deviation for triplicate reactions.

Table 4. Percent mortality following a winter exposure period of hard red winter wheat plants inoculated with one of four *Phoma sclerotoides* isolates from alfalfa during fall 2004

Treatment ^a	Live plants (%)		Plant mortality (%)		Missing plants (%)
	Ps (+) ^b	Ps (-)	Ps (+)	Ps (-)	
Noninoculated control	0	36	0	64	
Inoculated					
TRF isolate ^c	8	8	44	28	12
RLF isolate	8	8	64	8	
Holt isolate	0	0	40	60	
FF isolate	4	0	52	28	16

^a Twenty-five plants were inoculated with *P. sclerotoides* at 20 days of age and placed outside for one winter exposure period. The following spring, percent mortality was calculated and roots were observed for lesions and pycnidia.

^b Ps (+) = pycnidia observed microscopically on roots that were characteristic of those produced by *P. sclerotoides*; Ps (-) = no lesions or pycnidia observed.

^c TRF, Thief River Falls isolate; RLF, Red Lake Falls isolate; FF, Fergus Falls isolate.

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