Genetic and Morphological Evidence that *Phoma sclerotoides*, Causal Agent of Brown Root Rot of Alfalfa, Is Composed of a Species Complex

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Accepted for publication 11 October 2010.

ABSTRACT

Wunsch, M. J., and Bergstrom, G. C. 2011. Genetic and morphological evidence that *Phoma sclerotoides*, causal agent of brown root rot of alfalfa, is composed of a species complex. *Phytopathology* 101:594-610.

Phoma sclerotoides, causal agent of brown root rot of alfalfa, causes severe root and crown lesions on alfalfa and other perennial forage legumes in regions with harsh winters. Isolates of *P. sclerotoides* exhibit diverse cultural morphologies on potato dextrose agar (PDA), suggesting that they may exhibit a high degree of genetic diversity. To investigate the genetic relatedness of *P. sclerotoides* isolates, 154 isolates from North America were sequenced at 10 loci. Maximum parsimony and maximum likelihood analyses of the complete 10-locus data set placed isolates into

multiple strongly supported clades, and analyses of gene-jackknife and single-gene partitions of the data set indicated robust support for six major clades and three subclades. Genetic differences corresponded closely to differences in conidial size and septation, pycnidial neck length, mycelial pigmentation, and growth rate in axenic culture at 18 and 25°C. Isolates exhibited morphologies broadly consistent with the species description of *P. sclerotoides*, and new species were not designated. On the basis of genetic and morphological differences, we propose establishing seven infraspecific varieties within *P. sclerotoides*: *P. sclerotoides* var. *sclerotoides*, *champlainii*, *viridis*, *obscurus*, *steubenii*, *macrospora*, and *saskatchewanii*. All varieties of *P. sclerotoides* caused brown root rot of alfalfa and grew well at low temperatures.

Diseases associated with plant-pathogenic fungi within the genus *Phoma* are frequently caused by species complexes, the components of which may have subtle or pronounced differences in morphology. *Phoma medicaginis* var. *medicaginis* and *P. medicaginis* var. *macrospora*, which differ in conidial size and septation, both contribute to spring black stem and leaf spot of alfalfa (*Medicago sativa* L.) (9). At least eight subtypes of *P. lingam* cause black leg of oilseed rape (*Brassica napus* L.), with two subtypes corresponding to the teleomorphic state *Leptosphaeria maculans* and six corresponding to *L. biglobosa* (24,35). *P. destructiva* var. *destructiva* and *P. destructiva* var. *diversispora*, differing in conidial size and septation, both contribute to a leaf and stem rot of tomato (*Solanum lycopersicum* L.) (13). Similar species complexes associated with specific diseases have been recognized within *P. enteroleuca*, an opportunistic pathogen of trees and shrubs; within *P. exigua* causing foliar blight of *Vinca* sp. and foliar and bark lesions on *Forsythia* hybrids; within *P. macrostoma*, an opportunistic parasite of woody plants; and within *P. pomorum*, a plurivorous pathogen commonly associated with leaf spots on members of the family *Rosaceae* (8). For those *Phoma* species complexes in which genetic differentiation has been evaluated, morphological differences correspond closely to genetic differences (1,24,35).

From a phytopathology perspective, *Phoma* species complexes are significant because individual components of a complex can differ in aggressiveness, timing of infection, and temperature adaptation. On alfalfa, *P. medicaginis* var. *macrospora* is associ-

ated with more severe spring black stem and leaf spot symptoms than *P. medicaginis* var. *medicaginis* (9). On oilseed rape, *L. maculans* produces damaging cortical lesions and cankers on stems while *L. biglobosa* produces mild cortical lesions and blackening of pith tissues but no cankers, and infection by *L. maculans* starts later but progresses more rapidly than infection by *L. biglobosa* (20). Of three genetically distinct subtypes of *P. cucurbitacearum* causing gummy stem blight of cucurbits, one causes severe disease symptoms while the others produce only mild symptoms (30). On members of the family *Rosaceae*, *P. pomorum* var. *pomorum* and *P. pomorum* var. *calorpreferens* are both associated with leaf spots but *P. pomorum* var. *calorpreferens* is more heat tolerant (8).

Brown root rot (BRR) of alfalfa and other perennial legumes, caused by *P. sclerotoides* G. Preuss ex Sacc. (synonym *Plenodomus meliloti* Dearn. & Sanford), is associated with light to dark brown root and crown lesions, often with a darker border, that develop as plants emerge from winter dormancy. Lesions that girdle the taproot at or near the crown lead to plant mortality. In North America, BRR is most severe in central and northern Canada, where it is one of the most important diseases of sweet clover and causes considerable alfalfa mortality (11). In central Saskatchewan, alfalfa mortality associated with BRR can exceed 60% on susceptible alfalfa cultivars 2 years after seeding and can reach 40% 1 year after seeding (6). In the contiguous United States, BRR has been reported in the Rocky Mountain states south to New Mexico (17,38), in the upper Midwest (21,22), and in the northeast south to Pennsylvania (39,40).

Phoma sclerotoides displays pronounced variability in the pigmentation and abundance of aerial mycelium produced on potato dextrose agar (PDA). Single-conidium isolates identified as *P. sclerotoides* by pycnidial and conidial morphology on sterilized alfalfa root sections and autoclaved barley (40) produce white, tan, brown, green, or dark-gray mycelium on PDA, with

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doi:10.1094/PHYTO-04-10-0107

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production of aerial mycelium either abundant or sparse. The morphologies represent diversity among isolates and not morphological plasticity; when started from frozen stocks stored at -80°C , individual isolates repeatedly produce the same cultural characteristics on PDA (M. J. Wunsch, *personal observation*). Variability in cultural morphology of *P. sclerotioides* on PDA has also been reported by Gray et al. (12).

The pronounced morphological variability of *P. sclerotioides* isolates suggests that BRR may be caused by a species complex. The current study attempts to address this question by (i) rigorously evaluating whether isolates representing diverse cultural morphologies correspond to the species description of *P. sclerotioides*, (ii) confirming that isolates representing diverse cultural morphologies cause BRR and are adapted to low temperatures, and (iii) assessing morphological and genetic differentiation among isolates. Because components of species complexes can differ in properties such as aggressiveness and host range, investigating whether BRR is composed of a species complex is critical for developing effective management strategies for BRR. The species description of *P. sclerotioides* is based primarily on conidial and pycnidial characteristics in vivo (8). Pathogenicity testing was conducted under controlled conditions using single-conidium isolates, and diseased plants from pathogenicity testing were used for in vivo characterization of isolates. Morphological characteristics in vivo and in vitro were compared across isolates, and in vitro growth at 3, 10, 18, and 25°C was assessed. A broad collection of isolates was sequenced at multiple loci, and phylogenetic analyses of the sequence data were conducted by maximum parsimony and maximum likelihood.

MATERIALS AND METHODS

Fungal strains and culture maintenance. Isolates WI LMH-B, WI LMH-C, MN JNL-1a, and WI LMN-D from Minnesota and Wisconsin were isolated from symptomatic roots sent by D. A. Samac (United States Department of Agriculture–Agricultural Research Service, St. Paul, MN), and isolate WyoBerg from Wyoming was provided by F. A. Gray (University of Wyoming, Laramie). All other isolates were obtained from previously described surveys of alfalfa production fields (38–40) and additional surveys conducted in Montana and Wisconsin in 2006, New York in 2007, and Saskatchewan in 2007. Isolates were obtained from alfalfa root, crown, leaf, and overwintered (dead) stem tissues, and one isolate was obtained from reed canarygrass (*Phalaris arundinacea* L.) roots. Symptomatic tissue segments were excised, surface sterilized in 0.6% sodium hypochlorite and 70% ethanol, rinsed in sterile distilled water, plated onto 1.5% water agar, and incubated for 3 to 6 months at 10°C under continuous white fluorescent light with average luminescence of 2,000 lx. Pycnidia characteristic of *Phoma sclerotioides* were harvested and single-conidium isolates were established on PDA (Difco Laboratories, Becton, Dickson and Co., Sparks, MD). Single-conidium isolates were grown for 2 to 4 months at 10°C under continuous white fluorescent light, and cultures were harvested and stored in 15% glycerol at -80°C . For a subset of the isolates from Montana, New York, Vermont, and New Hampshire, pathogen identity was confirmed by assessing pycnidial morphology on autoclaved barley and on surface-disinfested healthy root segments obtained from greenhouse-grown alfalfa (40). Diagnostic polymerase chain reaction (PCR) was conducted on all isolates using pathogen-specific primers (23).

DNA extraction, amplification, purification, and sequencing. Cultures of 155 *P. sclerotioides* isolates and 1 *P. medicaginis* isolate were established on PDA from frozen stocks, transferred to potato dextrose broth (PDB) (Difco Laboratories, Becton, Dickson and Co.), and grown for 4 to 8 weeks at 10°C . Mycelium was harvested by decanting the broth, rinsing once in sterile distilled water, and centrifuging. Rinsed mycelium was ly-

ophilized and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

Ten loci were amplified and sequenced: the internal transcribed spacer (ITS) 1, 5.8S, and ITS2 of the rDNA; intron-spanning regions of actin (ACT), α -tubulin (AT), β -tubulin (BT), elongation factor 1- α (EF1 α), glyceraldehyde 3-phosphate dehydrogenase (G3P), and histone (HIS) genes; a portion of a mitochondrial NADH dehydrogenase (NAD) gene; and regions of the rDNA intergenic spacer flanking the 28S ribosomal subunit (IGS-NL) and flanking the 18S ribosomal subunit (IGS-NS). Sequences were generated as a part of a broader effort to investigate the population genetics of *P. sclerotioides* and to generate diagnostic primers for subtypes of *P. sclerotioides*, projects that required sequence data from a relatively large number of loci. Amplifications were conducted in 25- μl reactions containing 1.5 μl of template DNA, 1 $\times$ Takara *Ex Taq* buffer (Clontech Laboratories, Mountain View, CA), 0.8 mM dNTPs, 1 μM each of the forward and reverse primers, and 0.625 units of *Ex Taq* DNA polymerase, hot start version (Clontech Laboratories). Amplification and sequencing were conducted with a combination of previously designed primers (5,27,37) and novel primers; all primers are presented in the supplemental materials. For *P. sclerotioides*, PCR was conducted in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) with the following melting, annealing, and extension temperatures: 94, 54, and 67°C for ACT; 94, 58, and 69°C for AT; 94, 54, and 67°C for BT; 94, 58, and 68°C for EF1 α ; 94, 63, and 71°C for G3P; 94, 59, and 71°C for HIS; 94, 50, and 60°C for NAD; 94, 61, and 70°C for ITS; 94, 59.5, and 68°C for IGS with primers CNS1 and NL11; and 94, 62, and 70°C for IGS with primers CNS1 and R10. For *P. medicaginis*, the extension temperature was lowered to 68°C for AT and to 67°C for EF1 α but otherwise thermocycler conditions were the same. For all loci except NAD and IGS, an initial 60 s at the melting temperature was followed by 40 cycles of 45 s at the melting temperature, 45 s at the annealing temperature, and 60 s at the extension temperature; PCR was concluded with 5 min at the extension temperature and a 4°C bath. For NAD, the extension temperature was held for 80 s during the 40 cycles and, for IGS, the melting, annealing, and extension temperatures were held for 30 s, 30 s, and 3 min 40 s, respectively, during the 40 cycles, and the final extension temperature was held for 6 min. Otherwise, thermocycler conditions were the same as for other loci. Primers ITS1 and ITS4 (37) were used to amplify and sequence the ITS region; a combination of published and novel primers were used for other loci. Novel primers were designed by manual comparison of conserved regions among sequences from Ascomycete genera. Isolate Fr 1-3-IRC was sequenced at 6 loci; all other isolates and a *P. medicaginis* isolate were sequenced at all 10 loci. Sequencing was conducted at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY). Trace files were visualized with Finch TV (Geospiza, Seattle, WA) and SeqMan (Lasergene 7.2.1; DNASTAR, Madison, WI), and sequences were edited in SeqMan. The edited sequences were deposited in GenBank under accession numbers HM157290 to HM158849.

Phylogenetic analyses. Sequences were aligned with Clustal W in MegAlign (Lasergene 7.2.1; DNASTAR) using default gap and gap-length parameters of 15.00 and 6.66, respectively. Alignments were manually edited to resolve inconsistencies in the alignment of individual regions of sequence. The edited sequence alignment has been deposited in TreeBASE (submission ID 10892).

To evaluate genetic differentiation within *P. sclerotioides*, maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses were conducted on a 10-locus data set of 154 *P. sclerotioides* isolates (all except Fr 1-3-IRC) and 1 *P. medicaginis* isolate. MP and ML analyses were conducted on the 10-locus

concatenated data set, with each single locus, and with every combination of nine concatenated loci as well as the eight loci excluding the IGS region (gene-jackknife analyses) (14). Gene-jackknife analyses permit assessment of whether support for individual clades in the full (10-locus) analysis is driven by a single locus or by a signal derived from multiple loci (14). *P. medicaginis* was utilized as an outgroup in all analyses; *P. medicaginis* is a member of the *Didymellaceae*, a subclade within the genus *Phoma* that is sister to the *Leptosphaeriaceae*, the subclade to which *P. sclerotioidea*s pertains (2).

To evaluate the relationship of *P. sclerotioidea*s to other *Phoma* spp., MP and ML phylogenetic analyses were conducted on a data set of ITS sequences. The data set included 25 *P. sclerotioidea*s isolates with unique ITS haplotypes, 83 isolates of other *Phoma* spp. and related genera, and an outgroup (*Saccharomyces cerevisiae* strain CBS 1171). For all non-*P. sclerotioidea*s isolates except *P. medicaginis*, sequences were generated in previously published studies (3,4,10,19,24,25,31) and obtained from GenBank.

Unweighted MP analyses were conducted with PAUP* version 4.0b10 (32). Heuristic searches for the most parsimonious trees were conducted with 1,000 random addition replicates and tree bisection with reconnection branch swapping. To reduce computational time, rearrangements per replicate were limited to 10,000,000. Gaps were treated as missing data, and trees were rooted with *P. medicaginis*. Clade stability was assessed with 1,000 bootstrap replicates. Bootstrap analysis was implemented with a heuristic search in PAUP* using 1,000 random addition sequences per replicate and tree bisection with reconnection branch swapping. To reduce computational time, random additions per replicate were limited to 10,000 during bootstrap analyses.

ML analyses were conducted with GARLI v. 0.96b8 (42). A minimum of 20 runs was conducted until at least two searches from different starting points converged within one likelihood unit of the best tree. For each analysis, results from the best run were chosen. Nodal support was assessed by analyzing 1,000 bootstrap replicates, with a single run conducted per replicate. Nucleotide substitution models for ML analyses were selected with the Akaike Information Criterion implemented in ModelTest 3.7 (28). For the analysis of the ITS region of *Phoma* spp. and related genera, a TrN + I (estimation of invariant sites) + G (nucleotide substitution rates assumed to be gamma distributed) model of nucleotide substitution was used. For the analysis of genetic diversity within *P. sclerotioidea*s, a TrN+I+G model of nucleotide substitution was used for the full concatenated 10-locus data set. For gene-jackknife analyses, the following models were utilized: HKY+I+G when excluding ACT, K81uf+I+G when excluding AT, K81+I+G when excluding BT, TIM+I+G when excluding EF1 α , HKY+I+G when excluding G3P, TrN+I+G when excluding HIS, K81uf+I+G when excluding both IGS regions, TIM+I+G when excluding IGS-NL, SYM+I+G when excluding IGS-NS, HKY+I+G when excluding ITS, and TrN+I+G when excluding NAD. For single-gene analyses, the following models were utilized: TrN+I for ACT, TIM+I+G for AT, HKY+G for BT, TrN+G for EF1 α , TrN+G for G3P, TrN+I+G for HIS, TVM+G for IGS-NL, GTR+G for IGS-NS, TVM+G for ITS, and GTR+I+G for NAD.

Congruence of 50% bootstrap majority trees generated by MP and ML analyses were assessed with Kishino-Hasegawa (KH) and Templeton Wilcoxon signed rank (WS-R) tests implemented in PAUP*. Congruence was evaluated for trees generated from the ITS data set of *Phoma* isolates and the concatenated 10-locus data set of *P. sclerotioidea*s isolates.

Pathogenicity testing and morphological characterization in vivo. Individual plants of 'Vernal' alfalfa were grown in the greenhouse in SC10 Cone-Tainers (3.8 cm in diameter, 21 cm deep; Stuewe and Sons, Inc., Corvallis, OR) in a 1:2:1 mix (by

volume) of peat moss, autoclaved loam soil, and autoclaved sand. Six months after seeding, soil was removed from the first 4 cm of the root, five to six barley grains either colonized by *P. sclerotioidea*s or soaked and autoclaved (control) were placed against the taproot, and soil was replaced. Barley grain inoculum was prepared by transferring single-conidium isolates (established on PDA from frozen stocks) to soaked, autoclaved barley grains and incubating for 3 months at 10°C under continuous white fluorescent light. Fourteen treatments were evaluated: isolates NY Chz1-7r, NY Chz2-14cr, NY Chz7-2r, NY Chz10-18r, NY Chz17-19cr, CO 7-2-10a, NY Fr1-3-1RC, ME 5-2-5r, ME 1-3-11lf, MT 2-1-3, MT 3-1-5, MT 3-3-2a, MT 4-4-12, and an autoclaved barley control. Isolates were selected that represented the breadth of genetic diversity within *P. sclerotioidea*s. From the seven major clades identified in phylogenetic analyses of *P. sclerotioidea*s, one isolate was selected from each of clades 2, 6, and 7 (*P. sclerotioidea*s var. *champlainii*, *macrospora*, and *saskatchewanii*); two isolates were selected from each of clades 3 and 4 (var. *viridis* and *obscurus*); and three isolates were selected from each of clades 1 and 5 (var. *sclerotioidea*s and *steubenii*). Two to three isolates were tested from each of clades 1, 3, 4, and 5 because of their broader geographic distribution relative to clades 2, 6, and 7. Twelve plants were subjected to each treatment in each replicate of the experiment, and the experiment was repeated three times. A randomized complete block design was used. After inoculation, plants were moved to a growth chamber and incubated at 4°C for 6 weeks, -1 to +1°C for 5 weeks, -2 to +1°C for 12 weeks, 3°C for 4 weeks, and 10°C for 4 weeks. At the coldest temperature treatment (-2 to +1°C), plants were incubated in complete darkness; at all other temperatures, plants were incubated under a cycle of 16 h of light and 8 h of darkness. At the conclusion of the experiment, plant mortality was recorded, plants were uprooted, taproots were washed, and root and crown rot severity was assessed. Disease severity was evaluated on a 0 to 10 scale, with 0 = no disease, 1 = 0.1 to 10% of taproot lesioned, 2 = 10.1 to 20% of taproot lesioned,..., and 10 = 90.1 to 100% of taproot lesioned. Pycnosclerotia, immature pycnidia composed of outer cells with scleroplectenchymatous cell walls and inner cells with thin cell walls but no conidiogenous cells or conidia (8), were produced in or on lesions in all inoculated treatments but no mature pycnidia were found.

To generate mature pycnidia for in vivo morphological characterization, lesioned taproot segments bearing pycnosclerotia were transferred to moist chambers and incubated at 10°C under continuous light. Moist chambers were arranged in a complete randomized design in the incubator. After 1 month in moist chambers, pycnidial sections were prepared with a freezing microtome for all isolates, and conidial and pycnidial morphology of all isolates except NY Chz7-2r and NY Chz17-19cr were assessed. Pycnidia of isolates NY Chz7-2r and NY Chz17-19cr matured more slowly, and these isolates were assessed after 3 months in moist chambers. Digital photographs of mature pycnidia were taken under a dissecting microscope and digital photographs of conidia suspended in water were taken with a compound microscope. Conidial and pycnidial dimensions, conidial guttule numbers, and pycnidial neck numbers were assessed from the digital images. PDF Measure It v.1.06 (Traction Software, South Devon, UK), a plug-in to Adobe Acrobat 9 Pro (Adobe Systems, Inc., San Jose, CA), was used for measurements. Pycnidia and conidia used for morphological characterization were drawn from all three replicates of the inoculation experiment. Isolate NY Fr1-3-1RC was not evaluated morphologically because of insufficient production of pycnidia.

Morphological characterization in vitro. Thirty-three isolates representing all major clades identified in phylogenetic analyses were grown on PDA from frozen stocks. Isolates were selected to represent the breadth of genetic diversity within *P. sclerotioidea*s. Four isolates were included from each major clade identified in

phylogenetic analyses except clades 1, 2, and 5 (*P. sclerotoides* var. *sclerotoides*, *champlainii*, and *steubenii*). Nine isolates were included from clade 1 (var. *sclerotoides*) because of the prevalence of the clade in western North America and the high genetic diversity within the clade, only three isolates were included from clade 2 (var. *champlainii*) because a fourth was not available, and five isolates were included from clade 5 (var. *steubenii*) because of the prevalence of the clade in eastern North America. After assessing cultures for contamination, each culture was transferred to PDA and grown at 10°C under continuous white fluorescent light with average luminescence of 2,000 lx for 1 month. Two cultures of each isolate were established, and a completely randomized design was used for incubation of the cultures. Two months after culture establishment, each isolate was photographed to document culture appearance. Three months after culture establishment, in vitro conidial and pycnidial morphology of each isolate was evaluated using a mixture of conidia and pycnidia from both cultures of each isolate. Pycnidia were excised and placed individually on slides, and pycnidial dimensions and neck numbers were assessed directly under a compound microscope or from digital photographs taken under a compound microscope. Conidia were suspended in water, and digital photographs taken under a compound microscope were used to measure conidial dimensions and record conidial guttule numbers.

In vitro growth rates. Cultures of 21 isolates representing all major clades identified in phylogenetic analyses were grown on PDA from frozen stocks. Isolates were selected that represented the breadth of genetic diversity within *P. sclerotoides*. From the major clades identified in phylogenetic analyses of *P. sclerotoides*, three isolates were selected from each of clades 3 and 5 (var. *viridis* and *steubenii*), seven isolates were selected from clade 1 (var. *sclerotoides*), and two isolates were evaluated from clades 2, 4, 6, and 7 (var. *champlainii*, *obscurus*, *macrospora*, and *saskatchewanii*). A higher number of isolates was evaluated from clades 1, 3, and 5 because of the prevalence of these clades within North America and because of the high genetic diversity found within clade 1 (var. *sclerotoides*). Three months after culture establishment, conidia were harvested from each isolate, suspended in water, plated onto PDA, and incubated for 24 h under continuous fluorescent light at 10°C. Single germinating conidia were transferred to PDA. Single-conidium cultures were incubated at 3, 10, 18, or 25°C in complete darkness for 24 days, and radial growth of the cultures was measured. The 10 and 18°C temperature treatments were evaluated because they approximate the temperatures previously reported as optimal for *P. sclerotoides* (16,29), the 3°C treatment was included to assess growth at temperatures near freezing, and the 25°C temperature was included because it approximates the temperature previously reported as the upper threshold for *P. sclerotoides* growth (29). The incubation period of 24 days was selected to maximize the ability to differentiate treatments; after 24 days, cultures of vigorously growing isolates were near the edge of the petri dish. At each temperature, nine single-conidium cultures of each isolate were assessed in each of four replicates of the experiment, for a total of 36 single-conidium cultures of each isolate. A randomized complete block design was utilized.

Statistical analyses. *Pycnidial and conidial morphology.* Differences in conidial length, conidial width, pycnidial diameter, and pycnidial neck length were assessed with analysis of variance. For each data set, the assumptions of constant variance and normality were assessed by plotting residuals against predicted values and evaluating their variance and by plotting residuals against their ranks and examining their linearity. In all data sets, the assumptions were met, and no transformations were applied to the data. Single-degree-of-freedom contrasts were performed for all pairwise combinations of isolates; to control the type I error rate at the level of the experiment, the Tukey multiple comparison procedure (26) was employed. Analyses were conducted at both

the level of isolate and the level of infraspecific variety (using pooled data from corresponding isolates). Analyses were implemented in PROC GLM of SAS (version 9.2; SAS Institute, Cary, NC).

Conidial guttule number and pycnidial neck number were analyzed with cumulative logistic regression (18), and single-degree-of-freedom contrasts of all possible pairwise combinations of isolates or infraspecific varieties were conducted with Wald χ^2 tests. Analyses were conducted at both the level of isolate and the level of infraspecific variety (using pooled data from corresponding isolates). Analyses were implemented in PROC GENMOD of SAS (version 9.2; SAS Institute). The Bonferroni multiple comparison procedure (26) was used to control the type I error rate at the level of the experiment across the 528 pairwise contrasts of individual isolates for the in vitro morphology data, 66 pairwise contrasts of individual isolates for the in vivo morphology data, and 21 pairwise contrasts of infraspecific varieties.

Differences in conidial septation across isolates and across infraspecific varieties were evaluated with χ^2 tests of independence (41). Pairwise contrasts of isolates and infraspecific varieties were conducted with single-degree-of-freedom χ^2 tests, and the Bonferroni multiple comparison procedure (26) was used to control the type I error rate at the level of the experiment.

In vitro growth rates. In vitro growth rates of isolates were evaluated with analysis of variance, and analysis was conducted controlling for the effect of experimental replicate. Single-degree-of-freedom contrasts were performed for all pairwise combinations of isolates; to control the type I error rate at the level of the experiment, the Tukey multiple comparison procedure (26) was employed. Analyses were implemented in PROC GLM of SAS (version 9.2; SAS Institute).

Pathogenicity testing. Root rot severity was analyzed with cumulative logistic regression (18), with analysis conducted controlling for the effect of experimental replicate. Single-degree-of-freedom contrasts between the control and each of the 13 inoculated treatments were conducted with Wald χ^2 tests, and the Bonferroni multiple comparison procedure (26) was used to control the type I error rate at the level of the experiment. Analyses were implemented in PROC GENMOD of SAS (version 9.2; SAS Institute).

RESULTS

Fungal strains. All isolates produced large pycnidia with long necks for spore discharge and generated white cirri that turned yellow with age. Diagnostic PCR resulted in amplicons of the expected size ($\approx$ 500 bp) from 151 of 154 *P. sclerotoides* isolates evaluated. Diagnostic PCR was repeated for isolates ME 2-6cr, MT 3-3-2a, and NY Wyo1-3r but always failed to produce an amplification product.

Phylogenetic analyses. Alignment and concatenation of ACT, AT, BT, EF1 α , G3P, HIS, IGS-NS, IGS-NL, ITS, and NAD sequences of *P. sclerotoides* isolates and a *P. medicaginis* outgroup resulted in a data set with a length of 8,132 characters, of which 539 were parsimony informative. Parsimony analysis generated 445,997 MPTs with 2,481 steps (CI = 0.7864, RI = 0.9603). Likelihood analysis generated a best tree with log-likelihood score of -23,921.57. MP and ML analyses placed *P. sclerotoides* isolates into 29 strongly supported clades (bootstrap values $\geq$ 70%), and 22 strongly supported clades were congruent between MP and ML trees (Fig. 1; labeled as uppercase letters to left of bootstrap values). Of the 22 clades, 9 had bootstrap values $\geq$ 70% in all MP and ML gene-jackknife analyses (Fig. 2) and were assigned formal clade or subclade designations (Figs. 1 and 2); others lacked support in one or more gene-jackknife analyses. Gene-jackknife and single-gene analyses permit assessment of whether support for individual clades in the full (10-locus) data set is driven by a single locus or by a signal derived from multiple

A

MAXIMUM PARSIMONY ANALYSIS

ACT + AT + BT + EF1a +
G3P + HIS + IGS-NL +
IGS-NS + ITS + NAD

Bootstrap values > 70%
indicated at internodes.
Rooted with *Phoma medicaginis*.

8132 bp
1 of 445,997 MP trees
2481 steps
CI = 0.7864 RI = 0.9603

100 steps

B

MAXIMUM LIKELIHOOD ANALYSIS

ACT + AT + BT + EF1a +
G3P + HIS + IGS-NL +
IGS-NS + ITS + NAD

Bootstrap values > 70%
indicated at internodes.
Rooted with *Phoma medicaginis*.

8132 bp

0.02
NUCLEOTIDE SUBSTITUTIONS
PER SITE

Fig. 1. A, Maximum parsimony (MP) and B, maximum likelihood (ML) ($\ln = -23,921.57$) analyses of concatenated sequence data from 10 loci. Trees rooted with *Phoma medicaginis*, and bootstrap values >70% (1,000 replications) indicated at nodes. Clades that are congruent in MP and ML analyses are indicated with capital letters to the left of the bootstrap values. Clades that have >70% bootstrap support in all MP and ML gene-jackknife analyses are indicated by shading of bootstrap values and associated letters at nodes.

A GENE-JACKKNIFE ANALYSES

CLADE DESIGNATION	NODE	ALL LOCI	NOACT	NOAT	NOBT	NOEF	NOG3P	NOHIS	NOIGS	NOIGS-NS	NOIGS-NL	NOITS	NONAD	base pairs
		8132	7491	7300	7481	7654	7506	7616	6453	6927	7858	7623	5723	
<i>P. sclerotoides</i> var. <i>sclerotoides</i> (SUBCLADE 1A)	A	75 / 92	74 / 91	79 / 92	78 / 92	74 / 90	72 / 93	75 / 92			75 / 93	78 / 91	76 / 89	
	B	98 / 93	99 / 92	98 / 91	100 / 96	98 / 95	98 / 92	94 / 76	90 / 90	90 / 92	97 / 91	98 / 91	97 / 86	
	C	85 / 87	87 / 86	84 / 86		87 / 87	86 / 86	85 / 87	86 / 89	87 / 88	87 / 87	97 / 98	85 / 88	
	D	76 / 84	80 / 83	78 / 82	67 / 75	78 / 84	79 / 83	78 / 81			77 / 89	74 / 71	74 / 92	
<i>P. sclerotoides</i> var. <i>champlainii</i> (CLADE 2)	E	100 / 91	100 / 89	100 / 93	100 / 92	100 / 97	100 / 89	100 / 91	100 / 93	100 / 90	100 / 95	100 / 97	100 / 99	
<i>P. sclerotoides</i> var. <i>viridis</i> (CLADE 3)	F	100 / 99	100 / 99	100 / 97	100 / 99	100 / 96	100 / 98	100 / 100	99 / 100	100 / 93	100 / 100	100 / 99	100 / 99	
	G	98 / 98	97 / 99	98 / 98	100 / 100		97 / 97	100 / 100	97 / 98	97 / 98	98 / 98	97 / 97	97 / 98	
	H	71 / 83	71 / 82	67 / 76		71 / 80	70 / 82	63 / 71	70 / 78	71 / 82	72 / 80	70 / 83	70 / 78	
	J	98 / 97	98 / 96	98 / 96	94 / 93	97 / 97	97 / 96	95 / 95			98 / 97	98 / 96	99 / 95	
<i>P. sclerotoides</i> var. <i>viridis</i> (SUBCLADE 3A)	K	100 / 100	100 / 100	100 / 99	100 / 100	73 / 73	100 / 100	100 / 100	99 / 100	100 / 99	99 / 100	100 / 100	100 / 100	
<i>P. sclerotoides</i> var. <i>obscurus</i> (CLADE 4)	L	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100	100 / 99	100 / 95	100 / 100	100 / 100	100 / 100	
<i>P. sclerotoides</i> var. <i>obscurus</i> (SUBCLADE 4A)	M	90 / 97	88 / 92	92 / 95	90 / 94	92 / 95	76 / 88	87 / 93	79 / 92	79 / 90	90 / 95	91 / 95	90 / 94	
	N	100 / 79	100 / 82	100 / 85	100 / 87	100 / 75	100 / 88	100 / 84	97 / 77	88 / 66	100 / 88	100 / 78	100 / 82	
<i>P. sclerotoides</i> var. <i>steubenii</i> (CLADE 5)	O	99 / 99	100 / 97	96 / 98	98 / 97	99 / 98	97 / 95	98 / 96	94 / 86	97 / 90	99 / 98	100 / 99	99 / 98	
	P	97 / 92	94 / 83	100 / 98	98 / 89	98 / 85	95 / 85	91 / 83		73 / 50	97 / 91	97 / 93	97 / 85	
	Q	86 / 84	92 / 83	81 / 82	84 / 81	86 / 79	75 / 72			73 / 55	86 / 84	85 / 87	84 / 81	
	R	80 / 75	89 / 79		80 / 71	79 / 73	79 / 72				81 / 76	79 / 77	81 / 71	
	S	83 / 86	96 / 84		83 / 82	82 / 84	84 / 85	78 / 82	81 / 85	82 / 80	85 / 87	83 / 87	83 / 86	
	T	98 / 96	97 / 94		98 / 96	97 / 93	98 / 94	98 / 91	89 / 77	91 / 85	99 / 96	97 / 97	98 / 95	
<i>P. sclerotoides</i> var. <i>saskatchewanii</i> (CLADE 7)	U	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100	100 / 97	100 / 99	100 / 100	100 / 100	100 / 100	
	V	90 / 92	82 / 98	91 / 94	90 / 98	90 / 94		93 / 88	96 / 96	96 / 97	89 / 94	97 / 95	90 / 85	
<i>P. sclerotoides</i> var. <i>macrospora</i> (CLADE 6)	W	100 / 85	100 / 87	100 / 74	100 / 69	100 / 84	100 / 77	100 / 85	100 / 96	100 / 89	100 / 84	100 / 87	100 / 87	

B SINGLE GENE ANALYSES

CLADE DESIGNATION	NODE	ALL LOCI	ACT	AT	BT	EF	G3P	HIS	IGS-NS	IGS-NL	ITS	NAD	base pairs
		8132	641	832	651	478	626	516	1295	274	509	2498	
<i>P. sclerotoides</i> var. <i>sclerotoides</i> (SUBCLADE 1A)	A	75 / 92							87 / 98				
	B	98 / 93						88 / 92	81 / 71				
	C	85 / 87			95 / 96								
	D	76 / 84											
<i>P. sclerotoides</i> var. <i>champlainii</i> (CLADE 2)	E	100 / 91		91 / 77		97 / 99	97 / 100	95 / 83	100 / 92			97 / 100	
<i>P. sclerotoides</i> var. <i>viridis</i> (CLADE 3)	F	100 / 99		98 / 99		74 / 93			100 / 95	98 / 99	99 / 100	98 / 97	
	G	98 / 98				100 / 100							
	H	71 / 83											
	J	98 / 97							71 / 73				
<i>P. sclerotoides</i> var. <i>viridis</i> (SUBCLADE 3A)	K	100 / 100				100 / 99				87 / 79			
<i>P. sclerotoides</i> var. <i>obscurus</i> (CLADE 4)	L	100 / 100		92 / 98	92 / 100	96 / 95	85 / 80	75 / 80	100 / 100	81 / 94		83 / 72	
<i>P. sclerotoides</i> var. <i>obscurus</i> (SUBCLADE 4A)	M	90 / 97					74 / 76						
	N	100 / 79		89 / 93					100 / 82				
<i>P. sclerotoides</i> var. <i>steubenii</i> (CLADE 5)	O	99 / 99	79 / 89		84 / 82		85 / 94		74 / 84				
	P	97 / 92							100 / 100				
	Q	86 / 84						92 / 86					
	R	80 / 75											
	S	83 / 86											
	T	98 / 96											
<i>P. sclerotoides</i> var. <i>saskatchewanii</i> (CLADE 7)	U	100 / 100	95 / 94	100 / 100	94 / 93	86 / 88		100 / 99		89 / 76			
	V	90 / 92					99 / 100						
<i>P. sclerotoides</i> var. <i>macrospora</i> (CLADE 6)	W	100 / 85						97 / 97		84 / 95			

Numbers at left of forward slash: bootstrap values from maximum parsimony analysis

Numbers at right of forward slash: bootstrap values from maximum likelihood analysis

Color Key: MP and ML bootstrap values ≥ 95% 95% > MP and ML bootstrap values ≥ 70% MP and ML bootstrap values < 70%
 MP bootstrap value ≥ 95% and 95% > ML bootstrap value ≥ 70% ML bootstrap value ≥ 95% and 95% > MP bootstrap value ≥ 70%
 MP bootstrap value ≥ 95% and ML bootstrap value < 70% ML bootstrap value ≥ 95% and MP bootstrap value < 70%
 MP bootstrap value ≥ 70% and ML bootstrap value < 70% ML bootstrap value ≥ 70% and MP bootstrap value < 70%

Fig. 2. Congruence of clades as determined from maximum parsimony (MP) and maximum likelihood (ML) analyses of **A**, gene-jackknife and **B**, single-gene data partitions. Size of each partition is given below its name. Nodes (identified by capital letters) correspond to clades that are congruent in MP and ML analyses of the full 10-locus data set. In each analysis, 1,000 bootstrap replicates were performed. Single-gene analyses that did not provide strong bootstrap support (≥70%) for specific infraspecific varieties did not assign the infraspecific varieties to conflicting, strongly supported clades, with two exceptions: MP and ML analyses of the α -tubulin (AT) partition, which delineated clade 5 as a paraphyletic group with clade 6 monophyletic within it, and ML analysis of the internal transcribed spacer (ITS) partition, which excluded a single isolate of clade 5 (ME 3-4-16cr) from a node containing isolates of clades 5, 6, and 7.

loci. Clades with strong bootstrap support in all gene-jackknifing analyses are robust, with support derived from multiple loci, and strong bootstrap support in multiple single-gene partitions strengthens this conclusion. Of the nine clades with strong bootstrap support in all gene-jackknife analyses, five had bootstrap support $\geq 70\%$ at four to seven individual loci in MP or ML analyses, and all but one (subclade 4A) (Fig. 2) had bootstrap support $\geq 70\%$ at two or more individual loci in MP or ML analyses. The nine clades were not assigned to conflicting nodes (bootstrap support $\geq 70\%$) in analyses of single-gene partitions with two exceptions: MP and ML analyses of the AT partition, which delineated clade 5 as a paraphyletic group with clade 6 monophyletic within it, and ML analysis of the ITS partition, which excluded a single isolate of clade 5 (ME 3-4-16cr) from a node containing isolates of clades 5, 6, and 7. Of the nine clades, formal taxonomic designations were assigned only if at least three representative isolates were morphologically characterized in vitro on PDA, at least one representative isolate was morphologically characterized in vivo on alfalfa, and at least two representative isolates were assessed for their ability to grow at 3, 10, 18, and 25°C. Clade 2 was designated *P. sclerotoides* var. *champlainii*; clade 3, *P. sclerotoides* var. *viridis*; clade 4, *P. sclerotoides* var. *obscurus*; clade 5, *P. sclerotoides* var. *steubenii*; clade 6, *P. sclerotoides* var. *macrospora*; and clade 7, *P. sclerotoides* var. *saskatchewanii*. Subclade 1A (Figs. 1 and 2) falls within a broad group of isolates that is basal to all other isolates in ML analysis and has 100% bootstrap support in MP analysis (Fig. 1), and it was treated as a subclade of the broader group. This broad group of isolates was designated *P. sclerotoides* var. *sclerotoides*; it includes *P. sclerotoides* isolate WyoBerg (American Type Culture Collection [ATCC] MYA-295) from Wyoming (Fig. 1) characterized in previous studies (12,15), and sequence identity at the ITS and G3P loci (20) (GenBank accession numbers DQ525737 and DQ525733) suggests that it also contains *P. sclerotoides* reference isolate CBS 148.84 (ATCC 56515) from British Columbia, Canada. Subclades 3A and 4A, the two remaining clades with strong support in all gene jack-knifing analyses, form subclades of *P. sclerotoides* var. *viridis* and var. *obscurus* (Fig. 1). Insufficient morphological characterization of representative isolates precluded the assignment of formal names to subclades 1A, 3A, or 4A. KH and WS-R tests indicated that the MP and ML trees were not congruent ($P < 0.0001$), and both trees are presented (Fig. 3). Intraspecific varieties *champlainii*, *steubenii*, and *saskatchewanii* were named for New York and Vermont's Champlain Valley, New York's Steuben County, and the Canadian province of Saskatchewan, respectively. Intraspecific varieties *viridis*, *obscurus*, and *macrospora* were named for morphological characteristics (green-pigmented mycelium on PDA, dark pigmented mycelium on PDA, and large spores, respectively).

Alignment of ITS sequences for *P. sclerotoides*, other *Phoma* spp., and related genera resulted in a data set 517 characters long, of which 215 were parsimony informative. Phylogenetic analysis by MP generated 3,695 MPTs with 1,232 steps (CI = 0.4513, RI = 0.8781). Phylogenetic analysis by ML generated a best tree with log-likelihood score -5,913.94. In both MP and ML analyses, all *P. sclerotoides* isolates were placed in a strongly supported monophyletic group with *L. dolium* (Fig. 3; bootstrap support $\geq 98\%$), and *P. sclerotoides* and *L. dolium* (anamorph *P. acuta*) were grouped with other members of *Phoma* section *Plenodomus*. Section *Plenodomus*, the division within *Phoma* in which *P. sclerotoides* is placed on the basis of morphological characteristics, is associated with thick scleroplectenchymatous pycnidial walls, glabrous pycnidia, one- to two-celled conidia, and, when known, a *Leptosphaeria* teleomorph (8). KH and WS-R tests indicated that the MP and ML trees were not congruent ($P < 0.0001$), and both trees are presented (Fig. 3).

In vivo morphology. The pycnidial morphology of all isolates was consistent with the species description (8). Pycnidia were

glabrous, subglobose, and superficial (Fig. 4). Pycnidia were composed of outer polygonal cells with thick, scleroplectenchymatous cell walls that overlay thin-walled, smaller, polygonal cells in the interior (Fig. 5). In mature pycnidia, the thin-walled cells in the interior gave rise to conidiogenous cells. Cells at the central base of pycnidia were scleroplectenchymatous and either polygonal or elongated, forming a pallisade. Pycnidial diameters fell within the range of species description ([200–]350–800[–1,000] μm) (8) but only isolates of *P. sclerotoides* var. *sclerotoides* and *champlainii* produced pycnidia with diameters $\leq 1,000 \mu\text{m}$. Isolates of other varieties of *P. sclerotoides* exhibited maximum diameters of 600 to 800 μm but considerable isolate-isolate variability existed within varieties (Fig. 6). Pycnidia of all isolates produced long necks for spore discharge, with maximum neck lengths of 500 to 800 μm (Figs. 4 and 6), but *P. sclerotoides* var. *champlainii* isolate NY Chz1-7r produced significantly longer necks than the others ($P < 0.01$) (Fig. 6). Pycnidia with multiple necks were common in all isolates but were most common in isolates of *P. sclerotoides* var. *sclerotoides*, *champlainii*, and *obscurus* (Fig. 6). Pycnidial necks of *P. sclerotoides* var. *sclerotoides* and *viridis* isolates were glabrous to semipilose; pycnidial necks of *P. sclerotoides* var. *champlainii* isolate NY Chz1-7r were glabrous to pilose; and pycnidial necks of *P. sclerotoides* var. *obscurus*, *steubenii*, *macrospora*, and *saskatchewanii* isolates were semipilose to pilose (Fig. 4). All isolates produced cream-colored cirrus that yellowed with age.

Conidial morphology of isolates approximated the species description (8) with a few exceptions. No eguttulate conidia were observed, conidia were not always aseptate, and conidial lengths were more variable. Conidia were hyaline and subcylindrical to ellipsoidal, with one to three guttules in *P. sclerotoides* var. *champlainii*, *steubenii*, and *saskatchewanii* and one to four guttules in all others (Fig. 7). Guttules were mostly polar. *P. sclerotoides* var. *macrospora* produced both one- and two-celled conidia, and others produced only aseptate conidia. Conidial lengths were highly variable. All varieties of *P. sclerotoides* produced a range of conidial lengths that partially overlapped the species description but none corresponded to the species description completely. *P. sclerotoides* var. *sclerotoides* and *champlainii* produced short conidia (3.5 to 5.6 μm), *P. sclerotoides* var. *obscurus* and *macrospora* produced long conidia (4.3 to 8.1 μm), and others were intermediate. *P. sclerotoides* var. *sclerotoides* produced significantly shorter conidia than the others ($P < 0.01$) (Fig. 7). Conidial widths were broadly consistent with the species description (1.5 to 3.0 μm) for all varieties of *P. sclerotoides* except *P. sclerotoides* var. *champlainii*, in which less variability in conidial widths (1.4 to 1.9 μm) was observed. Conidia of *P. sclerotoides* var. *champlainii* were significantly narrower than conidia of other varieties ($P < 0.01$) (Fig. 7).

In vitro morphology. Cultural morphology of isolates varied widely on PDA. At 10°C under continuous white fluorescent light, aerial mycelium of *P. sclerotoides* var. *sclerotoides* isolates was sparse and white; aerial mycelium of var. *champlainii* isolates was sparse to moderately abundant and white; aerial mycelium of var. *viridis* isolates was moderately abundant to abundant and green to green-gray; aerial mycelium of var. *obscurus* isolates was moderately abundant to abundant and green-gray to dark gray; and aerial mycelium of varieties *steubenii*, *macrospora*, and *saskatchewanii* was sparse to moderately abundant and tan, brown, or gray (Fig. 4). Isolates of *P. sclerotoides* var. *steubenii*, *macrospora*, and *saskatchewanii* deposited a tan to yellow-brown pigmentation in the agar surrounding cultures (Fig. 4). Other isolates did not appreciably change the pigmentation of the surrounding agar. In mature cultures, deposition of a dark band of mycelium within the agar at the growing edge of the culture near petri dish walls frequently occurred but was particularly pronounced in isolates of *P. sclerotoides* var. *sclerotoides* and

A

MAXIMUM PARSIMONY ANALYSIS

ITS

Bootstrap values > 70%
indicated at internodes.

Rooted with
Saccharomyces cerevisiae

100
STEPS

CI = 0.4513 RI = 0.8781
Tree Length = 1232
1 of 3695 MP trees

517 bp

B

MAXIMUM LIKELIHOOD ANALYSIS

ITS

Bootstrap values > 70%
indicated at internodes.

Rooted with
Saccharomyces cerevisiae

0.4
NUCLEOTIDE SUBSTITUTIONS
PER SITE

517 bp

Fig. 3. A, Maximum parsimony and B, maximum likelihood ($\ln = -5,913.94$) analyses of sequence data of the complete internal transcribed spacer (ITS) 1, 5.8S rDNA, and ITS 2. Trees rooted with *Saccharomyces cerevisiae* and bootstrap values >70% (1,000 replications) indicated at internodes. GenBank accession numbers (underlined) are provided for sequences generated in previous studies (3,4,10,19,24,25,31). Section *Plenodomus* is a division within the genus *Phoma* associated with glabrous, thick-walled pycnidia; scleroplectenchymatous pycnidial cells; one- to two-celled conidia; and, when known, a *Leptosphaeria* teleomorph (8).

IN-VITRO CULTURE MORPHOLOGY (on potato dextrose agar)

A-G: *P. sclerotoides* var. *sclerotoides* isolates 'NY Chz2-14cr' (A), 'MT 3-1-5' (B), 'ON 4-28st' (C), 'SK 2-11' (D), 'SK 7-8' (E), 'NY Chz7-2r' (F), 'MT 4-4-4' (G)
H-J: *P. sclerotoides* var. *champlainii* isolates 'NY Chz1-7r' (H), 'NY Chz1-15r' (I), 'VT 3-2-1-7r' (J)
K-N: *P. sclerotoides* var. *viridis* isolates 'NY Chz17-19cr' (K), 'SK 4-21' (L), 'WI 5-1-5' (M), 'SK 4-21' (N)
O-R: *P. sclerotoides* var. *obscurus* isolates 'MT 1-4-2' (O), 'MT 4-1-3a' (P), 'MT 4-4-12' (Q), 'CO 7-2-10a' (R)
S-V: *P. sclerotoides* var. *steubeni* isolates 'NY Chz10-18r' (S), 'VT 2-2-3-8r' (T), 'ME 1-3-11lf' (U), 'NH 4-2-1-8r' (V)
W-Z: *P. sclerotoides* var. *macrospora* isolates 'NY Chz7-25cr' (W), 'ON 5-6' (X), 'ME 5-2-5r' (Y), 'NY Wyo18-1r' (Z)
AA-AD: *P. sclerotoides* var. *saskatchewanii* isolates 'SK 7-34' (AA), 'SK 4-6' (AB), 'SK 3-1' (AC), 'MT 2-1-3' (AD)

IN-VIVO PYCNIDIAL MORPHOLOGY (on roots of *Medicago sativa*)

A: *P. sclerotoides* var. *sclerotoides* 'NY Chz2-14cr'
B: *P. sclerotoides* var. *sclerotoides* 'MT 3-1-5'
C: *P. sclerotoides* var. *sclerotoides* 'NY Chz7-2r'
D: *P. sclerotoides* var. *champlainii* 'NY Chz1-7r'
E: *P. sclerotoides* var. *viridis* 'NY Chz17-19cr'
F: *P. sclerotoides* var. *viridis* 'MT 3-3-2a'
G: *P. sclerotoides* var. *obscurus* 'CO 7-2-10a'
H: *P. sclerotoides* var. *obscurus* 'MT 4-4-12'
I: *P. sclerotoides* var. *steubeni* 'NY Chz10-18r'
J: *P. sclerotoides* var. *steubeni* 'ME 1-3-11lf'
K: *P. sclerotoides* var. *macrospora* 'ME 5-2-5r'
L: *P. sclerotoides* var. *saskatchewanii* 'MT 2-1-3'

Fig. 4. Images illustrating that infraspecific varieties of *Phoma sclerotoides* differ morphologically yet produce pycnidia that are consistent with the species description of *P. sclerotoides* (8). In vitro (top), mycelium pigmentation differs by infraspecific variety. In vivo (bottom), all infraspecific varieties produce glabrous or nearly glabrous, subglobose, and superficial pycnidia with long necks for spore discharge. In vitro morphology was assessed by growing single-conidium isolates on potato dextrose agar at 10°C under continuous white fluorescent light for 2 months. In vivo morphology was assessed by inoculating healthy alfalfa with single-conidium isolates, harvesting diseased roots, and incubating symptomatic roots in moist chambers until pycnidial maturity.

champlainii (Fig. 4). Differences in culture appearance were repeatable, with isolates consistently showing the same phenotype when started from frozen stocks.

All isolates produced large pycnidia (maximum diameter 600 to 1,350 μm) with long necks for spore discharge. Pycnidial diameters were most variable in isolates of *P. sclerotoides* var. *viridis* (270 to 1,300 μm) and *champlainii* (320 to 1,350 μm), least variable in isolates of *P. sclerotoides* var. *steubenii* (300 to 760 μm), and intermediate (200 or 300 to 900 or 1,100 μm) in the others (Fig. 6). Pycnidial necks were ≈ 150 to 800 μm long, except in isolates of *P. sclerotoides* var. *champlainii*, which produced significantly longer necks (280 to 1,240 μm) ($P < 0.01$). Pycnidia with multiple necks were produced by all isolates but were most common in isolates of *P. sclerotoides* var. *sclerotoides*, *champlainii*, and *obscurus* (Fig. 6). All isolates produced cream-colored cirrus that yellowed with age.

Conidia were hyaline and subcylindrical to ellipsoidal, with one to four guttules in *P. sclerotoides* var. *champlainii*, *viridis*, and *saskatchewanii* and one to five guttules in all others (Fig. 7). Guttules were mostly polar. *P. sclerotoides* var. *macrospora* produced both aseptate and one-septate conidia, and others produced only aseptate conidia. Conidial lengths were highly variable. *P. sclerotoides* var. *sclerotoides* and *champlainii* produced short conidia (usually 4 to 7.5 μm but sometimes 2.5 to 7.9 μm), *P. sclerotoides* var. *macrospora* produced long conidia (6 to 12.4 μm), and others were intermediate. *P. sclerotoides* var. *macrospora* produced significantly longer conidia than the others

($P < 0.01$) (Fig. 7), and *P. sclerotoides* var. *sclerotoides* and *champlainii* produced the shortest conidia ($P < 0.01$). Conidia were ≈ 2 to 4 μm wide in *P. sclerotoides* var. *viridis*, *obscurus*, *steubenii*, *macrospora*, and *saskatchewanii* but significantly narrower (1.5 to 3.2 μm) in var. *champlainii* ($P < 0.010$) (Fig. 7). Conidial widths of *P. sclerotoides* var. *sclerotoides* were intermediate between var. *champlainii* and the others.

Pathogenicity testing. Of 13 isolates, 11 caused significantly more disease on Vernal alfalfa than the control, and at least one isolate of each variety of *P. sclerotoides* caused a significant increase in root rot severity relative to the control (Table 1). The highest levels of root rot severity and plant mortality were associated with isolates of *P. sclerotoides* var. *sclerotoides*, *viridis*, and *obscurus* (Table 1). Lesions were characteristic of BRR: light to dark brown, often with a darker border, and with abundant immature pycnidia produced on the surface of lesions. Root rot severity in the control was very low, suggesting that little cross-contamination occurred across treatments.

In vitro growth. On PDA, isolates grew well at 10°C and moderately well at 3°C but differed in their growth at 18 and 25°C. Optimal growth of isolates of *P. sclerotoides* var. *macrospora* occurred at 10°C, with growth almost completely inhibited at 25°C (Table 2). Isolates of *P. sclerotoides* var. *viridis* showed optimal growth at 10 to 18°C but also vigorous growth at 25°C. The temperature adaptation of all other isolates corresponded closely to previous reports (16,29), showing optimal growth at 10 and 18°C and limited growth at 25°C. At 25°C, isolates of *P.*

Fig. 5. Pycnidial cross-sections illustrating that all infraspecific varieties of *Phoma sclerotoides* have internal pycnidial morphologies consistent with the species description of *P. sclerotoides* (8). Outer cells are large with thick, scleroplectenchymatous cell walls, and inner cells are smaller, polygonal, and thin walled. Pycnidia were generated by inoculating healthy alfalfa with single-conidium isolates, harvesting diseased roots, and incubating symptomatic roots in moist chambers until pycnidial maturity.

IN-VITRO PYCNIDIAL MORPHOLOGY (on potato dextrose agar)

IN-VIVO PYCNIDIAL MORPHOLOGY (on roots of *Medicago sativa*)

Fig. 6. In vivo and in vitro pycnidial diameters, neck lengths, and neck numbers of *Phoma sclerotioides* isolates. Thin line of box plots represents the range of data, thick line represents middle 80% of observations, and asterisk denotes median. Neck number data are given as the percentage of pycnidia observed. Box plots and neck number data followed by different letters are significantly different ($P < 0.01$). The type I error rate was controlled across pairwise comparisons with the Tukey multiple comparison procedure (pycnidial diameter, neck length) or the Bonferroni procedure (neck number).

IN-VITRO CONIDIAL MORPHOLOGY (on potato dextrose agar)

IN-VIVO CONIDIAL MORPHOLOGY (on roots of *Medicago sativa*)

Fig. 7. In vivo and in vitro conidial lengths, widths, guttule numbers, and septation of *Phoma sclerotoides* isolates. Thin line of box plots represents the range of data, thick line represents middle 80% of observations, and asterisk denotes median. Guttule number and septation data are given as the percentage of conidia observed. Box plots, guttule number data, and septation data followed by different letters are significantly different ($P < 0.01$). The type I error rate was controlled across pairwise comparisons with the Tukey multiple comparison procedure (conidial length, width) or the Bonferroni procedure (guttule number, septation).

sclerotioides var. *viridis* isolates showed significantly more growth than other isolates ($P < 0.01$) (Table 2), and isolates of *P. sclerotioides* var. *macrospora* showed significantly less growth than all other isolates except ME 1-3-11lf of *P. sclerotioides* var. *steubenii* ($P < 0.01$). At 10 and 18°C, isolates of *P. sclerotioides* var. *sclerotioides* and *champlainii* grew significantly more than isolates of *P. sclerotioides* var. *obscurus*, *macrospora*, and *saskatchewanii* ($P < 0.01$) (Table 2) and, at 18°C, isolates of *P. sclerotioides* var. *steubenii* were intermediate ($P < 0.01$). At 3°C, in vitro growth was similar across all varieties of *P. sclerotioides* (Table 2), although moderate but significant ($P < 0.01$) differences were present among individual isolates.

DISCUSSION

The *Phoma* isolates evaluated in this study are genetically and morphologically differentiated but all broadly correspond to the published description of *P. sclerotioides* (8). In vivo, they produce glabrous, subglobose, thick-walled pycnidia with scleroplectenchymatous outer cells, thin-walled inner cells, and long necks for spore discharge. Conidia are ellipsoidal to subcylindrical, 1.5 to 3 µm wide, and with one to three or one to four mostly polar guttules. All are adapted to low temperatures, and they are pathogenic to alfalfa as it emerges from winter dormancy. Phylogenetic analysis of sequence data from the ITS region places the

TABLE 1. Aggressiveness of *Phoma sclerotioides* isolates to Vernal alfalfa

Isolate	Plant mortality ^y	Root rot severity ^z
<i>P. sclerotioides</i> var. <i>sclerotioides</i> NY Chz2-14cr	9/36	3.08 ****
<i>P. sclerotioides</i> var. <i>sclerotioides</i> MT 3-1-5	10/36	3.83 ****
<i>P. sclerotioides</i> var. <i>sclerotioides</i> NY Chz7-2r	5/36	2.08 **
<i>P. sclerotioides</i> var. <i>champlainii</i> NY Chz1-7r	5/36	2.08 ***
<i>P. sclerotioides</i> var. <i>viridis</i> NY Chz17-19cr	10/36	3.17 ****
<i>P. sclerotioides</i> var. <i>viridis</i> MT 3-3-2a	12/36	3.44 ****
<i>P. sclerotioides</i> var. <i>obscurus</i> MT 4-4-12	11/36	2.28 ****
<i>P. sclerotioides</i> var. <i>obscurus</i> CO 7-2-10a	4/36	1.81 **
<i>P. sclerotioides</i> var. <i>steubenii</i> NY Chz10-18r	7/36	1.11 NS
<i>P. sclerotioides</i> var. <i>steubenii</i> ME 1-3-11lf	1/36	0.61 NS
<i>P. sclerotioides</i> var. <i>steubenii</i> Fr 1-3-1RC	3/36	1.36 *
<i>P. sclerotioides</i> var. <i>macrospora</i> ME 5-2-5r	4/36	1.42 *
<i>P. sclerotioides</i> var. <i>saskatchewanii</i> MT 2-1-3	2/36	1.36 *
Control	1/36	0.19

^y Proportion of plants dead at conclusion of a replicated experiment conducted on potted alfalfa grown under controlled conditions.

^z Average root necrosis. Necrosis was rated on a 0 to 10 scale: 0, no disease; 1, 0.1 to 10% of tap root necrotic; 2, 11.1 to 20% of tap root necrotic; ..., 10, 90.1 to 100% of tap root necrotic. Asterisks represent significant differences relative to the control: *, **, ***, and **** indicates $P < 0.05$, 0.01, 0.001, and 0.0001, respectively; NS = not significant. The experiment-wide error rate was controlled with the Bonferonni correction.

TABLE 2. Adaptation of *Phoma sclerotioides* to low temperatures, as assessed by in vitro growth of *P. sclerotioides* at 3, 10, 18, and 25°C on potato dextrose agar (PDA) 24 days after culture establishment^z

Isolate	3°C (mm)	10°C (mm)	18°C (mm)	25°C (mm)
<i>P. sclerotioides</i> var. <i>sclerotioides</i>				
NY Chz2-14cr	16.62 (2.25) defgh	33.38 (1.25) jk	31.59 (1.58) hi	10.98 (1.81) gh
MT 3-1-5	16.94 (1.91) efghi	36.22 (1.47) l	36.76 (1.37) n	10.82 (1.65) g
NY Chz8-12r	16.85 (1.75) efghi	34.87 (1.61) kl	34.59 (1.46) klm	12.01 (2.21) ghi
NY Chz7-2r	15.67 (1.69) bcde	32.90 (1.61) ij	31.00 (3.06) gh	10.76 (0.79) g
MT 4-4-4	14.49 (1.59) ab	31.46 (1.03) hi	29.34 (3.29) fg	3.15 (0.55) b
SK 2-11	15.98 (1.44) cdef	33.70 (1.43) jk	35.82 (1.92) mn	5.60 (1.14) cd
SK 7-8	15.39 (1.69) bcd	30.54 (1.15) gh	32.79 (2.79) hijk	7.05 (0.88) def
<i>P. sclerotioides</i> var. <i>champlainii</i>				
NY Chz1-7r	15.01 (2.07) abc	30.25 (1.53) fgh	33.62 (3.61) jkl	8.28 (1.24) f
VT 3-2-1-7r	13.78 (1.11) a	29.54 (1.33) fg	33.01 (1.99) ijk	7.32 (1.38) def
<i>P. sclerotioides</i> var. <i>viridis</i>				
NY Chz17-19cr	16.27 (0.78) cdefg	26.16 (1.66) cd	25.37 (2.21) cd	24.55 (1.54) k
MT 3-3-2a	17.25 (0.58) fghij	32.06 (2.46) hij	35.41 (1.90) lmn	21.06 (2.74) j
WI 5-1-5	18.29 (1.00) jk	28.60 (1.13) ef	32.00 (2.14) hij	25.88 (1.28) k
<i>P. sclerotioides</i> var. <i>obscurus</i>				
CO 7-2-10a	17.85 (1.28) hijk	23.35 (2.70) a	23.79 (2.02) c	13.42 (3.24) i
MT 4-4-12	17.74 (1.63) hijk	26.96 (2.47) de	23.62 (2.00) c	6.04 (0.91) cde
<i>P. sclerotioides</i> var. <i>steubenii</i>				
NY Chz10-18r	20.42 (1.54) l	30.3 (3.70) fgh	27.72 (1.66) ef	11.51 (2.38) ghi
ME 1-3-11lf	17.89 (1.40) hijk	30.95 (2.67) gh	27.01 (2.02) de	4.16 (3.27) bc
NH 4-2-1-8r	17.37 (0.73) ghij	26.48 (3.37) cd	26.88 (1.25) de	6.17 (5.64) de
<i>P. sclerotioides</i> var. <i>macrospora</i>				
ME 5-2-5r	18.01 (1.07) ijk	27.03 (1.35) de	24.28 (1.73) c	2.76 (1.46) b
NY Wyo18-1r	18.96 (1.87) k	24.89 (2.13) abc	14.93 (2.21) a	0.27 (0.57) a
<i>P. sclerotioides</i> var. <i>saskatchewanii</i>				
MT 2-1-3	16.10 (0.99) cdefg	24.03 (2.04) ab	18.11 (2.05) b	12.91 (1.17) hi
SK 7-34	14.95 (0.92) abc	25.63 (1.81) bcd	24.44 (1.47) c	7.73 (2.43) ef

^z Mean and standard deviation of radial growth of cultures 24 days after transfer of single conidia to PDA. At each temperature, 36 cultures of each isolate were assessed (nine cultures per isolate in each of four replicates). Within temperature treatments, isolates with shared letters are not significantly different ($\alpha = 0.01$, experiment-wide error rate controlled with the Tukey multiple comparison procedure).

isolates in a single monophyletic group, confirming their close relationship.

We propose designating the genetic and morphological variants of *P. sclerotiioides* as infraspecific varieties. Within the genus *Phoma*, there is a long-standing convention of designating morphological variants of anamorph species as varieties of that species rather than independent species. The variety designation is utilized when variants broadly conform to the morphological characteristics of a species but differ in specific traits, such as temperature adaptation, conidial size or septation, culture morphology in vitro, or production of metabolites (8,34). Infraspecific variety designations are common among *Phoma* spp. (8,34) and variety designations are maintained even when morphological variants are also shown to be genetically differentiated (1). Although infraspecific varieties, not new species, have been designated within *P. sclerotiioides*, the term “species complex” has been retained for describing the seven infraspecific varieties of *P. sclerotiioides* as they relate to causing BRR. Consistent with other species complexes, individual BRR lesions can be caused by a single infraspecific variety of *P. sclerotiioides* or by a combination of infraspecific varieties.

We have proposed the designation of seven varieties within *P. sclerotiioides*, each of which is strongly supported by phylogenetic analyses of multilocus sequence data. The clades corresponding to the infraspecific varieties have strong bootstrap support in MP and ML analyses of a 10-locus sequence data set, and phylogenetic gene-jackknife analyses indicate that this support is driven by a signal across the genome and not by individual loci. Bootstrap support for the infraspecific varieties is also strong in phylogenetic analyses of many single gene partitions and, with only minor exceptions, analyses of single gene partitions did not place isolates of the infraspecific varieties into conflicting clades with strong bootstrap support. Genetic differences among infraspecific varieties correspond to differences in temperature adaptation and morphology. Culture morphology on PDA, pycnidial neck length, conidial length, conidial width, and conidial septation are the most reliable morphological characteristics for delimitation of the varieties. *P. sclerotiioides* var. *sclerotiioides* and *champlainii* produce short conidia and have white aerial mycelium on PDA; *P. sclerotiioides* var. *champlainii* produces narrow conidia and very long necks for spore discharge; *P. sclerotiioides* var. *viridis* produces green aerial mycelium on PDA and grows well at 25°C; *P. sclerotiioides* var. *obscurus* produces dark aerial mycelium on PDA; *P. sclerotiioides* var. *steubenii*, *macrospora*, and *saskatchewanii* produce tan, brown, or gray aerial mycelium on PDA and cause the surrounding agar to turn tan or yellow-brown; *P. sclerotiioides* var. *steubenii* grows more rapidly at 18 and 25°C than var. *macrospora*; and *P. sclerotiioides* var. *macrospora* produces very long conidia, some of which are one-septate, and its growth is nearly completely inhibited at 25°C.

The designation of additional varieties of *P. sclerotiioides* may be warranted in the future. Phylogenetic analyses indicate the existence of at least four additional genetically distinct subtypes of *P. sclerotiioides*, one distinct from the seven proposed varieties of *P. sclerotiioides* and three within those varieties. One isolate, SK 7-12, was closely related to other *P. sclerotiioides* isolates (Figs. 1 and 3) yet genetically too differentiated to be placed within any of the proposed varieties (Fig. 1); and *P. sclerotiioides* var. *sclerotiioides*, *viridis*, and *obscurus* each contain robust, strongly supported subclades (Fig. 1; designated 1a, 3a, and 4a). Variety designations were not assigned to these genetically differentiated subtypes because of limited or incomplete characterization. Evaluation of additional isolates from Saskatchewan is required to evaluate the placement of isolate SK 7-12; in vivo morphological characterization of isolates of subclade 1a was not possible due to permit restrictions on the use of Canadian isolates; and morphological characterization of subclades 3a and 4a was

conducted using only a single isolate from each, thereby precluding an assessment of isolate-isolate variability.

We have chosen to designate the morphologically and genetically distinct subtypes of *P. sclerotiioides* as varieties rather than independent species out of recognition of the subtlety of their morphological differences. In vivo on symptomatic alfalfa, many of the subtypes cannot be readily differentiated; even where statistically significant differences exist, considerable overlap in morphological characteristics is found. Differentiation of the subtypes requires either sequencing at informative loci or conducting a combination of in vitro culturing at 10°C, measurement and assessment of large numbers of conidia and pycnidia, and in vitro growth assays at 18 and 25°C. Maintenance of a broad species concept for *P. sclerotiioides* will facilitate ready identification of the pathogen and minimize confusion associated with improperly identified isolates. Designation of the subtypes as varieties is also consistent with prior precedent in the genus *Phoma* (7,9,13,33). A multiplex diagnostic PCR protocol is under development to facilitate accurate diagnosis of *P. sclerotiioides*; the diagnostic primers currently available for *P. sclerotiioides* (23) occasionally give false negative results and do not distinguish among intraspecific varieties.

The current study provides a framework for investigating differences in agriculturally significant properties of *P. sclerotiioides* isolates. In other diseases caused by *Phoma* spp., genetic differentiation of isolates has been associated with differences in pathogen aggressiveness, disease symptomology, and the timing of disease onset (20,30). Previous research suggests that isolates of *P. sclerotiioides* vary in their aggressiveness to alfalfa (12), and this study indicates that infraspecific varieties of *P. sclerotiioides* differ in their temperature adaptation. Research is currently in progress to evaluate whether infraspecific varieties of *P. sclerotiioides* differ in their aggressiveness to alfalfa roots and crowns, assess whether differences in aggressiveness depend on alfalfa genotype, and evaluate how the composition of the *P. sclerotiioides* species complex differs across North America. *P. sclerotiioides* can also contribute to spring black stem and leaf spot of alfalfa (36) and infect roots of overwintering grasses (11,21), and research is also in progress to evaluate which components of the *P. sclerotiioides* species complex infect roots of overwintering grasses and contribute to foliar disease on alfalfa. Because variation within *P. sclerotiioides* in aggressiveness, host range, geographic distribution, and temperature adaptation could influence alfalfa breeding strategies and crop rotations, the current study provides a framework for investigating the improved management of BRR.

The following descriptions are proposed for the varieties of *P. sclerotiioides*. All isolates identified as representative cultures have been deposited in the University of Minnesota Mycological Culture Collection (University of Minnesota Herbarium) as a resource to alfalfa breeders, pathologists, and other researchers.

***P. sclerotiioides* Preuss ex Sacc. var. *sclerotiioides*.** In radices *M. sativae*, conidia eiseptata, (3.5–)4–5(–5.6) × 1.5–2.7(–3.0) µm. Colla pycnidii vel una vel numerosa, vel glabra vel paullo pilosa; in longitudinem (120–)200–600(–830) µm. In agara solani et sacchari, mycelia aëria alba et sparsa; conidia eiseptata, (2.5–)4.5–6.5(–7.5) × (1.7–)2–3.5 µm. Colla pycnidii vel una vel numerosa, in longitudinem (120–)200–600(–830) µm. Aput 18°C, auctus medius coloniae 29 ad 37 mm post 24 dies. Aput 25°C, auctus medius coloniae 3 ad 12 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate, (3.5–)4–5(–5.6) × 1.5–2.7(–3.0) µm with one to four guttules. Pycnidial necks glabrous to semipilose, single or multiple, with lengths up to (50–)100–700(–800) µm.

In vitro (PDA): Under continuous light at 10°C, aerial mycelium white and sparse. Conidia aseptate, (2.5–)4.5–6.5(–7.5) × (1.7–)2–3.5 µm with one to five guttules. Pycnidial necks up to (120–)200–600(–830) µm long and frequently multiple. After

24 days in complete darkness at 18 and 25°C, average radial growth 29 to 37 mm and 3 to 12 mm, respectively.

Representative cultures. NY Chz2-14cr ex *M. sativa* (Leguminaceae), New York, United States; NY Chz7-2r ex *M. sativa* (Leguminaceae), New York, United States; and SK 7-8 ex *M. sativa* (Leguminaceae), Saskatchewan, Canada.

***P. sclerotoides* var. *champlainii* Wunsch & Bergstrom, var. nov.** In radices *M. sativae*, conidia eiseptata, 4–5.6 × 1.4–1.9 µm. Colla pycnidii plerumque numerosa, vel glabra vel pilosa; in longitudinem (250–)350–720(–810) µm. In agar solani et sacchari, mycelia aerea alba et sparsa vel paullo abunda; conidia eiseptata, (4.3–)5.4–7.2(–7.9) × (1.5–)1.8–2.8(–3.2) µm. Colla pycnidii plerumque numerosa, in longitudinem (300–)400–1,000(–1,100) µm. Aput 18°C, auctus medius coloniae 33 ad 34 mm post 24 dies. Aput 25°C, auctus medius coloniae 7 ad 8 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate, 4–5.6 × 1.4–1.9 µm. Pycnidial necks glabrous to pilose, usually multiple, with lengths up to (250–)350–720(–810) µm.

In vitro (PDA): Under continuous light at 10°C, aerial mycelium white and sparse to moderately abundant. Conidia aspetate, (4.3–)5.4–7.2(–7.9) × (1.5–)1.8–2.8(–3.2) µm. Pycnidial necks up to (300–)400–1,000(–1,100) µm long and usually multiple. After 24 days in complete darkness at 18 and 25°C, average radial growth 33 to 34 mm and 7 to 8 mm, respectively.

Representative culture. NY Chz1-7r ex *M. sativa* (Leguminaceae), New York, United States.

***P. sclerotoides* var. *viridis* Wunsch & Bergstrom, var. nov.** In radices *M. sativae*, conidia eiseptata, (4.2–)4.9–6.8(–7.5) × 1.7–2.5 µm. Colla pycnidii vel una vel numerosa, vel glabra vel paullo pilosa; in longitudinem (100–)150–450(–540) µm. In agar solani et sacchari, mycelia aerea viride et abunda vel paullo abunda; conidia eiseptata, (5.9–)6.5–9.0(–9.6) × (2.0–)2.2–3.5(–3.9) µm. Colla pycnidii vel una vel numerosa, in longitudinem (100–)150–680(–800) µm. Aput 18°C, auctus medius coloniae 25 ad 35 mm post 24 dies. Aput 25°C, auctus medius coloniae 21 ad 26 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate, (4.2–)4.9–6.8(–7.5) × 1.7–2.5 µm. Pycnidial necks glabrous to semipilose, single or multiple, with lengths up to (100–)150–450(–540) µm.

In vitro (PDA): Under continuous light at 10°C, aerial mycelium green to green-gray and moderately abundant to abundant. Conidia aspetate, (5.9–)6.5–9.0(–9.6) × (2.0–)2.2–3.5(–3.9) µm. Pycnidial necks single or multiple and up to (100–)150–680(–800) µm long. After 24 days in complete darkness at 18 and 25°C, average radial growth 25 to 35 mm and 21 to 26 mm, respectively.

Representative cultures. NY Chz17-19cr ex *M. sativa* (Leguminaceae), New York, United States; MT 3-3-2a ex *M. sativa* (Leguminaceae), Montana, United States; WI 5-1-5 ex *M. sativa* (Leguminaceae), Wisconsin, United States.

***P. sclerotoides* var. *obscurus* Wunsch & Bergstrom, var. nov.** In radices *M. sativae*, conidia eiseptata, (4.3–)5.2–6.5(–7.9) × 1.8–2.6 µm. Colla pycnidii vel una vel numerosa, vel paullo pilosa vel pilosa, in longitudinem (90–)150–550(–670) µm. In agar solani et sacchari, mycelia aerea obscura et paullo abunda vel abunda; conidia eiseptata, (5.5–)6.5–9.0(–9.6) × 2.2–3.5(–4.1) µm. Colla pycnidii plerumque numerosa, in longitudinem (150–)260–680(–750) µm. Aput 18°C, auctus medius coloniae 23 ad 24 mm post 24 dies. Aput 25°C, auctus medius coloniae 6 ad 13 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate, (4.3–)5.2–6.5(–7.9) × 1.8–2.6 µm. Pycnidial necks semipilose to pilose, single or multiple, with lengths up to (90–)150–550(–670) µm.

In vitro (PDA): Under continuous light at 10°C, aerial mycelium green-gray to dark gray and moderately abundant to

abundant. Conidia aspetate, (5.5–)6.5–9.0(–9.6) × 2.2–3.5(–4.1) µm. Pycnidial necks up to (150–)260–680(–750) µm long and usually multiple. After 24 days in complete darkness at 18 and 25°C, average radial growth 23 to 24 mm and 6 to 13 mm, respectively.

Representative cultures. CO 7-2-10a ex *M. sativa* (Leguminaceae), Colorado, United States; MT 4-4-12 ex *M. sativa* (Leguminaceae), Montana, United States.

***P. sclerotoides* var. *steubenii* Wunsch & Bergstrom, var. nov.** In radices *M. sativae*, conidia eiseptata, (3.9–)4.2–5.3(–6.2) × (1.8–)2.0–2.9(–3.6) µm. Colla pycnidii vel una vel numerosa, vel paullo pilosa vel pilosa; in longitudinem (100–)160–490(–800) µm. In agar solani et sacchari, mycelia aerea bruna vel cana et sparsa vel paullo abunda. Agarum cingen coloniae flavum vel paullo brunum. Conidia eiseptata, (5.0–)6.2–8.5(–8.9) × (2.3)2.6–3.7(–4.2) µm. Colla pycnidii vel una vel numerosa, in longitudinem (130–)200–530(–750) µm. Aput 18°C, auctus medius coloniae 27 ad 28 mm post 24 dies. Aput 25°C, auctus medius coloniae 4 ad 12 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate, (3.9–)4.2–5.3(–6.2) × (1.8–)2.0–2.9(–3.6) µm. Pycnidial necks semipilose to pilose, single or multiple, with lengths up to (100–)160–490(–800) µm.

In vitro (PDA): Under continuous light at 10°C, aerial mycelium sparse to moderately abundant and tan, brown, or gray. In mature colonies, the agar surrounding the colony is pigmented tan or yellow-brown. Conidia aspetate, (5.0–)6.2–8.5(–8.9) × (2.3)2.6–3.7(–4.2) µm. Pycnidial necks single or multiple and up to (130–)200–530(–750) µm long. After 24 days in complete darkness at 18 and 25°C, average radial growth 27 to 28 mm and 4 to 12 mm, respectively.

Representative cultures. NY Chz10-18r ex *M. sativa* (Leguminaceae), New York, United States; NH 4-2-1-8r ex *M. sativa* (Leguminaceae), New Hampshire, United States.

***P. sclerotoides* var. *macrospora* Wunsch & Bergstrom, var. nov.** In radices *M. sativae*, conidia eiseptata et uniseptata, (4.6–)5.4–6.8(–7.1) × (1.8–)2.1–2.7(–3.2) µm. Colla pycnidii vel una vel numerosa, vel paullo pilosa vel pilosa; in longitudinem (120–)180–410(–480) µm. In agar solani et sacchari, mycelia aerea bruna vel cana et sparsa vel paullo abunda. Agarum cingen coloniae flavum vel paullo brunum. Conidia eiseptata et uniseptata, (6.0–)8.2–11.1(–12.4) × (2.0–)2.3–4.0(–4.3) µm. Colla pycnidii plerumque solitaria, in longitudinem (150–)220–600(–750) µm. Aput 18°C, auctus medius coloniae 15 ad 24 mm post 24 dies. Aput 25°C, auctus medius coloniae 0 ad 3 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate and 1-septate, (4.6–)5.4–6.8(–7.1) × (1.8–)2.1–2.7(–3.2) µm. Pycnidial necks semipilose to pilose, single or multiple, with lengths up to (120–)180–410(–480) µm.

In vitro (PDA): Under continuous light at 10°C, aerial mycelium sparse to moderately abundant and tan, brown, or gray. In mature colonies, the agar surrounding the colony is pigmented tan or yellow-brown. Conidia aspetate and one-septate, (6.0–)8.2–11.1(–12.4) × (2.0–)2.3–4.0(–4.3) µm. Pycnidial necks usually single and up to (150–)220–600(–750) µm long. After 24 days in complete darkness at 18 and 25°C, average radial growth 15 to 24 mm and 0 to 3 mm, respectively.

Representative culture. ME 5-2-5r ex *M. sativa* (Leguminaceae), Maine, United States.

***P. sclerotoides* var. *saskatchewanii* Wunsch & Bergstrom, var. nov.** In radices *M. sativae*, conidia eiseptata, (4.0–)5.0–6.1(–7.1) × 1.7–2.8 µm. Colla pycnidii vel una vel numerosa, vel paullo pilosa vel pilosa; in longitudinem (190–)260–550(–780) µm. In agar solani et sacchari, mycelia aerea bruna vel cana et sparsa vel paullo abunda. Agarum cingen coloniae flavum vel paullo brunum. Conidia eiseptata, (4.0–)6.0–8.6(–9.1) × (1.8–)2.2–3.5(–4.2) µm. Colla pycnidii plerumque solitaria, in longi-

tudinem (110–)200–450(–620) μm . Aput 18°C, auctus medius coloniae 18 ad 24 mm post 24 dies. Aput 25°C, auctus medius coloniae 8 ad 13 mm post 24 dies.

Differentiating characteristics. In vivo (alfalfa roots): Conidia aseptate, (4.0–)5.0–6.1(–7.1) $\times$ 1.7–2.8 μm . Pycnidial necks semipilose to pilose, single or multiple, with lengths up to (190–)260–550(–780) μm .

In vitro (PDA): Under continuous light at 10°C, aerial mycelium sparse to moderately abundant and tan, brown, or gray. In mature colonies, the agar surrounding the colony is pigmented tan or yellow-brown. Conidia aspetate, (4.0–)6.0–8.6(–9.1) $\times$ (1.8–)2.2–3.5(–4.2) μm . Pycnidial necks up to (110–)200–450(–620) μm long and usually single. After 24 days in complete darkness at 18 and 25°C, average radial growth 18 to 24 mm and 8 to 13 mm, respectively.

Representative cultures. MT 2-1-3 ex *M. sativa* (*Leguminaceae*), Montana, United States; SK 7-34 ex *M. sativa* (*Leguminaceae*), Saskatchewan, Canada.

ACKNOWLEDGMENTS

This research was funded in part by Cornell University Hatch Project NYC 153433, the Northern New York Agricultural Development Program, and a Keickhefer Adirondack Fellowship to the senior author. We thank E. Burrichter for her assistance with the laboratory work; R. Korf and K. Hodge (Department of Plant Pathology and Plant-Microbe Biology, Cornell University) for their help with microscopy and pycnidial sectioning; J. Hansen (Department of Plant Breeding and Genetics, Cornell University) for providing seeds of Vernal alfalfa for pathogenicity testing; K. Bassendowski (Agriculture and Agri-Food Canada; Saskatoon, SK), H. Darby (University of Vermont Extension), M. Dillon (Colorado Agricultural Experiment Station, San Luis Valley Research Center), B. Gossen (Agriculture and Agri-Food Canada; Saskatoon, SK), C. Grau (University of Wisconsin-Madison), M. Hall (The Pennsylvania State University), R. Kersbergen (University of Maine), D. Samac (United States Department of Agriculture–Agricultural Research Service, St. Paul, MN), H. Schwartz (Colorado State University), A. Tenuta (Ontario Ministry of Agriculture, Food, and Rural Affairs), R. Torres (New Mexico State University Extension, Taos) for their assistance in survey efforts; extension educators from Cornell Cooperative Extension, Colorado State University Extension, Montana State University Extension, University of New Hampshire Extension, New Mexico State University Extension, The Pennsylvania State University Extension, and University of Wisconsin Extension for their assistance in survey efforts; and the late F. Gray (University of Wyoming) for donating the *P. sclerotoides* Berg isolate.

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