CHARACTERIZATION OF *FUSARIUM OXYSPORUM* AND *PHOMA SCLEROTIOIDES*, PATHOGENS OF BIRDSFOOT TREFOIL AND ALFALFA

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Michael Johannes Wunsch

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CHARACTERIZATION OF *FUSARIUM OXYSPORUM* AND *PHOMA SCLEROTIOIDES*, PATHOGENS OF BIRDSFOOT TREFOIL AND ALFALFA

Michael Johannes Wunsch, Ph. D.

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Soil-borne pathogens causing root and crown rots and vascular wilts are important causes of stand decline of perennial forage legumes. Fusarium wilt of birdsfoot trefoil (*Lotus corniculatus*), caused by an aggressive strain of *Fusarium oxysporum*, is associated with severe vascular discoloration, wilting and chlorosis, and rapid plant death of birdsfoot trefoil. *Phoma sclerotioides*, causal agent of brown root rot (BRR), causes root and crown lesions as alfalfa (*Medicago sativa*) and other perennial legumes emerge from winter dormancy, leading to increased plant mortality, reduced spring regrowth, and diminished yields.

Fusarium wilt of birdsfoot trefoil has been a serious problem for trefoil production in parts of New York and Vermont since the 1970s. Analysis of *F. oxysporum* isolates causing this disease indicated that the pathogen has a unique host range relative to previously designated *F. oxysporum* formae speciales. Vegetative compatibility analysis and phylogenetic analysis of multilocus sequence data suggest that the pathogen is monophyletic. We propose designating the fungus *F. oxysporum* f. sp. *loti*.

P. sclerotioides has long been known to be an important constraint to alfalfa production in Alaska and central and western Canada. In eastern North America, it

has been reported only in Nova Scotia. Surveys of alfalfa production fields in Maine, New Hampshire, New York, and Ontario in 2004, 2005, and 2007 indicate that the pathogen is widespread in eastern North America, often occurring at high incidence levels. Patterns of infected alfalfa within fields suggest that the pathogen was not recently introduced. Surveys of alfalfa production fields in Colorado, New Mexico, and Pennsylvania conducted in 2006 and 2007 suggest that central Pennsylvania and high mountain valleys at the latitude of northern New Mexico may delineate the southern extent of the distribution of *P. sclerotioides* within alfalfa production regions of eastern and western North America, respectively. Phylogenetic analyses of multilocus sequence data places North American isolates of *P. sclerotioides* into multiple strongly supported clades, and morphological differences among isolates correspond to genetic differences. On the basis of genetic and morphological differences, we established seven infraspecific varieties within *P. sclerotioides*: *P. sclerotioides* var. *sclerotioides, champlainii, viridis, obscurus, steubenii, macrospora,* and *saskatchewanii*.

BIOGRAPHICAL SKETCH

Michael was born in Erie, Pennsylvania, and he grew up in Pennsylvania, Arizona, and Montana. He received a B.S. in biology and a B.A. in economics from the University of Missouri, Columbia in August 2000. Subsequent to his undergraduate studies, Michael served with the U.S. Peace Corps in the small community of El Cantoral, D.C., F.M. in central Honduras, where he facilitated efforts by vegetable crop growers to diversify their production and sell directly to supermarkets. In August 2003, Michael began his graduate work in plant pathology at Cornell University, where his research has focused on soil-borne fungal pathogens. At Cornell, Michael was an active member of the plant pathology graduate student association, helping with graduate student recruitment and other activities, and served as a teaching assistant in university's introductory biology program for multiple years, instructing the laboratory component of introductory biology courses for both biology majors and non-majors. He is a member of the American Phytopathological Society and has presented his doctoral research at annual meetings of the American Phytopathological Society and the North American Alfalfa Improvement Conference.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

FUSARIUM WILT OF BIRDSFOOT TREFOIL AND BROWN ROOT ROT OF ALFALFA AND OTHER PERENNIAL LEGUMES: BACKGROUND AND RESEARCH OBJECTIVES

1.1 PERENNIAL FORAGE LEGUMES

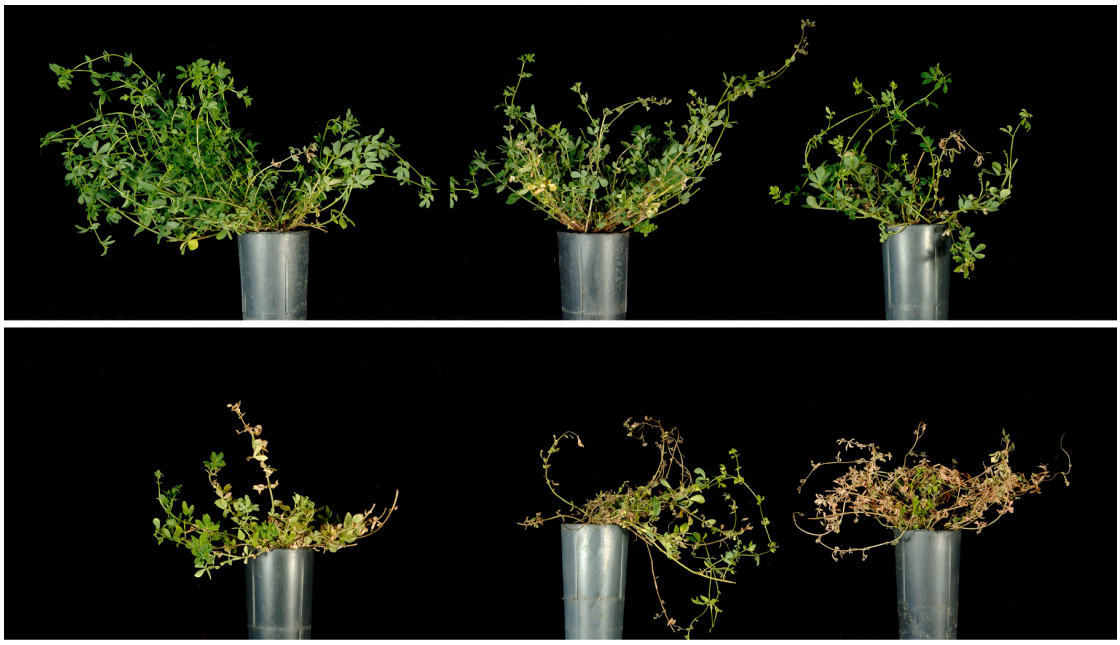
Perennial forage legumes, valued as livestock feed for their high nutritional content, are important to the dairy economy of the northeastern United States. As perennials that form symbioses with nitrogen-fixing *Rhizobium* bacteria, forage legumes have lower fertilization and/or stand establishment costs than comparable feed sources such as perennial grasses and corn, and they serve as key rotational crops that help replenish the soil after multiple seasons of seedings to annual crops. Alfalfa (*Medicago sativa* L), the most widely planted forage legume, is planted on nearly 2.5 million acres in the Northeast. Seeded either by itself or in a mixture with grasses, alfalfa is the most widely planted crop in Massachusetts, New York, and Vermont and the second most widely planted crop in Pennsylvania (NASS 2002). Alfalfa, high in crude protein and rich in vitamins and minerals, is an integral ingredient in the dietary ration of dairy cattle, as well as an important feed source for other livestock (Orloff et al. 1995). Other perennial legumes planted for forage in the Northeast include red clover (*Trifolium pratense* L.) and birdsfoot trefoil (*Lotus corniculatus* L.). Birdsfoot trefoil, valued for its production of condensed tannins that prevent bloat and improve the efficiency of protein utilization in ruminant animals (Kendall 1966, Terrill et al.

1992), is particularly well adapted to poorly drained, acidic, and drought-prone soils not suited for alfalfa production (Chevrette et al. 1960, Heinrichs 1970, Keeney 1985).

Profitable production of forage legumes depends on successful stand establishment and stand persistence. Stand establishment costs are high for alfalfa, birdsfoot trefoil and other perennial legumes, and three to four years of harvests are generally needed to provide adequate returns. Soil-borne pathogens are particularly important agents of stand decline (Orloff et al. 1995); as causal agents of root and crown rots and of vascular wilts, they reduce the vigor of infected plants and lower overall stand density, requiring that stands be renewed frequently. Soil-borne pathogens can also contribute to plant mortality when alfalfa is dormant during winter and when alfalfa emerges from winter dormancy, both by predisposing plants to weather-related plant stresses and by further weakening plants already injured by winter conditions.

1.2 FUSARIUM WILT OF BIRDSFOOT TREFOIL

Fusarium wilt of birdsfoot trefoil, caused by the soilborne fungal pathogen *Fusarium oxysporum*, is associated with high rates of plant mortality in the seeding year, causing rapid stand decline of birdsfoot trefoil (Gotlieb and Doriski 1983, Murphy et al. 1985, Tillapaugh 1991). Vascular tissues of infected plants are colonized by *F. oxysporum,* leading to chlorosis, stunting and wilting, severe vascular discoloration, and death of birdsfoot trefoil (Figures 1.1 and 1.2), often within weeks of planting. Problems with Fusarium wilt contributed to a sharp reduction in acreage planted to birdsfoot trefoil in the Champlain Valley of New York and Vermont in the 1970s and early 1980s (Murphy et al. 1985), a region that had previously produced



A typical progression of wilt symptoms on birdsfoot trefoil.

FIGURE 1.1 Wilting and chlorosis of foliar tissues associated with Fusarium wilt of birdsfoot trefoil.

FIGURE 1.2 Vascular necrosis of root tissues associated with Fusarium wilt of birdsfoot trefoil.

most of the certified birdsfoot trefoil seed in the United States (Seaney and Henson 1970). Fusarium wilt continues to limit birdsfoot trefoil production in New York and Vermont today (Bergstrom et al. 1995, Litchfield-Kimber 1996, Tillapaugh 1991, Wunsch pers. obs.).

1.3 BROWN ROOT ROT OF PERENNIAL FORAGE LEGUMES

Phoma sclerotioides G. Preuss ex Sacc. (syn. *Plenodomus meliloti* Dearn. & G. B. Sanford), the causal agent of brown root rot (BRR) of alfalfa and other perennial legumes, is a low-temperature fungal pathogen that causes root and crown lesions (Figure 1.3) as plants emerge from winter dormancy. BRR is associated with alfalfa yield loss, slow emergence from winter dormancy and stand decline, and it causes sporadic but severe plant mortality in alfalfa production fields (Berkenkamp et al. 1991, Hollingsworth et al. 2003). BRR has long been known to be a constraint to

FIGURE 1.3 Characteristic symptoms of brown root rot of alfalfa, caused by *P. sclerotioides.*

alfalfa production in Alaska and western Canada, and it is particularly severe in central and northern Alberta, Manitoba, Saskatatchewan, and the Yukon (Davidson 1990). It was not known to occur in the contiguous United States until it was found in Wyoming in 1996 (Hollingsworth and Gray 1999). *P. sclerotioides* was first detected in the eastern United States in 2003 in Clinton County, NY when a lesioned root tested positive for the pathogen.

1.4 DISEASE MANAGEMENT

Fusarium wilt and brown root rot must be managed by host resistance. The application of fungicides is noneconomical in forage crop production, and crop rotation is not effective for either disease. *P. sclerotioides* and *F. oxysporum* have broad host ranges; *P. sclerotioides* infects the roots of winter wheat, perennial grasses and other perennials (Davidson 1990), and strains of *F. oxysporum* that cause vascular wilt on a specific host can colonize roots of other hosts either endophytically without causing symptoms or pathogenically by causing a range of symptoms from root and crown necrosis to vascular wilt (Armstrong and Armstrong 1975, Gordon et al. 1989, Wang and Jeffers 2000). *P. sclerotioides* and *F. oxysporum* can also colonize plant debris, and *P. sclerotioides* forms resting structures that permit it to persist in the soil in the absence of a suitable substrate for growth (Sanford 1933).

1.5 OBJECTIVES OF DISSERTATION RESEARCH

The objectives of my dissertation research were to characterize the distribution and impact of *P. sclerotioides* in the northeastern United States and other parts of North America and to characterize populations of *F. oxysporum* causing wilt of

birdsfoot trefoil and *P. sclerotioides*. The goals of my work with *P. sclerotioides* were to (1) characterize the distribution and incidence of the pathogen in alfalfa production regions of the northeastern United States and parts of eastern Canada, (2) identify the southern extent of distribution of *P. sclerotioides* within the eastern and western United States, (3) evaluate the genetic diversity of *P. sclerotioides* and whether genetic differences correspond to differences in morphology. The goals of my work with *F. oxysporum* were to (1) evaluate the distribution of *F. oxysporum* strains causing wilt of birdsfoot trefoil, (2) characterize the aggressiveness and genetic relatedness of geographically diverse isolates of the *F. oxysporum* wilt pathogen, and (3) determine whether the wilt pathogen warrants designation as a unique forma specialis of *F. oxysporum.*

alfalfa

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CHAPTER 2

CHARACTERIZATION OF *FUSARIUM OXYSPORUM* F. SP. *LOTI* FORMA SPECIALIS NOV., A MONOPHYLETIC PATHOGEN CAUSING VASCULAR WILT OF BIRDSFOOT TREFOIL*

2.1 ABSTRACT

A vascular wilt caused by *Fusarium oxysporum* has been a serious problem for birdsfoot trefoil (*Lotus corniculatus*) production in parts of New York and Vermont, USA since the 1970s, causing wilt, severe root necrosis, and rapid plant death. Analysis of *F. oxysporum* isolates causing this disease indicated that the pathogen has a unique host range relative to previously designated *F. oxysporum* formae speciales and is monophyletic. Pathogenic isolates from New York and Vermont caused severe vascular wilt of trefoil and moderate vascular wilt of pea but no disease on alfalfa, red clover, soybean or dry bean. The host range of trefoil isolates was distinct from *F. oxysporum* isolates pathogenic to other legumes. *F. oxysporum* isolates pathogenic to trefoil belonged to a single vegetative compatibility group separate from nonpathogenic isolates and shared identical mitochondrial small subunit rDNA, translation elongation factor 1-alpha, and nuclear rDNA intergenic spacer haplotypes. Phylogenetic analysis of the concatenated sequence data assigned isolates pathogenic to trefoil to a single, well-supported clade distinct from other pathogenic *F.*

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oxysporum. We propose designating the fungus *Fusarium oxysporum* Schlechtendahl emend. Snyder & Hansen f. sp. *loti* forma specialis nova.

2.2 INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum*, has been a serious problem for birdsfoot trefoil (*Lotus corniculatus* L.; BFT) production in parts of New York and Vermont since the 1970s. BFT is a forage legume valued for its production of condensed tannins (Terrill et al. 1992), which prevent bloat and improve the efficiency of protein utilization in ruminant animals (Kendall 1966), and for its adaptation to soils and field conditions poorly suited for alfalfa production (Chevrette et al. 1960, Heinrichs 1970, Keeney 1985). Through the early 1970s, BFT was widely planted in the Champlain Valley of New York and Vermont for the production of certified seed, and BFT continues to be planted in New York and Vermont for forage production. Reduced persistence of BFT contributed to a sharp reduction in acreage planted to BFT in the Champlain Valley in the 1970s and early 1980s (Murphy et al. 1985), and problems with rapid stand decline continue to limit BFT production in New York and Vermont (Bergstrom et al. 1995, Litchfield-Kimber 1996, Tillapaugh 1991; Wunsch, pers. obs.). In affected fields, BFT seedlings display chlorosis, stunting and wilting, severe vascular discoloration, and plant death within weeks of planting. Vascular tissues of infected plants are colonized by *F. oxysporum*, which is readily isolated from both roots and stems, and high rates of BFT mortality are observed in the seeding year (Gotlieb and Doriski 1983, Murphy et al. 1985, Tillapaugh 1991). Fusarium wilt has certain similarities to a *Fusarium* root and crown rot of BFT described in Argentina and Uruguay (Altier 1997a, Chao et al. 1992) but progresses more rapidly.

The disease prevalent in South America is also characterized by vascular wilt and severe root necrosis caused by *F. oxysporum*, but the wilt symptoms develop more slowly, with significant plant mortality not observed until the second and third production years (Altier 1997b, Altier et al. 2000). Fusarium wilt is distinct from general root and crown rot of BFT (Berkenkamp et al. 1972, Drake 1958), which is associated with a complex of pathogens, including various *Fusarium* species, and does not lead to vascular wilt. Fusarium wilt of BFT is only known to occur in New York and Vermont, USA.

F. oxysporum is a ubiquitous fungus in both agricultural and nonagricultural soils worldwide (Gordon and Martyn 1997). It can colonize plant debris, but it is generally found in close association with plant roots (Windels and Kommedahl 1974). *F. oxysporum* can colonize roots endophytically without causing disease symptoms, colonize roots pathogenically and contribute to root and crown rot, or colonize the vascular system and cause severe root necrosis, vascular wilt, and rapid plant death (Armstrong and Armstrong 1975, Gordon et al. 1989, Wang and Jeffers 2000). Isolates causing vascular wilt are host-specific, causing disease on only one to several plant species and colonizing other species endophytically or not at all (Armstrong and Armstrong 1975, Gordon et al. 1989). Pathogenic *F. oxysporum* isolates differing in host range are morphologically indistinguishable and are organized into specialized forms, or formae speciales, on the basis of the host(s) on which they cause vascular wilt (Armstrong and Armstrong 1975).

Bergstrom and Kalb (1995) proposed that the Fusarium wilt pathogen of BFT found in New York be designated a new forma specialis, *F. oxysporum* f. sp. *loti*. No previously designated formae speciales are known to have a host range encompassing

BFT (Armstrong and Armstrong 1981), and preliminary greenhouse experiments with a single monoconidial isolate from New York suggested the pathogen was specific to BFT. However, host range experiments were not repeated, and the proposed taxonomic designation could not be finalized.

Management of Fusarium wilt of BFT has been limited by a lack of resistant cultivars. A greenhouse evaluation of five commercially available BFT cultivars indicated that all were highly susceptible to Fusarium wilt (Zeiders and Hill 1988). Likewise, field and greenhouse trials conducted in the 1990s indicated that widely planted Norcen and 11 other BFT cultivars available to growers all exhibited high susceptibility to Fusarium wilt (Bergstrom and Kalb 1995). A cultivar with moderate resistance to Fusarium wilt was released in 2004 (Viands et al. 2004), and efforts to breed BFT with improved resistance to Fusarium wilt are ongoing. The breeding efforts, however, have utilized a single pathogenic isolate to screen for resistance, and the appropriateness of this protocol has not been evaluated. Many formae speciales of *F. oxysporum* are composed of two or more clonal evolutionary lineages (Baayen et al. 2000, Mbofung et al. 2007, O'Donnell et al. 1998, Skovgaard et al. 2001), and clonal lineage is closely related to pathogenic race in multiple formae speciales of *F. oxysporum* (Kim et al. 2005, Mbofung et al. 2007, Ploetz and Pegg 2000, Skovgaard et al. 2001). Because races differ in their virulence to specific host genotypes, use of a single pathogenic isolate to breed for resistance is only appropriate when multiple races of the pathogen are not present. The genetic diversity of the pathogen causing Fusarium wilt of BFT has not been evaluated, and it is unclear whether the BFT pathogen is composed of a single or multiple evolutionary lineages across its known geographic distribution. If multiple evolutionary lineages are present, differences in

pathogenic properties among the isolates are more likely, and the use of a multiple isolates, one from each lineage, may be needed to screen for host resistance.

The objectives of this study were to evaluate whether the Fusarium wilt pathogen of BFT warrants designation as a forma specialis nova and to assess the genetic diversity of the pathogen population across the pathogen's known geographic distribution. Replicated host range experiments were conducted to assess the taxonomic designation of the BFT pathogen; the forma specialis concept in *F. oxysporum* is based on unique host range, and cross-inoculation experiments utilizing multiple hosts and multiple formae speciales of *F. oxysporum* are required to demonstrate that a Fusarium wilt pathogen warrants designation as a forma specialis nova (Armstrong and Armstrong 1975). Vegetative compatibility group (VCG) analysis (Correll et al. 1987) and multilocus sequence typing (MLST) were conducted to evaluate the genetic diversity of the BFT pathogen population. Both techniques have proven useful for identifying clonal lineages of *F. oxysporum* (Baayen et al. 2000, Koenig et al. 1997, Mbofung et al. 2007, O'Donnell et al. 2004). MLST analysis was conducted using three loci widely used in intraspecific studies of *F. oxysporum* (Mbofung et al. 2007, O'Donnell et al. 1998, O'Donnell et al. 2004): the complete intergenic spacer (IGS) of the nuclear rDNA repeat, an intron-spanning region of the translation elongation factor 1-alpha (EF-1α), and the mitochondrial small subunit rDNA (mtSSU). For illustrative purposes, the MLST data were combined with publicly available sequences generated by previous researchers and presented as a phylogeny; maximum parsimony was used for phylogenetic inference.

2.3 MATERIALS AND METHODS

***F. oxysporum* isolates used in this study.** *F. oxysporum* cultures isolated from symptomatic BFT in 1985, 1990, 1991, 1995, and 2004 were utilized in this study. Cultures were either isolated directly from symptomatic BFT collected in commercial production fields or were baited from naturally infested field soil. Twenty-one BFT production fields were evaluated for symptomatic BFT, and soil samples were collected from an additional four fields with a history of BFT production. Baiting was conducted by seeding the susceptible BFT cultivar Georgia-1 (Deer Creek Seed, Ashland, WI) into a mix of field soil and autoclaved sand (1:1, v/v) in greenhouse flats and evaluating plants for vascular wilt weekly for eight weeks. *F. oxysporum* isolates were collected from BFT throughout the known geographic distribution of the Fusarium wilt pathogen in New York and Vermont. Monoconidial cultures were established from each isolate and assessed for morphological characteristics on carnation leaf agar (CLA) and for gross cultural characteristics on potato dextrose agar (PDA; Difco Laboratories, Becton, Dickson and Co., Sparks, MD); cultures were grown 7 days (on PDA) or 14 days (on CLA) at approximately 25 C under a mix of fluorescent cool white and ultraviolet light (12 hr/day).

Pathogenicity Trial. All single-conidium cultures identified as *F. oxysporum* were assessed for pathogenicity to BFT in the greenhouse. Individual trefoil plants (cv. Georgia-1) were established in cells of greenhouse flat inserts (806 narrow series deep insert; Hummert International, Earth City, MO) in a 1:2:1 mix (by volume) of peat moss, autoclaved sand, and autoclaved loam soil. Three to four seeds were placed in each pot, and seeds were covered with approximately 0.1 g of *Rhizobium* inoculant for birdsfoot trefoil (Trace Chemicals LLC, Pekin, IL). Three weeks after

seeding, plants were thinned to one plant per pot. Plants were grown at approximately 25 to 35°C in the greenhouse under 16 hours of light/day and fertilized monthly with a 200 ppm solution of 21-5-20 fertilizer (364 g/gal) and Epsom salts (113 g/gal). Plants were inoculated 12 to 13 weeks after seeding using a procedure adapted from pathogenicity assays developed for other *F. oxysporum* wilt pathogens (Brumrner and Nygaard 1995, Pastor-Corrales and Abawi 1987, Venuto et al. 1995). Inoculum was prepared by growing cultures in Czapek-Dox broth (C-Dox; Difco Laboratories, Becton, Dickson and Company, Sparks, MD) on a rotating shaker (150 revolutions/min) for three to four days and passing the cultures through two layers of cheese cloth. Foliage was trimmed approximately 6 cm above the crown, plants were removed from the pots, and the root mass (complete with its associated soil) was severed 4 to 5 cm below the crown. The lower portion of the root mass was returned to the pots, the upper portion was soaked for 30 minutes in a conidial suspension adjusted to 1 x 10^6 spores/ml or in a water or dilute C-Dox (1:3 C-Dox to water, v/v) control, and plants were repotted. In every replicate of the experiment, 64 unique isolates and two control treatments were assessed, and each treatment was evaluated on 12 plants. The experiment was repeated three times. The spatial organization of treatments was randomized within each replicate. Plants were assessed for wilting, chlorosis and stunting once to twice weekly after repotting. Stems of symptomatic plants were collected at least 1 cm above the crown, stripped of leaves, surface sterilized in 0.6% hypochlorite and 70% ethanol, cut into segments, and plated onto ¼-PDA. Cultures growing from the ends of stem segments and displaying gross morphological characteristics typical of *F. oxysporum* were transferred to a modified Nash-Snyder *Fusarium*-selective media (FSM) prepared as described by Nelson et al.

(1983) but with 0.3 g/L streptomycin sulfate and 0.175 g/L neomycin sulfate. A plant was considered positive for Fusarium wilt only if cultures isolated from the ends of stem segments grew on FSM. Fourteen weeks after inoculation, plants were uprooted, and the first 4 cm of each plant's tap root was split longitudinally to evaluate root necrosis. Necrosis was rated on a scale of 0 to 4: 0, no discoloration of stele; 1, small dark specks or strands in the stele (0.1 to 33% of stele necrotic); 2, moderate to large dark strands forming arcs or rings in the stele cross-section (33.1% to 66% of stele necrotic); 3, extensive discoloration of the stele (66.1 to 99% of stele necrotic); 4, stele completely necrotic and plant dead (100% necrosis). Isolates were considered pathogenic to trefoil if they caused Fusarium wilt in at least two of the three replicates of the experiment and were considered nonpathogenic if they failed to cause Fusarium wilt in any replicate.

Host range. Cross-inoculations were conducted in the greenhouse with economically important legumes grown in New York and, when available, corresponding pathogenic *F. oxysporum* strains. Birdsfoot trefoil (cv. Georgia-1; Deer Creek Seed, Ashland, WI), red clover (*Trifolium pretense,* cv. Chesapeake; H. Riday, USDA Dairy Research Center, Madison, WI), alfalfa (*Medicago sativa,* cv. MNGN-1; USDA Western Regional Plant Introduction Station, Pullman, WA), soybean (*Glycine max,* cv. Essex; K. Rainey, Virginia Tech, Blacksburg, VA), pea (*Pisum sativum,* cv. M410; Brotherton Seed, Moses Lake, WA), and bean (*Phaseolus vulgaris,* cv. U.I. 114; S. Singh, University of Idaho Research and Extension Center, Kimberly, ID) were tested. All cultivars have documented susceptibility to *F. oxysporum* (Brumrner and Nygaard 1995, Kraft 1994, Salgado et al. 1994, Sandler et al. 1988, Venuto et al. 1995). One isolate each of *F. oxysporum* f. sp. *medicaginis* (Fom004, from

Pennsylvania), *F. oxysporum* f. sp. *pisi* (Fopi001, from New York), and *F. oxysporum* pathogenic to red clover (Fo062, from Wisconsin), and five *F. oxysporum* isolates pathogenic to BFT, including isolates from both New York (Fo012, Fo069, FoMK61) and Vermont (FoVt3a and FoVt71), were evaluated. Dilute C-Dox broth (1:3 C-Dox to water, v/v) was used as a control. Individual plants were established in SC10 Cone-Tainers (3.8 cm diameter, 21 cm deep; Stuewe and Sons, Inc, Corvallis, OR) in a 1:2:1 mix (by volume) of peat moss, autoclaved sand, and autoclaved loam soil. Three to four seeds were placed in each pot, and seeds were covered with approximately 0.1 g of the appropriate *Rhizobium* inoculant (Trace Chemicals LLC, Pekin, IL). One to three weeks after seeding, plants were thinned to one plant per pot. A 200 ppm solution of 21-5-20 fertilizer (96 g/liter) and Epsom salts (30 g/liter) was applied monthly. The plants were grown in the greenhouse at approximately 25 to 35°C under 16 hours of light/day.

Protocols specific to each host were used for inoculations (Brumrner and Nygaard 1995, Ferrant and Carroll 1981, Kraft 1994, Pastor-Corrales and Abawi 1987, Venuto et al. 1995). Spore suspensions were established with C-Dox broth, as described above. Individual trefoil, alfalfa, clover, bean, pea, and soybean plants were inoculated with spore suspensions ($1x10^6$ spores/ml) and the C-Dox control 12 weeks, 10 weeks, 6 weeks, 10 days, 10 days, and 8 days after seeding, respectively. Planting dates were coordinated such that inoculations of the different hosts were conducted concurrently in each replicate of the experiment. Prior to inoculation, foliage was trimmed approximately 8 to 10 cm above the crown for alfalfa, clover, and trefoil but not trimmed for pea, bean or soybean. Plants were removed from their pots, and the lower third to quarter of the roots were removed. Soil was shaken free of alfalfa,

clover, pea, bean and soybean but not trefoil roots. Trefoil, alfalfa, and clover were soaked in spore suspensions or the C-Dox control for 30 min; bean and soybean plants, for 5 min; and pea plants, for 1-2 min. Twelve plants of each host species were subjected to each treatment in each replicate of the experiment; the experiment was repeated three times. The spatial organization of treatments within each replicate was randomized.

Plants were assessed for wilting, chlorosis and stunting once to twice weekly after repotting. Stems of symptomatic plants were collected at least 1 cm above the crown, surface sterilized, and assessed for *F. oxysporum*, as conducted during pathogenicity testing (above). A plant was considered positive for *Fusarium* wilt only if cultures isolated from the ends of stem segments grew on FSM. Root necrosis was evaluated 65 days after inoculation by splitting the tap root of each plant longitudinally. Necrosis was rated on a scale of 0 to 5: 0, no root necrosis; 1, small dark specks or strands in the stele (0.1 to 10% necrosis); 2, moderate dark strands forming small arcs or rings in the stele cross-section (10.1 to 35% necrosis); 3, large dark strands in the stele (35.1 to 65% necrosis); 4, most of stele discolored (65.1 to 90% necrosis); 5, stele and part or all of cortex discolored and/or plant dead (90.1 to 100% necrosis).

Statistical analysis. To evaluate the incidence of *Fusarium* wilt of inoculated versus control treatments in the host range experiment, contingency tables were constructed for the binary wilt response across hosts and treatments within each experimental replicate. Heterogeneity chi-square analysis (Zar 1999) resulted in a failure to reject the null hypothesis that the replicates of the experiment were homogeneous ($\chi^2 = 105.87$, df = 105, p = 0.457), and results were pooled across

replicates. The proportion of wilted plants was calculated for each treatment, and the proportions were arcsine transformed (Zar 1999). The transformed data were analyzed with the Dunnett test for multiple comparisons, except that rather than comparing all treatments to each other, only comparisons between the control (C-Dox) and the inoculated treatments were made (Zar 1999).

Root necrosis data were analyzed with cumulative logistic regression (Hosmer and Lemeshow 2000). Heterogeneity chi-square analysis (Zar 1999) resulted in a rejection of the null hypothesis that experimental replicates were homogeneous (χ^2 = 335.62, df = 256, p = 0.0006, pathogenicity testing experiment; χ^2 = 587.01, df = 525, p = 0.0312, host range experiment), and results were not pooled across replicates. Logistic regression was conducted controlling for the effect of experimental replicate. Individual contrasts of pairs of treatments were performed with Wald chi-square tests. In the pathogenicity testing experiment, contrasts were established between the C-Dox control and each of 32 other treatments (28 pathogenic isolates, 3 nonpathogenic isolates, and the water control). In the host range experiment, contrasts were established between the C-Dox control and each inoculated treatment for each host. All analyses were implemented in PROC GENMOD (SAS, version 9.1, SAS Institute, Cary, NC). The Bonferroni multiple comparison procedure (Neter et al. 1996) was used to control the Type I error rate at the level of the experiment across the 32 contrasts conducted in the pathogenicity testing experiment and the 48 contrasts conducted in the host range experiment.

Vegetative compatibility. Nitrogen nonutilizing (*nit*) mutants were produced as previously described (Correll et al. 1987, Puhalla 1985) for 31 *F. oxysporum*

TABLE 2.1 Geographic origin, vegetative compatibility group, and pathogenicity of *Fusarium oxysporum* isolates from birdsfoot trefoil used in this study.

			Pathogenicity [c]		
Isolate [a]	**Origin (year isolated)**	**VCG [b]**	Wilt	Necrosis	**Designation [d]**
Fo012	Wyoming Co., NY (1990)	0480	8/36	1.31 ***	*Fusarium oxysporum* f. sp. *loti*
Fo036	Essex Co., NY (1985)	0480	2/36	0.81 *	*F. oxysporum* f. sp. *loti*
Fo054	Wyoming Co., NY (1991)	0480	3/36	1.08 ***	*F. oxysporum* f. sp. *loti*
Fo055	Wyoming Co., NY (1991)	0480	3/36	0.75 *	*F. oxysporum* f. sp. *loti*
Fo068	Wyoming Co., NY (1995)	0480	5/36	1.22 **	*F. oxysporum* f. sp. *loti*
Fo069	Wyoming Co., NY (1995)	0480	12/36	1.50 ****	*F. oxysporum* f. sp. *loti*
Fo070	Wyoming Co., NY (1995)	0480	4/36	1.32 ***	*F. oxysporum* f. sp. *loti*
Fo071	Wyoming Co., NY (1995)	0480	5/36	1.20 **	*F. oxysporum* f. sp. *loti*
Fo072	Wyoming Co., NY (1995)	0480	7/36	1.28 ***	*F. oxysporum* f. sp. *loti*
FoFT1a	Wyoming Co., NY (2004)	0480	7/36	1.17 ***	*F. oxysporum* f. sp. *loti*
FoFT3a1	Wyoming Co., NY (2004)	0480	11/36	1.37 ****	*F. oxysporum* f. sp. *loti*
FoGF3c	Tompkins Co., NY (2004)	0480	5/36	1.33 ****	*F. oxysporum* f. sp. *loti*
FoMK41	Wyoming Co., NY (2004)	0480	4/36	1.39 ****	*F. oxysporum* f. sp. *loti*
FoMK61	Wyoming Co., NY (2004)	0480	9/36	1.65 ****	*F. oxysporum* f. sp. *loti*
FoMK7b1	Wyoming Co., NY (2004)	0480	7/36	1.31 ***	*F. oxysporum* f. sp. *loti*
FoPlc1b	Wyoming Co., NY (2004)	0480	4/36	1.28 ***	*F. oxysporum* f. sp. *loti*
FoPlc14a	Wyoming Co., NY (2004)	0480	4/36	1.06 **	*F. oxysporum* f. sp. *loti*
FoVH52	Wyoming Co., NY (2004)	0480	2/36	0.89 **	*F. oxysporum* f. sp. *loti*
FoVH7c1	Wyoming Co., NY (2004)	0480	6/36	0.86 *	*F. oxysporum* f. sp. *loti*
FoVH17a	Wyoming Co., NY (2004)	0480	7/36	1.65 ****	*F. oxysporum* f. sp. *loti*
FoVt3a	Addison Co., VT (2004)	0480	2/36	1.08 ***	*F. oxysporum* f. sp. *loti*
FoVt63	Addison Co., VT (2004)	0480	7/36	1.09 ***	*F. oxysporum* f. sp. *loti*
FoVt71	Addison Co., VT (2004)	0480	7/36	1.17 ***	*F. oxysporum* f. sp. *loti*
FoVt81	Addison Co., VT (2004)	0480	2/36	1.06 **	*F. oxysporum* f. sp. *loti*
FoVt112b	Addison Co., VT (2004)	0480	4/36	1.20 ***	*F. oxysporum* f. sp. *loti*
FoVt12b	Addison Co., VT (2004)	0480	4/36	0.81 *	*F. oxysporum* f. sp. *loti*
FoVt14	Addison Co., VT (2004)	0480	2/36	0.58 NS	*F. oxysporum* f. sp. *loti*
FoVt17a	Addison Co., VT (2004)	0480	4/36	1.03 **	*F. oxysporum* f. sp. *loti*
FoChz10c	Clinton Co., NY (2004)	Unique	0/36	0.08 NS	*F. oxysporum*
FoStL1a	St. Lawrence Co., NY (2004)	Unique	0/36	0.11 NS	*F. oxysporum*
FoVal42	Columbia Co., NY (2004)	Unique	0/36	0.03 NS	*F. oxysporum*

[a] All isolates were obtained from roots or stems of birdsfoot trefoil.

[b] Vegetative compatibility group; "unique" indicates that the isolate is vegetatively compatible with itself but with none of the other isolates listed.

[c] Proportion of plants exhibiting *Fusarium* wilt within 98 days of inoculation and mean root necrosis of plants 98 days after inoculation in a replicated greenhouse experiment with the susceptible trefoil cultivar Georgia-1. The root mass was cut 4 to 5 cm below the crown and soaked 30 min in a suspension of 1.0×10^6 spores/ml. Two control treatments, water and dilute Czapek-Dox broth, were included. Plants were only considered positive for *Fusarium* wilt if *Fusarium* sp. was reisolated from surface sterilized sections of symptomatic stems collected at least 1 cm above the crown. Root necrosis was rated on a 0 to 4 scale: 0, no root necrosis; 1, small dark specks or strands in the stele; 2, moderate to large dark strands forming arcs or rings in the stele cross-section; 3, extensive discoloration of the stele; 4, stele completely necrotic and plant dead. Asterisks represent a significant difference relative to the control ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ for one, two, three and four asterisks, respectively); "NS" represents no significant difference ($P>0.05$) relative to the control. Of the 36 plants subjected to each control treatment, none developed vascular wilt, and mean root necrosis was 0.06.

[d] All isolates designated *F. oxysporum* f. sp. *loti* caused *Fusarium* wilt in one or more of 12 plants in at least two of the three replicates of the experiment assessing pathogenicity.

isolates: 28 isolates pathogenic to trefoil and three isolates (FoChz10c, FoStL1a and FoVal42) obtained from trefoil but nonpathogenic to trefoil (Table 2.1). Reciprocal pairwise crosses were established on minimal media between *nit1* and NitM mutants for nine of the isolates, six pathogenic isolates (Fo036, Fo068, FoMK41, FoPlc14a, FoVH17a and FoVt63) and three nonpathogenic isolates (FoChz10c, FoStL1a and FoVal42). Tester NitM strains were selected representing each unique vegetative compatibility group identified in the reciprocal crosses, and *nit*1 mutants of the remaining 22 pathogenic trefoil isolates were crossed with each tester strain.

DNA extraction, amplification, purification and sequencing. *F. oxysporum* isolates were grown 7 days at room temperature on ¼-PDA, transferred to 50 ml centrifuge tubes containing 35 ml half-strength potato dextrose broth (½-PDB; 12 grams PDB/liter water), and placed on a rotary shaker (150 revolutions/min) for 4 to 5 days. The mycelium was harvested by centrifuging, decanting the broth, and rinsing twice in sterile distilled water (adding 35 ml water, centrifuging, and decanting). The rinsed mycelium was lyophilized, and DNA extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions.

The complete nuclear ribosomal DNA intergenic spacer (IGS) region and portions of the nuclear elongation factor 1-α (EF-1α) and mitochondrial small subunit rDNA (mtSSU) were amplified and sequenced for 37 isolates. Three isolates (FoChz10c, FoStL1a, FoVal42) obtained from trefoil but nonpathogenic to trefoil, all 28 isolates identified as pathogenic to trefoil (Table 2.1), two isolates (Fom001, Fom004) of *F. oxysporum* f. sp. *medicaginis*, two isolates (Fopi001, Fopi002) of *F. oxysporum* f. sp. *pisi*, one isolate (Fo062) of *F. oxysporum* pathogenic to red clover

(*Trifolium pratense*), and one isolate (FoGa2) of *F. oxysporum* f. sp. *tulipae* were analyzed. Pathogenicity testing of isolates Fom001, Fom004, Fopi001, Fopi002, and Fo062 resulted in rapid wilt and severe root necrosis on the corresponding hosts (Tables 2.2 and 2.3; Wunsch, unpublished data). Amplification of EF-1α was conducted in 50-µl reactions containing 3 µl template DNA, 1x Takara Ex Taq buffer (Takara Bio, Madison, WI), 200 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM each of primers EF1a and EF2ag (O'Donnell et al. 1998), and 1.25 units *Ex Taq* DNA polymerase, hot start version (Takara Bio, Madison, WI). PCR was conducted in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) as follows: 30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C for 40 cycles, followed by a 5-min extension at 72°C and a 4°C bath. Amplification of mtSSU was conducted in 50-µl reactions

TABLE 2.2. Incidence[a] of *Fusarium* wilt on agriculturally important legumes grown in New York after inoculation with *Fusarium oxysporum* isolates from trefoil, alfalfa, red clover and pea.

	F. oxysporum f. sp. *loti*					*Fo medi-caginis*	*Fo* red clover	*Fo pisi*	
Host[b]	**FoVt3a**[c]	**FoVt71**[c]	**FoMK61**[c]	**Fo069**[c]	**Fo012**[c]	**Fom004**[d]	**Fo062**[d]	**Fopi001**[d]	**Control**[e]
Trefoil	**44.4** **	**52.8** **	**58.3** **	**58.3** **	**63.9** **	0	0	2.8	0
Alfalfa	0	0	0	0	0	**97.2** **	0	0	2.8
Red Clover	0	0	0	0	0	0	**19.4** *	0	0
Pea	2.8	8.3	**19.4** *	11.1	11.1	2.8	11.1	**97.2** **	0
Bean	0	0	0	0	0	0	0	0	0
Soybean	0	0	0	0	0	0	0	0	0

[a] The percent of plants out of 36 tested exhibiting *Fusarium* wilt within 65 days of inoculation in a replicated greenhouse experiment. Trimmed bare roots (alfalfa, clover, pea, bean and soybean) or trimmed root masses with soil (trefoil) were soaked in a suspension of 1.0 x 10^6 spores/ml. Results were compared with a control treatment where root masses were soaked in dilute Czapek-Dox broth. Asterisks represent a significant difference relative to the control ($P<0.05$ and $P<0.01$ for one and two asterisks, respectively). Plants were only considered positive for Fusarium wilt if *Fusarium* sp. was reisolated from surface sterilized sections of symptomatic stems collected at least 1 cm from the crown.

[b] Cultivars with known susceptibility to *F. oxysporum* were tested: trefoil *(Lotus corniculatus)* cv. Georgia-1**,** alfalfa *(Medicago sativa)* cv. MNGN-1**,** red clover *(Trifolium pratense)* cv. Chesapeake**,** pea *(Pisum sativum)* cv. M410**,** pinto bean *(Phaseolus vulgaris)* cv. U.I. 114**,** soybean *(Glycine max)* cv. Essex

[c] Isolates of *F. oxysporum* f. sp. *loti.*

[d] Fom004 is an isolate of *F. oxysporum* f. sp. *medicaginis* obtained from symptomatic alfalfa in Pennsylvania; Fo062, an isolate of *F. oxysporum* with heightened virulence to red clover obtained from red clover in Wisconsin; Fopi001, an isolate of *F. oxysporum* f. sp. *pisi* obtained from pea in New York.

[e] Sterile Czapek-Dox broth diluted 1:3 with water. Czapek-Dox broth was the medium used to generate spores.

TABLE 2.3. Root necrosis[a] of agriculturally important legumes grown in New York after inoculation with *Fusarium oxysporum* isolates from trefoil, alfalfa, red clover and peas.

	F. oxysporum f. sp. *loti*					*Fo medi-caginis*	*Fo* red clover	*Fo pisi*	
Host[b]	**FoVt3a**[c]	**FoVt71**[c]	**FoMK61**[c]	**Fo069**[c]	**Fo012**[c]	**Fom004**[d]	**Fo062**[d]	**Fopi001**[d]	**Control**[e]
Trefoil	**2.39** ****	**2.64** ****	**3.00** ****	**2.50** ****	**2.81** ****	0.17	0.06	0.56	0.17
Alfalfa	0.08	0.17	0.39	0.17	0	**4.78** ****	0.11	0.06	0.19
Red Clover	0.33	0.67	**0.97** **	0.42	0.47	**1.97** ****	**2.53** ****	**0.94** **	0.11
Pea	0.47	**1.08** *	**1.31** **	**1.22** *	0.97	**1.08** *	0.86	**4.67** ****	0.03
Bean	0.58	0.47	0.61	0.56	0.19	0.53	0.58	0.67	0.06
Soybean	0.03	0.08	0.03	0.03	0.06	0.03	0.06	0	0.06

[a] Mean root necrosis 65 days after inoculation in a replicated greenhouse experiment; 36 plants were tested for each host-isolate combination. Trimmed bare roots (alfalfa, clover, pea, bean and soybean) or trimmed root masses with soil (trefoil) were soaked in a suspension of 1.0 x 10^6 spores/ml. Root necrosis was rated on a 0 to 5 scale: 0, no root necrosis; 1, small dark specks or strands in the stele; 2, moderate dark strands forming small arcs or rings in the stele cross-section; 3, large dark strands in the stele; 4, most of stele discolored; 5, stele and part or all of cortex discolored and/or plant dead. Results were compared with a control treatment where root masses were soaked in dilute Czapek-Dox broth. Asterisks represent a significant difference relative to the control ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ for one, two, three and four asterisks, respectively).

[b] Cultivars with known susceptibility to *F. oxysporum* were tested: trefoil *(Lotus corniculatus)* cv. Georgia-1**,** alfalfa *(Medicago sativa)* cv. MNGN-1**,** red clover *(Trifolium pratense)* cv. Chesapeake**,** pea *(Pisum sativum)* cv. M410**,** pinto bean *(Phaseolus vulgaris)* cv. U.I. 114**,** soybean *(Glycine max)* cv. Essex.

[c] Isolates of *F. oxysporum* f. sp. *loti.*

[d] Fom004 is an isolate of *F. oxysporum* f. sp. *medicaginis* obtained from symptomatic alfalfa in Pennsylvania; Fo062, an isolate of *F. oxysporum* with heightened virulence to red clover obtained from red clover in Wisconsin; Fopi001, an isolate of *F. oxysporum* f. sp. *pisi* obtained from pea in New York.

[e] Sterile Czapek-Dox broth diluted 1:3 with water. Czapek-Dox broth was the medium used to generate spores.

with 3 µl template DNA, 1x Takara Ex Taq buffer, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.3 µM each of primers MS1 and MS2 (White et al. 1990), and 1.25 units *Ex Taq* DNA polymerase, hot start version. Thermocycling parameters were 40 cycles of 94°C for 35 sec, 52°C for 55 sec and 72°C for 2 min, followed by a 7-min extension at 72°C and a 4°C bath. Amplification of the IGS region was conducted in 25-µl reactions with 1.5 µl template DNA, 1x Takara Ex Taq buffer, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.3 µM each of primers LR11 (5'-TGGTTTTTGCGGCTGTCTGA-3') and CNS3 (White et al. 1990), and 0.625 units *Ex Taq* DNA polymerase, hot start version. Thermocycling parameters were 90 sec at 94°C and 35 cycles of 94°C for 30 sec, 72.5°C for 30 sec and 73.5°C for 3 min 40 sec, followed by a 6-min extension at 73.5°C and a 4°C soak. To ensure sufficient PCR

product for sequencing, five 25-µl reactions amplifying the IGS region were conducted for each isolate and pooled prior to PCR product purification. Negative controls (no template) were included in every assay. Amplification products were visualized under UV light on 1.5% agarose gels stained with ethidium bromide. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. Sequencing was conducted at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY). The amplification primers were used to sequence EF-1a and mtSSU, and primers CLR12 (Anderson and Stasovski 1992), CNS1 (White et al. 1990), ONL13B (O'Donnell 2004), IGS 2r (5'-CACCAGCCAAACCACCTCTTC-3'), and IGS 3c (5'-TCCGAGACCGTTTTAGTGGGCC-3') were used to sequence the IGS region. Trace files were visualized with FinchTV (Geospiza, Seattle, WA), and sequences were edited in EditSeq (Lasergene 7.2.1, DNASTAR Inc., Madison, WI). The edited sequences were deposited in GenBank under accession numbers EU313430 to EU313540. Additional mtSSU, EF-1α, and IGS sequences of other *Fusarium* isolates generated by Mbofung et al. (2007), Baayen et al. (2000), and K. O'Donnell (USDA-ARS, Peoria, IL) were obtained from GenBank or directly from K. O'Donnell.

Phylogenetic analysis. Sequences were aligned with Clustal W in MegAlign (Lasergene 7.2.1, DNASTAR, Madison, WI) 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.

Unweighted parsimony analysis was conducted with PAUP* version 4.0b10 (Swofford 2002). Heuristic searches for the most parsimonious trees were conducted

with 1,000 random addition replicates and tree bisection with reconnection branch swapping. Gaps were treated as missing data, and trees were rooted with *F. subglutinans* based on previous phylogenetic analysis (Mbofung et al. 2007). 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, rearrangements per bootstrap replicate were limited to 10,000. Congruence of the mtSSU, EF-1α, and IGS data partitions was assessed with the Kishino-Hasegawa (KH) and Templeton Wilcoxon signed rank (WS-R) tests in PAUP* using 70% bootstrap majority trees from each partition as constraints. The KH test indicated that all partitions were congruent except mtSSU and IGS ($P<0.001$ constraining mtSSU to the IGS bootstrap tree). The WS-R test indicated that the EF-1α and IGS partitions were congruent ($P=0.2482$ constraining EF-1α to the IGS bootstrap tree, $P=0.0402$ constraining IGS to the EF-1α bootstrap tree) but that the EF-1α and IGS partitions were incongruent with the mtSSU partition ($P<0.02$). However, incongruence was limited to the placement of taxa distantly related to isolates pathogenic to BFT; isolates pathogenic to BFT were placed in a single, well-supported clade (>70% bootstrap support) in all mtSSU, EF-1α and IGS most parsimonious trees (MPTs), and all taxa placed in a separate clade from the BFT isolates in the MPT of the mtSSU partition were placed in separate, well supported clades in the MPTs of the EF-1α and IGS partitions. Consequently, mtSSU, EF-1α and IGS sequences were concatenated, and global parsimony analysis of the three loci was conducted.

2.4 RESULTS

***F. oxysporum* isolates.** Twenty-eight *F. oxysporum* isolates causing vascular wilt and severe root necrosis of BFT were collected (Table 2.1). The isolates were obtained throughout the pathogen's known geographic distribution, with 17 from western New York (Wyoming County), one from central New York (Tompkins County), one from eastern New York (Essex County), and eight from western Vermont (Addison County). The isolates were collected from eight fields in Wyoming County, one field in Tompkins County, one field in Essex County, and one field in Addison County. Isolates from two fields in Wyoming County and one field in Addison County were obtained by baiting the pathogen from naturally infested field soil. All other isolates were obtained directly from symptomatic BFT collected in production fields. Wilt symptoms, including flagging, chlorosis, and stunting, generally developed within 20 and 60 days of inoculation. Wilt incidence (Table 2.1), however, was low to moderate because applications of MilStop (BioWorks, Inc., Victor, NY) to control powdery mildew led to death of mildew-infected leaves and stems, making wilt symptom diagnosis difficult. Root necrosis was elevated relative to the control at the conclusion of the experiment, 98 days after inoculation (Table 2.1). Nonpathogenic isolates (isolates that did not induce vascular wilt of BFT) did not cause root necrosis (Table 2.1). No wilt symptoms developed in either the water or the C-Dox broth control, and mean root necrosis was 0.06 in both controls.

Cultural and morphological characteristics of the isolates fit the type description of *F. oxysporum* (Nelson et al. 1983). On PDA, isolates produced abundant aerial mycelia with peach colored sporodochia. The underside of colonies was cream to peach colored. On CLA, isolates produced slightly curved macroconidia

with a boot shaped basal cell and attenuated apical cell that were generally three-septate but occasionally two- or four-septate. Microconidia were oval or kidney shaped and generally single celled but occasionally two-celled. Microconidia were borne in false heads on monophialides. Chlamydospores were terminal or intercalary, mostly single but occasionally in pairs.

Host range. The *F. oxysporum* isolates pathogenic to BFT displayed a unique host range relative to the other pathogenic *F. oxysporum* tested. Of the five *F. oxysporum* isolates pathogenic to BFT tested, all caused moderate to high incidence of vascular wilt on BFT, low incidence of vascular wilt on peas, and no vascular wilt on alfalfa, red clover, bean or soybean (Table 2.2). The incidence of vascular wilt on BFT was significantly different from the control ($P<0.01$) for all five isolates; the incidence of vascular wilt on pea, however, was significantly different from the control ($P<0.05$) for only one isolate (Table 2.2). The root necrosis response to inoculation by *F. oxysporum* isolates pathogenic to BFT was similar to the wilt response (Table 2.3); all five isolates pathogenic to BFT caused severe root necrosis on BFT, and three of the isolates caused moderate root necrosis on pea. One isolate also caused moderate root necrosis on red clover. Root necrosis was significantly higher than the control for all five isolates on BFT ($P<0.0001$), for three isolates on pea ($P<0.05$), and for one isolate on red clover ($P<0.01$). The *F. oxysporum* isolates pathogenic to alfalfa, red clover, and pea caused severe root necrosis and moderate to high incidence of vascular wilt on their respective hosts but not on BFT, bean, or soybean (Tables 2.2 and 2.3). The isolate pathogenic to alfalfa caused higher root necrosis on red clover and on pea relative to the control ($P<0.05$); the isolates pathogenic to pea and to red clover caused a low incidence of vascular wilt on pea that

was not significantly different from the control (P>0.05). The isolate pathogenic to pea caused a low incidence of vascular wilt on BFT not significantly different from the control (P>0.05). In the control, root necrosis was low, but one plant developed vascular wilt (Tables 2.2 and 2.3).

Vegetative compatibility. All isolates pathogenic to BFT were vegetatively compatible. Reciprocal pairwise crosses between NitM and *nit1* mutants of isolates Fo036, Fo068, FoMK41, FoPlc14a, FoVH17a, FoVt63, FoChz10c, FoStL1a, and FoVal42 produced heterokaryons only between mutants of pathogenic isolates and between mutants of the same isolate. Four VCGs were identified; all pathogenic isolates shared a single VCG, and nonpathogenic isolates each represented unique VCGs. All isolates were self-compatible. Crosses between *nit1* mutants of the remaining 22 pathogenic isolates and Fo036, FoChz10c, FoStL1a, and FoVal42 NitM tester strains (representing each of the four VCGs) resulted in heterokaryon formation only with the Fo036 NitM tester strain. *Nit1* mutants of each of the remaining 22 pathogenic isolates were compatible with the Fo036 NitM tester strain; none were compatible with the FoChz10c, FoStL1a or FoVal42 NitM tester strains. All pathogenic isolates were assigned to a single VCG distinct from the VCGs represented by the nonpathogenic isolates (Table 2.1). The VCG of the pathogenic isolates was assigned VCG code 0480 by T.R. Gordon (University of California, Davis), the current *F. oxysporum* VCG numbering coordinator, in accordance to the numbering system proposed by Puhalla (1985), Kistler et al. (1998) and Katan (1999).

Phylogenetic analysis. Amplification of the mtSSU, EF-1α, and IGS regions resulted in PCR products approximately 730 bp, 700 bp, and 3,100 bp long,

respectively. All of the isolates pathogenic to BFT shared identical sequence at the mtSSU, EF-1α, and IGS loci.

The aligned mtSSU, EF-1α, and IGS data sets consisted of 714, 695, and 2,565 characters, respectively, of which 17, 38, and 220 characters, respectively, were parsimony-informative. Maximum parsimony analysis of the concatenated sequences resulted in three MPTs with 839 steps (CI=0.784, RI=0.882). The MPTs differed with respect to the placement of non-pathogenic isolate FoStL1a obtained from BFT and the subclade containing *F. oxysporum* f. sp. *pisi* isolates Fopi001 and Fopi002. Isolates pathogenic to BFT plus one nonpathogenic isolate were placed in a clade (76% bootstrap support) distinct from isolates pathogenic to other hosts (Figure 2.1). The isolates pathogenic to BFT were placed in a single subclade (75% bootstrap support); no other isolates were in the clade (Figure 2.1).

2.5 DISCUSSION

We propose that the fungus causing Fusarium wilt of BFT be designated *Fusarium oxysporum* Schlechtendahl emend. Snyder & Hansen f. sp. *loti* forma specialis nov., as suggested by Bergstrom and Kalb (1995). Among legumes of economic importance in New York, the fungus displays a unique host range, causing severe vascular wilt of BFT but not of alfalfa, red clover, dry bean, or soybean. None of the other pathogenic *F. oxysporum* tested caused vascular wilt of BFT. A single BFT plant inoculated with *F. oxysporum* f. sp. *pisi* developed vascular wilt, but because wilt developed in only one replicate of the experiment, cross-contamination likely caused this result. The host range of *F. oxysporum* f. sp. *loti* may extend to pea. All five isolates of the BFT pathogen tested caused vascular wilt of peas, with four of

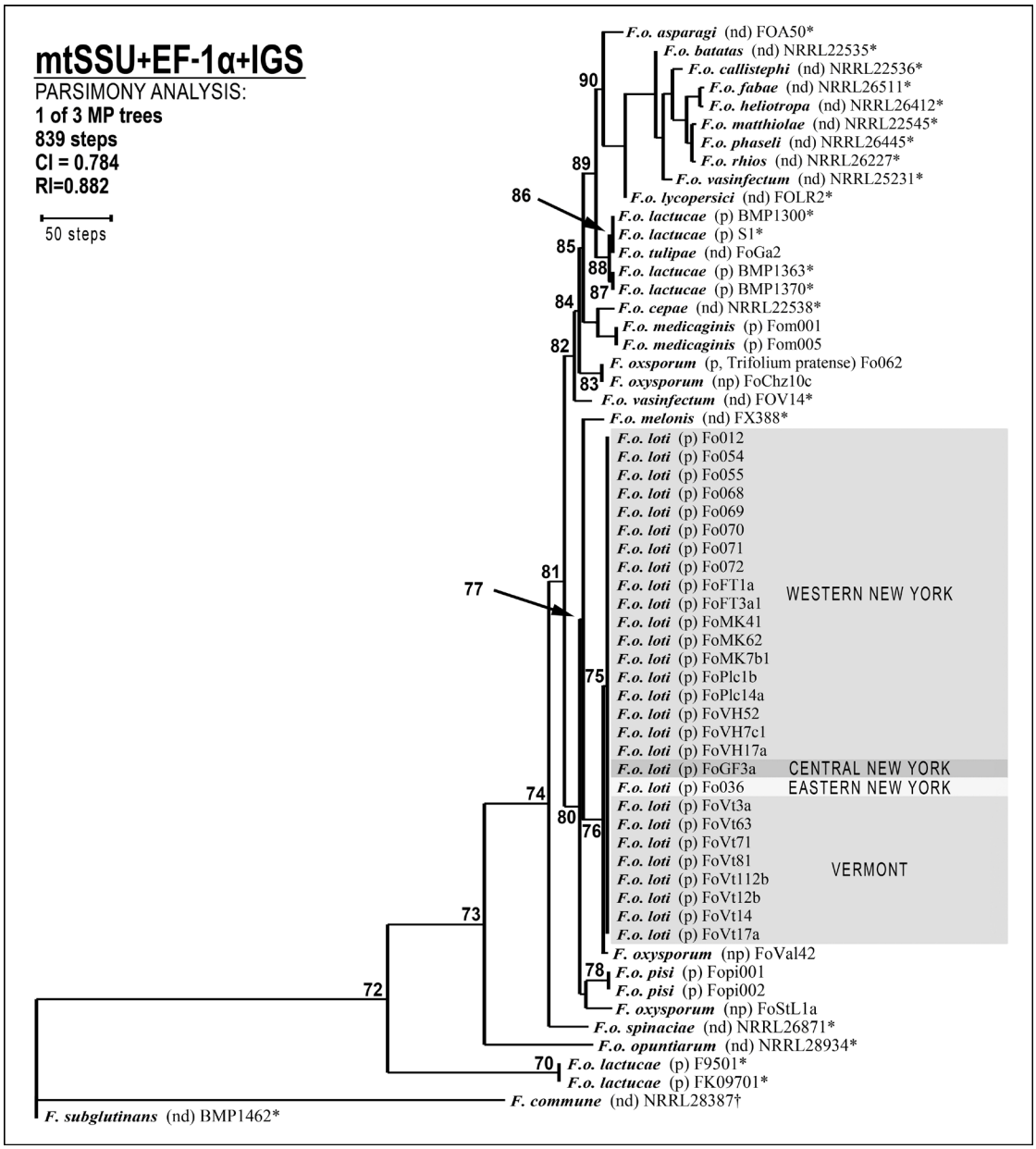

FIGURE 2.1. Maximum parsimony analysis of the concatenated mtSSU, EF-1α, and IGS partitions and rooted with *F. subglutinans* isolate BMP1462. Shown is one of three most parsimonious trees; parsimony bootstrap values greater than 70% (1,000 replications) are indicated at the internodes. Isolates tested for pathogenicity in this study or in Mbofung et al. (34) and found to be pathogenic or nonpathogenic are denoted with "(p)" or "(np)", respectively; isolates not tested for pathogenicity are denoted with "(nd)". Isolates sequenced by Mbofung et al. (34) are noted with an asterisk, and isolates sequenced by Baayen et al. (7) or K. O'Donnell are noted by the symbol "†"; all other isolates were sequenced in this study.

the five isolates causing vascular wilt in at least two of the three replicates of the experiment. A host range encompassing both BFT and pea would not be surprising; many formae speciales of *F. oxysporum*, including *vasinfectum, apii,* and *medicaginis*, are known to cause vascular wilt on multiple hosts (Armstrong and Armstrong 1981). However, additional experiments will be needed to confirm this result. Low levels of vascular wilt were also observed in pea inoculated with *F. oxysporum* isolates pathogenic to alfalfa and red clover, and it is likely that the inoculation technique used for peas was unduly harsh and predisposed plants to infection. Disease development associated with *F. oxysporum* can depend on inoculation technique (Armstrong and Armstrong 1975), and the host range exhibited by the pathogen under field conditions in naturally infested soils could be different.

F. oxysporum f. sp. *loti* is a monophyletic pathogen composed of a single clonal lineage. All *F. oxysporum* f. sp. *loti* isolates collected across New York and Vermont over a 20-year period were vegetatively compatible, shared identical mtSSU, EF-1α and IGS sequence haplotypes, and were placed into a single, well-supported clade by maximum parsimony analysis. In *F. oxysporum*, isolates belonging to the same VCG are traditionally interpreted as descendants of a common ancestor (Klein and Correll 2001). Sexual recombination, which could shuffle heterokaryon incompatibility alleles and obscure the relationship between VCG and evolutionary lineage, is not known to occur in *F. oxysporum* (Gordon and Martyn 1997), and meiotic and/or mitotic recombination, if present, are believed to be rare events. That *F. oxysporum* f. sp. *loti* is a monophyletic pathogen characterized by a single clonal lineage is not surprising. Other formae speciales, including *albedinis* and *ciceris*

(Jiménez-Gasco et al. 2002, Tantaoui et al. 1996), have been reported with a similar population structure.

Wilt diseases caused by *F. oxysporum* are generally managed by host resistance. Related species of *Lotus* may be valuable sources of resistance to Fusarium wilt for improvement of birdsfoot trefoil. Three cultivars (Columbia, Maku, and Marshfield) of *L. uliginosus* were highly resistant, and one accession (PI 316270) of *L. glaber* was moderately resistant to a New York isolate of *F. oxysporum* from BFT (Bergstrom et al. 1995, unpublished data). Zeiders and Hill (1988) demonstrated that the potential for development of resistant lines exists within the gene pool of *Lotus corniculatus*. Pardee, a BFT cultivar with moderate resistance to Fusarium wilt, has recently been registered (Viands et al. 2004) and may provide an alternative to growers interested in planting BFT in fields with a history of Fusarium wilt. Because of the observed clonality of *F. oxysporum* f. sp. *loti,* the current study suggests this cultivar, which was developed using a single isolate of the pathogen to screen for resistance, may exhibit moderate resistance to Fusarium wilt throughout the known geographic range of the disease. However, breeders should be cautious in interpreting the apparent clonality of *F. oxysporum* f. sp. *loti* within the context of virulence; high genetic similarity of *F. oxysporum* isolates does necessarily preclude the existence of race-level variability (Elias et al. 1993, Jiménez-Gasco et al. 2002, Mes et al. 1994).

To date, the occurrence of Fusarium wilt of BFT and of the pathogen *F. oxysporum* f. sp. *loti* is known only in New York and Vermont, USA. Movement of infected trefoil hay and infested soil are the most likely mechanisms for geographic spread of the pathogen. Based on inoculation of flowering BFT plants, Litchfield-Kimber (1996) demonstrated that *F. oxysporum* f. sp. *loti* did not infect seed, but that

seed lots artificially contaminated with dry fragments of infected pods and other vegetative tissues could serve to disseminate the viable pathogen. Yet she did not detect the Fusarium wilt pathogen in commercial BFT seed lots produced in Michigan, Minnesota, western New York, or Wisconsin, USA or in Alberta, Canada (Litchfield-Kimber 1996). The fungal gene sequence and vegetative compatibility tools identified in this study should prove useful in detecting *F. oxysporum* f. sp. *loti* in plants and soil and in tracking any future expansion in the pathogen's geographic range. We have designated isolate Fo069 as the type culture for *F. oxysporum* f. sp. *loti* and deposited it with the American Type Culture Collection (Manassas, VA). Isolates Fo069, FoMK61, and FoVt63 have also been deposited in the culture collection of the Fusarium Research Center at the Pennsylvania State University (State College, PA).

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CHAPTER 3

DISTRIBUTION, IMPACT, AND SOIL ENVIRONMENT OF *PHOMA SCLEROTIOIDES* IN NORTHEASTERN U.S. ALFALFA FIELDS*

3.1 ABSTRACT

We report brown root rot (BRR) of alfalfa, caused by the fungal pathogen *Phoma sclerotioides*, for the first time in the eastern United States. Alfalfa production fields in New York, Vermont and New Hampshire were sampled in spring 2005, and soil characteristics were related to variability in BRR incidence and severity in two New York fields sampled extensively. BRR was detected in eight of ten fields sampled in New York, six of seven fields sampled in Vermont, and five of six fields sampled in New Hampshire. Lesions on both roots and crowns were common in all three states, and most BRR lesions extended into the cortical tissues. Diagnostic PCR of *P. sclerotioides* isolates produced a single amplicon of the expected size. In-vivo conidia and pycnidia morphology of Northeastern isolates was consistent with published descriptions of *P. sclerotioides*, and *P. sclerotioides* was re-isolated from symptomatic lesions after pathogenicity testing. In two New York fields sampled extensively, BRR severity varied with soil strength, soil texture, soil saturation and alfalfa stand density. The spatial pattern of BRR within fields suggests the pathogen was not recently introduced. The results suggest BRR is widespread in alfalfa

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production fields in New York, Vermont and New Hampshire.

3.2 INTRODUCTION

Phoma sclerotioides G. Preuss ex Sacc. (syn. *Plenodomus meliloti* Dearn. & G. B. Sanford) is a low-temperature fungal pathogen of overwintering legumes (Cormack 1934, Hollingsworth et al. 2005, Hwang and Flores 1987, Sanford 1933) and cereals (Larsen et al. 2007). It causes particularly severe damage to alfalfa (*Medicago sativa* L.) and sweet clover (*Melilotus alba* Desr.). Causal agent of brown root rot (BRR) of alfalfa, sweet clover and other forage legumes, *P. sclerotioides* causes lesions on both roots and crowns, and it is associated with yield loss (Berkenkamp and Baenziger 1969, Berkenkamp et al. 1991), slow emergence from winter dormancy (Hollingsworth et al. 2003), and winterkill (Hollingsworth and Gray 1999, Salonen 1962, Sanford 1933). It is primarily pathogenic to alfalfa and sweet clover roots in late winter and early spring, as plants emerge from winter dormancy (Cormack 1934, Sanford 1933); it is not pathogenic during summer (Cormack 1934). *P. sclerotioides* is a saprophyte of canola (*Brassica* sp.) and annual cereal debris (Davidson 1990), and it has been found in association with a wide range of other herbaceous plants, including redroot amaranth (*Amaranthus retroflexus*), pigweed (*Axyris amaranthoides*), red fescue (*Festuca rubra*), Kentucky bluegrass (*Poa pratensis*), and winter rye (*Secale cereale*) (Gaudet and Bhalla 1988, Lebeau and Logsdon 1958, Sanford 1933).

Within North America, *P. sclerotioides* has long been known to be a problem in alfalfa and sweet clover production in Alaska and in Alberta, Saskatchewan, Manitoba and the Yukon Territory of Canada (Lebeau and Logsdon 1958, McDonald

1955, Sanford 1933). It was first observed in the contiguous United States in 1996, when it was isolated from symptomatic alfalfa in Wyoming (Hollingsworth and Gray 1999), and it has subsequently been associated with diseased alfalfa plants in Idaho (Hollingsworth et al. 2003), Montana (Mikkelson 1997), Minnesota (Larsen et al. 2007), and Wisconsin (Larsen et al. 2004). However, its occurrence and distribution in the eastern United States has not been studied.

Preliminary work conducted by the authors in 2003 and 2004 suggested that *P. sclerotioides* is present in New York (Wunsch et al. 2006). In spring 2003, a symptomatic alfalfa root collected in northeastern New York tested positive for the pathogen (Wunsch et al. 2006) using PCR-based sequence-characterized amplified region (SCAR) primers developed by Larsen et al. (2002). In a subsequent survey of New York alfalfa production fields in 2004 (Wunsch et al. 2006), cultures characteristic of *P. sclerotioides* were isolated from diseased alfalfa roots and crowns in multiple fields. Pathogenicity testing was not conducted, but diagnostic PCR of axenic single-conidium cultures yielded a single amplicon of the size expected for *P. sclerotioides*. The survey, however, did not permit an evaluation of the importance of *P. sclerotioides* to alfalfa production in New York. The severity of the lesions from which *P. sclerotioides* was isolated was not recorded, and roots were collected in some regions of the state during the summer, when *P. sclerotioides* is not isolated as efficiently (McDonald 1955), thereby precluding accurate assessments of BRR incidence.

In eastern North America, *P. sclerotioides* has been previously reported only in Nova Scotia (Davidson 1990). It has not been reported in Ontario, Quebec, Newfoundland or New Brunswick, and it is unclear if the observed occurrence of BRR

in New York in 2003 and 2004 was the result of a recent introduction of *P. sclerotioides*. Further, many farms in New York have a wide range of soil types both across and within production fields, and it is unclear which soil environments, and thus which fields, might be at highest risk of severe BRR losses. Soil characteristics are known to impact the severity of diseases caused by many fungal soilborne pathogens, including *Fusarium solani* (Harveson et al. 2005), *Rhizoctonia solani* (Gill et al. 2000, Gill et al. 2004) and *Sclerotinia sclerotiorum* (Singh and Singh 1983), but the relationship between soil environment and BRR severity has not been studied.

Analysis of spatial patterns can be useful for inferring underlying processes, such as the origin of local epidemics (Legendre and Fortin 1989, Sanford 1933), and for developing testable hypotheses regarding environmental conditions favorable to an organism (Legendre and Fortin 1989, Perry et al. 2002). When a pathogen has been recently introduced at a limited number of foci within a field, as occurs when soilborne fungi are introduced with soil on unwashed equipment, the initial distribution of disease will be aggregated, with disease gradients away from the foci of introduction (Madden 1989). Likewise, when underlying environmental heterogeneity differentially affects a pathogen's fitness, spatial distribution of the pathogen will correspond, at least partly, to the spatial distribution of environmental conditions favorable to the organism (Legendre and Fortin 1989, Perry et al. 2002). Multiple New York fields sampled in 2004 appeared to have an uneven distribution of alfalfa BRR (Wunsch, unpublished), suggesting possible aggregation due to recent introduction of *P. sclerotioides* or to underlying environmental heterogeneity; however, small sample sizes precluded further analysis.

The objectives of this study were (i) to confirm the presence of *P. sclerotioides* in the northeastern United States by completion of Koch's postulates, (ii) to examine the occurrence, spatial distribution, and impact of BRR in New York, Vermont and New Hampshire alfalfa production fields, and (iii) to relate soil heterogeneity to observed spatial patterns of alfalfa BRR within affected fields. The impact of BRR was estimated by assessing BRR incidence, evaluating the frequency and severity of BRR lesions on both roots and crowns, and examining the relationship between BRR severity and spring regrowth.

3.3 MATERIALS AND METHODS

Survey. Ten fields in New York, seven fields in Vermont and six fields in New Hampshire were sampled in spring 2005. In general, only fields that had passed through at least two winters were selected, and fields were selected arbitrarily by cooperators. However, two fields (New York field 2, Clinton Co., and New Hampshire field 3, Coös Co.; Table 3.1) were selected because of heavy winterkill losses that spring, and one field (New York field 4, Lewis Co.; Table 3.1) had passed through only one winter. Plants were collected at multiple sites within each field; within each sampling site, plants were arbitrarily selected without regard to vigor or apparent health. All foliage and at least 15 cm of the root were collected. Two fields in New York, one in Wyoming County (hereafter, "field Wyo1") and another in Clinton County (hereafter, "field Chz1"), were sampled extensively, with approximately 30 plants collected at each of 23 sites (field Wyo1) or 24 sites (field Chz1) on an equidistant 60 m x 100 m or 180 m x 300 m grid, respectively. In all other fields, approximately 20 to 40 plants were collected from two to four sampling

TABLE 3.1 Brown root rot (BRR) incidence and severity in New York, Vermont, and New Hampshire alfalfa production fields in spring 2005.

						BRR lesion severity, root [e]						BRR lesion severity, crown [g]				
Field	County	Year[a]	BRR[b]	Tot[c]	Rt[d]	1	2	3	4	5	Cr[f]	1	2	3	4	5
New York																
1	Clinton	2001	**120**	**749**	**102**	28	44	12	11	7	**27**	8	13	5	1	0
2	Clinton	2002	**1**	**15**	**1**	0	1	0	0	0	**0**	0	0	0	0	0
3	Lewis	2003	**1**	**27**	**0**	0	0	0	0	0	**1**	0	1	0	0	0
4	Lewis	2004	**0**	**18**	**0**	0	0	0	0	0	**0**	0	0	0	0	0
5	Steuben	2002	**37**	**55**	**28**	1	12	6	4	5	**18**	1	9	5	1	2
6	Steuben	2003	**5**	**37**	**2**	0	1	0	1	0	**3**	0	3	0	0	0
7	St. Lawrence	2002	**6**	**46**	**4**	2	2	0	0	0	**2**	0	0	1	1	0
8	St. Lawrence	2003	**0**	**41**	**0**	0	0	0	0	0	**0**	0	0	0	0	0
9	St. Lawrence	2003	**6**	**51**	**4**	2	1	0	0	1	**3**	1	2	0	0	0
10	Wyoming	2001	**170**	**703**	**156**	66	74	14	1	1	**23**	3	9	7	3	1
Vermont																
1	Addison	2001	**21**	**44**	**11**	2	6	2	1	0	**12**	0	6	4	2	0
2	Addison	2002	**4**	**42**	**3**	1	2	0	0	0	**2**	1	0	0	0	1
3	Addison	2002	**22**	**43**	**10**	3	2	3	2	0	**16**	0	6	2	7	1
4	Franklin	2000	**2**	**41**	**1**	0	1	0	0	0	**1**	0	0	1	0	0
5	Grand Isle	2002	**17**	**38**	**16**	8	7	1	0	0	**3**	0	2	0	1	0
6	Orleans	2001	**0**	**22**	**0**	0	0	0	0	0	**0**	0	0	0	0	0
New Hampshire																
1	Coös	2000	**2**	**59**	**2**	1	1	0	0	0	**0**	0	0	0	0	0
2	Coös	2003	**5**	**33**	**3**	0	0	1	2	0	**2**	0	1	1	0	0
3	Coös	2003	**7**	**53**	**3**	1	0	2	0	0	**4**	1	2	0	0	1
4	Grafton	2000	**15**	**40**	**1**	1	0	0	0	0	**15**	0	9	3	2	1
5	Grafton	2002	**17**	**43**	**9**	2	4	0	2	1	**10**	0	6	2	2	0
6	Grafton	2003	**0**	**45**	**0**	0	0	0	0	0	**0**	0	0	0	0	0

[a] Seeding year of field.

[b] Number of sampled plants from which *Phoma sclerotioides* was successfully isolated.

[c] Total number of plants sampled.

[d] Number of plants from which *P. sclerotioides* was successfully isolated from root lesions.

[e] Number of plants with BRR root lesions in severity classes 1 to 5, where 1 = lesions restricted to the epidermal tissues, 2 = lesions extend into the cortical tissues but impact ≤ 33% of root diameter, 3 = lesions affect 34 – 66% of root diameter, 4 = lesions affect 67 – 99% of root diameter, and 5 = lesions completely girdle root.

[f] Number of plants from which *P. sclerotioides* was successfully isolated from crown lesions. Lesions occurring on woody tissues at or above the soil line and on the first 3 to 4 cm of the tap root were considered crown lesions.

[g] Number of plants with BRR crown lesions in severity classes 1 to 5, where 1 = lesions restricted to the epidermal tissues, 2 = lesions extend into the cortical tissues but impact ≤ 33% of crown diameter, 3 = lesions affect 34 – 66% of crown diameter, 4 = lesions affect 67 – 99% of crown diameter, and 5 = lesions completely girdle crown.

sites, with roughly 10 plants collected from each site; in fields with heterogeneous topography and/or soil type, sampling sites were selected such that all major field environments were represented. Fields Chz1 and Wyo1 were large commercial alfalfa production fields seeded in 2001; stand density was low in wet spots, but otherwise the fields were productive and appeared healthy. Both fields were rain-fed; average precipitation, as measured at nearby Burlington, VT and Rochester, NY, respectively, is 7.9 to 10.3 cm/month and 6.9 to 8.6 cm/month, respectively, from May through August (data from the Northeast Regional Climate Center; Cornell University, Ithaca, NY). All fields, except two in Lewis County, NY, were sampled in late April or early May 2005, shortly after alfalfa broke dormancy. The fields in Lewis County were sampled May 31. Collected plants were double-bagged, placed on ice, transported to the laboratory, and stored at 3ºC until they were processed.

Pathogen isolation. Tissue segments were excised from roots and crowns of all alfalfa plants collected, regardless of symptoms, and surface sterilized with 0.6% hypochlorite and 70% ethanol, rinsed with sterile distilled water, plated onto 1.5% water agar, and incubated for 3 to 6 months at 10ºC under continuous white fluorescent light with average illuminance of 2000 lux. Two to four tissue pieces were placed in each Petri dish; 100-mm diameter dishes were used. For each plant, separate isolations were conducted for root and for crown tissue; the crown was defined as the woody parts just above the soil line and the upper 3 to 4 cm of the tap root found at or just below the soil line. All plants were processed within 50 days of collection.

After the incubation period, pycnidia were harvested from the root and crown segments and/or the surrounding water agar with a sterile toothpick, dipped briefly into 95% ethanol, and transferred to potato dextrose agar (PDA; Difco Laboratories,

Becton, Dickson and Company, Sparks, MD) amended with 0.3 g/L streptomycin sulfate salt (Sigma-Aldrich, Inc., St. Louis, MO). Because *P. sclerotioides* sometimes fails to develop the diagnostic long necks on its pycnidia during the initial incubation period on WA, two to ten pycnidia were transferred to PDA for each root and each crown sampled regardless of pycnidial morphology. When beaked pycnidia characteristic of *P. sclerotioides* were present, spore suspensions from harvested pycnidia were used to directly establish single-conidium isolates. The cultures were incubated at 10ºC under continuous white fluorescent light, and gross morphological characteristics were assessed 2, 4 and 8 weeks after establishment. For all cultures with gross morphological characteristics typical of *P. sclerotioides,* single-conidium isolates were established on PDA.

The single-conidium isolates were assessed for gross colony morphology and pycnidial morphology. *P. sclerotioides* pycnidia are relatively large (0.2-1.0 mm diameter), have one to several rostra, exude a liquid exudate from the necks prior to spore discharge, and produce a white cirrus that darkens to yellow, tan and then brown with increasing age (Boerema et al. 1994, Hollingsworth et al. 2002). Because morphological characteristics of *P. sclerotioides* are variable on PDA (Hollingsworth et al. 2002), all isolates were also transferred to oatmeal agar (Boerema et al. 1994) and to 1.5% water agar amended with autoclaved barley grains. Water agar amended with autoclaved barley grains was particularly useful for assessing *P. slcerotioides* pycnidial morphology. *P. sclerotioides* pycnidia are variable in axenic culture and are best assessed in-vivo on host tissue (Boerema et al. 1994). However, screening all of the isolates on alfalfa or sweet clover root tissue was not practical; over 500 unique single-conidium isolates originating from different roots and crowns were evaluated.

A subset of approximately 50 single-conidium isolates was grown on 1.5% water agar amended with both autoclaved barley grains and surface-sterilized segments of greenhouse-grown healthy alfalfa roots, incubated for 8 weeks at 10°C under constant light, and pycnidial morphology on the two substrates was compared. The gross morphological characteristics of *P. sclerotioides* pycnidia on barley were consistent and highly similar to the pycnidial characteristics observed on alfalfa tissue, and the remaining isolates were screened on water agar amended with barley grains. The barley grain medium was prepared by soaking hulled barley in distilled water for 4 to 6 hours, decanting the water, and autoclaving the soaked barley grains in glass Petri dishes for 60 minutes twice. After cooling, approximately 5 to 20 barley grains were transferred to hardened 1.5% water agar in Petri dishes. Mycelial disks, taken from the growing edge of single-conidium isolates growing on PDA, were transferred to the water agar, and cultures were incubated at 10ºC under continuous white fluorescent light for 1.5 to 2 months.

BRR incidence. For each field, root, crown and total BRR incidence were calculated by dividing the number of plants from which *P. sclerotioides* was successfully isolated from the corresponding part of the plant by the total number of plants sampled.

Assessment of BRR severity. All roots and crowns were labeled before processing. Immediately after isolations were made, the labeled roots and crowns were placed in storage at -17ºC. After the final assessment of culture identity was completed, the samples were removed from storage, and the severity of the lesions from which *P. sclerotioides* was successfully isolated was rated. The following scale was used: 0, *P. sclerotioides* not successfully isolated from root or crown; 1, lesions

restricted to the epidermal tissues; 2, lesions extend into the cortical tissues but affect ≤ 33% of root or crown diameter; 3, lesions affect 34 – 66% of root or crown diameter; 4, lesions affect 67 – 99% of root or crown diameter; and 5, lesions completely girdle root or crown. BRR severity ratings were made separately for roots and crowns, and total BRR severity of individual plants was designated by assigning the more severe rating (the root versus the crown) to the plant.

Confirmation of pathogen identity. *Diagnostic PCR.* For every field from which *P. sclerotioides* was successfully isolated, morphological identification of at least one single-conidium *P. sclerotioides* culture was confirmed using *P. sclerotioides*-specific PCR-based sequence-characterized amplification region (SCAR) markers (Larsen et al. 2002). The culture subjected to the PCR test was arbitrarily selected from the single-conidium isolates collected from that field. Cultures were established on PDA, transferred to potato dextrose broth (PDB; Difco Laboratories, Becton, Dickson and Company, Sparks, MD), and grown for 3 to 5 weeks at 10ºC under continuous white fluorescent light. After incubation, the cultures were centrifuged, the PDB decanted, and the mycelium rinsed twice in sterile distilled water. The mycelium was lyophilized, and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA) according to manufacturer instructions. Diagnostic PCR was conducted in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA). Each reaction included 14.375 µl water, 0.25 µl Qiagen Hot Star Taq (5 U/µl), 0.5 µl primer PSB12-FS-24 (50 µM), 0.5 µl primer PSB12-RS-26 (50 µM), 0.25 µl dNTPs (10mM), 2.5 µl Qiagen 10x PCR buffer, and 2.5 µl $MgCl_2$ (25 mM). The following thermocycler parameters were used: 2 min at 95ºC, followed by 35 cycles of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min, and a final extension of 1

min at 72ºC, followed by a 4ºC bath. Positive and negative controls were included. The *P. sclerotioides* "Berg" isolate (American Type Culture Collection isolate MYA-295), isolated from a diseased alfalfa plant near Farson, WY was used as a positive control; a no-template reaction was included as a negative control.

In-vivo pycnidia and conidia characterisitics. In-vivo confirmation of pathogen identity was conducted by evaluating pycnidia and conidia produced in-vivo on inoculated alfalfa. Six single-conidium isolates, two each from New York, Vermont and New Hampshire, were used to inoculate "Multi-plier" alfalfa, a variety highly susceptible to BRR (Hollingsworth et al. 2005). Individual plants were started in SC10 Cone-Tainers (Stuewe and Sons, Inc., Corvallis, OR) in an autoclaved mix of 50% sand and 50% loam soil. Three to four seeds were placed in each pot, approximately 0.1 g of Dormal alfalfa *Rhizobium* inoculant (Becker Underwood, Ames, IA) was sprinkled over the seed, and the seed and inoculant were lightly covered with peat moss. Three weeks after seeding, plants were thinned to one plant per pot, with the most vigorous seedlings selected. Before inoculation, plants were grown for five months in the greenhouse at 25ºC to 35ºC, with a 200 ppm solution of 21-5-20 fertilizer (96 g/L) and Epsom salts (30 g/L) applied monthly. Five months after seeding, the soil was removed from the first 3 cm of the root, two to four barley grains colonized by *P. sclerotioides* were placed against the root, and the soil was replaced. Barley grain inoculum was produced by growing *P. sclerotioides* on PDA in 100-mm diameter Petri dishes, covering the colonized PDA with autoclaved soaked barley, and incubating at 10°C under constant light for 8 to 10 weeks. Seven plants were inoculated with each isolate. Immediately after inoculation, plants were moved to growth chambers. To induce dormancy, they were maintained two weeks at 10ºC

with 16 h light/day, two weeks at 3ºC with 16 h light/day, and 10 days at alternating temperatures (20 to 21 h at 3ºC and 3 to 4 h at -17ºC) under complete darkness. To simulate winter and spring, plants were then kept two weeks in complete darkness at -17ºC, eight weeks at 3ºC with 16 h light/day and four weeks at 10ºC with 16 h light/day. Finally, to ensure that all plants had emerged from dormancy before winterkill assessments were made, plants were kept at 25ºC with 16 h light/day for one week. The roots were harvested and washed, and pycnidia were collected from the roots. For each isolate, the diameter of 40 randomly selected pycnidia and length, width, guttule number and guttule polarity of 40 randomly selected conidia were assessed with a compound microscope. Summary statistics (minimum, maximum, 10% quantile, 90% quantile, and median) of the pycnidium and conidium size data were calculated in PROC MEANS (SAS, version 9.1, SAS Institute, Cary, NC), and pycnidial and conidial characteristics were compared to published descriptions of *P. sclerotioides* (Boerema et al. 1991, Hollingsworth et al. 2002).

Pathogenicity testing. The roots of the inoculated greenhouse- and chamber-grown alfalfa (above) were thoroughly colonized by *P. sclerotioides,* with abundant pycnidia produced throughout. However, it was unclear whether the fungus colonized the roots pathogenically or saprophytically. All of the harvested roots were completely soft-rotted, and none of the plants emerged from dormancy, suggesting that the potted plants may have been killed by the -17ºC treatment. To test for pathogenicity, Vernal alfalfa, a variety susceptible to BRR (Tsukamoto 1965), was planted at field sites in New York's Clinton and Steuben Counties in spring 2006. Vernal was used because sufficient seed of Multi-plier was not available. Clinton County is in northeastern New York; Steuben County, in south central New York.

Neither field had been previously assessed for BRR, but both fields were located on farms where BRR was detected in 2005. The Clinton Co. and Steuben Co. fields are located at 44°54' and 42°15' north latitude, respectively, and 60 m and 510 m above sea level, respectively. Both sites receive ample spring and summer rainfall, and irrigation was not used. Five replicates, each consisting of five rows 1.4 m long and 0.25 m apart, were established at each site; one additional row and 0.3 m of row length were added to the edges of the plot as a border. In every row, 1.0 g of seed and 0.05 g of *Rhizobium* inoculant were used. Plots were seeded in the spring and thinned once in the early summer, selecting the most vigorous plants. Plants were weeded twice, and foliage was trimmed once in August. Plants were inoculated Sept. 26 and Oct. 6 at the Clinton Co. and Steuben Co. plots, respectively. Plants did not appear stressed. Barley grain inoculum was prepared by growing *P. sclerotioides* on PDA in Petri dishes, covering the cultures with autoclaved soaked barley, and incubating under continuous light for two months at 10°C. Single-spore isolates 'Chz5 9R' and 'StbI-1(2)17R', collected in 2005 from different fields on each respective farm, were used for the Clinton Co. and Steuben Co. plots, respectively. Soil was carefully removed from one side of the roots, barley grain inoculum was placed against the upper tap root 3 to 6 cm below the crown, and the soil was replaced. Four of the five rows in each replicate were inoculated; the fifth was not. Twelve plants, approximately three from the end of each inoculated row, were collected in each replicate (a total of 60 plants/plot) on Dec. 2 in Clinton Co. and Dec. 3 in Steuben Co. The soil was not frozen at either site. Plants were stored at 3°C until processing; all samples were processed within a week of collection. Plants were washed vigorously, and root and crown rot severity was rated for each plant using an ordinal scale from 0 to 5 (as

above). Root and crown segments were excised from all plants, surface sterilized, plated on WA and incubated under continuous light at 10°C for nine weeks. After incubation, tissue segments and surrounding agar were examined under a compound scope for the beaked pycnidia characteristic of *P. sclerotioides*. When pycnidium morphology was ambiguous, pycnidia were harvested and used to establish single-conidium cultures on PDA. The single-conidium cultures were incubated under continuous light at 10°C for four weeks, and culture identity assessed. Single-conidium cultures were also established from arbitrarily selected pycnidia characteristic of *P. sclerotioides*. Only lesions from which *P. sclerotioides* was successfully isolated were considered to be BRR.

Evaluation of spring regrowth. Before tissue segments were excised for *P. sclerotioides* isolation, the spring regrowth was evaluated for a subset of the collected plants in the two extensively sampled fields. The plants collected at 21 of the 23 sampled sites in field Wyo1 and 20 of the 24 sampled sites in field Chz1 were subjected to analysis; the sampling sites represented in the analysis were chosen randomly. Shortly after the plants were collected, the roots were labeled. The spring regrowth, including both stems and leaves, was removed from each plant approximately 1 cm above the crown and placed into a correspondingly labeled coffee filter. All dry stems and other debris from previous seasons were separated and discarded. The labeled roots were returned to refrigeration for subsequent pathogen isolation. The labeled coffee filters were stacked, stapled together to prevent spillage, placed in small paper bags, and dried at 50ºC for five days. Immediately after removing samples from the drier, the dry weight of spring regrowth was recorded to the nearest 0.01 g. The relationship between BRR severity and spring regrowth of

individual plants was assessed with least-squares regression. Because of very small sample sizes for the most severe disease severity category, the two highest disease severity categories (BRR severity 4 and 5) were pooled for the analysis. Regression was conducted in PROC GLM (SAS, version 9.1, SAS Institute, Cary, NC) with BRR severity as a categorical independent variable. Assumptions of least-squares regression were assessed, and the square root transformation was applied to spring regrowth to stabilize the variance of the residuals and normalize the distribution of the residuals.

Stand density. In the Wyoming and Clinton County fields where extensive sampling was conducted, the relative stand density at the various sampling locations was recorded when the plants were collected. Locations where plant density was less than approximately four plants/m^2 were noted as having low stand density.

Soil analysis. In the extensively sampled Wyoming and Clinton County fields, soil characteristics were analyzed at all sampling sites. Soil cores were collected in the center of the sampling location, and loose soil was obtained from around the roots as they were sampled. The soil cores (height 7.6 cm, internal diameter 7.6 cm) were taken from just below the soil surface. Two cores and one core were collected from each sampling site in fields Chz1 and Wyo1, respectively. The intact cores were placed in plastic bags and stored at 3ºC until processing. The loose soil was collected in plastic bags, dried on a greenhouse bench, and stored at 3ºC until processing.

Percent clay and particulate organic matter. Percent clay and particulate organic matter (POM) of the soil samples were analyzed with a protocol adapted from Kettler et al. (2001). The air-dried loose soil samples were crushed into a fine powder

with a large pestle. A subsample of each crushed soil sample was passed through a 2-mm sieve to remove rocks and debris and dried at 100ºC for approximately 24 hours. For each sample, 30 g of soil were transferred to a 125-ml wide-mouth bottle and 90 ml of an aqueous 0.5% sodium polyphosphate (Sigma-Aldrich, Inc., St. Louis, MO) solution were added. Samples were shaken vigorously and placed on a reciprocating shaker (180 reciprocations/min) for approximately 16 h. After dispersion, the samples were passed through a 0.053-mm mesh sieve to separate sand (>0.053 mm) and POM from silt and clay. Sand and POM were dried at 100ºC to constant weight, weighed to the nearest 0.01 gram, and heated at 450ºC overnight to assess POM by loss on ignition (LOI; 6). The soil slurry that passed through the 0.053-mm sieve was transferred to 1.2 L sedimentation cylinders. Water was added to bring the solution volume to 1.13 L, the cylinder was capped and inverted multiple times to fully mix the contents, and the uncapped cylinder was left undisturbed for 24 hours. After 24 hours, the unsedimented clay suspension was suctioned into weigh containers, dried at 100ºC to constant weight, and weighed to the nearest 0.01 g. The sedimented portion (silt) was also transferred to weigh containers, dried at 100ºC to constant weight, and weighed to the nearest 0.01 g. Clay and POM (by LOI) content were calculated by dividing their calculated weights by the sum of the calculated weights of POM, sand, clay and silt. Because LOI overestimates soil organic matter content (Cambardella et al. 2001), percent POM was corrected with a routine formula employed by the Cornell University Nutrient Analysis Laboratory:

$$\%\ \mathrm{POM}_{corrected} = (\%\ \mathrm{POM}_{by\ LOI} * 0.7) - 0.23 \qquad [1]$$

Soil texture analysis to assess clay and POM content was conducted twice for each sampling location, and results were averaged over the two repetitions. The

accuracy of the results for clay content was evaluated by sending six of the soil samples, crushed and sieved as described, to the Cornell Nutrient Analysis Laboratory (Cornell University, Ithaca, NY) for soil texture analysis using the pipette method (Gee and Bauder 1986), a standard method of particle-size analysis. The results from the two methods were compared by conducting linear regression with PROC REG (SAS, version 9.1, SAS Institute, Cary, NC); because the sample sizes were small and it was reasonable to conclude that both methods would yield values very close to 0% clay if no clay were present, the intercept was forced to zero in the analysis. The regression yielded the models $y = 0.919x$, $y = 0.825x$, and $y = 0.872x$ for repetition 1, repetition 2, and the average of repetitions 1 and 2 of the soil texture analysis, respectively, where x represents the results from the pipette method. The coefficient of correlation for the tests was 0.992 (n=6), 0.982 (n=6), and 0.990 (n=6), respectively.

Soil strength and relative saturation. The strength and relative saturation of the soil, measures of compaction and wetness, respectively, were assessed using the soil cores. The relative saturation of field-moist soil cores, not the relative saturation of the cores at a controlled matric potential, was used for analysis because it more realistically represents the variability in soil moisture observed in the fields, both of which had pronounced low spots and high spots. Soil cores were gently trimmed to volume, two layers of cheesecloth were fastened over the bottom of the cores with a rubber-band, and field weight of the cores was determined to the nearest 0.1 g. Cores were placed in a basin, gradually wetted from the bottom, and soaked for 2 days. The weight of the saturated cores was recorded, and the cores were transferred to a custom-made sand tension table controlled with vacuum pressure regulators (Topp et al. 1993)

to adjust the matric potential. Wet soil samples were placed atop 0.42-micron nylon filter membranes which rest on fine sand. The pressure in the sand bed was reduced to -10kPa for 1 day, drawing water out of the soil cores until hydraulic equilibrium was established (Klute 1986). Controlling the water potential of soil samples was required for meaningful comparisons of soil strength due to the strong dependence of measured soil strength values on water status. After matric potential equilibration, a micropenetrometer consisting of a 30º angle cone with a 4-mm diameter base soldered to a 2-mm diameter stainless steel rod was driven into the top of each soil core at a rate of 10 mm s^{-1} for 4 to 8 seconds using a modified drill press. The resistive force to the insertion was recorded to the nearest gram each second via the serial output from the balance to a Microsoft Excel spreadsheet using data acquisition software (WinWedge 3.0, TALtech, Philadelphia, PA). To avoid edge effects, the penetrometer was pushed 5 to 10 mm into the sample before resistance measurements were started. For each core, penetrometer readings were taken at three locations. If the penetrometer encountered a rock, the reading was discarded and replaced with a new reading. Penetration resistance of each core was averaged and converted to pressure units (MPa) by multiplying the penetration resistance by the force of gravity (9.8 m s^{-2}) and dividing by the penetrometer area (π *0.002^2 m^2). The soil cores were then dried at 100ºC to constant weight and weighed to the nearest 0.1g. Total porosity was estimated by dividing the volumetric water content of the saturated soil core (in cc) by the volume of the soil core (in cc); volumetric water content of the saturated soil core was found by subtracting the oven-dry core weight from the saturated core weight and dividing by the oven-dry core weight and multiplying by the dry bulk density. All pore spaces were assumed to be completely filled with water in the saturated core and

completely drained in the oven-dry core. The original field water content of the soil was estimated by subtracting the oven-dry soil core weight from the field-moist core weight and dividing the difference by soil core volume. Relative saturation was calculated by dividing original field water content of the soil (in cc) by total porosity of the soil (in cc). Results from the two soil cores collected at each site in the Clinton County field were averaged.

Distribution of BRR within fields. *Topographical maps.* The spatial pattern of BRR incidence within the sampled areas of fields Wyo1 and Chz1 was mapped with Surfer (version 8.0, Golden Software, Golden, CO). The 60 m x 100 m and 180 m x 300 m sampling grids of fields Wyo1 and Chz1, respectively, were plotted within the first quadrant of a Cartesian plane, with the origin representing the approximate southeast corner of each sampling grid, the x and y axes representing the approximate southern and eastern edges of the grids, and each unit along the axes representing one meter. Analogous maps were created to illustrate the spatial pattern of soil characteristics in the fields. Sampling locations with low stand density were marked with asterisks on the BRR incidence map. The machinery entry point to field Wyo1 corresponds to point (100, 60); entry points to field Chz1 correspond to points (0, 0) and (0, 60).

Spatial structure. As a preliminary test of spatial structure, contingency tables with the dichotomous response BRR (present or absent) and nominal response sampling site (1 to 23 or 24) were constructed, and chi-square analysis testing the null hypothesis that frequency of BRR is uniform across sampling locations was conducted using PROC FREQ (SAS, version 9.1, SAS Institute, Cary, NC). However, because chi-square analysis of contingency tables does not incorporate spatial components into

the analysis, it cannot evaluate positive autocorrelation (aggregation). To overcome this limitation, two tests of autocorrelation, Mantel statistics and Spatial Analysis by Distance IndicEs (SADIE), were also conducted. Both tests methods utilize permutation approaches to assess statistical significance and can be used with smaller sample sizes than comparable tests that assume an underlying normal distribution for assessing significance. Mantel tests can be used with sample sizes as small as 20 (Fortin and Gurevitch 2001), and both tests have been used to successfully detect autocorrelation in sample sizes as small as 12 (Schmale et al. 2005). Counts of BRR-positive plants per sampling location, the variable traditionally related to spatial location in ecological applications of Mantel and SADIE tests, could not be used because the total number of plants sampled differed across sampling sites within the fields; instead, as suggested by Perry et al. (2002) for SADIE tests, percent BRR-positive plants, rounded to the nearest integer, was used.

The Mantel test evaluates the correlation between two n x n distance matrices; in this study, one matrix represented the differences in spatial distances and the other, differences in the frequency of BRR among all possible pairs of sampling sites. To produce the normalized Mantel statistic (r), the standard normal transformation is applied to each matrix, the products of corresponding elements of the matrices are summed, and the sum is divided by $N - 1$, as follows:

$$r = \{\sum_{i=1}^{n}\sum_{j=1}^{n}[(x_{ij} - \overline{x})/s_x][(y_{ij}-\overline{y})/s_y]\}/(N-1) \quad \text{for } i \neq j \qquad [2]$$

where i and j, respectively, denote matrix rows and columns 1 to n, and N represents the number of distances, excluding the diagonal, in one of the matrices (Fortin and Gurevitch 2001, Legendre and Fortin 1989). The resulting test statistic ranges from -1 to 1, with values closer to 0 less likely to result in a rejection of the null hypothesis

that the two distance matrices are uncorrelated. Significance of the test statistic is determined with permutation techniques; one of the two matrices is permuted randomly, and *r* is computed with the various permutations, producing a set of values of *r* used to represent the sampling distribution of *r* under H_0 (Legendre and Fortin 1989). A SAS Program (SAS, version 9.1, SAS Institute, Cary, NC) written by Edgar Barry Moser (Louisiana State University, Baton Rouge, LA; available online from the LSU Department of Experimental Statistics) was used for the analysis.

SADIE describes ecological spatial structure by relating spatial pattern in a population to the minimum total movement required for individuals in that population to reach a completely uniform distribution (Perry et al. 1999). To establish a uniform distribution, individuals must be moved either from sources (points with more individuals than the mean) to sinks (points with fewer individuals than the mean) or, equivalently, from sinks to sources. The total distance that individuals in the population would need to move in order to establish a completely uniform distribution is denoted as total flow distance, D:

$$D = \sum_{i=1}^{p} \sum_{j=1}^{q} v_{ij} d_{ij} \qquad [3]$$

where p = the number of sampling locations with counts above the mean (sources), q = the number of locations with counts below the mean (sinks), i = source locations 1 to p, j = sink locations 1 to q, v_{ij} = the number of individuals flowing from source location i to sink location q, and d_{ij} = distance between source location i and sink location j (Dale et al. 2002). The minimum total flow distance, D_r (distance to regularity), is developed by an algorithm, and permutation techniques are used to evaluate the probability of observing that distance by chance. The sample is permuted randomly, D_r is calculated for each permutation, and the resulting set of D_r values is

used to construct a sampling distribution for testing the significance of the observed distance. Total aggregation of the sample is represented by an index of aggregation, I_a, calculated by dividing the observed distance to regularity by the mean minimum distance to regularity of the permutations of the sample, with values of $I_a < 1$, $= 1$, and > 1 suggesting uniform, random, and aggregated patterns, respectively (Perry 1998). SADIEShell (version 1.22, J. N. Perry, Rothamsted Research, Hertfordshire, U.K.; available online from Rothamsted Research) was used for the analysis.

Cumulative logistic regression. The distribution of BRR in both of the extensively sampled fields was non-uniform, and a proportional odds model was used to assess if variations in soil characteristics and stand density were significant explanators of BRR distribution. The proportional odds model is a logistic regression technique applicable for data with an ordinal response variable. It models several cumulative log-odds, one for each cut point in the polytomous response (i.e., BRR severity 5 vs. 4, 3, 2, 1, 0; BRR severity 5, 4 vs. 3, 2, 1, 0; etc.), fitting different intercepts for each cumulative log-odds but constraining the regression parameters of the independent variables to be equal across all cut points (Hosmer and Lemeshow 2000, Scott et al. 1997, Stokes et al. 2000):

$$\text{logit}\,(\theta_k) = \alpha_k + \sum_{g=1}^{t} \beta_g x_g + \qquad [4]$$

for dichotomous and continuous independent variables, where k represents levels 1, 2, …, k of the dependent variable; logit (θ_k), the cumulative log-odds for level k of the dependent variable; α_k, the intercept for level k; g, independent variables 1, 2, …, t; β_g, the regression parameter for independent variable g; and ε, error. Maximum likelihood is used to estimate the parameter values (Allison 2001, Stokes et al. 2000). PROC LOGISTIC (SAS, version 9, SAS Institute, Cary, NC) was used for the

analysis. A model with independent variables percent clay, percent soil organic matter, stand density, soil strength, and relative soil saturation was fit for each field, and all possible combinations of interactions and second-order nonlinearities were tested. Significant interactions ($P<0.05$) were added to the model; none of the nonlinearities were significant. Overdispersion, a product of the sampling design, was a problem in both models. Because modifying the model by adding interaction terms or nonlinearities did not resolve the problem, overdispersion was addressed by using the ratio of the Pearson goodness-of-fit chi-square to its degrees of freedom to scale the standard errors and chi-square values of the parameter estimates (Allison 2001). The proportional odds assumption was assessed with the score test produced by SAS (Allison 2001, Scott et al. 1997) and confirmed by conducting binary logistic regression separately for each cut-point of the dependent variable, estimating point estimates and 95% confidence intervals of the binary log-odds ratios corresponding to each cut-point, and assessing whether the point estimates for each model were within the corresponding confidence intervals of the other models (Scott et al. 1997). The proportional odds assumption was not met when BRR severity levels had small sample sizes ($n \leq 7$). To meet the model assumptions, BRR severity levels 3, 4 and 5 were pooled for the Wyoming County field, and levels 4 and 5 were pooled for the Clinton County field.

Correlation analysis. The results of the logistic regression were corroborated by conducting simple correlation analysis. Pearson's correlation coefficient was calculated in PROC CORR (SAS, version 9, SAS Institute, Cary, NC) for the relationship between BRR incidence and clay and POM content, soil saturation, and soil strength.

3.4 RESULTS

Distribution, incidence and severity of BRR. *Phoma sclerotioides* was successfully isolated from alfalfa roots and/or crowns from eight of ten alfalfa production fields surveyed in New York, six of seven fields surveyed in Vermont, and five of six fields surveyed in New Hampshire (Table 3.1), including fields from both western and northern New York and both central and northern Vermont and New Hampshire (Figure 3.1). Among fields from which *P. sclerotioides* was isolated, BRR incidence ranged from 4 to 67% in New York, 5 to 51% in Vermont, and 3 to 40% in New Hampshire (Figure 3.1). Both root and crown BRR lesions were common in all three states, and *P. sclerotioides* was successfully isolated from both roots and crowns

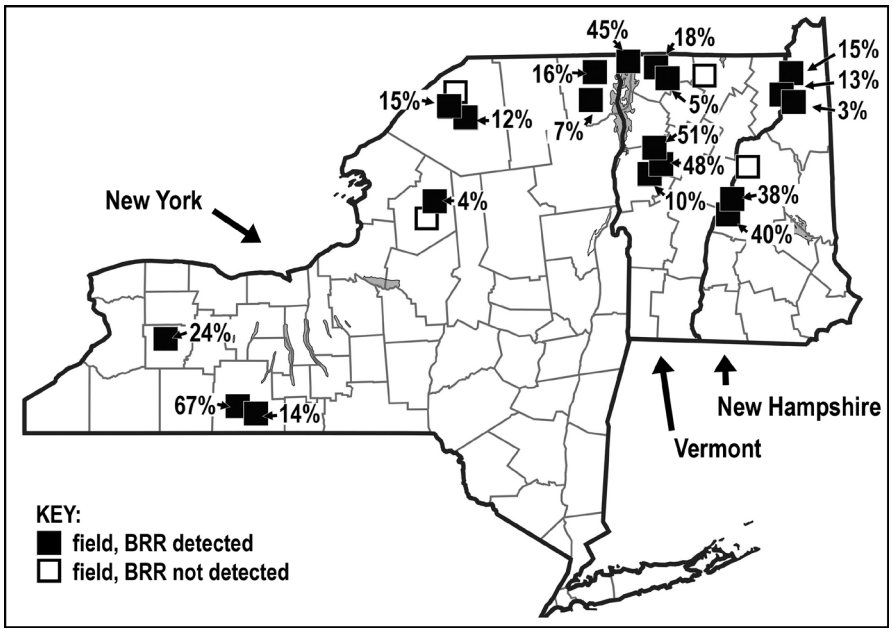

FIGURE 3.1 Distribution and incidence of brown root rot (BRR) in New York, Vermont and New Hampshire alfalfa production fields sampled in spring 2005. Filled boxes denote fields where *Phoma sclerotioides* was successfully isolated from at least one root and/or crown; empty boxes represent fields where *P. sclerotioides* was not successfully isolated. Percentages indicate BRR incidence; plants were considered positive for BRR only if *P. sclerotioides* was successfully isolated from the root and/or crown. Approximately 40 plants, selected randomly without regard to vigor or apparent health, were collected from most fields.

in all but two of the fields where BRR was detected. In most fields, the frequency of root versus crown BRR lesions was approximately equal; however, root lesions were much more prevalent in three fields, and crown lesions were much more prevalent in *sclerotioides* was isolated extended into the cortical tissues (Table 3.1).

Confirmation of pathogen identity. Diagnostic PCR resulted in amplicons of the expected size (approximately 500 bp) for both the positive control (the *P. sclerotioides* "Berg" isolate from Farson, WY, USA; American Type Culture Collection isolate MYA-295) and at least one randomly selected single-conidium *P. sclerotioides* isolate from each of the fields from which the pathogen was successfully isolated. The negative control (no template) resulted in no amplification product. In-vivo on roots of greenhouse- and growth chamber-grown alfalfa, New York, New Hampshire and Vermont *P. sclerotioides* isolates produced pycnidia with diameters from 300 to 850 µm and conidia with lengths from 4 to 8 µm and widths from 1.5 to 4 µm (Figure 3.2). Conidia guttule number ranged from 0 to 4, and most (>97%) conidia had polar guttules. Pathogenicity testing at field sites in New York's Clinton

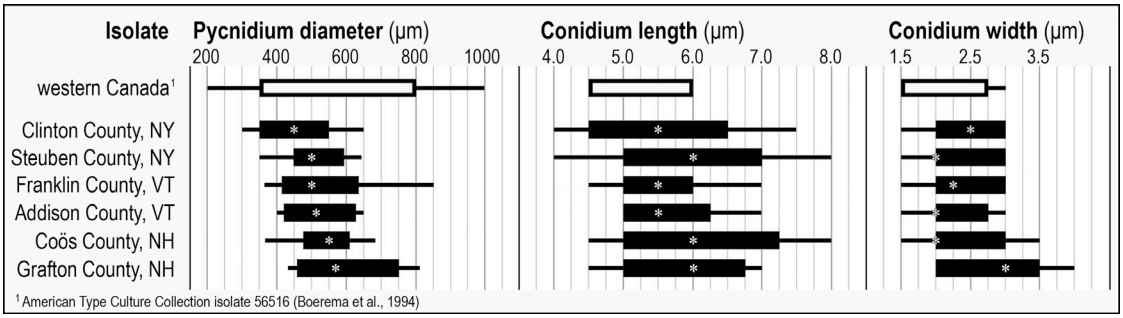

FIGURE 3.2 In-vivo pycnidium and conidium sizes of single-conidium *Phoma sclerotioides* isolates from New York, Vermont and New Hampshire compared to the published description of the species. For the Northeastern isolates, extent of the thin bars corresponds to minimum and maximum values, extent of the thick bars corresponds to 10% and 90% quantiles, and asterisks correspond to median values of 40 randomly selected pycnidia and 40 randomly selected conidia. The values for the western Canada isolate are taken from Boerema et al. (1994); the extent of the thin lines represents the minimum and maximum values, and the boxes represent the most common values.

and Steuben Counties resulted in reddish-brown to dark-brown epidermal lesions and light- to dark-brown lesions progressing into the cortical tissues. The lesions developed at the site where inoculum was placed against the root. However, *P. sclerotioides* was isolated from both lesioned and nonlesioned roots. Nine weeks after plating surface-sterilized root segments onto water agar, abundant beaked pycnidia characteristic of *P. sclerotioides* were produced in the agar or directly on root segments. In the Clinton Co. plot, *P. sclerotioides* was isolated from 37 of 60 plants, of which 14 showed no lesions, 20 had epidermal lesions, and three had lesions progressing into the cortex. In the Steuben Co. plot, *P. sclerotioides* was isolated from 20 of the 60 plants, of which three showed no lesions, 15 had epidermal lesions, and two had lesions extending into the cortex. Single-conidium cultures established on PDA from pycnidia harvested from WA plates were characteristic of *P. sclerotioides.*

Evaluation of alfalfa spring regrowth. Spring regrowth of individual plants varied widely, particularly when BRR severity levels were low, and zero or near-zero spring regrowth was observed for plants at all BRR severity levels (Figure 3.3). Linear regression of BRR severity (with severity levels 4 and 5 pooled because of small sample sizes) against the dry weight of spring regrowth (subjected to the square-root transformation to meet model assumptions) yielded correlation coefficients of 0.0125 (n=615) and 0.0079 (n=628) for the Wyoming and Clinton County fields, respectively. Evaluation of the null hypothesis that spring regrowth was unrelated to BRR severity resulted in F-values of 1.92 and 1.23 with 4 degrees of freedom and corresponding *p*-values of 0.1045 and 0.2955. For field Wyo1 (Wyoming County), contrasts between the highest BRR severity level (4, lesions affect ≥ 67% of root

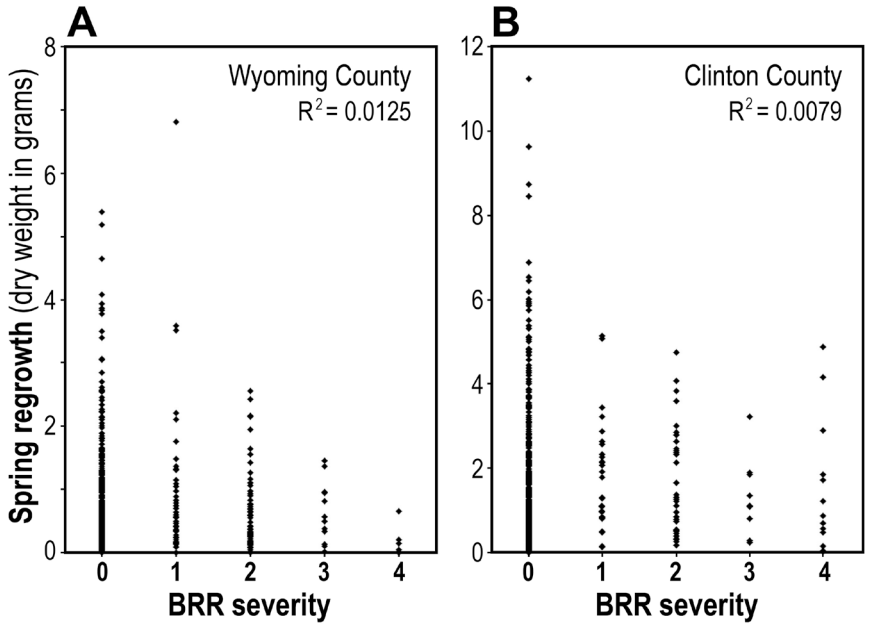

FIGURE 3.3 Scatter plots of the spring regrowth (dry weight in grams) of individual alfalfa plants with brown root rot (BRR) severity of 0 (*Phoma sclerotioides* not successfully isolated from the root or crown), 1 (*P. sclerotioides* isolated; lesions epidermal), 2 (*P. sclerotioides* isolated; lesions extend into cortex but affect ≤33% of root or crown diameter), 3 (*P. sclerotioides* isolated; lesions affect between 34 and 66% of root or crown diameter), and 4 (*P. sclerotioides* isolated; lesions affect ≥67% of root or crown diameter) in the extensively sampled fields **A**, Wyoming County, NY, and **B**, Clinton County, NY.

and/or crown diameter) and other BRR severity levels indicated a significant ($P<0.05$) or marginally significant ($P=0.0676$) decrease in spring regrowth for plants with BRR severity 4 relative to plants with BRR severity 0, 1 and 2, but not 3 (Table 3.2). For field Chz 1 (Clinton County), contrasts between the highest BRR severity level (Berkenkamp et al. 1991, lesions affect ≥ 67% of root and/or crown diameter) and other BRR severity levels indicated no significant decrease in spring regrowth ($P>0.05$) for plants with BRR severity 4 relative to plants with BRR severity 0, 1, 2 or 3 (Table 3.2).

Distribution of BRR within fields. Among the 17 fields sampled at four sites each, BRR was not detected in three fields. In the remaining 14 fields, BRR was

TABLE 3.2 Parameter estimates of the least-squares regression models assessing the relationship between brown root rot severity and square-root transformed spring regrowth (dry weight in grams). Over 600 plants were evaluated in each field.

Field	Variable[a]	Parameter estimate[b]	Standard error	*P*-value
Wyoming County, NY	Intercept	0.3353	0.1871	0.0736
	BRRsev 0	0.3922	0.1883	0.0377
	BRRsev 1	0.4645	0.1972	0.0188
	BRRsev 2	0.3567	0.1948	0.0676
	BRRsev 3	0.2505	0.2176	0.2502
	BRRsev 4	0.0000	*	*
Clinton County, NY	Intercept	1.0561	0.1570	<0.0001
	BRRsev 0	0.0005	0.1589	0.9976
	BRRsev 1	0.2208	0.1911	0.2484
	BRRsev 2	0.1092	0.1832	0.5514
	BRRsev 3	0.0008	0.2319	0.9971
	BRRsev 4	0.0000	*	*

[a] BRRsev 0, 1, 2, and 3 are dummy variables for BRR severity, where 0 indicates *P. sclerotioides* not detected; 1, *P. sclerotioides* detected, BRR lesions epidermal; 2, *P. sclerotioides* detected, BRR lesions extend into cortex but affect ≤33% of root or crown diameter; 3, *P. sclerotioides* detected, BRR lesions affect between 34 and 66% of root or crown diameter; and 4, *P. sclerotioides* detected, BRR lesions affect ≥67% of root or crown diameter.

[b] Each of the parameter estimates for the dummy variables BRRsev 0, 1, 2 and 3 represents a comparison between that particular BRR severity level and the highest severity level, BRRsev 4.

detected at one of the sampling sites in one field, at two of the sampling sites in four fields, at three of the sampling sites in one field, and at all four sampling sites in eight fields. In the two fields (Wyo1 and Chz1) sampled at 23 to 24 sites each, the highest BRR incidence was not observed at the machinery entry points to the fields, and gradients of decreasing BRR incidence from the machinery entry points were not observed (Figure 3.4). The chi-square test and SADIE both indicated that the observed distribution of BRR within fields Wyo1 and Chz1 was random, not uniform; likewise, the Mantel test and SADIE both concluded that the distribution of BRR was random, not aggregated (Table 3.3).

Stand density. In the extensively sampled fields Wyo1 and Chz1 (seeded in 2001), 5 of 23 and 10 of 24 sampling sites, respectively, had low alfalfa stand densities (Figure 3.4).

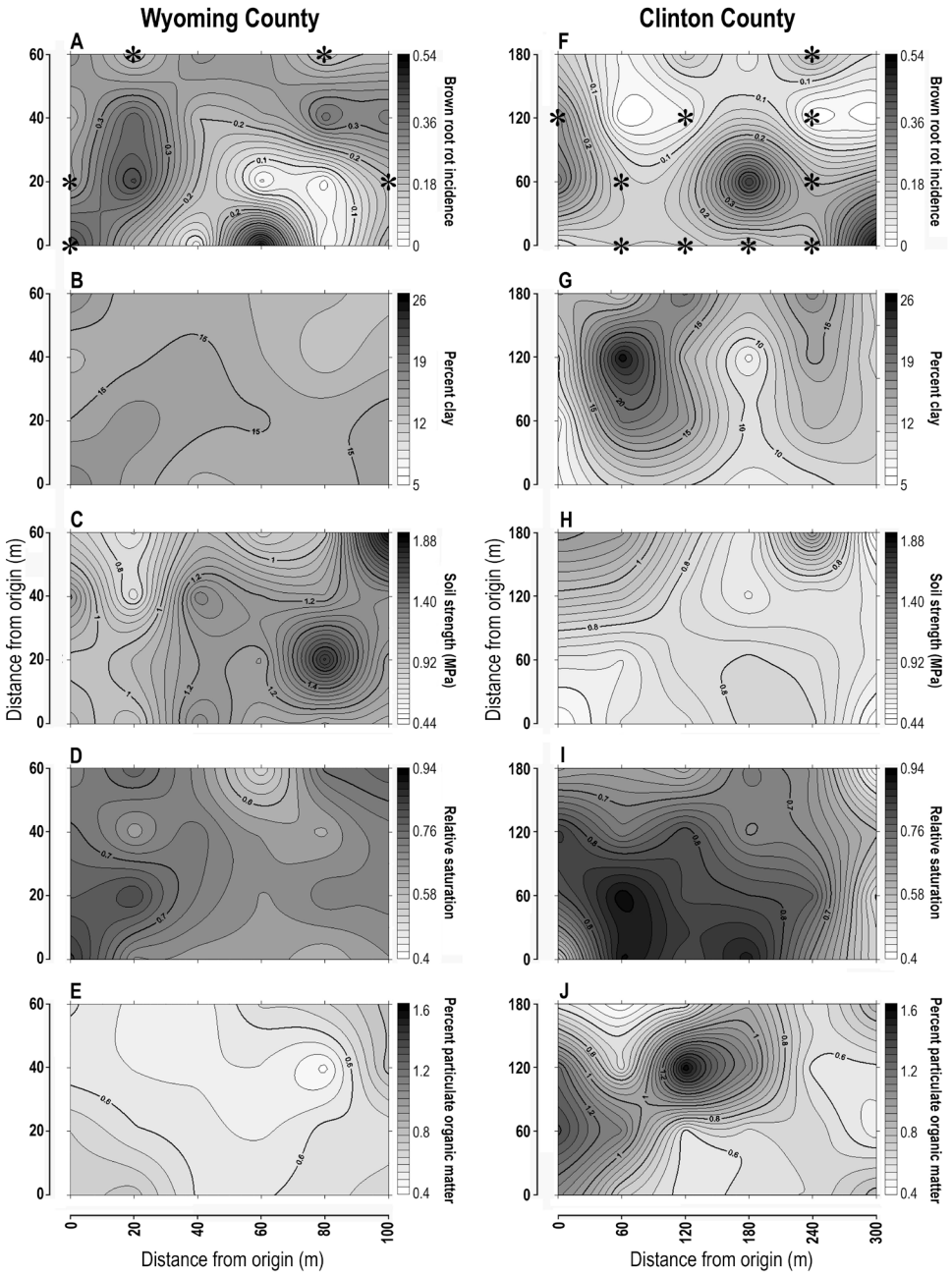

FIGURE 3.4 Contour maps of the spatial patterns of brown root rot incidence, stand density and soil characteristics in two New York alfalfa production fields, one in Wyoming County and another in Clinton County. Contour maps **A** to **E** refer to the Wyoming County field; maps **F** to **J,** to the Clinton County field; asterisks refer to the sampling sites where alfalfa stand density was low. The entry point for machinery into the Wyoming County field corresponds to point (100,60) on the Cartesian plane of the respective maps; entry points into the Clinton County field correspond to points (0,0) and (0,60). Approx. 30 plants were collected from each of 23 or 24 sites on an equidistant grid; only 23 sites were sampled in the Wyoming County field because the plants collected at point (40,20) were accidentally left in the field. Soil analyses were conducted on loose soil collected from the rhizosphere of sampled plants and on soil cores taken at the center of each sampling site.

Soil characteristics. Clay and particulate organic matter content (POM) varied widely across sampling sites in field Chz1 but varied little across sampling sites in field Wyo1 (Table 3.4); six different soil types were represented in the Clinton County field, and only one soil type was represented in the Wyoming County field (Table 3.4). Soil strength varied widely in both fields, but it was more variable in field Wyo1 than field Chz1 (Table 3.4); heavy compaction by farm equipment was observed on one end of the Wyoming County field but not observed in the Clinton County field. Relative soil saturation varied moderately across sampling sites in field Chz1 but was somewhat less variable in field Wyo1 (Table 3.4). Both fields exhibited topographic variation, with low spots where water collected and high spots where water drained

TABLE 3.3 Assessment of spatial distribution of brown root rot (BRR) in Clinton and Wyoming county, NY, fields sampled at 24 and 23 sites on a grid, respectively.

Chi-square test			
Field	X^2 (df)	P[a]	Pattern[b]
Clinton	98.81 (23)	< 0.0001	Not uniform; random or aggregated.
Wyoming	61.70 (22)	< 0.0001	Not uniform; random or aggregated.
Mantel test			
Field	r[c]	P[d]	Pattern[b]
Clinton	0.0486	0.5998	Not aggregated; random or uniform.
Wyoming	-0.0655	0.4436	Not aggregated; random or uniform.
SADIE			
Field	I_a[e]	P_a[f]	Pattern[b]
Clinton	1.054	0.3205	Random; not uniform or aggregated.
Wyoming	0.900	0.6282	Random; not uniform or aggregated.

[a] P-value from a test of the null hypothesis (H_0) that that BRR frequency is independent of sampling site.
[b] Spatial pattern of BRR incidence suggested by the corresponding test. As proposed by Perry et al. (38), BRR frequencies, rounded to the nearest integer, were used in the place of counts for SADIE. The same approach was extended to the Mantel tests.
[c] Normalized Mantel statistic (10); values of r approaching ±1 from unity represent progressively greater aggregation.
[d] P-value from a test of H_0 that differences in BRR frequency between all possible pairs of sampling sites are not correlated to the Euclidian distances between the corresponding pairs of sampling sites.
[e] Index of aggregation (36); values of $I_a < 1$, $= 1$, and > 1 represent uniform, random and aggregated spatial patterns.
[f] P-value from a test of H_0 that the minimum distance that individuals would need to be moved from their corresponding sampling sites to achieve a uniform spatial distribution is not different from the minimum distance expected under a random assignment of the individuals to the sites.

more readily. However, topographic variability was greater in the Clinton County than in the Wyoming County field.

Cumulative logistic regression indicated that, controlling for the other variables in the model, relative saturation, stand density, clay content, and soil strength were all significant ($P < 0.05$) or marginally significant ($P < 0.08$) explanators of the observed variability in BRR incidence and severity within the Wyoming and Clinton County fields (Table 3.5). In both fields, the odds of more severe BRR increased as relative soil saturation increased, and they decreased as alfalfa stand density decreased (Table 3.5). However, the effects of percent clay and of soil strength on BRR severity varied by field. In Clinton County, the odds of more severe BRR decreased as soil clay content increased. In Wyoming County, the odds of more severe BRR increased as soil clay content increased, but only among sites with low alfalfa stand density; across the entire field, no significant relationship was detected (Table 3.5). In Wyoming County, the odds of more severe BRR decreased as soil strength increased.

TABLE 3.4 Mean and standard deviation of brown root rot (BRR) incidence and soil characteristics across sampling locations within two fields sampled extensively in spring 2005.

Field[a]	BRR[b]	Clay[c]	POM[d]	Str[e]	Satur[f]	Soil types[g]
Wyoming County, NY	**0.24** (0.13)	**14.69** (1.37)	**0.63** (0.10)	**1.13** (0.28)	**0.67** (0.06)	Conesus gravelly silt-loam
Clinton County, NY	**0.16** (0.14)	**12.51** (4.62)	**0.81** (0.32)	**0.78** (0.21)	**0.71** (0.13)	Hoagensburg loam, Mino fine sandy loam, Muskellunge silty clay loam, Nickelville very fine sandy loam, Swanton loam, and Trout River gravelly loam.

[a] Sampling was conducted at 23 sites in the Wyoming County field and 24 sites in the Clinton County field.
[b] BRR incidence; approximately 30 plants were analyzed for *Phoma sclerotioides* at each sampling site.
[c] Clay content (percent).
[d] Particulate organic matter content (percent).
[e] Soil strength (MPa), a measure of soil compaction.
[f] Soil saturation (proportion of available soil pore space filled with water) of field-moist soil at time of plant sampling.

TABLE 3.5 Parameter estimates of the cumulative logistic regression models used to evaluate the influence of soil characteristics and stand density on brown root rot severity in extensively sampled New York alfalfa production fields. Over 700 plants, collected at 23 to 24 sites, were assessed in each field.

Field	Variable[a]	Parameter estimate[b]	Standard error[c]	*P*-value
Wyoming County, NY	Intercept 1	-6.365	1.617	< 0.0001
	Intercept 2	-4.865	1.601	0.0024
	Intercept 3	-4.250	1.596	0.0078
	Clay	0.0097	0.100	0.9230
	POM	0.914	1.157	0.4299
	Stand 0	-4.126	1.431	0.0039
	Strength	-2.033	0.619	0.0010
	Rel Satur	6.338	2.610	0.0152
	Clay*Stand	0.241	0.091	0.0078
Clinton County, NY	Intercept 1	-5.187	1.200	< 0.0001
	Intercept 2	-4.522	1.183	0.0001
	Intercept 3	-3.565	1.171	0.0023
	Intercept 4	-3.178	1.168	0.0065
	Clay	-0.068	0.036	0.0596
	POM	-0.245	0.498	0.6223
	Stand 0	-1.921	0.724	0.0080
	Strength	0.904	0.772	0.2415
	Rel Satur	2.355	1.337	0.0782
	Str*Stand	2.045	0.826	0.0133

[a] Intercept 1, 2, 3, 4 = intercepts for the cumulative log-odds models, listed in order of ascending BRR severity cut-point; clay = percent clay; POM = percent particulate organic matter; stand 0 = dummy variable for low (relative to average) stand density; strength = soil strength, a measure of compaction; rel satur = relative soil saturation; clay*stand = interaction between clay and stand density; and str*stand = interaction between soil strength and stand density. BRR severity was rated on a 0 (*P. sclerotioides* not detected) to 5 (*P. sclerotioides* detected; lesions completely girdle root or crown) scale; because of small sample sizes at the highest severity levels, severity levels 3, 4 and 5 were pooled for the Wyoming County field and levels 4 and 5 were pooled for the Clinton County field in the analysis.

[b] Wald chi-square, corrected for overdispersion by dividing the original Wald chi-square the ratio of the Pearson goodness-of-fit chi-square to its degrees of freedom (Allison 2001).

[c] Corrected for overdispersion by multiplying the original standard error by the ratio of the Pearson goodness-of-fit chi-square to its degrees of freedom (Allison 2001).

In Clinton County, the odds of more severe BRR increased as soil strength increased, but only among sites with low stand density; across the entire field, no significant relationship was detected (Table 3.5). The likelihood ratio test of the overall significance of the regression models resulted in *p*-values of 0.0077 and 0.0250 for the Wyoming and Clinton County fields, respectively.

Maps illustrating the spatial distribution of BRR incidence, stand density, and soil characteristics show similar trends (Figure 3.4). A general tendency for areas of low stand density to have lower BRR incidence, at least relative to the adjacent sampling sites, can be seen for both fields (Figure 3.4, maps A and F; points with low stand density marked with asterisks), though it is somewhat less pronounced for the Wyoming County field than the Clinton County field. A negative relationship between percent clay and BRR incidence was evident in the Clinton County field (r^2 = -0.31, n=24), and a strong positive relationship between percent clay and BRR incidence existed among the sampling sites with low stand density in the Wyoming County field (r^2 = 0.89, n=5). A negative relationship between soil strength and BRR incidence was evident in the Wyoming County field (r^2 = -0.32, n=23), and a positive relationship between soil strength and BRR incidence existed among sampling sites with low stand density in the Clinton County field (r^2 = 0.59, n=10). Finally, a weak correlation between soil saturation and BRR incidence existed for the Wyoming County field (r^2 = 0.20, n=23). No correlation, however, was observed between soil saturation and BRR incidence in the Clinton County field (r^2 = -0.01, n=24). All soil variables identified as nonsignificant explanators of BRR severity by logistic regression (Table 3.5) also showed low correlation with BRR incidence ($r^2 \leq 0.10$).

3.5 DISCUSSION

The combination of identification of all isolates on three types of agar, in-vivo assessment of morphological characteristics of the fungus, diagnostic PCR, and pathogenicity testing confirmed that *P. sclerotioides* is prevalent in alfalfa fields in New York, Vermont and New Hampshire. Though the conidia produced on

inoculated alfalfa roots by isolates from the Northeast were somewhat larger than those described by Boerema et al. (1994), similarly large conidia have also been reported from the state of Wyoming (Hollingsworth et al. 2002), and other characteristics of the isolates in-vivo, such as conidial guttule number and polarity and pycnidial diameter, were consistent with published descriptions of the pathogen (Boerema et al. 1994, Hollingsworth et al. 2002). The predominance of epidermal lesions in pathogenicity testing was not surprising. The plants were collected in early December, before the ground froze and just two months after inoculation, and previous researchers (Cormack 1934, Sanford 1933) have observed that BRR lesions develop primarily in the late winter and early spring. Likewise, re-isolation of *P. sclerotioides* from inoculated alfalfa roots with no visible lesions supports previous research suggesting that the pathogen can be harbored by roots lacking symptoms (Samac and Hollingsworth 2004).

The results from the spring 2005 survey of New York, Vermont and New Hampshire alfalfa production fields suggest that BRR is widespread in the region and that the disease may be economically important. Moderate (10-24%) and high (38-67%) BRR incidence was observed in many fields. A majority of the lesions associated with *P. sclerotioides* extended into the cortical tissues, suggesting that BRR may negatively impact root function and overall plant vigor in the region. Finally, BRR lesions were common on crowns as well as roots, suggesting that BRR may also directly reduce the shoot production potential of individual plants.

However, further work is needed to fully assess the impact of BRR on alfalfa production in the region. BRR has been observed to fluctuate considerably across years (Berkenkamp et al. 1991, Hollingsworth et al. 2003), and the BRR incidence and

severity observed in 2005 may be either lower or higher than average. Further, the morphology of lesions from which *P. sclerotioides* was isolated varied widely, and it is unlikely that *P. sclerotioides* was the sole cause of all of the lesions from which it was successfully isolated. Alfalfa root and crown lesions are frequently the product of the interaction of multiple pathogens (Grau 1996, Leath et al. 1988). *P. sclerotioides* was rarely, if ever, the only fungus growing on or from the surface-sterilized tissue segments from which *P. sclerotioides* was isolated on water agar, though identity and pathogenicity of other fungi were not assessed. Finally, the relationship between BRR severity and spring regrowth was weak in the two fields sampled extensively. However, it is important to note several factors that may have limited our ability to detect an overall significant relationship if it existed. Sampling was conducted very early in the spring, which may have precluded our ability to successfully differentiate winterkill from normal variability in alfalfa emergence from dormancy. Plants remaining in both fields may have been those most tolerant to BRR; both fields had already passed through four winters, and plants highly susceptible to BRR may have been killed in previous years. Finally, as noted earlier, *P. sclerotioides* activity fluctuates annually. If 2005 was not particularly conducive to BRR development, many of the observed lesions may have developed primarily in a previous year, thereby giving plants at least one season to compensate.

The non-aggregated distribution of BRR observed in the extensively sampled Clinton and Wyoming County fields suggests that *P. sclerotioides* was not introduced recently to the fields on equipment with contaminated soil. The distribution of BRR was not aggregated at the field level, and the highest incidence of BRR was not found at the machinery entry points to the fields. The detection of BRR at all sampling

locations in many of the fields sampled at four sites further supports the conclusion that *P. sclerotioides* was not introduced recently. The results suggest that *P. sclerotioides* previously escaped detection or that it was introduced by another means. In Wyoming, where BRR was first reported in 1996, *P. sclerotioides* is widespread in non-agricultural soils (Reedy et al. 2006), suggesting that the pathogen has long been present and could be native. Recent Canadian research indicates that *P. sclerotioides* contributes to alfalfa spring blackstem and leaf spot, causing foliar lesions similar to those produced by *P. medicaginis* (Hwang et al. 2006, Wang et. 2004). If foliar infection is indeed common, aerial dispersal of conidia of *P. sclerotioides* may also occur.

The strong relationship between BRR incidence and soil factors suggests that the observed distribution of BRR within the Clinton and Wyoming County fields may have been driven by differences in soil microenvironments. Variation in relative soil saturation was a significant explanator of the non-uniform distribution of BRR in both fields. While soil strength and soil clay content were not consistent explanators across fields, they were significant explanators of BRR either across sampling sites with low stand density or across all sampling sites in individual fields. Whether high levels of the factors promoted or inhibited BRR, however, is not immediately apparent. Because both alfalfa fields had already passed through four winters and incurred considerable stand decline prior to spring 2005, the plants most susceptible to BRR may have already been killed in the field microenvironments particularly conducive to BRR by the time sampling was conducted. Indeed, sites with low alfalfa stand density exhibited lower BRR severity in both fields. The present observational study suggests that it is reasonable to hypothesize that edaphic factors influence BRR, but

experimental approaches will be needed to confirm that relative soil saturation, and possibly soil strength and soil clay content, influence BRR incidence.

Although *P. sclerotioides* has never been previously reported in the eastern United States, the widespread distribution of *P. sclerotioides* in New York, Vermont and New Hampshire observed in this study is not surprising. Despite a climate conducive to low-temperature fungi, research on low-temperature pathogens of alfalfa in the northern United States has been lacking (Leath 1989). Further, the slow rate of growth that *P. sclerotioides* exhibits in culture at room temperature (Sanford 1933), and the low frequency with which *P. sclerotioides* is isolated during the summer months (McDonald 1955) make it unlikely that the pathogen would have been recovered from samples submitted to diagnostic labs or from roots collected in other survey efforts.

The widespread distribution and high incidence of BRR observed in New York, Vermont and New Hampshire alfalfa production fields coupled with the prevalence of BRR lesions extending into the root and crown cortex suggest that further work is needed to develop management strategies for the disease in the Northeast. Resistance to BRR in alfalfa is heritable (Hollingsworth et al. 2005), and significant differences in BRR resistance have been observed in alfalfa varieties grown in Alberta (Berkenkamp et al. 1991). Identification of the relative BRR susceptibility of alfalfa varieties adapted to the Northeast is needed to assess which commercially available varieties are best suited to fields with a history of severe BRR and to identify sources of resistance for future breeding.

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CHAPTER 4

FIRST REPORT OF BROWN ROOT ROT OF ALFALFA, CAUSED BY *PHOMA SCLEROTIOIDES,* IN COLORADO, MAINE, NEW MEXICO, ONTARIO, AND PENNSYLVANIA*

4.1 ABSTRACT

Brown root rot (BRR), caused by the fungal pathogen *Phoma sclerotioides*, is associated with root and crown lesioning of alfalfa (*Medicago sativa* L.) and plant mortality in regions with severe winters. In spring 2006 or 2007, alfalfa plants were collected in seven production fields in Colorado, five fields in Maine, five fields in New Mexico, 10 fields in southern Ontario, 10 fields in Pennsylvania, and two fields in West Virginia. In Ontario, some lesions girdled the crown; in three fields in Maine, large pycnidia characteristic of *P. sclerotioides* were present on alfalfa crowns and overwintered stems; in two fields in Colorado, high incidence of plant mortality associated with severe crown lesions was observed; and in all other fields, plants exhibited a range of root and crown rot symptoms. *P. sclerotioides* was isolated from one field in Colorado, five fields in Maine, one field in New Mexico, eight fields in Ontario, and four fields in Pennsylvania. In fields with *P. sclerotioides,* incidence of

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alfalfa root or crown infection by *P. sclerotioides* was low in Colorado and New Mexico (2 to 3%) and moderate in Maine, Ontario, and Pennsylvania (5 to 29%). *P. sclerotioides* isolates had conidial and pycnidial morphology characteristic of *P. sclerotioides*, tested positive for *P. sclerotioides* in diagnostic PCR, and had ITS sequences 95.5 to 100% identical to *P. sclerotioides* ATCC isolate 56515 over a 488-bp alignment. Pathogenicity testing of representative isolates from each state resulted in light brown, dark brown, or nearly black lesions, often with a darker border. Abundant pycnidia were found on the surface of lesions, and plant mortality was associated with lesions that girdled the root or crown. To our knowledge, this is the first report of BRR in Colorado, Maine, New Mexico, Ontario, and Pennsylvania, and the southernmost report of *P. sclerotioides* in eastern and western North America.

4.2 INTRODUCTION

Phoma sclerotioides G. Preuss ex Sacc. (synonym *Plenodomus meliloti* Dearn. & G. B. Sanford), causal agent of brown root rot (BRR) of alfalfa, is associated with decreased yield and reduced stand longevity of alfalfa in regions with harsh winters. Pathogenic in the late winter and early spring, *P. sclerotioides* causes necrotic lesions that can girdle alfalfa roots and crowns before plants emerge from winter dormancy. It causes sporadic but severe losses in alfalfa production (Berkenkamp et al. 1991, Hollingsworth et al. 2003), and alfalfa plant mortality associated with BRR can exceed 60 percent on susceptible alfalfa varieties two years after seeding and can reach 40 percent one year after seeding (Berkenkamp et al. 1991).

In North America, *P. sclerotioides* has long been known to occur in Alaska, the Yukon, Alberta, British Columbia, Manitoba, and Saskatchewan (Davidson 1990,

Lebeau and Logsdon 1958, McDonald 1955, Sanford 1933). *P. sclerotioides* was first reported in the contiguous United States in Wyoming (Hollingsworth and Gray 1999) and has subsequently been detected in alfalfa production fields in much of the northern United States, including Idaho, Minnesota, Montana, New Hampshire, New York, Wisconsin, and Vermont (Hollingsworth et al. 2003, Larsen et al. 2004, Larsen et al. 2007, Wunsch et al. 2007).

The distribution of *P. sclerotioides* in North America remains poorly understood. The pathogen is a major constraint on alfalfa production in parts of central and western Canada, but its occurrence in eastern Canada has been documented only in Nova Scotia (Davidson 1990). The pathogen is common in alfalfa production fields in northern New Hampshire and Vermont (Wunsch et al. 2007), but it is unknown if it occurs in Maine. Finally, *P. sclerotioides* is adapted to low temperatures and occurs only in region with cold climates (Davidson 1990), but the southern extent of its range is unknown.

The objectives of this study were to evaluate the distribution of *P. sclerotioides* within Maine and Ontario and to identify the southern extent of the pathogen's range within alfalfa production regions of eastern and western North America. Alfalfa production fields were sampled in Colorado, Maine, New Mexico, Ontario, Pennsylvania, and West Virginia in 2006 and 2007; the incidence of alfalfa root and crown infection by *P. sclerotioides* was assessed; isolates were subjected to diagnostic PCR, sequenced at the ITS region, and characterized morphologically to confirm pathogen identity; and pathogenicity testing was completed.

4.3 MATERIALS AND METHODS

Surveys of alfalfa production fields. In May 2006, alfalfa plants were collected from two fields in Otero Co., CO; three fields in Huerfano Co., CO; two fields in Rio Grande Co., CO; two fields in Taos Co., NM; and three fields in Rio Arriba County, NM. Sampling was conducted at four sites within each field, and approximately 20 or 40 plants were collected per field. In April and May 2007, alfalfa plants were collected from one field each in Penobscot and Somerset Counties, ME; three fields in Waldo Co., ME; five fields south of Ottawa, ON; one field each near Belleville, Binbrook, Caledonia, Lindsay, and Woodstock, ON; one field each in Armstrong, Columbia, Jefferson, Mercer, and Sullivan Counties, PA; two fields each in Centre and Crawford Counties, PA; and in two fields in Mason Co., WV. All fields existed at least two winters. Approximately 10 to 15 plants were sampled per field in Pennsylvania and West Virginia, approximately 10 to 70 plants were sampled per field in Maine, and approximately 20 to 30 plants were sampled per field in Ontario. In each field, plants were collected from multiple sites.

Pathogen isolation and identification. Tissue segments were excised from roots and crowns of all alfalfa plants collected, regardless of symptoms, and surface-sterilized with 0.6% hypochlorite and 70% ethanol, rinsed with sterile distilled water, and incubated for 3 to 6 months at 10°C under continuous white fluorescent light with average luminescence of 2,000 lx. After the incubation period, pycnidia characteristic of *P. sclerotioides* were harvested from tissue segments or the surrounding agar and utilized to establish pure cultures from single conidia. Single conidia were transferred to potato dextrose agar (PDA; Difco Laboratories, Becton, Dickson and Company,

Sparks, MD) and incubated at 10°C under continuous white light for 2 to 3 months, and morphology of the isolates was assessed.

Diagnostic PCR. Single-conidium cultures were transferred to potato dextrose broth (PDB; Difco Laboratories, Becton, Dickson and Company, Sparks, MD) and grown at 10ºC under continuous white fluorescent light. After 3 to 5 weeks, the cultures were centrifuged, PDB was decanted, and mycelium was rinsed in sterile distilled water. The mycelium was lyophilized, and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA) according to manufacturer instructions. Diagnostic PCR was conducted in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) with primers developed by Larsen et al. (2002). Each reaction included 14.375 µl water, 0.25 µl Qiagen Hot Star Taq (5 U/µl), 0.5 µl primer PSB12-FS-24 (50 µM), 0.5 µl primer PSB12-RS-26 (50 µM), 0.25 µl dNTPs (10mM), 2.5 µl Qiagen 10x PCR buffer, and 2.5 µl $MgCl_2$ (25 mM). The following thermocycler parameters were used: 2 min at 95ºC, followed by 35 cycles of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min, and a final extension of 1 min at 72ºC, followed by a 4ºC bath. Positive and negative controls were included. The *P. sclerotioides* "Berg" isolate (American Type Culture Collection isolate MYA-295), isolated from a diseased alfalfa plant near Farson, WY was used as a positive control; a no-template reaction was included as a negative control. Diagnostic PCR was conducted on one isolate each from Colorado and New Mexico, 13 isolates from Ontario, 21 isolates from Maine, and 5 isolates from Pennsylvania.

Sequencing and alignment of ITS region. The internal transcribed spacer (ITS) 1, 5.8S, and ITS2 of the rDNA of all *P. sclerotioides* isolates were amplified and sequenced using primers ITS1 and ITS4 (White et al., 1990). Amplification was

conducted in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) in 25 µl reactions with 0.625 units/reaction hot start *Ex Taq* DNA polymerase (Takara Bio, Madison, WI), 1x *Ex Taq* buffer, 0.5 µM of each primer, and 0.5 mM dNTPs. Thermocycler parameters were 94°C for 1 min and 40 cycles of 94°C for 45 seconds, 61°C for 45 seconds and 70°C for 1 min, followed by 70°C for 5 min and a 4°C soak. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA), and sequencing was conducted at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY). Trace files were visualized with FinchTV (Geospiza, Seattle, WA), and sequences were edited in EditSeq (Lasergene 7.2.1, DNASTAR Inc., Madison, WI). Sequences of *P. sclerotioides* ATCC isolate 56515 and *P. medicaginis* ATCC isolate 16929 were generated previously by Larsen et al. (2007) and obtained from GenBank. Sequence alignment was conducted in MegAlign (Lasergene 7.2.1, DNASTAR Inc., Madison, WI) using the default gap and gap length penalties of 15.00 and 6.66, respectively. The sequence alignment was manually edited to resolve inconsistencies in the alignment of the ends of the sequences.

Pathogenicity testing. Pathogenicity testing was conducted on alfalfa cv. Vernal grown in SC10 Cone-Tainers (Stuewe and Sons, Inc, Corvallis, OR) in a 1:1 mix (v/v) of autoclaved sand and autoclaved loam soil. Barley grain inoculum was prepared by growing *P. sclerotioides* on potato dextrose agar in Petri dishes, covering the cultures with autoclaved soaked barley (barley was soaked approximately 4 hr and autoclaved for 40 minutes twice), and incubating under continuous light for two months at 10°C. One isolate from Colorado, six isolates from Maine, one isolate from New Mexico, six isolates from Ontario, and one isolate from Pennsylvania were evaluated. Three pathogenicity testing experiments were conducted, one for isolates from Colorado and

New Mexico, another for isolates from Maine and Pennsylvania, and a third for isolates from Ontario. Pathogenicity testing was conducted on 14 to 23 plants per isolate for Colorado and New Mexico, 18 plants per isolate for Maine and Pennsylvania, and 2 to 4 plants per isolate for Ontario. Roots were inoculated approximately four months after the alfalfa was seeded. Soil was carefully removed from one side of the roots, barley grain inoculum was placed against the upper tap root 3 to 6 cm below the crown, and the soil was replaced. Autoclaved barley grains were used in the control. After inoculation, plants were maintained at 15°C for 3.5 weeks, 4°C for 5.5 weeks, 0 to -2°C for 12 weeks, 4°C for 3 weeks, and 15°C for 2 weeks under 12 hours light/day (except at 0 to -2°C, when plants received no light). To confirm that *P. sclerotioides* caused the lesions, re-isolation of the pathogen was attempted from all plants. Tissue segments were excised from lesioned roots and crowns, surface sterilized with 0.6% hypochlorite and 70% ethanol, plated onto water agar, and incubated at 10°C under continuous light. After two months, the tissue segments and surrounding water agar were evaluated for the pycnidia characteristic of *P. sclerotioides.*

4.4 RESULTS

Surveys of alfalfa production fields. Fields sampled in Rio Grande County, Colorado exhibited severe winterkill, with most plants completely girdled by crown lesions. In three fields in Maine, large pycnidia characteristic of *P. sclerotioides* were present on alfalfa crowns and overwintered stems, and in Ontario, some lesions girdled the crown. Plants from other fields exhibited a range of root and crown rot symptoms.

P. sclerotioides was isolated from 1 of 7 fields sampled in Colorado, 5 of 5 fields sampled in Maine, 1 of 5 fields sampled in New Mexico, 8 of 10 fields sampled in Ontario, 4 of 9 fields sampled in Pennsylvania, and 0 of 2 fields sampled in West Virginia (Table 4.1). Incidence of *P. sclerotioides* within individual alfalfa production fields was as high as 2 percent in Colorado, 29 percent in Maine, 3 percent in New Mexico, 29 percent in Ontario, and 22 percent in Pennsylvania (Table 4.1). Root and crown lesions associated with *P. sclerotioides* extended into the cortex but were generally only moderate in severity (Table 4.1).

Pathogen identification. On PDA, single-conidium isolates from Colorado and New Mexico produced large pycnidia (0.33 to 0.80 mm diameter) with long necks for spore discharge, white cirri darkening to yellow with age, and unicellular, hyaline, ovoid conidia 5 to 7 μm long by 2 μm wide. Representative single-conidium isolates from Maine, Ontario, and Pennsylvania also produced large pycnidia (0.33 to 1.15 mm diameter) with long necks for spore discharge, white cirri darkening to yellow with age, and unicellular, hyaline, ovoid conidia 5 to 8 μm long by 2 to 3 μm wide. Conidial and pycnidial morphology was consistent with previous reports (Boerema et al. 2004, Hollingsworth et al. 2002).

Diagnostic PCR. PCR conducted with *P. sclerotioides*-specific primers (Larsen et al. 2002) resulted in a single amplification product for 2 of 2 isolates evaluated from Colorado and New Mexico, 12 of 13 isolates evaluated from Ontario, 21 of 21 isolates evaluated from Maine, and 5 of 5 isolates evaluated from Pennsylvania. For one isolate from Ontario, no amplification product was produced. Morphologically, the isolate was indistinguishable from others collected in Ontario, but diagnostic PCR repeatedly gave a negative result.

TABLE 4.1 Incidence of alfalfa root and crown infection by *P. sclerotioides* and severity of lesions associated with *P. sclerotioides* in fields sampled in Colorado, Maine, New Mexico, Ontario, and Pennsylvania.

			Root [e]		Crown [f]		Stem [g]
Field (year seeded)	**Plants[a]**	**BRR[b]**	**Incid.[c]**	**Sev. [d]**	**Incid.[c]**	**Sev. [d]**	**Incid [c]**
Colorado (sampled in 2006)							
Otero County, field 1 (2000)	40	0	0	NA	0	NA	ND
Otero County, field 2 (2001)	40	0	0	NA	0	NA	ND
Huerfano County, field 1 (mixed age)	46	0	0	NA	0	NA	ND
Huerfano County, field 2 (2001)	20	0	0	NA	0	NA	ND
Huerfano County, field 3 (2002)	24	0	0	NA	0	NA	ND
Rio Grande County, field 1 (2003)	50	0	0	NA	0	NA	ND
Rio Grande County, field 2 (2001)	44	2	0	NA	2	2.00	ND
Maine (sampled in 2007)							
Penobscot County (2005)	69	19	10	1.43	9	2.00	9
Somerset County (2001)	32	19	13	1.50	6	2.00	0
Waldo County, field 1 (2001)	11	9	0	NA	9	3.00	17
Waldo County, field 2 (2003)	30	13	10	2.00	7	2.50	0
Waldo County, field 3 (2005)	52	29	10	1.60	21	2.18	33
New Mexico (sampled in 2006)							
Taos County, field 1 (2001)	37	3	0	NA	3	2.00	ND
Taos County, field 2 (1998)	42	0	0	NA	0	NA	ND
Rio Arriba County, field 1 (2004)	42	0	0	NA	0	NA	ND
Rio Arriba County, field 2 (1998)	37	0	0	NA	0	NA	ND
Rio Arriba County, field 3 (2001)	21	0	0	NA	0	NA	ND
Ontario (sampled in 2007)							
Caledonia (unknown)	28	0	ND	ND	ND	ND	ND
Binbrook (unknown)	25	8	ND	ND	ND	ND	ND
Lindsay (unknown)	29	17	ND	ND	ND	ND	ND
Belleville (unknown)	32	9	ND	ND	ND	ND	ND
Woodstock (unknown)	28	7	ND	ND	ND	ND	ND
South of Ottawa, field 1 (unknown)	20	0	ND	ND	ND	ND	0
South of Ottawa, field 2 (unknown)	21	29	ND	ND	ND	ND	19
South of Ottawa, field 3 (unknown)	18	0	ND	ND	ND	ND	0
South of Ottawa, field 4 (unknown)	20	5	ND	ND	ND	ND	5
South of Ottawa, field 5 (unknown)	29	13	ND	ND	ND	ND	9
Pennsylvania (sampled in 2007)							
Armstrong County (2004)	14	0	0	NA	0	NA	ND
Centre County, field 1 (2004)	9	0	0	NA	0	NA	ND
Centre County, field 2 (2005)	11	0	0	NA	0	NA	ND
Columbia County (2003)	12	8	8	2.00	0	NA	ND
Crawford County, field 1 (2003)	9	22	11	1.00	11	2.00	ND
Crawford County, field 2 (2004)	13	8	0	NA	8	2.00	ND
Jefferson County (2005)	13	8	0	NA	8	2.00	ND
Mercer County (2005)	16	0	0	NA	0	NA	ND
Sullivan County (2004)	14	0	0	NA	0	NA	ND
West Virginia (sampled in 2007)							
Mason County, field 1 (2004)	12	0	0	NA	0	NA	ND
Mason County, field 1 (2005)	11	0	0	NA	0	NA	ND

[a] Number of plants sampled in field

[b] Percentage of plants from which *P. sclerotioides* was isolated

[c] Percentage of plants from which *P. sclerotioides* was isolated from root, crown, or stem tissues.

[d] Average severity of lesions from which *P. sclerotioides* was isolated, rated on a 1 to 5 scale: 1 = lesions restricted to epidermal tissues, 2 = lesions extend into cortical tissues but impact <33% of root or crown diameter, 3 = lesions affect 33 to 66% of root or crown diameter, 4 = lesions affect >66% of root or crown diameter, and 5 = lesions completely girdle root or crown. Fields where data on root and crown rot were not recorded are indicated with "ND", and fields where *P. sclerotioides* was not isolated from root or crown tissues are indicated with "NA".

[e] Root tissues; includes lateral roots and all but the first 3 to 4 cm of the taproot.

[f] Crown tissues; defined as the first 3 to 4 cm of the taproot.

[g] Dead overwintered stem tissues. Fields in which stem tissues were not evaluated are indicated with "ND".

ITS sequence identity. Isolates from Maine and Ontario showed 95.5% to 100% sequence identity to *P. sclerotioides* isolate 'ATCC 56515' (GenBank accession number DQ525733; Larsen et al. 2007) over 488 bp of aligned sequence (Table 4.2). Isolates from Pennsylvania, Colorado, and New Mexico showed 95.5%, 98.4%, and 98.2% sequence identity, respectively, to *P. sclerotioides* isolate 'ATCC 56515'. Nine unique ITS haplotypes were represented among the sequences, seven from eastern North America, and one each from Colorado and New Mexico (Table 4.2).

Pathogenicity testing. Inoculation of alfalfa cv. Vernal with isolates from Maine and Pennsylvania resulted in light brown lesions with a darker border or dark brown to black lesions. Abundant pycnidia were frequently produced on the surface of lesions, and many lesions completely girdled crowns or tap roots, leading to plant mortality. Inoculation of Vernal alfalfa with an isolate from New Mexico resulted in dark brown to nearly black lesions that girdled the crown and upper tap root of many plants, leading to plant mortality. Abundant pynidia were found on the surface of lesions. Inoculation with an isolate from Colorado resulted in light brown tap root lesions with a darker border, and dark brown to black lesions girdling the crowns of some plants. Inoculations with isolates from Ontario resulted in dark brown lesions, often with a darker border. Abundant pycnidia were produced on the surface of lesions, and many lesions girdled the crowns. For diseased plants from all inoculations, *P. sclerotioides* was successfully re-isolated from surface-sterilized segments of lesioned roots and crowns. For all isolates, lesion severity was more severe on the crowns than the roots (Table 4.3), and differences between root rot and crown rot severity likely reflected the placement of inoculum near the crown. Many crown lesions girdled the plant, and high levels of crown rot were associated with elevated alfalfa mortality (Table 4.3).

TABLE 4.2 Single-nucleotide polymorphisms and insertion-deletions in a 488-bp alignment of ITS sequence data from *P. sclerotioides* ATCC isolate 56515 collected in British Columbia and representative *P. sclerotioides* isolates collected in Colorado, Maine, New Mexico, Ontario, and Pennsylvania.

Isolate (accession no.)[a]	**Alignment position**[b]														
Position	6	7	10	11	12	13	14	15	16	17	20	21	22	23	24
Concensus	–	–	**G**	**C**	–	–	**G**	**T**	**G**	**C**	**T**	–	**G**	–	**G**
ATCC 56515 (DQ525733)	T	T	G	C	–	G	G	T	G	C	T	T	G	T	G
Colorado															
CO 7-2-10a (EU265669)	–	–	G	C	–	–	G	T	G	C	C	G	G	T	G
Maine															
ME 3-4-16cr (FJ179157)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–
ME 5-1-5r (FJ179159)	T	–	G	C	–	G	G	T	G	C	T	T	G	T	G
ME 4-1-9st (FJ179158)	–	–	G	C	C	G	G	T	G	C	C	G	G	C	G
ME 1-2-9cr (FJ179156)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–
ME 5-2-5r (FJ179160)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–
ME 1-2-8cr (FJ179155)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–
ME 5-4-6cr (FJ179161)	T	T	G	C	–	G	G	T	G	C	T	T	G	T	G
New Mexico															
NM 1-1-5c (EU265670)	–	–	G	C	–	–	G	T	G	C	C	G	G	T	G
Ontario															
ON Q5-21st (FJ179154)	–	–	G	C	C	G	G	T	G	C	C	G	G	C	G
ON Q2-3 (FJ179153)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–
ON 4-24 (FJ179151)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–
ON 4-28 (FJ179152)	T	T	G	C	–	G	G	T	G	C	T	T	G	T	G
Pennsylvania															
Pa 2-9cr (FJ179162)	–	–	A	–	–	–	–	–	–	–	T	–	–	–	–

Isolate[a]	**Alignment position**[b]														**bp**[c]
Position	26	28	31	32	33	42	87	92	97	138	358	372	373	446	
Concensus	**C**	**T**	**A**	**C**	**G**	**C**	**A**	**G**	**T**	**A**	**C**	**C**	**A**	**G**	
ATCC 56515	C	A	A	T	G	T	A	G	T	C	C	C	A	G	488
Colorado															
CO 7-2-10a	C	C	A	C	G	T	A	G	T	A	C	C	A	G	480
Maine															
ME 3-4-16cr	G	T	A	C	T	T	A	T	G	A	C	C	G	G	467
ME 5-1-5r	C	C	A	T	G	T	A	G	T	C	C	C	A	G	486
ME 4-1-9st	C	C	C	T	G	C	C	G	T	C	C	C	A	G	478
ME 1-2-9cr	G	T	A	C	T	C	A	T	C	A	C	T	A	G	466
ME 5-2-5r	G	T	A	C	T	C	A	T	C	A	C	C	A	A	466
ME 1-2-8cr	G	T	A	C	T	C	A	T	C	A	C	C	A	G	467
ME 5-4-6cr	C	A	A	T	G	T	A	G	T	C	C	C	A	G	488
New Mexico															
NM 1-1-5c	C	C	A	C	G	T	A	G	T	A	T	C	A	G	479
Ontario															
ON Q5-21st	C	C	C	T	G	C	C	G	T	C	C	C	A	G	478
ON Q2-3	G	T	A	C	T	C	A	T	C	A	C	T	A	G	466
ON 4-24	G	T	A	C	T	C	A	T	C	A	C	C	A	G	467
ON 4-28	C	A	A	T	G	T	A	G	T	C	C	C	A	G	488
Pennsylvania															
Pa 2-9cr	G	T	A	C	T	C	A	T	C	A	C	T	A	G	466

[a] GenBank accession number given in parentheses. Sequence data for *P. sclerotioides* isolate 'ATCC 56515' was generated in a previous study (Larsen et al. 2007).

[b] Alignment position in a 488-bp alignment of the 15 sequences. Gaps denoted by a dash (–).

[c] Number of base pairs identical to *P. sclerotioides* isolate ATCC 56515; total alignment length was 488 bp.

TABLE 4.3 Plant mortality and root and crown rot severity on Vernal alfalfa inoculated with *P. sclerotioides* isolates from Colorado, New Mexico, Maine, Ontario, and Pennsylvania.

		Disease severity[b]	
Isolate (geographic origin)	**Plant mortality[a]**	**Root**	**Crown**
Colorado and New Mexico			
CO 7-2-10 (Rio Grande Co., CO)	8/14	1.29	3.21
NM 1-1-5 (Taos Co., NM)	16/23	1.87	4.09
Ontario			
ON Q2-3 (near Ottawa, ON)	0/3	1.00	2.67
ON Q2-6 (near Ottawa, ON)	0/3	1.67	3.67
ON Q5-21 (near Ottawa, ON)	1/2	1.00	4.00
ON 4-21 (near Lindsay, ON)	3/3	2.00	5.00
ON 4-24 (near Lindsay, ON)	1/3	1.00	4.33
ON 4-28 (near Lindsay, ON)	3/4	1.25	4.75
Maine and Pennsylvania			
ME 1-2-9cr (Waldo Co., ME)	0/18	1.22	2.00
ME 3-3-2r (Waldo Co., ME)	5/18	2.33	2.67
ME 4-1-9st (Penobscot Co., ME)	3/18	1.11	2.50
ME 4-3-5r (Penobscot Co., ME)	4/18	1.06	2.39
ME 5-4-6r (Somerset Co., ME)	1/18	1.33	2.11
ME 5-2-5r (Somerset Co., ME)	3/18	0.78	2.61
PA 2-9cr (Jefferson Co., PA)	5/18	1.22	3.11
Control	0/18	0.94	1.89

[a] Expressed as a proportion of plants inoculated.
[b] Rated on a 0 to 5 scale: 0 = no disease, 1 = lesions restricted to epidermal tissues, 2 = lesions extend into cortical tissues but impact <33% of root or crown diameter, 3 = lesions affect 33 to 66% of root or crown diameter, 4 = lesions affect >66% of root or crown diameter, and 5 = lesions completely girdle root or crown.

4.5 DISCUSSION

Within alfalfa production regions, high-altitude mountain valleys at the latitude of northern New Mexico and southern Colorado appear to delineate the southern extent of *P. sclerotioides* in western North America. Central Pennsylvania appears to delineate the southern extent of *P. sclerotioides* in eastern North America. Among the fields sampled in Colorado and New Mexico, *P. sclerotioides* was only isolated in the San Luis Valley, the coldest and highest altitude region represented in the survey. The fields where *P. sclerotioides* was detected were located at approx. 2300 meters above

sea level; fields at somewhat lower altitude in the Huerfano River drainage (approx. 2100 m) of Colorado's Rocky Mountain Front, along the Arkansas River in Colorado's southeastern plains (approx. 1250 m), and along the Rio Chama near Abiquiu, NM (approx. 1800 m) did not appear to harbor the pathogen. Among the fields sampled in Pennsylvania, *P. sclerotioides* was most frequently isolated in the colder, high-altitude regions of the northeastern corner of the state and was only isolated from one field in the central part of the state.

P. sclerotioides was isolated from all alfalfa production fields sampled in Maine and nearly all fields sampled in Ontario. Incidence of infection was similar to levels observed in New York, New Hampshire, and Vermont (Wunsch et al. 2007); in most fields, *P. sclerotioides* was recovered from approximately 5 to 30% of plants. Although BRR had not been previously reported in Maine or Ontario, the high frequency with which the pathogen was isolated from alfalfa roots and crowns is not surprising. Using standard culturing techniques at room temperature, the pathogen can rarely be isolated. *P. sclerotioides,* which grows best at low temperatures, is out-competed by other pathogens and saprophytes at room temperature. Furthermore, alfalfa root and crown lesions are frequently caused by multiple pathogens, and, consequently, lesion morphology is not always diagnostic.

P. sclerotioides is unlikely to be of economic importance in New Mexico, southern Colorado, or Pennsylvania. Incidence levels were generally very low in alfalfa production fields of these regions, and all lesions associated with the pathogen impacted less than a third of the diameter of the root or crown. The impact of *P. sclerotioides* on alfalfa production in Maine and Ontario is unclear. The severity of lesions associated with *P. sclerotioides* was not recorded for Ontario but was relatively

low in Maine. However, BRR is generally a sporadic problem in Wyoming and Alberta (Berkenkamp et al. 1991, Hollingsworth et al. 2003), causing severe losses some years but not others, and multiple years of observation may be required to properly evaluate its impact.

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CHAPTER 5

GENETIC AND MORPHOLOGICAL EVIDENCE THAT *PHOMA SCLEROTIOIDES*, CAUSAL AGENT OF BROWN ROOT ROT OF ALFALFA, IS COMPOSED OF A SPECIES COMPLEX.*

5.1 ABSTRACT

Phoma sclerotioides, 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. sclerotioides* exhibit diverse gross 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. sclerotioides* isolates, 154 North American isolates were sequenced at ten loci. Maximum parsimony and maximum likelihood analyses of the complete 10-locus dataset placed isolates into multiple strongly supported clades, and analyses of gene-jackknife and single-gene partitions of the dataset indicated robust support for six major clades and three subclades. Isolates exhibited morphologies broadly consistent with the species description of *P. sclerotioides* but differed in specific traits. Genetic differences corresponded closely to differences in conidial size and septation, pycnidial neck length, gross cultural morphology on PDA, and growth rate in axenic culture at 18°C and 25°C. On the basis of genetic and morphological differences, we propose establishing seven infraspecific varieties within *P. sclerotioides*: *P. sclerotioides* var.

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sclerotioides, champlainii, viridis, obscurus, steubenii, macrospora, and *saskatchewanii.* All varieties of *P. sclerotioides* caused brown root rot of alfalfa and grew well at low temperatures.

5.2 INTRODUCTION

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.; Boerema et al. 1993). 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* (Mendes-Pereira et al. 2003, Vincenot et al. 2007). *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 (*Lycopersicon esculentum* Mill.; de Gruyter et al. 2002). Similar species complexes associated with specific diseases have been recognized within *P. enteroleuca*, an opportunistic pathogen of trees and shrubs; within *P. exigua* causing with 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 Rosaceae (Boerema et al. 2004). For those *Phoma* species complexes in which genetic differentiation has been evaluated, morphological differences correspond

closely to genetic differences (Abeln et al. 2002, Mendes-Pereira et al. 2003, Vincenot et al. 2007).

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 associated with more severe spring black stem and leaf spot symptoms than *P. medicaginis* var. *medicaginis* (Boerema et al. 1993). 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* (Johnson and Lewis 1994). 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 (Somai et al. 2002). On members of the 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 (Boerema et al. 2004).

Brown root rot of alfalfa and other perennial legumes (BRR), caused by *P. sclerotioides* G. Preuss ex Sacc. (synonym *Plenodomus meliloti* Dearn. & G. B. 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 it causes considerable alfalfa mortality (Davidson, 1990). In central Saskatchewan, alfalfa mortality associated with BRR can exceed 60 percent

on susceptible alfalfa varieties two years after seeding and can reach 40 percent one year after seeding (Berkenkamp et al. 1991). In the contiguous United States, BRR has been reported in the Rocky Mountain states south to New Mexico (Hollingsworth et al. 2003, Wunsch et al. 2008), in the upper Midwest (Larsen et al. 2004, Larsen et al. 2007), and in the Northeast south to Pennsylvania (Wunsch et al. 2007, Wunsch et al. 2009).

P. sclerotioides displays pronounced variability in gross cultural morphology when grown in axenic culture on potato dextrose agar (PDA). Single-conidium isolates identified as *P. sclerotioides* by pycnidial and conidial morphology on sterilized alfalfa root sections and autoclaved barley (Wunsch et al. 2007) produce white, tan, brown, green, or dark gray mycelium on PDA, with 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 gross cultural characteristics on PDA (Wunsch, pers. obs.). Variability in gross cultural morphology of *P. sclerotioides* has also been reported by Gray et al. (2008).

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 [1] rigorously evaluating whether isolates representing diverse cultural morphologies correspond to the species description of *P. sclerotioides*, [2] confirming that isolates representing diverse cultural morphologies cause brown root rot and are adapted to low temperatures, and [3] assessing morphological and genetic differentiation among isolates. The species description of *P. sclerotioides* is based primarily on conidial and pycnidial characteristics in-vivo (Boerema et al. 2004).

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.

5.3 MATERIALS AND METHODS

Fungal strains and culture maintenance. Isolates 'LMH-B', 'LMH-C', 'JNL-1a', and 'LMN-D' from Minnesota and Wisconsin (Table 5.1) were established from symptomatic roots sent by D. A. Samac (USDA-ARS, St. Paul, MN), and isolate 'WyoBerg' from Wyoming was contributed by F. A. Gray (University of Wyoming, Laramie). All other isolates were obtained from previously described surveys of alfalfa production fields (Wunsch et al. 2007, Wunsch et al. 2008, Wunsch et al. 2009) 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 (Table 5.1). Symptomatic tissue segments were excised, surface sterilized in 0.6% hypochlorite and 70% ethanol, rinsed in sterile distilled water, plated onto 1.5% water agar, and incubated 3 to 6 months at 10°C under continuous white fluorescent light with average luminescence of 2,000 lx. Pycnidia characteristic of *P. sclerotioides* were harvested, and single-conidium isolates were established on potato dextrose agar (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-sterilized healthy root segments obtained from greenhouse-grown alfalfa (Wunsch et al. 2007). Diagnostic PCR was conducted on all isolates using pathogen-specific primers (Larsen et al. 2002).

DNA extraction, amplification, purification, and sequencing. Cultures of 155 *P. sclerotioides* isolates (Table 5.1) and one *P. medicaginis* isolate were established on PDA from frozen stocks, transferred to potato dextrose broth (PDB; Difco Laboratories, Becton, Dickson and Co., Sparks, MD), and grown 4 to 8 wks at 10°C. Mycelium was harvested by decanting the broth, rinsing once in sterile distilled water, and centrifuging. Rinsed mycelium was lyophilized, and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to 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), alpha tubulin (AT), beta tubulin (BT), elongation factor 1-alpha (EF1a), 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). Amplifications were conducted in 25-µl reactions containing 1.5 µl of template DNA, 1x Takara *Ex Taq* buffer (Clontech Laboratories, Mountain View, CA), 0.8 mM

TABLE 5.1 *Phoma sclerotioides* isolates used in this study.

Isolate	Origin (year isolated)	Isolated from		Diag. PCR[c]	Designation
		Host plant[a]	Organ[b]		
CO 7-2-10a	Rio Grande Co., CO (2006)	*Ms*	CR	+	var. *obscurus*
ME 1-2-4st	Waldo Co., ME (2007)	*Ms*	ST	+	var. *viridis*
ME 1-2-8cr	Waldo Co., ME (2007)	*Ms*	CR	+	var. *macrospora*
ME 1-2-9cr	Waldo Co., ME (2007)	*Ms*	CR	+	var. *steubenii*
ME 1-3-8st	Waldo Co., ME (2007)	*Ms*	ST	+	var. *sclerotioides*
ME 1-3-11lf	Waldo Co., ME (2007)	*Ms*	Leaf	+	var. *steubenii*
ME 2-6cr	Waldo Co., ME (2007)	*Ms*	CR	–	var. *steubenii*
ME 2-8st	Waldo Co., ME (2007)	*Ms*	ST	+	var. *macrospora*
ME 3-1-7r	Waldo Co., ME (2007)	*Ms*	RT	+	var. *sclerotioides*
ME 3-3-2r	Waldo Co., ME (2007)	*Ms*	RT	+	var. *sclerotioides*
ME 3-4-15cr	Waldo Co., ME (2007)	*Ms*	CR	+	var. *sclerotioides*
ME 3-4-16cr	Waldo Co., ME (2007)	*Ms*	CR	+	var. *steubenii*
ME 4-1-9st	Penobscot Co., ME (2007)	*Ms*	ST	+	var. *viridis*
ME 4-3-5r	Penobscot Co., ME (2007)	*Ms*	RT	+	var. *steubenii*
ME 4-3-12cr	Penobscot Co., ME (2007)	*Ms*	CR	+	var. *sclerotioides*
ME 4-4-4cr	Penobscot Co., ME (2007)	*Ms*	CR	+	var. *steubenii*
ME 5-1-4cr	Somerset Co., ME (2007)	*Ms*	CR	+	var. *steubenii*
ME 5-1-5r	Somerset Co., ME (2007)	*Ms*	RT	+	var. *sclerotioides*
ME 5-2-5r	Somerset Co., ME (2007)	*Ms*	RT	+	var. *macrospora*
ME 5-3-8r	Somerset Co., ME (2007)	*Ms*	RT	+	var. *viridis*
ME 5-4-1r	Somerset Co., ME (2007)	*Ms*	RT	+	var. *macrospora*
ME 5-4-6r	Somerset Co., ME (2007)	*Ms*	RT	+	var. *sclerotioides*
MN JNL-1a	Goodhue Co., MN (2005)	*Ms*	RT	+	var. *viridis*
MT 1-3-4	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 1-4-2	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *obscurus*
MT 2-1-3	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *saskatchewanii*
MT 2-1-4	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *obscurus*
MT 2-2-11	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 2-3-6	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *obscurus*
MT 3-1-2	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *viridis*
MT 3-1-5	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 3-2-9	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 3-3-1	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *obscurus*
MT 3-3-2a	Beaverhead Co., MT (2006)	*Ms*	RT/CR	–	var. *viridis*
MT 3-4-8	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 4-1-3a	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *obscurus*
MT 4-2-1	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 4-2-7	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *viridis*
MT 4-4-4	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
MT 4-4-12	Beaverhead Co., MT (2006)	*Ms*	RT/CR	+	var. *obscurus*
MT 5-2-4	Phillips Co., MT (2006)	*Ms*	CR	+	var. *viridis*
MT 6-2-6	Phillips Co., MT (2006)	*Ms*	CR	+	var. *viridis*
MT 6-5-10	Phillips Co., MT (2006)	*Ms*	CR	+	var. *obscurus*
NH 1-1-2-4cr	Coös Co., NH (2005)	*Ms*	CR	+	var. *viridis*

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[a] *Ms* = *Medicago sativa* (alfalfa), *Pa* = *Phalaris arundinacea* (reed canarygrass)
[b] CR = crown, RT = root, ST = overwintered stem, RT/CR = root or crown
[c] PCR conducted with *P. sclerotioides*-specific primers (Larsen et al. 2007). A positive sign (+) indicates that diagnostic PCR resulted in a single amplicon of expected size (approx. 500 bp), a negative sign (–) indicates that no amplicon was produced, and "ND" indicates that diagnostic PCR was not performed.

TABLE 5.1 (Continued)

Isolate	Origin (year isolated)	Isolated from		Diag. PCR[c]	Designation
		Host plant[a]	Organ[b]		
continued from previous page					
NH 1-1-2-10cr	Coös Co., NH (2005)	*Ms*	CR	+	var. *steubenii*
NH 1-2-1-3r	Coös Co., NH (2005)	*Ms*	RT	+	var. *viridis*
NH 1-2-1-9r	Coös Co., NH (2005)	*Ms*	RT	+	var. *steubenii*
NH 2-1-1-13cr	Coös Co., NH (2005)	*Ms*	CR	+	var. *viridis*
NH 2-1-2-2cr	Coös Co., NH (2005)	*Ms*	CR	+	var. *steubenii*
NH 2-1-2-8r	Coös Co., NH (2005)	*Ms*	RT	+	var. *viridis*
NH 4-1-1-5cr	Grafton Co., NH (2005)	*Ms*	RT	+	var. *steubenii*
NH 4-2-1-8r	Grafton Co., NH (2005)	*Ms*	RT	+	var. *steubenii*
NH 4-2-2-2r	Grafton Co., NH (2005)	*Ms*	RT	+	var. *sclerotioides*
NH 4-2-2-5cr	Grafton Co., NH (2005)	*Ms*	CR	+	var. *sclerotioides*
NH 4-2-3-5r	Grafton Co., NH (2005)	*Ms*	RT	+	var. *viridis*
NH 4-2-3-8r	Grafton Co., NH (2005)	*Ms*	RT	+	var. *sclerotioides*
NH 4-2-4-2cr	Grafton Co., NH (2005)	*Ms*	CR	+	var. *sclerotioides*
NH 4-2-4-3cr	Grafton Co., NH (2005)	*Ms*	CR	+	var. *sclerotioides*
NH 4-2-4-5cr	Grafton Co., NH (2005)	*Ms*	CR	+	var. *sclerotioides*
NH 4-2-4-10cr	Grafton Co., NH (2005)	*Ms*	CR	+	var. *sclerotioides*
NM 1-1-5c	Taos Co., NM (2006)	*Ms*	CR	+	var. *obscurus*
NY 1-2	Wyoming Co., NY (2007)	*Ms*	RT/CR	+	var. *steubenii*
NY Chz1-7r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *champlainii*
NY Chz1-15r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *champlainii*
NY Chz1-16r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Chz1-30r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *viridis*
NY Chz2-14cr	Clinton Co., NY (2005)	*Ms*	CR	+	var. *sclerotioides*
NY Chz4-2r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *sclerotioides*
NY Chz5-32r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Chz7-2r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *sclerotioides*
NY Chz7-25cr	Clinton Co., NY (2005)	*Ms*	CR	+	var. *macrospora*
NY Chz8-7r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *sclerotioides*
NY Chz8-12r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *sclerotioides*
NY Chz10-18r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Chz12-18r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *sclerotioides*
NY Chz12-22r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *viridis*
NY Chz12-30r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Chz17-19cr	Clinton Co., NY (2005)	*Ms*	CR	+	var. *viridis*
NY Chz21-19cr	Clinton Co., NY (2005)	*Ms*	CR	+	var. *viridis*
NY Chz22-14cr	Clinton Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Chz22-21r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *sclerotioides*
NY Chz23-18r	Clinton Co., NY (2005)	*Ms*	RT	+	var. *viridis*
NY Fr1-3-1RC	Franklin Co., NY (2008)	*Pa*	RT	ND	var. *steubenii*
NY Stb1-1-1-4r	Steuben Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Stb1-1-2-17r	Steuben Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Stb1-1-4-13r	Steuben Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Stb1-2-1-4cr	Steuben Co., NY (2005)	*Ms*	CR	+	var. *steubenii*
NY Stb1-2-1-8cr	Steuben Co., NY (2005)	*Ms*	CR	+	var. *steubenii*
					continued on next page

[a] *Ms* = *Medicago sativa* (alfalfa), *Pa* = *Phalaris arundinacea* (reed canarygrass)
[b] CR = crown, RT = root, ST = overwintered stem, RT/CR = root or crown
[c] PCR conducted with *P. sclerotioides*-specific primers (Larsen et al. 2007). A positive sign (+) indicates that diagnostic PCR resulted in a single amplicon of expected size (approx. 500 bp), a negative sign (–) indicates that no amplicon was produced, and "ND" indicates that diagnostic PCR was not performed.

TABLE 5.1 (Continued)

Isolate	Origin (year isolated)	Isolated from		Diag. PCR[c]	Designation
		Host plant[a]	Organ[b]		
continued from previous page					
NY Wyo1-3r	Wyoming Co., NY (2005)	*Ms*	RT	–	var. *viridis*
NY Wyo10-4r	Wyoming Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
NY Wyo18-1r	Wyoming Co., NY (2005)	*Ms*	RT	+	var. *macrospora*
NY Wyo20-8r	Wyoming Co., NY (2005)	*Ms*	RT	+	var. *steubenii*
ON 3-7	Binbrook, ON (2007)	*Ms*	RT/CR	+	var. *viridis*
ON 3-24	Binbrook, ON (2007)	*Ms*	RT/CR	+	var. *steubenii*
ON 4-21	Lindsay, ON (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
ON 4-24	Lindsay, ON (2007)	*Ms*	RT/CR	+	var. *macrospora*
ON 4-28	Lindsay, ON (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
ON 5-6	Belleville, ON (2007)	*Ms*	RT/CR	+	var. *macrospora*
ON 5-8	Belleville, ON (2007)	*Ms*	RT/CR	+	var. *steubenii*
ON 9-25	Woodstock, ON (2007)	*Ms*	RT/CR	+	var. *steubenii*
ON Q2-6r	South of Ottawa, ON (2007)	*Ms*	RT	+	var. *sclerotioides*
ON Q2-13st	South of Ottawa, ON (2007)	*Ms*	ST	+	var. *steubenii*
ON Q4-12st	South of Ottawa, ON (2007)	*Ms*	ST	+	var. *viridis*
ON Q5-16st	South of Ottawa, ON (2007)	*Ms*	ST	+	var. *macrospora*
ON Q5-21st	South of Ottawa, ON (2007)	*Ms*	ST	+	var. *viridis*
PA 1-5r	Columbia Co., PA (2007)	*Ms*	RT	+	var. *steubenii*
PA 2-9cr	Jefferson Co., PA (2007)	*Ms*	CR	+	var. *steubenii*
PA 3-11cr	Crawford Co., PA (2007)	*Ms*	CR	+	var. *steubenii*
PA 4-1r	Crawford Co., PA (2007)	*Ms*	RT	+	var. *steubenii*
PA 4-5cr	Crawford Co., Pa (2007)	*Ms*	CR	+	var. *steubenii*
SK 2-1	Macdowall., SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 2-2	Macdowall., SK (2007)	*Ms*	RT/CR	+	var. *saskatchewanii*
SK 2-11	Macdowall., SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 2-17	Macdowall., SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 2-20	Macdowall., SK (2007)	*Ms*	RT/CR	+	var. *viridis*
SK 2-26	Macdowall., SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 3-1	Nipawin., SK (2007)	*Ms*	RT/CR	+	var. *saskatchewanii*
SK 4-1	Carrot River, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 4-2	Carrot River, SK (2007)	*Ms*	RT/CR	+	var. *viridis*
SK 4-6	Carrot River, SK (2007)	*Ms*	RT/CR	+	var. *saskatchewanii*
SK 4-21	Carrot River, SK (2007)	*Ms*	RT/CR	+	var. *viridis*
SK 5-9	Arborfield, SK (2007)	*Ms*	RT/CR	+	var. *viridis*
SK 6-2	Tisdale, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 6-3	Tisdale, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 6-7	Tisdale, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 6-12	Tisdale, SK (2007)	*Ms*	RT/CR	+	var. *viridis*
SK 7-8	Valparaiso, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 7-9	Valparaiso, SK (2007)	*Ms*	RT/CR	+	var. *saskatchewanii*
SK 7-10	Valparaiso, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
SK 7-12	Valparaiso, SK (2007)	*Ms*	RT/CR	+	*P. sclerotioides*
SK 7-34	Valparaiso, SK (2007)	*Ms*	RT/CR	+	var. *saskatchewanii*
SK 9-16	Langham, SK (2007)	*Ms*	RT/CR	+	var. *sclerotioides*
					continued on next page

[a] *Ms* = *Medicago sativa* (alfalfa), *Pa* = *Phalaris arundinacea* (reed canarygrass)
[b] CR = crown, RT = root, ST = overwintered stem, RT/CR = root or crown
[c] PCR conducted with *P. sclerotioides*-specific primers (Larsen et al. 2007). A positive sign (+) indicates that diagnostic PCR resulted in a single amplicon of expected size (approx. 500 bp), a negative sign (–) indicates that no amplicon was produced, and "ND" indicates that diagnostic PCR was not performed.

TABLE 5.1 (Continued)

Isolate	Origin (year isolated)	Isolated from: Host plant[a]	Isolated from: Organ[b]	Diag. PCR[c]	Designation
continued from previous page					
VT 2-1-1-7r	Franklin Co., VT (2005)	*Ms*	RT	+	var. *steubenii*
VT 2-1-4-5r	Franklin Co., VT (2005)	*Ms*	RT	+	var. *sclerotioides*
VT 2-2-3-8r	Franklin Co., VT (2005)	*Ms*	RT	+	var. *steubenii*
VT 3-2-1-7cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *viridis*
VT 3-2-1-7r	Addison Co., VT (2005)	*Ms*	RT	+	var. *sclerotioides*
VT 3-2-2-3cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *sclerotioides*
VT 3-2-4-1r	Addison Co., VT (2005)	*Ms*	RT	+	var. *viridis*
VT 3-2-4-3r	Addison Co., VT (2005)	*Ms*	RT	+	var. *sclerotioides*
VT 3-2-4-6r	Addison Co., VT (2005)	*Ms*	RT	+	var. *sclerotioides*
VT 3-2-4-9cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *steubenii*
VT 3-2-4-10cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *sclerotioides*
VT 3-3-1-5cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *steubenii*
VT 3-3-1-7cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *viridis*
VT 3-3-2-5cr	Addison Co., VT (2005)	*Ms*	CR	+	var. *viridis*
VT 3-3-4-9r	Addison Co., VT (2005)	*Ms*	RT	+	var. *steubenii*
VT 3-3-4-11r	Addison Co., VT (2005)	*Ms*	RT	+	var. *viridis*
WI 3-2-9	Shawano Co., WI (2006)	*Ms*	RT/CR	+	var. *sclerotioides*
WI 4-2-7	St. Croix Co., WI (2006)	*Ms*	CR	+	var. *sclerotioides*
WI 5-1-5	St. Croix Co., WI (2006)	*Ms*	CR	+	var. *viridis*
WI LMN-B	St. Croix Co., (2005)	*Ms*	RT	+	var. *sclerotioides*
WI LMH-C	St. Croix Co., WI (2005)	*Ms*	RT	+	var. *sclerotioides*
WI LMN-D	Dunn Co., WI (2005)	*Ms*	RT	+	var. *viridis*
WyoBerg/ATCC MYA-295	Sweetwater Co., WY (unknown)	*Ms*	RT/CR	+	var. *sclerotioides*

[a] *Ms* = *Medicago sativa* (alfalfa), *Pa* = *Phalaris arundinacea* (reed canarygrass)
[b] CR = crown, RT = root, ST = overwintered stem, RT/CR = root or crown
[c] PCR conducted with *P. sclerotioides*-specific primers (Larsen et al. 2007). A positive sign (+) indicates that diagnostic PCR resulted in a single amplicon of expected size (approx. 500 bp), a negative sign (–) indicates that no amplicon was produced, and "ND" indicates that diagnostic PCR was not performed.

dNTPs, 1 μM each of the forward and reverse primers (Table 5.2), and 0.625 units *Ex Taq* DNA polymerase, hot start version (Clontech Laboratories). 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°C, 54°C, and 67°C for ACT; 94°C, 58°C, and 69°C for AT; 94°C, 54°C, and 67°C for BT; 94°C, 58°C, and 68°C for EF1a; 94°C, 63°C, and 71°C for G3P; 94°C, 59°C, and 71°C for HIS; 94°C, 50°C, and 60°C for NAD; 94°C, 61°C, and 70°C for ITS; 94°C, 59.5°C, and 68°C for IGS with primers CNS1 and NL11; and 94°C, 62°C, 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° for EF1a, but otherwise thermocycler conditions were the same. For all loci except NAD and IGS, an initial 60 sec at the melting temperature was followed by 40 cycles of 45 seconds at the melting temperature, 45 seconds at the annealing temperature, and 60 sec 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 sec, 30 sec, and 3 min 40 sec, 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 (White et al. 1990) were used to amplify and sequence the ITS region; a combination of published and novel primers were used for other loci (Table 5.2). Novel primers were designed by manual comparison of conserved regions among sequences from Asocomycete genera. Isolate 'Fr 1-3-1RC' was sequenced at six loci; all other isolates (Table 5.1) 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.

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.

Phylogenetic analyses were conducted using maximum parsimony (MP) and

TABLE 5.2 Primers used in this study.

Locus (reference[z])	Primer sequence
Actin	
ACT-77tF [a,Ps,Pm]	5'-AGT CCA ACC GTG AGA AGA TGA-3'
ACT-800R [a,Ps,Pm]	5'-ACC GAT CCA GAC RGA GTA CTT-3'
Alpha tubulin	
AT-228F [a,Ps,Pm]	5'-TCG CAA ACT CTT GCT GGG AG-3'
AT-1253R [a,Ps,Pm]	5'-AGT TGG TCT GGA ACT CGT T-3'
Beta tubulin	
BT-743F [a,Ps,Pm]	5'-AYC ATG CGT GAG ATY GTA CGT-3'
BT-1443R [a,Ps,Pm]	5'-CCT CRC GGA TCT TGG AGA TCA-3'
Elongation factor 1-alpha	
EF-124F [a,Pm]	5'-ATC AAC GTG GTC GTT ATC GG-3'
EF-2ag [a,Pm] (O'Donnell et al. 1998)	5'-GGA AGT ACC AGT GAT CAT GTT-3'
EF-145F [a,Ps]	5'-CAC GTT GAC TCC GGA AAG TC-3'
EF-687R [a,Ps]	5'-ATG GTG ATA CCA CGC TCA CG-3'
Glyceraldehyde 3-phosphate dehydrogenase	
GPD2 [a,Ps,Pm] (Berbee et al. 1999)	5'-GCC AGG CAG TTG GTT GTG C-3'
GPD3b [a,Pm]	5'-CAT CAT GGT CGT CAA GGT-3'
GPD3c [a,Ps]	5'-CAT GGT CGT CAA GGT ANG CT-3'
Histone	
HIS-99F [a,Ps,Pm]	5'-CAA GCA GAC TGC CCG TAA GT-3'
HIS-898R [a,Ps,Pm]	5'-CGG GCG AGC TGG ATA TCC TT-3'
rDNA intergenic spacer	
CNS1 [a,Ps,Pm] (White et al. 1990)	5'-GAG ACA AGC ATA TGA CTA CTG-3'
R10 [a,Ps,Pm]	5'-TGG GTT TAG ACC GTC GTG AG-3'
NL11 [a,Ps]	5'-CTG AAC GCC TCT AAG TCA G-3'
IGSNS-F476 [s,Ps]	5'-CAA AAT CGC TCG CCA AAT-3'
IGSNS-F567 [s,Ps]	5'-TTT CCA AAG TCA CTG CCC TAC ACA-3'
IGSNS-F572 [s,Pm]	5'-CCG AAG GCG AGA AAT CCA GAA AG-3'
IGSNS-F694 [s,Ps]	5'-CCA ATA GGC CAG GGG ACC AC-3'
IGSNS-F704 [s,Ps]	5'-CGA GCA GCC CGA TCA ACT ACC TAC-3'
IGSNS-F705 [s,Ps]	5'-GGG GGA TAG CCA GAT TCA T-3'
IGSNS-F827 [s,Ps]	5'-GCG GGG TTA ATG TGT CGG AAA AG-3'
mtDNA NADH dehydrogenase	
NAD-248F [a,Ps,Pm]	5'-GTT TCT ATG CTT ATA CCT GT-3'
NAD-740R [a,Ps]	5'-TAC CAT AGT GGC AGC GTG TA-3'
NAD-907R [a,Pm]	5'-TGG CTC ATT GTA GAA TAA GC-3'
NAD-127 [s,Ps]	5'-ACG TAG GTG GCT GAA GTA AT-3'
NAD-785 [s,Ps]	5'-TTC CCT CAG CGC TAA CAA AAC CT-3'

[z] Primers for which no reference is given were developed in this study.
[a] Amplification and sequencing primer
[s] Internal sequencing primer
[Ps] Primer used for *P. sclerotioides*
[Pm] Primer used for *P. medicaginis*

maximum likelihood (ML). To evaluate genetic differentiation among isolates of *P. sclerotioides*, analyses were conducted on a dataset of 154 *P. sclerotioides* isolates and a *P. medicaginis* outgroup (isolate 'Phm001NY-83' collected in New York) sequenced

at 10 loci. Analyses were conducted with the full concatenated data set, with every combination of nine concatenated loci as well as the eight loci excluding the IGS region (gene-jackknife analyses), and with each of the single loci. Gene-jackknife analyses permitted assessment of whether support for individual clades in the full (10-locus) analysis was driven by a single locus or by a signal derived from multiple loci. To evaluate the placement of genetically diverse *P. sclerotioides* among other *Phoma* species, analyses of the ITS region were conducted with a dataset of 25 *P. sclerotioides* isolates with unique ITS haplotypes, 83 isolates of other *Phoma* species and related genera, and an outgroup (*Saccharomyces cerevisae* strain 'CBS 1171'). For all non-*P. sclerotioides* isolates except *P. medicaginis,* sequences were generated in previously published studies (Aveskamp et al. 2009, Balmas et al. 2005, Camara et al. 2002, Irinyi et al. 2009, Mendes-Pereira et al. 2003, Morales et al. 1993, Sugita et al. 1999) and obtained from GenBank.

Unweighted MP analyses were conducted with PAUP* version 4.0b10 (Swofford 2002). 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 (Zwickl 2006). A minimum of 20 runs were 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 (Posada and Crandall 1998). For the analysis of the ITS region of *Phoma* species 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. sclerotioides,* a TrN+I+G model of nucleotide substitution was used for the full concatenated 10-locus dataset. For gene-jackknife analyses, the following models were utilized: HKY+I+G when exluding ACT, K81uf+I+G when excluding AT, K81+I+G when excluding BT, TIM+I+G when excluding EF1a, 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 EF1a, 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.

Pathogenicity testing and morphological characterization in-vivo. Individual plants of Vernal alfalfa were established in the greenhouse in SC10 Cone-Tainers (3.8 cm 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. sclerotioides* 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. Twelve plants were subjected to each treatment in each replicate of the experiment, and the experiment was replicated three times. After inoculation, plants were moved to a growth chamber and incubated at 4°C for 6 weeks, at -1 to 1°C for 5 weeks, at -2 to 1°C for 12 weeks, at 3°C for 4 weeks, and at 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 16 hr light / 8 hr dark cycle. 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, … , 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 (Boerema et al. 2004), 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. After 1 month in moist chambers, pycnidial sections were prepared with a freezing microtome for all isolates, and conidial morphology and external 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.

Morphological characterization in-vitro. Thirty-three isolates representing all major clades identified in phylogenetic analyses were started on PDA from frozen stocks. 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 months after culture establishment, each isolate was photographed to document gross cultural morphology. Three months after culture establishment, in-vitro conidial and pycnidial morphology of each isolate was evaluated. Pycnidia were excised, 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. Cultures of 21 isolates representing all major clades identified in phylogenetic analyses were started on PDA from frozen stocks. Three months after culture establishment, conidia were harvested from each isolate, suspended in water, plated onto PDA, and incubated for 24 hr under continuous fluorescent light at 10°C. Single germinating conidia were transferred to PDA. Single-conidium cultures were incubated at 3°C, 10°C, 18°C, or 25°C in complete darkness for 24 days, and radial growth of the cultures was measured. 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.

Statistical analyses. Differences in conidial length, conidial width, pycnidial diameter, pycnidial neck length, and in-vitro radial growth of isolates were assessed with analysis of variance, with analysis of in-vitro growth conducted controlling for the effect of experimental replicate. For each data set, the assumptions of constant variance and normality were assessed by plotting the residuals against predicted values and evaluating their variance and by plotting the residuals against their ranks and examining their linearity. In all data sets, the assumptions were met, and no transformations were applied to the data. All pairwise combinations of isolates were compared, and the Tukey multiple comparison procedure (Neter el at. 1996) was employed to control the Type I error rate at the level of the experiment. Analyses were implemented in PROC GLM (SAS, version 9.2; SAS Institute, Cary, NC).

Root rot severity, conidial guttule number, and pycnidial neck number of isolates were analyzed with cumulative logistic regression (Hosmer and Lemeshow

2000). In the pathogenicity testing experiment, logistic regression was conducted controlling for the effect of experimental replicate. Individual isolates were compared by performing contrasts with Wald chi-square tests. For root rot severity, contrasts were established between the control and each of the 13 inoculated treatments; for conidial guttule number and pycnidial neck number, contrasts were established for all possible pairwise combinations of isolates. All analyses were implemented in PROC GENMOD (SAS, version 9.2; SAS Institute, Cary, NC). The Bonferroni multiple comparison procedure (Neter et al. 1996) was used to control the Type I error rate at the level of the experiment across the 13 contrasts conducted in the pathogenicity testing experiment, the 528 contrasts evaluating conidial guttule number of isolates, and the 528 contrasts evaluating pycnidial neck length.

5.4 RESULTS

Fungal strains. All isolates produced large pycnidia with long necks for spore discharge and generated white cirrus that turned yellow with age. Diagnostic PCR resulted in amplicons of the expected size (approximately 500 bp) from 151 of 154 *P. sclerotioides* isolates evaluated (Table 5.1). 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, EF1a, G3P, HIS, IGS-NS, IGS-NL, ITS, and NAD sequences of *P. sclerotioides* isolates and a *P. medicaginis* outgroup resulted in a dataset 8132 characters long, of which 539 were parsimony-informative. Parsimony analysis generated 445,997 MPTs with 2481 steps (CI = 0.7864, RI = 0.9603). Likelihood analysis generated a best tree with log-

likelihood score -23921.57. MP and ML analyses placed *P. sclerotioides* isolates into multiple strongly supported clades (bootstrap values ≥ 70%), and 22 strongly supported clades were congruent between MP and ML trees (Figure 5.1; labeled as upper-case letters to left of bootstrap values). Nine of the 22 clades had bootstrap values ≥ 70% in all MP and ML gene-jackknife analyses (Figure 5.2); others lacked support in one or more gene-jackknife analyses. Of the nine clades with strong bootstrap support in all gene-jackknife analyses, five had bootstrap support ≥ 70% at four to seven individual loci in MP or ML analyses, and all but one (subclade 4A; Figure 5.2) had bootstrap support ≥ 70% at two or more individual loci in MP or ML analyses. Gene-jackknife and single-gene analyses permit assessment of whether support for individual clades in the full (10-locus) dataset is driven by a single locus or by a signal derived from multiple 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. Clades with strong bootstrap support in all gene-jackknife analyses were given specific clade or subclade designations (Figures 5.1, 5.2). Formal names were assigned to clades with strong phylogenetic support 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°C, 10°C, 18°C, and 25°C. Clade 2 was designated *P. sclerotioides* var. *champlainii*; clade 3, *P. sclerotioides* var. *viridis*; clade 4, *P. sclerotioides* var. *obscurus*; clade 5, *P. sclerotioides* var. *steubenii*; clade 6, *P. sclerotioides* var. *macrospora*; and clade 7, *P. sclerotioides* var. *saskatchewanii*. Subclade 1A (Figures 5.1, 5.2) falls within a broad

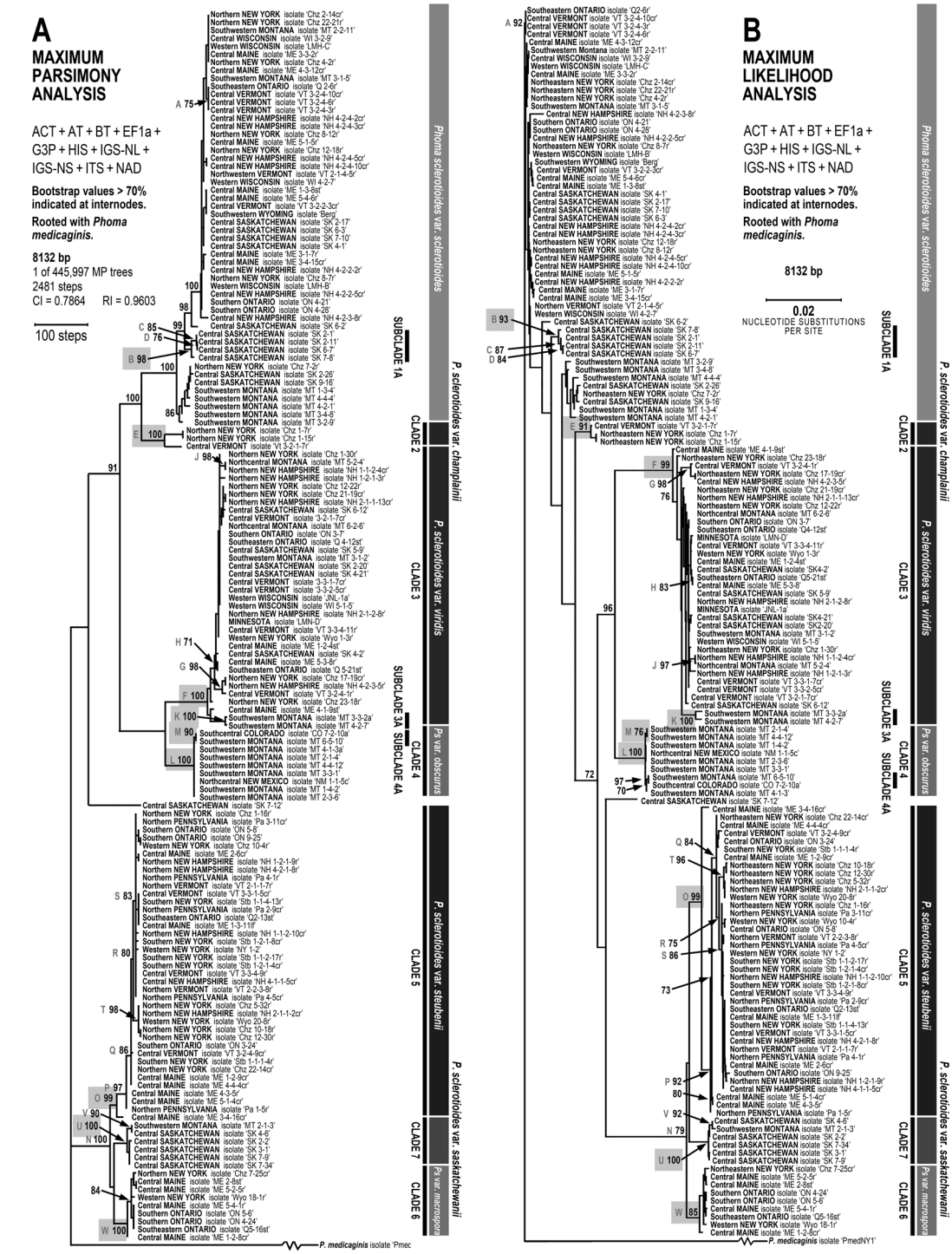

FIGURE 5.1 (A) Maximum parsimony and (B) maximum likelihood (ln = -23921.57) analyses of concatenated sequence data from 10 loci. Trees rooted with *P. medicaginis*, and bootstrap values greater than 70% (1,000 replications) indicated at nodes. Clades that are congruent in MP and ML analyses are indicated with capital letters to the left of the bootstrap values. Clades that have >70% bootstrap support in all MP and ML gene –jackknife analyses (Figure 5.2) are indicated by shading of bootstrap values and associated letters at nodes.

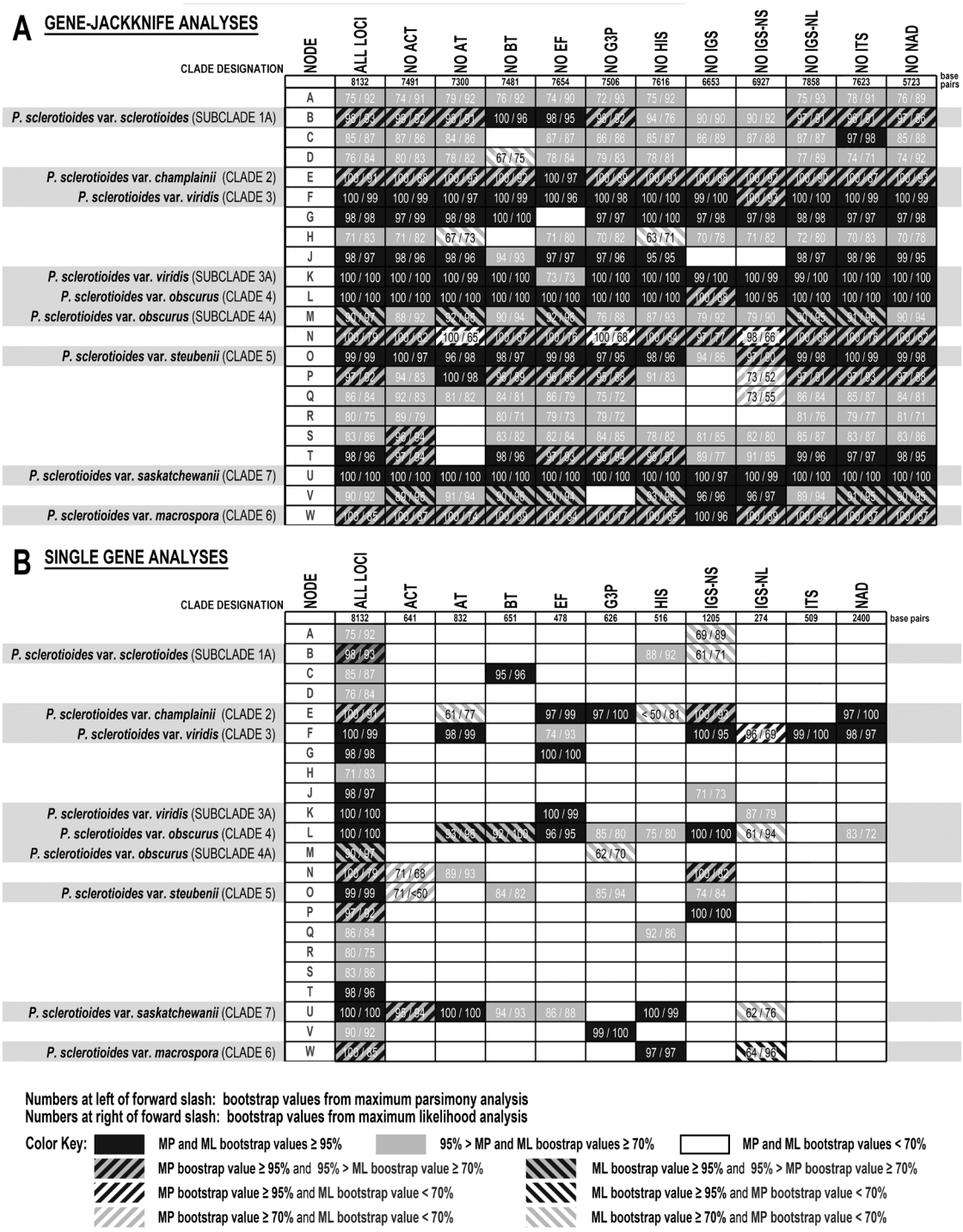

FIGURE 5.2 Congruence of clades as determined from maximum parsimony and maximum likelihood analyses of (A) gene-jackknife and (B) single-gene data partitions. The size of each partition is given below its name. Nodes, identified by capital letters, correspond to clades that are congruent in MP and ML analyses of the full 10-locus dataset (Figure 5.1). In each analysis, 1,000 bootstrap replicates were performed.

group of isolates that is basal to all other isolates in ML analysis and that has 100% bootstrap support in MP analysis (Figure 5.1), and it was treated as a subclade of the broader group. This broad group of isolates was designated *P. sclerotioides* var. *sclerotioides*; it includes *P. sclerotioides* isolate 'WyoBerg' (ATCC MYA-295) from Wyoming, USA (Figure 5.1) characterized in previous studies (Gray et al. 2008, Hollingsworth et al. 2005), and sequence identity at the ITS and G3P loci (Larsen et al. 2007; GenBank accession numbers DQ525737 and DQ525733) suggests it also contains *P. sclerotioides* 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. sclerotioides* var. *viridis* and var. *obscurus* (Figure 5.1). Insufficient morphological characterization of representative isolates precluded the assignment of formal names to subclades 1A, 3A, or 4A.

Alignment of ITS sequences for *P. sclerotioides,* other *Phoma* species, and related genera resulted in a dataset 517 characters long, of which 215 were parsimony-informative. Phylogenetic analysis by MP generated 3695 MPTs with 1232 steps (CI = 0.4513, RI = 0.8781). Phylogenetic analysis by ML generated a best tree with log-likelihood score -5913.94. In both MP and ML analyses, all *P. sclerotioides* isolates were placed in a strongly supported monophyletic group with *Leptosphaeria doliolum* (Figure 5.3; bootstrap support ≥ 98%), and *P. sclerotioides* and *L. doliolum* (anamorph *P. acuta*) were grouped with other members of *Phoma* section *Plenodomus*. Section *Plenodomus*, the division within *Phoma* in which *P. sclerotioides* is placed on the basis of morphological characters, is associated with thick scleroplectenchymatous

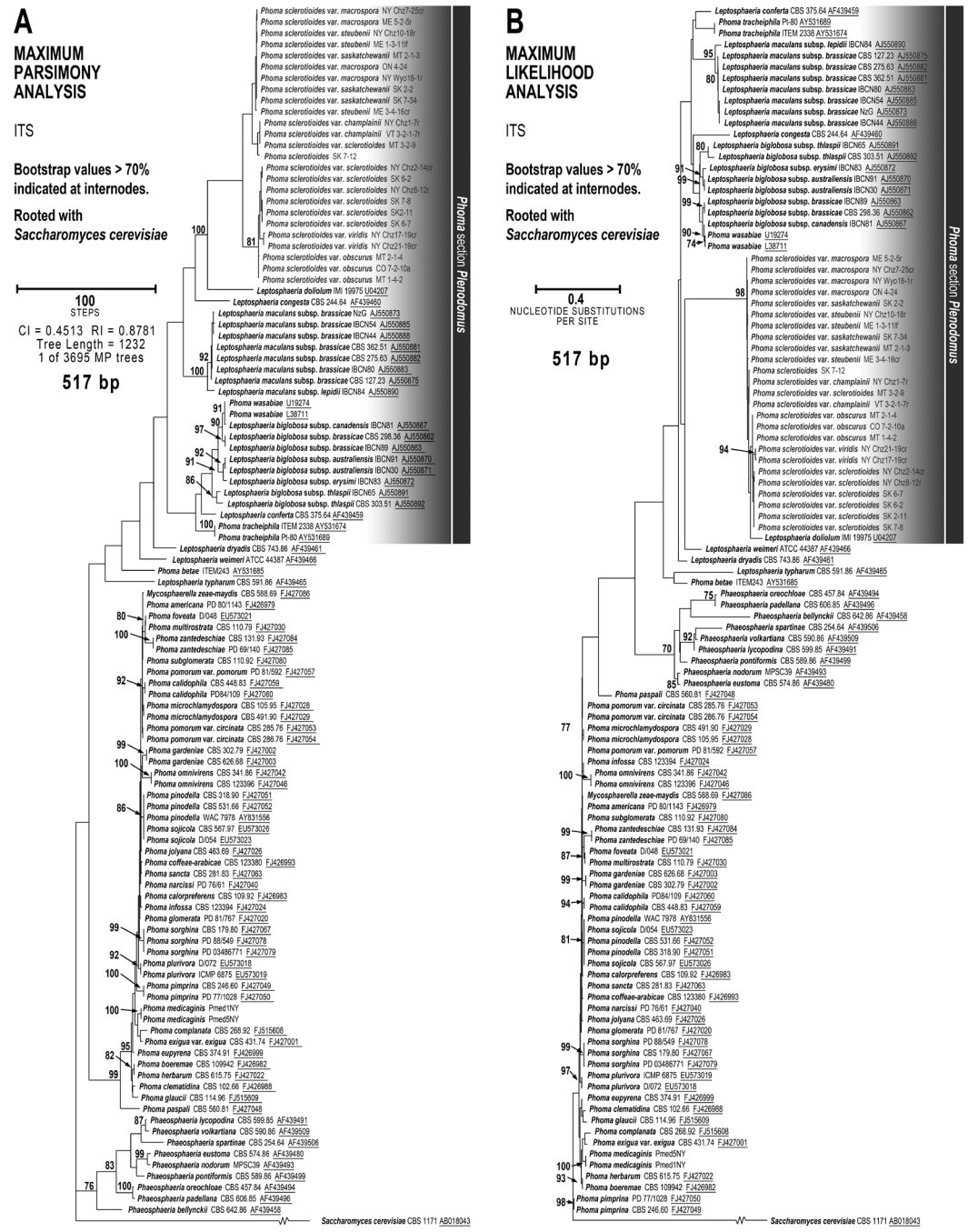

FIGURE 5.3 (A) Maximum parsimony and (B) maximum likelihood (ln = -5913.94) analyses of sequence data of the complete internal transcribed spacer (ITS) 1, 5.8S rDNA, and ITS 2. Trees rooted with *Saccharomyces cerevisiae*, and bootstrap values greater than 70% (1,000 replications) indicated at internodes. GenBank accession numbers (underlined) are provided for sequences generated in previous studies (Aveskamp et al. 2009, Balmas et al. 2005, Camara et al. 2002, Irinyi et al. 2009, Mendes-Pereira et al. 2003, Morales et al. 1993, Sugita et al. 1999). Section *Plenodomus* is a division within the genus *Phoma* associated with glabrous, thick-walled pycnidia; scleroplectenchymatous pycnidial cells; 1- to 2-celled conidia; and, when known, a *Leptosphaeria* telemorph (Boerema et al. 2004).

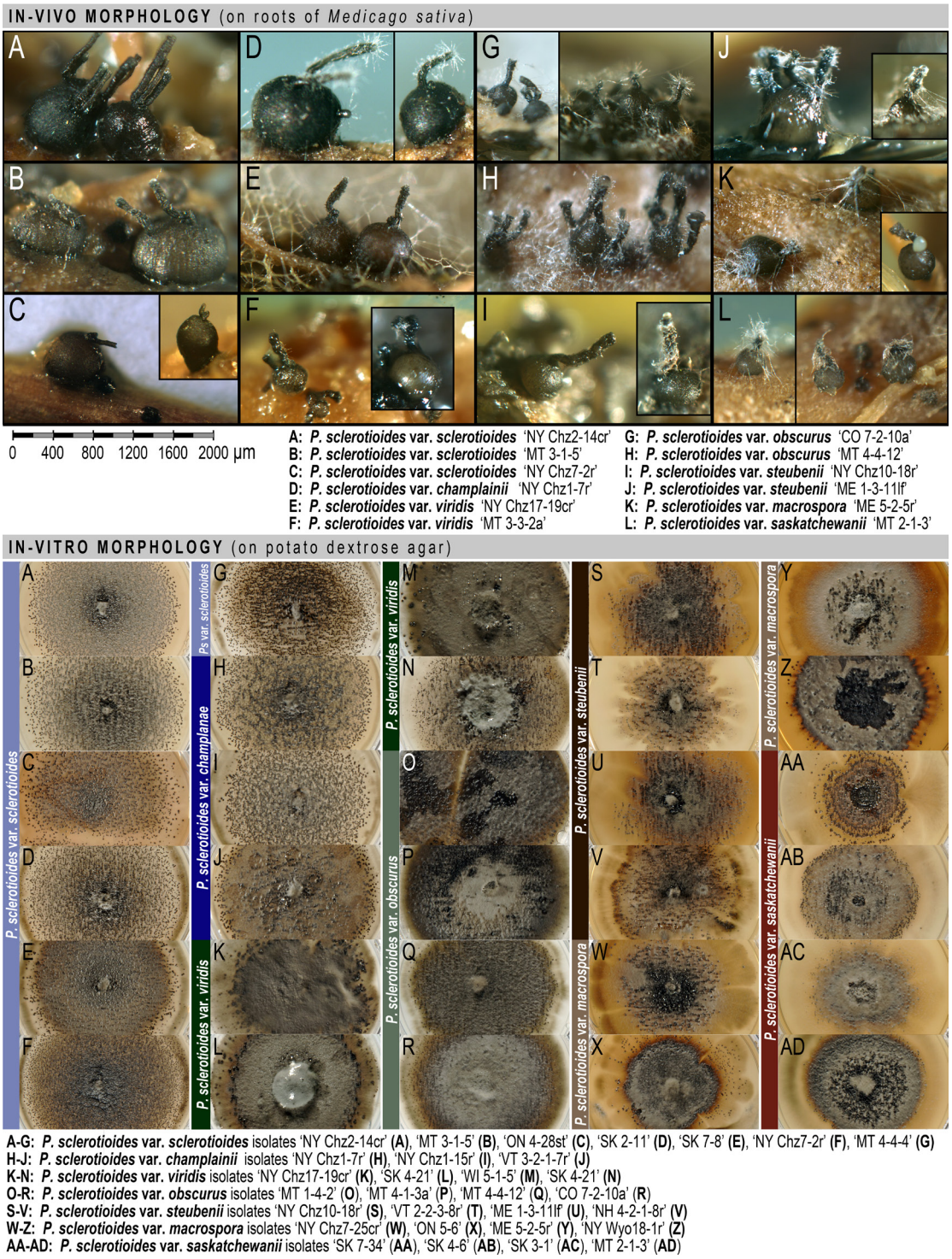

FIGURE 5.4 In-vivo and in-vitro morphology *P. sclerotioides* isolates. In-vivo morphology was assessed by inoculating healthy alfalfa with single-conidium isolates, harvesting diseased roots, and incubating symptomatic roots in moist chambers until pynidial maturity. In-vitro morphology was assessed by growing single-conidium isolates on potato dextrose agar at 10°C under continuous white fluorescent light for 2 months.

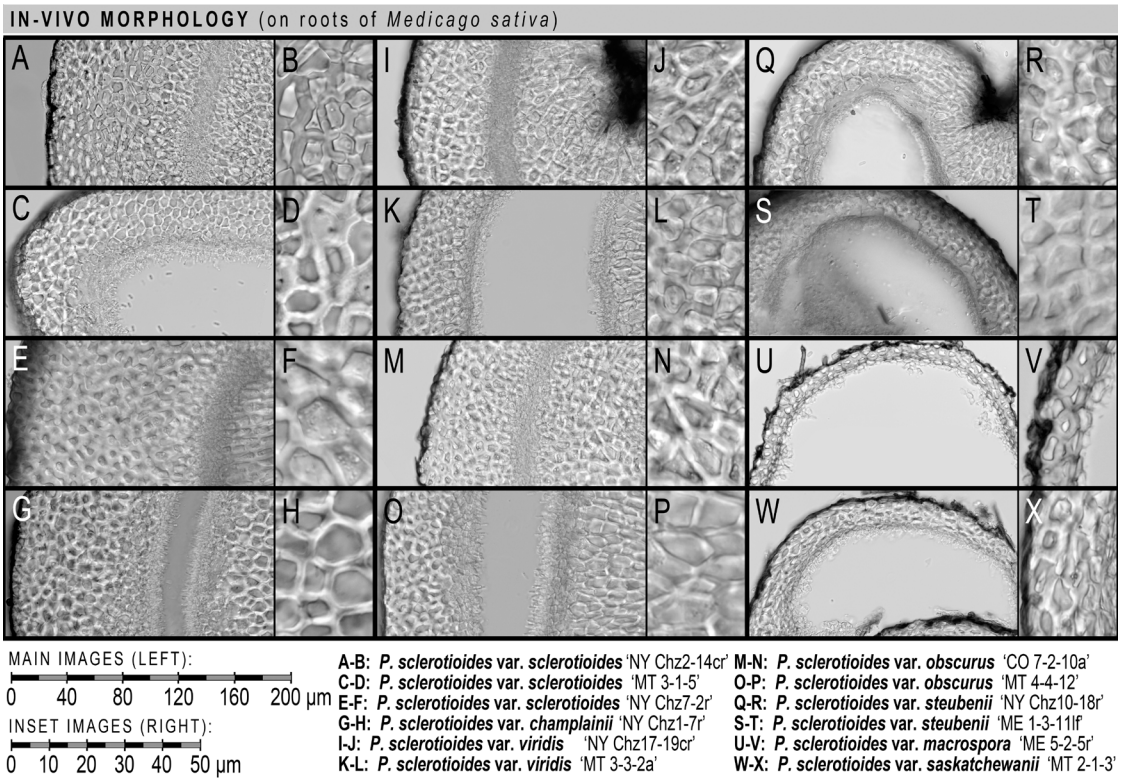

FIGURE 5.5 Cross-sections of pycnidia produced in-vivo on alfalfa roots. In-vivo morphology was assessed by inoculating healthy alfalfa with single-conidium isolates, harvesting diseased roots, and incubating symptomatic roots in moist chambers until pynidial maturity.

pycnidial walls, glabrous pycnidia, 1- to 2-celled conidia, and, when known, a *Leptosphaeria* teleomorph (Boerema et al. 2004).

In-vivo morphology. The pycnidial morphology of all isolates was consistent with the species description (Boerema et al. 2004). Pycnidia were glabrous, subglobose, and superficial (Figure 5.4). Pycnidia were composed of outer polygonal cells with thick, scleroplectenchymatous cell walls that overlay thin-walled, smaller, polygonal cells in the interior (Figure 5.5). 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(-1000) µm; (Boerema et al. 2004)], but only isolates of *P. sclerotioides* var. *sclerotioides* and

var. *champlainii* produced pycnidia with diameters up to 1000 µm. Isolates of other varieties of *P. sclerotioides* exhibited maximum diameters between 600 and 800 µm, but considerable isolate-to-isolate variability existed within varieties (Figure 5.6). Pycnidia of all isolates produced long necks for spore discharge, with maximum neck lengths of 500 to 800 µm (Figures 5.4, 5.6), but *P. sclerotioides* var. *champlainii* isolate 'NY Chz1-7r' produced significantly longer necks than the others ($p < 0.01$; Figure 5.6). Pycnidia with multiple necks were common in all isolates but were most common in isolates of *P. sclerotioides* var. *sclerotioides, champlainii* and *obscurus* (Figure 5.6). Pycnidial necks of *P. sclerotioides* var. *sclerotioides* and var. *viridis* isolates were glabrous to semi-pilose; pycnidial necks of *P. sclerotioides* var. *champlainii* isolate 'NY Chz1-7r' were glabrous to pilose, and pycnidial necks of *P. sclerotioides* var. *obscurus, steubenii, macrospora,* and *saskatchewanii* isolates were semi-pilose to pilose (Figure 5.4). All isolates produced cream-colored cirrus that yellowed with age.

Conidial morphology of isolates approximated the species description (Boerema et al. 2004) with a few exceptions. No eguttalate conidia were observed, conidia were not always aseptate, and conidial lengths were more variable. Conidia were hyaline and subcylindrical to ellipsoidal, with 1 to 3 guttules in *P. sclerotioides* var. *champlainii, steubenii,* and *saskatchewanii* and 1 to 4 guttules in all others (Figure 5.7). Guttules were mostly polar. *P. sclerotioides* var. *macrospora* produced both one- and two-celled conidia, and others produced only aseptate condia. Conidial lengths were highly variable. All varieties of *P. sclerotioides* produced a range of conidial lengths that partially overlapped the species description, but none corresponded to the species description completely. *P. sclerotioides* var. *sclerotioides* and var. *champlainii*

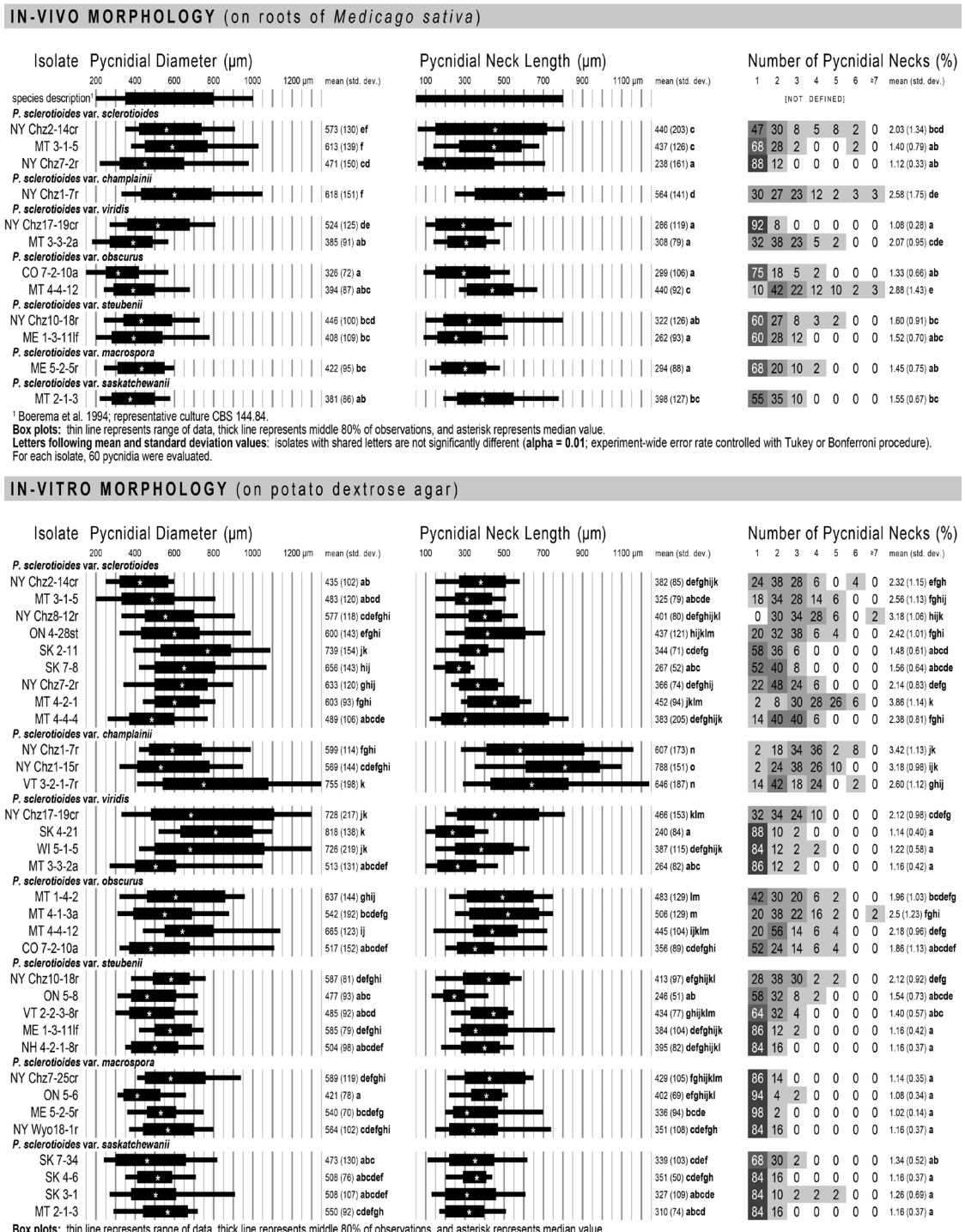

FIGURE 5.6 In-vivo and in-vitro pycnidial diameters, neck lengths, and neck numbers of *P. sclerotioides* isolates. Neck number data are given as the percentage of pycnidia observed.

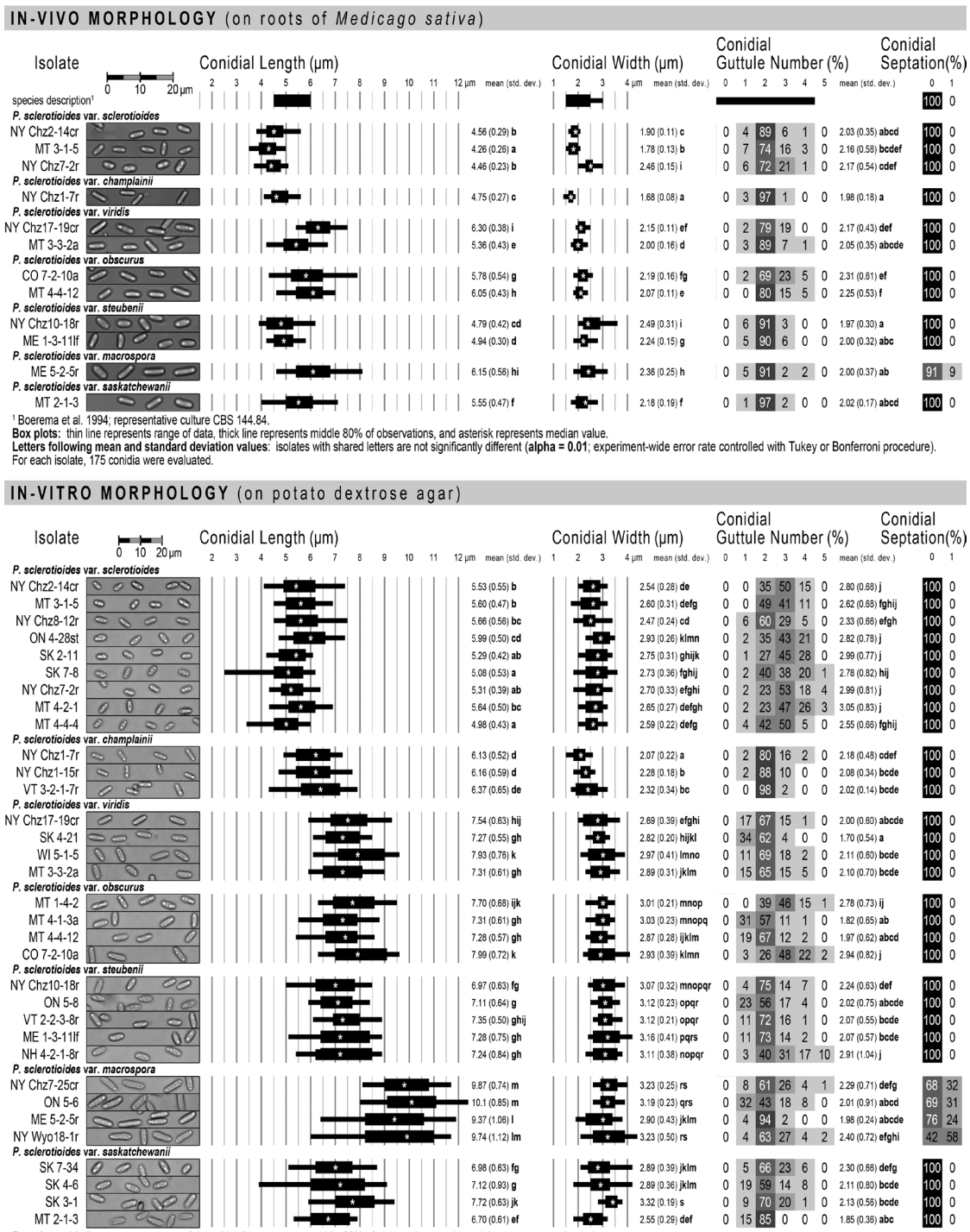

FIGURE 5.7 In-vivo and in-vitro conidial lengths, widths, guttule numbers, and septation of *P. sclerotioides* isolates. Guttule number and septation data are given as the percentage of conidia observed.

produced short conidia (3.5 – 5.6 μm), *P. sclerotioides* var. *obscurus* and var. *macrospora* produced long conidia (4.3 – 8.1 μm), and others were intermediate. *P. sclerotioides* var. *sclerotioides* produced significantly shorter conidia than the others ($p < 0.01$; Figure 5.7). Conidial widths were broadly consistent with the species description (1.5 – 3.0 μm) for all varieties of *P. sclerotioides* except *P. sclerotioides* var. *champlainii*, in which less variability in conidial widths (1.4 – 1.9 μm) was observed. Conidia of *P. sclerotioides* var. *champlainii* were significantly narrower than conidia of other varieties ($p < 0.01$; Figure 5.7).

In-vitro morphology. Gross cultural morphology of isolates varied widely on PDA. At 10°C under continuous white fluorescent light, aerial mycelium of *P. sclerotioides* var. *sclerotioides* 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 (Figure 5.4). Isolates of *P. sclerotioides* var. *steubenii*, *macrospora*, and *saskatchewanii* deposited a tan to yellow-brown pigmentation in the agar surrounding cultures (Figure 5.4). 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. sclerotioides* var. *sclerotioides* and var. *champlainii* (Figure 5.4). Differences in gross cultural morphology were repeatable,

with isolates consistently showing the same phenotype when started from frozen stocks.

All isolates produced large pycnidia (maximum diameter 600 to 1350 μm) with long necks for spore discharge. Pycnidial diameters were most variable in isolates of *P. sclerotioides* var. *viridis* (270 to 1300 μm) and *champlainii* (320 to 1350 μm), least variable in isolates of *P. sclerotioides* var. *steubenii* (300 to 760 μm), and intermediate (200 or 300 μm to 900 or 1100 μm) in the others (Figure 5.6). Pycnidial necks were approximately 150 to 800 μm long except in isolates of *P. sclerotioides* var. *champlainii*, which produced significantly longer necks ($p < 0.01$; 280 to 1240 μm). Pycnidia with multiple necks were produced by all isolates but were most common in isolates of *P. sclerotioides* var. *sclerotioides, champlainii,* and *obscurus* (Figure 5.6). All isolates produced cream-colored cirrus that yellowed with age.

Conidia were hyaline and subcylindrical to ellipsoidal, with 1 to 4 guttules in *P. sclerotioides* var. *champlainii, viridis*, and *saskatchewanii* and 1 to 5 guttules in all others (Figure 5.7). Guttules were mostly polar. *P. sclerotioides* var. *macrospora* produced both aspetate and 1-septate conidia, and others produced only aseptate condia. Conidial lengths were highly variable. *P. sclerotioides* var. *sclerotioides* and var. *champlainii* produced short conidia (usually 4 to 7.5 μm but sometimes 2.5 to 7.9 μm), *P. sclerotioides* var. *macrospora* produced long conidia (6 to 12.4 μm), and others were intermediate. *P. sclerotioides* var. *macrospora* produced significantly longer conidia than the others ($p < 0.01$, Figure 5.7), and *P. sclerotioides* var. *sclerotioides* produced significantly shorter conidia than all others except var. *champlainii* ($p < 0.01$). Conidia were approximately 2 to 4 μm wide in *P. sclerotioides* varieties *viridis, obscurus, steubenii, macrospora,* and *saskatchewanii*

but significantly narrower (1.5 to 3.2 μm) in var. *champlainii* ($p < 0.01$, Figure 5.7). Conidial lengths of *P. sclerotioides* var. *sclerotioides* 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. sclerotioides* caused a significant increase in root rot severity relative to the control (Table 5.3). The highest levels of root rot severity and plant mortality were associated with isolates of *P. sclerotioides* var. *sclerotioides*, var. *viridis,* and var. *obscurus* (Table 5.3). Lesions were characteristic of brown root rot: 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

TABLE 5.3 Pathogenicity of *P. sclerotioides* isolates to Vernal alfalfa.

Isolate	Plant mortality[a]	Root rot severity[b]
P. sclerotioides var. *sclerotioides* 'MT 3-1-5'	10/36	3.83 ****
P. sclerotioides var. *sclerotioides* 'NY Chz2-14cr'	9/36	3.08 ****
P. sclerotioides var. *sclerotioides* 'NY Chz7-2r'	5/36	2.08 **
P. sclerotioides var. *champlainii* 'NY Chz1-7r'	5/36	2.08 ***
P. sclerotioides var. *viridis* 'MT 3-3-2a'	12/36	3.44 ****
P. sclerotioides var. *viridis* 'NY Chz17-19cr'	10/36	3.17 ****
P. sclerotioides var. *obscurus* 'CO 7-2-10a'	4/36	1.81 **
P. sclerotioides var. *obscurus* 'MT 4-4-12'	11/36	2.28 ****
P. sclerotioides var. *steubenii* 'ME 1-3-11lf'	1/36	0.61 NS
P. sclerotioides var. *steubenii* 'NY Chz10-18r'	7/36	1.11 NS
P. sclerotioides var. *steubenii* 'NY Fr1-3-1RC'	3/36	1.36 *
P. sclerotioides var. *macrospora* 'ME 5-2-5r'	4/36	1.42 *
P. sclerotioides var. *saskatchewanii* 'MT 2-1-3'	2/36	1.36 *
Control	1/36	0.19

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

[b] 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: $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$ for one, two, three, and four asterisks, respectively. The experiment-wide error rate was controlled with the Bonferonni correction.

TABLE 5.4 In-vitro growth of *P. sclerotioides* at 3°C, 10°C, 18°C and 25°C on potato dextrose agar 24 days after culture establishment.

Isolate	3°C (mm)[a]	10°C (mm)[a]	18°C (mm)[a]	25°C (mm)[a]
P. sclerotioides* var. *sclerotioides				
MT 3-1-5	16.94 (1.91) **efghi**	36.22 (1.47) **l**	36.76 (1.37) **n**	10.82 (1.65) **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**
NY Chz2-14cr	16.62 (2.25) **defgh**	33.38 (1.25) **jk**	31.59 (1.58) **hi**	10.98 (1.81) **gh**
NY Chz7-2r	15.67 (1.69) **bcde**	32.9 (1.61) **ij**	31.00 (3.06) **gh**	10.76 (0.79) **g**
NY Chz8-12r	16.85 (1.75) **efghi**	34.87 (1.61) **kl**	34.59 (1.46) **klm**	12.01 (2.21) **ghi**
SK 2-11	15.98 (1.44) **cdef**	33.70 (1.43) **jk**	35.82 (1.92) **mn**	5.6 (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**
P. sclerotioides* var. *champlainii				
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**
P. sclerotioides* var. *viridis				
MT 3-3-2a	17.25 (0.58) **fghij**	32.06 (2.46) **hij**	35.41 (1.90) **lmn**	21.06 (2.74) **j**
NY Chz17-19cr	16.27 (0.78) **cdefg**	26.16 (1.66) **cd**	25.37 (2.21) **cd**	24.55 (1.54) **k**
WI 5-1-5	18.29 (1.00) **jk**	28.60 (1.13) **ef**	32.00 (2.14) **hij**	25.88 (1.28) **k**
P. sclerotioides* var. *obscurus				
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**
P. sclerotioides* var. *steubenii				
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**
NY Chz10-18r	20.42 (1.54) **l**	30.3 (3.70) **fgh**	27.72 (1.66) **ef**	11.51 (2.38) **ghi**
P. sclerotioides* var. *macrospora				
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**
P. sclerotioides* var. *saskatchewanii				
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**

[a] 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).

differed in their growth at 18°C and 25°C. Optimal growth of isolates of *P. sclerotioides* var. *macrospora* occurred at 10°C, with growth almost completely inhibited at 25°C (Table 5.4). Isolates of *P. sclerotioides* 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 (Hollingsworth et al. 2002, Sanford 1933), showing optimal growth at 10 and 18°C and limited growth at 25°C. At 25°C, isolates of *P. sclerotioides* var. *viridis* isolates showed significantly more growth than other isolates ($p < 0.01$; Table 5.4), 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°C and 18°C, isolates of *P. sclerotioides* var. *sclerotioides* and var. *champlainii* grew significantly more than isolates of *P. sclerotioides* var. *obscurus, macrospora* and *saskachewanii* ($p < 0.01$; Table 4), 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 5.4), although moderate but significant ($p < 0.01$) differences were present among individual isolates.

5.5 DISCUSSION

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, gross cultural morphology in-vitro, or production of metabolites (van der AA et al. 1990, Boerema et al. 2004). Infraspecific variety designations are common among *Phoma* species (van der AA et al. 1990, Boerema et al. 2004), and variety designations are maintained even when morphological variants are also shown to be genetically differentiated (Abeln et al. 2002).

The *Phoma* isolates evaluated in this study are genetically and morphologically differentiated, but all broadly correspond to the published description of *P. sclerotioides* (Boerema et al. 2004). 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 1 to 3 or 1 to 4 mostly polar guttules. All are well adapted to low temperatures, and they are pathogenic to alfalfa as it emerges from winter dormancy. The isolates differ primarily in conidial size and septation, pycnidial neck length, adaptation to growth at high temperatures, and gross cultural morphology in-vitro. Phylogenetic analysis of sequence data from the ITS region places the isolates in a single monophyletic group, confirming their close relationship to each other.

We have proposed the designation of seven varieties within *P. sclerotioides*. Each is strongly supported by phylogenetic analyses of multilocus sequence data, and each corresponds to differences in morphology or temperature adaptation. Gross cultural morphology, pycnidial neck length, conidial length, conidial width, and conidial septation are the most reliable characters for delimitation of the varieties. *P. sclerotioides* var. *sclerotioides* and *champlainii* produce short conidia and have white aerial mycelium on PDA; *P. sclerotioides* var. *champlainii* produces narrow conidia and very long necks for spore discharge; *P. sclerotioides* var. *viridis* produces green aerial mycelium on PDA and grows well at 25°C; *P. sclerotioides* var. *obscurus* produces dark aerial mycelium on PDA; *P. sclerotioides* 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. sclerotioides* var. *steubenii* grows more rapidly at 18°C than *P. sclerotioides* var. *macrospora* or *saskatchewanii*; and *P. sclerotioides* var. *macrospora* produces very long conidia, some of which are 1-septate, and its growth is nearly completely inhibited at 25°C. Pycnidial diameter, pycnidial neck number, and conidial guttule number are less useful for taxonomic delimitation within *P. sclerotioides* due to high isolate-to-isolate variability; for these

characters, statistically significant differences were rarely observed that distinguished all isolates of one variety from all isolates of other varieties.

The designation of additional varieties of *P. sclerotioides* may be warranted in the future. Phylogenetic analyses indicate the existence of at least four additional genetically distinct subtypes of *P. sclerotioides*, one distinct from the seven proposed varieties of *P. sclerotioides* and three within those varieties. One isolate, 'SK 7-12', was closely related to other *P. sclerotioides* isolates (Figures 5.1, 5.3) yet genetically too differentiated to be placed within any of the proposed varieties (Figure 5.1), and *P. sclerotioides* var. *sclerotioides, viridis,* and *obscurus* each contain robust, strongly supported subclades (Figure 5.1; 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-to-isolate variability.

We have chosen to designate the morphologically and genetically distinct subtypes of *P. sclerotioides* 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 characters 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°C and 25°C. Maintenance of a broad species concept for *P. sclerotioides* 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* (van der AA et al. 2000, Boerema and Höweler 1967, Boerema et al. 1993, de Gruyter et al. 2002).

The biological significance of genetic differentiation within *P. sclerotioides* is unknown. In addition to causing brown root rot, *P. sclerotioides* can also contribute to spring black stem and leaf spot of alfalfa (Wang et al. 2004) and can infect roots of overwintering grasses (Davidson 1990, Larsen et al. 2007). Research is currently in progress to assess the distribution of the seven varieties of *P. sclerotioides* within North America, to evaluate if members of the *P. sclerotioides* species complex differ in their ability to cause brown root rot of alfalfa, and to assess if all components of the *P. sclerotioides* species complex infect roots of overwintering grasses and contribute to foliar disease on alfalfa.

The following descriptions are proposed for the varieties of *P. sclerotioides*:

Phoma sclerotioides Preuss ex Sacc. var. ***sclerotioides***

In radices *Medicago sativae*, conidia eseptata, (3.5-)4-5(-5.6) x 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 aeria alba et sparsa; conidia eseptata, (2.5-)4.5-6.5(-7.5) x (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) x 1.5-2.7(-3.0) µm with 1 to 4 guttules. Pycnidial necks glabrous to semi-pilose, 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 aspetate, (2.5-)4.5-6.5(-7.5) x (1.7-)2-3.5 µm with 1 to 5 guttules. Pycnidial necks up to (120-)200-600(-830) µm long and frequently multiple. After 24 days in complete darkness at 18°C and 25°C, average radial growth 29 to 37 mm and 3 to 12 mm, respectively.

Representative cultures:

'NY Chz2-14cr' ex *Medicago sativa* (Leguminaceae), New York, USA; 'NY Chz7-2r' ex *Medicago sativa* (Leguminaceae), New York, USA; and 'SK 7-8' ex *Medicago sativa* (Leguminaceae), Saskatchewan, Canada.

Phoma sclerotioides var. ***champlainii*** Wunsch & Bergstrom, *var. nov.*

In radices *Medicago sativae*, conidia eseptata, 4-5.6 x 1.4-1.9 µm. Colla pycnidii plerumque numerosa, vel glabra vel pilosa; in longitudinem (250-)350-720(-810) µm. In agara solani et sacchari, mycelia aeria alba et sparsa vel paullo abunda; conidia eseptata, (4.3-)5.4-7.2(-7.9) x (1.5-)1.8-2.8(-3.2) µm. Colla pycnidii plerumque numerosa, in longitudinem (300-)400-1000(-1100) 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 x 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) x (1.5-)1.8-2.8(-3.2) µm. Pycnidial necks up to (300-)400-1000(-1100) µm long and usually multiple. After 24 days in complete darkness at 18°C and 25°C, average radial growth 33 to 34 mm and 7 to 8 mm, respectively.

Representative culture:

'NY Chz1-7r' ex *Medicago sativa* (Leguminaceae), New York, USA.

Phoma sclerotioides var. ***viridis*** Wunsch & Bergstrom, *var. nov.*

In radices *Medicago sativae*, conidia eseptata, (4.2-)4.9-6.8(-7.5) x 1.7-2.5 µm. Colla pycnidii vel una vel numerosa, vel glabra vel paullo pilosa; in longitudinem (100-)150-450(-540) µm. In agara solani et sacchari, mycelia aeria viride et abunda vel paullo abunda; conidia eseptata, (5.9-)6.5-9.0(-9.6) x (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) x 1.7-2.5 µm. Pycnidial necks glabrous to semi-pilose, 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) x (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°C and 25°C, average radial growth 25 to 35 mm and 21 to 26 mm, respectively.

Representative cultures:

'NY Chz17-19cr' ex *Medicago sativa* (Leguminaceae), New York, USA; 'MT 3-3-2a' ex *Medicago sativa* (Leguminaceae), Montana, USA; 'WI 5-1-5' ex *Medicago sativa* (Leguminaceae), Wisconsin, USA.

Phoma sclerotioides var. ***obscurus*** Wunsch & Bergstrom, *var. nov.*

In radices *Medicago sativae*, conidia eseptata, (4.3-)5.2-6.5(-7.9) x 1.8-2.6 µm. Colla pycnidii vel una vel numerosa, vel paullo pilosa vel pilosa, in longitudinem (90-)150-550(-670) µm. In agara solani et sacchari, mycelia aeria obscura et paullo abunda vel abunda; conidia eseptata, (5.5-)6.5-9.0(-9.6) x 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) x 1.8-2.6 µm. Pycnidial necks semi-pilose 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) x 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°C and 25°C, average radial growth 23 to 24 mm and 6 to 13 mm, respectively.

Representative cultures:

'CO 7-2-10a' ex *Medicago sativa* (Leguminaceae), Colorado, USA; 'MT 4-4-12' ex *Medicago sativa* (Leguminaceae), Montana, USA.

Phoma sclerotioides var. ***steubenii*** Wunsch & Bergstrom, *var. nov.*

In radices *Medicago sativae*, conidia eseptata, (3.9-)4.2-5.3(-6.2) x (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 agara solani et sacchari, mycelia aeria bruna vel cana et sparsa vel paullo abunda. Agarum cingen coloniae flavum vel paullo brunum. Conidia eseptata, (5.0-)6.2-8.5(-8.9) x (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) x (1.8-)2.0-2.9(-3.6) µm. Pycnidial necks semi-pilose 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) x (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°C and 25°C, average radial growth 27 to 28 mm and 4 to 12 mm, respectively.

Representative cultures:

'NY Chz10-18r' ex *Medicago sativa* (Leguminaceae), New York, USA; 'NH 4-2-1-8r' ex *Medicago sativa* (Leguminaceae), New Hampshire, USA.

Phoma sclerotioides var. ***macrospora*** Wunsch & Bergstrom, *var. nov.*

In radices *Medicago sativae*, conidia eseptata et uniseptata, (4.6-)5.4-6.8(-7.1) x (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 agara solani et sacchari, mycelia aeria bruna vel cana et sparsa vel paullo abunda. Agarum cingen coloniae flavum vel paullo brunum. Conidia eseptata et uniseptata, (6.0-)8.2-11.1(-12.4) x (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) x (1.8-)2.1-2.7(-3.2) µm. Pycnidial necks semi-pilose 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 1-septate, (6.0-)8.2-11.1(-12.4) x (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°C and 25°C, average radial growth 15 to 24 mm and 0 to 3 mm, respectively.

Representative culture:

'ME 5-2-5r' ex *Medicago sativa* (Leguminaceae), Maine, USA.

Phoma sclerotioides var. ***saskatchewanii*** Wunsch & Bergstrom, *var. nov.*

In radices *Medicago sativae*, conidia eseptata, (4.0-)5.0-6.1(-7.1) x 1.7-2.8 µm. Colla pycnidii vel una vel numerosa, vel paullo pilosa vel pilosa; in longitudinem (190-)260-550(-780) µm. In agara solani et sacchari, mycelia aeria bruna vel cana et

sparsa vel paullo abunda. Agarum cingen coloniae flavum vel paullo brunum. Conidia eseptata, (4.0-)6.0-8.6(-9.1) x (1.8-)2.2-3.5(-4.2) µm. Colla pycnidii plerumque solitaria, in longitudinem (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) x 1.7-2.8 µm. Pycnidial necks semi-pilose 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) x (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°C and 25°C, average radial growth 18 to 24 mm and 8 to 13 mm, respectively.

Representative cultures:

'MT 2-1-3' ex *Medicago sativa* (Leguminaceae), Montana, USA; 'SK 7-34' ex *Medicago sativa* (Leguminaceae), Saskatchewan, Canada.

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CHAPTER 6

FUSARIUM WILT OF BIRDSFOOT TREFOIL AND BROWN ROOT ROT OF ALFALFA: DIRECTIONS FOR FUTURE RESEARCH

This thesis provides a detailed characterization of the distribution and genetic diversity of *Fusarium oxysporum* f. sp. *loti*, causal agent of Fusarium wilt of birdsfoot trefoil, in New York and Vermont; the distribution of *Phoma sclerotioides*, causal agent of brown root rot of alfalfa, in the northeastern United States, southern Ontario, southern Colorado, and northern New Mexico; and genetic and morphological diversity within *P. sclerotioides*. Our finding that *F. oxysporum* f. sp. *loti* isolates are vegetatively compatible and share identical multilocus sequence haplotypes indicates that the pathogen may be clonal across its known distribution in New York and Vermont and suggests that the relative susceptibility of birdsfoot trefoil cultivars to Fusarium wilt will be similar in infested fields throughout the region. Our finding that *P. sclerotioides* is widespread throughout much of the northeastern United States indicates that the distribution of the pathogen is much broader than previously understood, but it is not surprising. Research on low-temperature pathogens of alfalfa in the northern United States has been lacking (Leath 1989), and standard culturing protocols at room temperature do not permit successful isolation of *P. sclerotioides*. The high genetic and morphological diversity observed among North American isolates of *P. sclerotioides* provides strong evidence that the pathogen is comprised of a species complex, but the full implications of this finding are unknown. Within other pathogenic *Phoma* species, including *P. medicaginis, P. lingam,* and *P.*

cucurbitacearum, genetically and morphologically distinct varieties correspond to differences in aggressiveness (Boerema et al. 1993, Johnson and Lewis 1994, Somai et al. 2002). In this section, directions for future research on Fusarium wilt of birdsfoot trefoil and brown root rot of alfalfa are outlined.

6.1 FUSARIUM WILT OF BIRDSFOOT TREFOIL

Accurate identification of *F. oxysporum* f. sp. *loti* requires pathogen isolation and subsequent pathogenicity testing on birdsfoot trefoil, a process that takes a minimum of 3 months to complete and requires greenhouse facilities. Pathogenicity testing is required because no other approaches are currently available that are adequate for identifying the fungus. Formae speciales of *F. oxysporum* are indistinguishable morphologically from each other and from endophytic strains of *F. oxysporum* (Armstrong and Armstrong 1954, Armstrong and Armstrong 1965, Armstrong and Armstrong 1975, Armstrong and Armstrong 1981). DNA sequence data of an intron-spanning region of the elongation factor 1-alpha (EF1a) gene and of the complete intergenic spacer of the rDNA are useful for distinguishing some formae speciales, and an online database of EF1a and IGS sequences from a broad collection of vouchered *F. oxysporum* isolates has been established (Geiser et al. 2004). However, the EF1a and IGS haplotype shared by *F. oxysporum* f. sp. *loti* isolates is indistinguishable from EF1a and IGS haplotypes exhibited by strains of *F. oxysporum* f. sp. *apii*, a pathogen of celery, *F. oxysporum* f. sp. *conglutinans*, a pathogen of cole crops, and *F. oxysporum* f. sp. *pisi*, a pathogen of pea.

Future research should be directed toward the development of diagnostic PCR primers for rapid identification of *F. oxysporum* f. sp. *loti*. Diagnostic PCR would

permit rapid diagnosis of diseased plants collected in production fields, and it would facilitate efforts to breed trefoil for resistance by permitting rapid assessment of inoculation efficacy and accurate diagnosis of symptomatic plants. As a first step in the development of a diagnostic PCR protocol, pathogenicity testing should be conducted to confirm that isolates of *F. oxysporum* f. sp. *apii, F. oxysporum* f. sp. *conglutinans,* and *F. oxysporum* f. sp. *pisi* sharing an identical EF1a and IGS haplotype with *F. oxysporum* f. sp. *loti* do not cause Fusarium wilt on trefoil. Many formae speciales of *F. oxysporum* cause wilt symptoms on more than one host (Armstrong and Armstrong 1975), and confirmation is needed that *F. oxysporum* f. sp. *loti* is distinct from these formae speciales and that birdsfoot trefoil is not simply a previously undescribed host of *F. oxysporum* f. sp. *apii, F. oxysporum* f. sp. *conglutinans,* or *F. oxysporum* f. sp. *pisi*. *F. oxysporum* f. sp. *loti* did not cause wilt symptoms on pea (*Pisum sativum*) cultivar M410 (Chapter 2), susceptible to all known races of *F. oxysporum* f. sp. *pisi* (Kraft 1994), suggesting that the trefoil pathogen is distinct from all races of *F. oxysporum* f. sp. *pisi*; however, rigorous validation of this conclusion requires pathogenicity testing on trefoil with the *F. oxysporum* f. sp. *pisi* isolates sharing sequence identity with the trefoil pathogen. No information is available on whether *F. oxysporum* f. sp. *loti* is distinct from *F. oxysporum* f. sp. *apii* or *F. oxysporum* f. sp. *conglutinans*, and inoculations should be conducted on trefoil to evaluate if the host ranges of these formae speciales extend to trefoil.

Given the low DNA sequence variability observed within *F. oxysporum,* diagnostic primers specific for isolates causing Fusarium wilt of birdsfoot trefoil should be developed from unique PCR amplification products obtained from amplified fragment length polymorphisms (AFLPs). AFLPs are useful for detecting genetic

variation within *F. oxysporum* (Baayen et al. 2000, O'Donnell et al. 2004), and they able to differentiate isolates sharing identical EF1a and IGS haplotypes (O'Donnell et al. 2004). Diagnostic primers can be developed by sequence characterization of amplification products only generated for *F. oxysporum* pathogenic to trefoil. Diagnostic primers can also be developed using a similar approach based on unique products obtained from random amplified polymorphic DNA (RAPD) reactions (e.g., Jiménez-Gasco and Jiménez-Diaz, 2003). However, AFLPs are preferable to RAPDs; whereas RAPDs frequently generate different amplification products each time they are conducted, AFLPs are highly replicable (Mueller and Wolsenbarger 1999).

6.2 BROWN ROOT ROT OF ALFALFA

In other diseases caused by *Phoma* species complexes, components of the species complex have been documented to differ in aggressiveness, in the host tissues that they infect, and in their host range. *P. medicaginis* var. *macrospora* causes more severe stem and leaf blight on alfalfa than *P. medicaginis* var. *medicaginis* (Boerema et al. 1993); *P. exigua* var. *heteromorpha* and other strains of *P. exigua* both contribute to stem and leaf blight of *Vinca* sp. but only *P. exigua* var. *heteromorpha* has a host range extending to oleander (*Nerium oleander*; Boerema et al. 2004); 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 (Somai et al. 2002); and genetically differentiated isolates of *P. lingam* differ in their ability to form stem cankers on oilseed rape (*Brassica rapus*; Williams and Fitt 1999).

Efforts to breed alfalfa for resistance to brown root rot have utilized a single isolate of *P. sclerotioides* to screen for resistance (Hollingsworth et al. 2005), and the efficacy of that approach should be evaluated. An isolate of *P. sclerotioides* var. *sclerotioides* was used to screen for resistance in that study, and it is unknown whether alfalfa cultivars resistant to isolates of *P. sclerotioides* var. *sclerotioides* are also resistant to isolates of other varieties of *P. sclerotioides*. Addressing this question would help ensure that efforts to breed alfalfa for BRR resistance will result in the development of alfalfa cultivars that show resistance to BRR regardless of the composition of the *P. sclerotioides* species complex in a particular field.

It has recently been reported that *P. sclerotioides* can also contribute to spring black stem and leaf spot (SBS) of alfalfa (Wang et al. 2004), a disease historically ascribed solely to *P. medicaginis*. However, it is unknown whether all varieties of *P. sclerotioides* contribute to SBS and whether varieties of *P. sclerotioides* causing SBS differ in their aggressiveness; a single isolate of *P. sclerotioides* was used for pathogenicity testing (Wang et al. 2004). Addressing these questions will help us better understand the contribution and importance of *P. sclerotioides* in the development of SBS and will help us better understand *P. sclerotioides* epidemiology. Isolates of *P. sclerotioides* that can infect foliar tissues also have the potential to be disseminated by the movement of hay.

P. sclerotioides is known to infect the roots of winter wheat and perennial grasses, including grasses frequently grown in mixed seedings with alfalfa (Davidson 1990, Larsen et al. 2007, Lebeau and Logsdon 1958). However, it is not understood whether all varieties of *P. sclerotioides* have a host range extending to grasses and whether varieties of *P. sclerotioides* infecting grasses differ in their aggressiveness.

Addressing these questions will help us better understand the economic impact of *P. sclerotioides* to winter wheat and perennial grass production and will help us better assess the potential of perennial grasses and winter wheat to maintain inoculum levels of varieties of *P. sclerotioides*.

If isolates from different *P. sclerotioides* varieties differ in their host range or their ability to cause BRR or SBS, understanding the geographic distribution of the different *P. sclerotioides* varieties will be important. If alfalfa cultivars resistant to *P. sclerotioides* var. *sclerotioides* are susceptible to other *P. sclerotioides* varieties, understanding the geographic distribution of *P. sclerotioides* varieties will help predict the regions in which the cultivars will show BRR resistance. If SBS is caused only by certain varieties of *P. sclerotioides*, understanding the distribution of the varieties will help predict where the pathogen will have the greatest impact on alfalfa foliar blight. If only a subset of the *P. sclerotioides* varieties can infect the roots of winter wheat and perennial grasses, understanding the distribution of *P. sclerotioides* varieties will help predict where such crops are likely to maintain inoculum levels of *P. sclerotioides*.

If genetic and morphological diversity within *P. sclerotioides* corresponds to differences in host range or in the ability to cause BRR or SBS, the development of diagnostic primers specific to different *P. sclerotioides* varieties will be important. Distinguishing the different varieties of *P. sclerotioides* currently requires either sequencing at informative loci or conducting a combination of in-vitro culturing, assessment and measurement of large numbers of pycnidia and conidia, and evaluation of in-vitro growth rates at 18°C and 25°C. Both approaches are laborious and expensive. Development of a multiplex diagnostic PCR protocol would permit rapid

identification of all *P. sclerotioides* varieties contributing to BRR or SBS on symptomatic plant tissues. Sequence divergence among *P. sclerotioides* varieties was high at many loci sequenced as a part of the phylogenetic analysis of *P. sclerotioides* (Chapter 5), and diagnostic primer development should be possible using sequence differences at the various loci.

Finally, future research should be directed at better understanding the evolutionary potential of *P. sclerotioides*. The evolutionary processes of selection, genetic drift, mutation, recombination, and gene flow all contribute to the ability of pathogens to evolve (McDonald and Linde 2002), and the strength of these processes determines the rate at which agriculturally important characteristics such as aggressiveness, host range, and temperature adaptation can change. Though the strength of selection for aggressive genotypes is likely weakened by the ability of *P. sclerotioides* to survive as a saprophyte, the saprophytic ability of the pathogen also makes the loss of aggressive *P. sclerotioides* strains by genetic drift improbable. Because *P. sclerotioides* populations are unlikely to crash following crop rotations, genetic drift is improbable in the pathogen. Understanding gene flow within *P. sclerotioides* would permit an assessment of the potential for the movement of unique genotypes, such as those with elevated aggressiveness or different temperature adaptation, among fields and regions (McDonald and Linde 2002). Evaluating the occurrence of recombination within *P. sclerotioides* would permit an evaluation of the pathogen's potential to combine favorable alleles, including beneficial mutations and other rare traits, and its ability to create novel genotypes differing in aggressiveness, host range, or other characteristics (Anderson and Kohn 1998). A teleomorphic state has not been identified for *P. sclerotioides,* but the absence of a known sexual state

does not preclude its existence; in some fungi, development of perithecia occurs only on specific substrates, making the telomorphic state difficult to identify. Many closely related *Phoma* species are known to undergo sexual recombination; *Phoma* species in section *Plenodomus,* the subdivision of the genus *Phoma* to which *P. sclerotioides* has been assigned, generally have a *Leptosphaeria* teleomorph (Boerema et al. 2004).

The strength of recombination and gene flow within *P. sclerotioides* is unknown. In the absence of an known teleomorphic state, recombination can be detected indirectly in haploid fungi such as *P. sclerotioides* by evaluating if alleles at multiple independent loci within populations display random association, as expected in recombining populations. Evaluation of the concordance of gene genealogies, tests for multilocus association, and Hudson's four-gamete test are techniques frequently employed for this purpose (Couch and Kohn 2000, Hudson and Kaplan 1985, Milgroom 1995). Gene flow can be evaluated by direct methods, such as observing the same clone in multiple locations (Couch and Kohn 2000) and by indirect methods that evaluate whether populations exhibit differentiation across geographic distances. If gene flow has not recently occurred between populations, isolates from different geographic regions tend to fall into separate phylogenetic clades (Couch and Kohn 2000), reflecting the accumulation of different mutations, differences in the selection pressure, and differences in the evolutionary history (e.g. bottleneck events) of each population. Likewise, if only limited gene flow occurs, the gene diversity at specific loci for a population from a limited geographic region will be lower than the total diversity at the corresponding loci for samples drawn from a larger geographic region (Milgroom 1995).

The completion of these studies would help alfalfa breeders more effectively screen for BRR and SBS resistance, help growers plan appropriate rotations for fields in which BRR is a problem, help growers assess the risk of introducing novel *P. sclerotioides* varieties when buying hay from other regions, and help researchers anticipate the likelihood that *P. sclerotioides* may overcome resistant cultivars.

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