Seed Rot and Damping-off of Alfalfa in Minnesota Caused by *Pythium* and *Fusarium* Species

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Abstract

Globally, 15 *Pythium* species have been found to cause damping-off and seed rot of alfalfa, although surveys of species causing disease on alfalfa in the midwestern United States are lacking. Pathogens were isolated by a seedling baiting technique from soil samples of five alfalfa fields in Minnesota with high levels of damping-off. Of the 149 organisms isolated, 93 (62%) were identified as *Pythium* spp. and 43 (29%) were identified as *Fusarium* species. *Pythium sylvaticum*, *P. irregulare*, and *P. ultimum* var. *ultimum* were aggressive pathogens on germinating alfalfa seedlings. Strains of seven *Pythium* spp. pathogenic on soybean and corn were also pathogenic on alfalfa. The majority of the *Fusarium* isolates

were identified as *F. solani* and *F. oxysporum* with a low number of *F. redolens* and *F. incarnatum-equiseti*. The *F. oxysporum* and *F. incarnatum-equiseti* strains were the most aggressive in causing seed and root rot. *Pythium* strains were sensitive to Apron XL (mefenoxam) and pyraclostrobin in vitro but efficacy varied when the fungicides were applied as a seed treatment. Seed treatments with Apron XL were more effective than treatments with Stamina against *Pythium*. The presence of aggressive, broad-host-range pathogens causing seed rot and damping-off suggests that new strategies are needed for managing this disease in alfalfa production systems.

Rapid and uniform seedling emergence is critical for obtaining a productive and persistent stand of alfalfa (*Medicago sativa*). However, a number of pathogens attack alfalfa seeds and seedlings, severely reducing plant establishment. In many locations, alfalfa seeds are planted into cold, wet soil conditions that are ideal for seed rot and damping-off to occur. A complex of soil-borne pathogens including *Pythium* spp., *Rhizoctonia solani*, *Phytophthora medicaginis*, and *Fusarium* spp. cause seed rot and damping-off of alfalfa (Hancock 1983; Leath et al. 1988) and may inflict damage to roots of surviving plants, resulting in reduced yields, decreased winter survival, and shortened stand life. Of these pathogens, *Pythium* spp. are considered the most damaging. Little research has focused on the role of *Fusarium* spp. in seed rot and damping-off of alfalfa.

Alfalfa seedlings infected by *Pythium* develop softened radicles and brownish lesions on cotyledons and lack root hairs. Hypocotyls and roots appear water-soaked and flaccid before seedlings collapse and die. Infection of older plants causes destruction of fine feeder roots, which interferes with nitrogen fixation, nutrient uptake from the soil, and water absorption. Injury to the root may cause root “forking” in which shallow adventitious roots form above the damaged primary root (Leath et al. 1988).

Currently, little is known about the species of *Pythium* attacking alfalfa in Minnesota. In California, *P. ultimum* was identified as a cause of damping-off in alfalfa (Hancock 1983) and was associated with root-let diseases, along with *P. irregulare* and *P. paroecandrum* (Hancock 1985). In Missouri, damping-off of alfalfa was found to be caused by *P. ultimum*, *P. sylvaticum*, and *P. irregulare*, while reductions in root length were caused by *P. dissotocum*, *P. acanthicum*, *P. torulosum*, and *P. rostratum* (Larkin et al. 1995). In Minnesota, *P. ultimum*,

P. paroecandrum, and a *P. sylvaticum*-like species were previously identified as alfalfa damping-off agents, and a culture plate assay was developed to screen alfalfa cultivars for resistance to the pathogens (Altier and Thies 1995). The identification of *Pythium* spp. causing disease on corn and soybean (Broders et al. 2007a; Matthiesen et al. 2016; Radmer et al. 2017; Alejandro Rojas et al. 2017; Zhang and Yang 2000), crops grown in rotation with alfalfa, raises questions about their potential to also cause disease on alfalfa.

Identification of *Pythium* spp. using morphological characteristics is difficult. The development of universal primers for the ribosomal ITS region have led to widespread use of this sequence for fungal and oomycete identification (Robideau et al. 2011). However, the ITS sequence of *Pythium* spp. can be riddled with repeats of varying length or single base insertions and deletions making alignment and comparison with reference databases difficult (Schroeder et al. 2013). The mitochondrial cytochrome oxidase *c* subunit I gene (COI) is commonly used for identification in oomycetes. However, it exhibits less interspecies variation than is seen for the ITS region (Robideau et al. 2011), and consequently there are species that cannot be differentiated by COI. Thus, ITS and COI sequences are complementary barcodes for identifying *Pythium* spp.

Damping-off organisms of alfalfa are managed with cultural practices, over-seeding to offset seed and seedling losses, and fungicide seed treatments. Apron XL (active ingredient mefenoxam; R,S-2[(2,6-dimethylphenyl)-methoxyacetylaminol]-propionic acid methyl ester), is widely used as a seed treatment and offers protection from *Pythium* spp. and *Phytophthora* spp. However, varying levels of sensitivity to mefenoxam or its enantiomer, metalaxyl, have been found among *Pythium* pathogens of many field crops, including corn and soybean (Broders et al. 2007a; Matthiesen et al. 2016; Radmer et al. 2017; Zhang and Yang 2000), wheat (Cook and Zhang 1985), pea (Parke et al. 1991), sugar beet (Brantner and Windels 1998), and potato (Taylor et al. 2002). Species with reduced sensitivity to mefenoxam were first reported on alfalfa three decades ago (Hwang 1988). Currently, little is known about the response of *Pythium* spp. causing disease on alfalfa to Apron XL. Recently, quinone outside inhibitor (QoI) fungicides such as Stamina (active ingredient pyraclostrobin; carbamic acid,2-[[[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxy]methyl]phenyl]methoxy-,methyl ester) have been labeled for managing seed and seedling diseases of alfalfa caused by *Fusarium* spp., *R. solani*, *Aphanomyces euteiches*, and *Phytophthora medicaginis* and *Pythium* diseases on other crops. Information on sensitivity to Stamina of *Pythium* spp. causing disease on alfalfa is lacking.

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The objectives of this study were to: (i) isolate and identify pathogens associated with seed rot and damping-off of alfalfa in Minnesota; (ii) evaluate the aggressiveness of these pathogens, as well as *Phytophthium* spp. and *Pythium* spp. known to be corn and soybean pathogens, for pathogenicity on alfalfa; and (iii) determine the sensitivity of *Pythium* spp. pathogenic on alfalfa to mefenoxam and pyraclostrobin.

Materials and Methods

Soil collection. Individual soil samples were collected in the fall of 2013 and the spring of 2014 from alfalfa production fields in Minnesota in the second or third production year as part of a survey for soilborne pathogens. Samples were collected using a soil probe to a depth of 15 cm in a “W” sampling pattern across each field to collect at least 16 soil cores. Each sample was sieved to remove stones and crop debris and stored at 4°C. In preliminary assays, soil samples from five fields (Becker, St. Hilaire, Fulda, Parkers Prairie, and Waseca) were found to have a high frequency of seed rot and damping-off when used for alfalfa seed germination in growth chamber assays. Soil samples from these fields were used in this study to isolate seed rot and damping-off pathogens.

Pathogen isolation. A rolled-towel method was used to bait pathogens from soil. Seeds of ‘Saranac,’ a *Pythium*-susceptible cultivar (Grau et al. 1989; Jones and Samac 1996), were surface sterilized by immersion in 70% ethanol for 1 min, immersion and agitation in 10% commercial household bleach for 5 min, followed by three rinses in sterile distilled water. A 2-mm layer of each soil was laid in a 40-mm square on germination paper and moistened with sterile distilled water, then 20 surface-sterilized alfalfa seeds were placed in a 4 × 5 grid pattern on the soil. A second germination paper was laid over the soil, moistened thoroughly, and then both papers were rolled together and placed in a plastic bag. Six rolled towels were prepared from each soil. Groups of three bagged rolls were placed in ziplock-sealed bags and incubated at either 18°C or 24°C in darkness for 3 days. Seeds and seedlings removed from the rolled-towel were rinsed in running distilled water to remove soil and surface-sterilized with the ethanol and bleach protocol described above. Seeds or seedlings recovered from each rolled-towel were placed on 1.5% water agar (WA), incubated at the baiting temperature, and observed daily for mycelial growth. Hyphal tips were removed and transferred to commercial agar plates (CMA; BD and Company, Sparks, MD) to obtain pure cultures. Each soil sample was baited at least twice at each temperature. Cultures on CMA were stored at 4°C.

A collection of 18 *Pythium* species and three *Phytophthium* species isolated from infected soybean seedlings or baited from soil removed from soybean fields in Minnesota (Radmer et al. 2017) were obtained from L. Radmer, J. Kurle, and D. Malvick (University of Minnesota, Twin Cities) for testing against alfalfa.

DNA extraction. Isolates were grown in 20 ml of V8 broth following a modified protocol from Matsumoto et al. (1999). Original V8 juice (Campbell’s Soup Co., Camden, NJ) was centrifuged to remove solids. The clarified juice (100 ml), 2.0 g CaCO₃, and 900 ml sterile distilled water were combined and then autoclaved. Plugs cut from the edge of a growing culture were placed in the center of a 100-mm-diameter Petri plate with 20 ml V8 broth, and incubated in darkness at the baiting temperature for 4 to 7 days. Mycelium was filtered from the broth using Miracloth (EMD Chemicals, San Diego, CA), rinsed with sterile distilled water, pressed between paper towels to remove excess liquid, and freeze-dried.

Approximately 1 cm² of lyophilized mycelium of each culture was processed using a FastDNA Kit (MP Biomedicals, Solon, OH) and a Precellys 24 FastPrep machine (Bertin Technologies, Washington, DC) according to the manufacturer’s instructions. DNA was stored at –20°C.

PCR amplification, DNA sequencing and analysis. A section of ribosomal DNA including a partial 18S region, ITS1, the 5.8S region, and ITS2 was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Reactions consisted of 25 µl 2X GoTaq Green (Promega Corp., Madison, WI), 0.5 µl each of 50 pmol/µl ITS1 and 50 pmol/µl ITS4, 21 µl of sterile distilled water, and 50 to 350 ng DNA. PCR parameters

were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 7 min and a 10°C hold. For strains identified as *Pythium* species by ITS sequencing, a further amplification of the COI gene was done using the primers and conditions reported by Robideau et al. (2011). A 50-µl reaction was run using the degenerate primers OomCox1-Levup (5′-TCAW CWMGATGGCTTTTTTCAAC-3′) and FM85mod (5′-RRHWAC KTGACTDATRATACCAAA-3′). Amplification of ITS and COI sequences was verified by gel electrophoresis using 10 µl of the reaction product on a 1% agarose gel, stained with ethidium bromide, and visualized with a GelDoc EZ Imager and Image Lab 5.1 software (BioRad, Hercules, CA). When COI amplification resulted in two bands on gel images, the electrophoresis was repeated with the remaining 40 µl of each reaction to separate the bands. Both excised bands were purified according to the manufacturer’s instructions with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The alternative reverse primer OomCox1-Levlo (5′-CYTCHGGRTGWCCRAAAAACCAAA-3′) (Robideau et al. 2011) was used for samples that did not amplify using FM85mod. DNA samples that did not amplify with these methods were amplified using OomCox1-Levup and OomCox1-Levlo with a gradient of annealing temperatures in a MyCycler (BioRad, Hercules, CA). Reaction parameters for this amplification were 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 50 to 60°C for 1 min, and 72°C for 1 min with a final extension of 72°C for 7 min, and a 10°C hold. For isolates identified as *Fusarium* spp. by ITS sequencing, amplification of translation elongation factor1-α (EF1-α) was done with the degenerate primer sequences EF1 (5′-ATGGGTAAGGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATG-3′) designed by O’Donnell et al. (1998). Reactions consisted of 5 µl 10X standard Taq reaction buffer, 1.0 µl each of 50 pmol/µl EF1 and 50 pmol/µl EF2, 1.0 µl of 10 mM dNTPs, 0.25 µl Hot Start Taq DNA polymerase (New England Biolabs, Ipswich, MA), 40.75 µl of sterile distilled water, and 1 µl DNA (50 to 200 ng). Reaction parameters for this amplification were 95°C for 30 s, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min with a final extension of 68°C for 7 min, and a 10°C hold. All PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. DNA concentrations were quantified with Nanodrop 2000 software and spectrometer (Thermo Fisher Scientific, Minneapolis, MN). Amplified and purified DNA samples were stored at –20°C.

Amplified DNA samples were sequenced in both directions by the University of Minnesota Biomedical Genomics Center (St. Paul, MN). Sequencher (version 4.10.1, Gene Codes Corporation, Ann Arbor, MI) base-calling software was used to align chromatograms, and generate consensus FASTA sequences for comparison with the NCBI nucleotide nonredundant (nr/nt) collection for identification of *Pythium* spp. The BLAST parameters for identification were *e*-values of 0.0, maximum identity match of 99% or greater with no gaps, and a query coverage of 98% or greater. For identification of *Fusarium* spp., the consensus FASTA sequences for both the ITS region and EF1-α were used for BLAST analysis on the *Fusarium* MLST website (<http://www.westerdijkinstitute.nl/fusarium/>) utilizing polyphasic identification as recommended by O’Donnell et al. (2015).

Pathogenicity assays. A culture plate method (Altier and Thies 1995) was used to test pathogenicity and aggressiveness of 21 *Pythium* strains baited from alfalfa field soils selected based on origin of the soil sample and species identification. In addition, 18 *Pythium* and three *Phytophthium* strains previously tested for pathogenicity on soybean and corn seedlings (Radmer et al. 2017) were tested for pathogenicity on alfalfa seeds and seedlings. Inoculum was prepared from pure cultures stored on CMA at 4°C. Fresh subcultures on CMA were incubated at 21°C until mycelia covered the surface of the plate, then 3-mm plugs from the edge of the colony were transferred to the centers of 100-mm-diameter plates of 1.5% WA. Plates were incubated at 21°C for 3 to 7 days until the agar surface was covered with mycelium. Twenty-five ‘Saranac’ alfalfa seeds were surface-sterilized with ethanol and bleach, as described above, and placed on the surface of each plate. Uninoculated plates with 25 seeds served as controls. The plates were incubated in a growth chamber for 5 days at 21°C, with a 16-h photoperiod. The experiment was arranged as a randomized complete

block with three replicates per isolate with each replicate as a block and the experiment was done twice. Seeds and seedlings were rated for disease severity on a 1 to 5 scale with 1 = the primary root free of necrosis; 2 = the primary root tip necrotic, but firm; 3 = the primary root tip soft and rotted; 4 = the seed germinated with an emerged, rotted radicle; and 5 = seeds ungerminated and rotted (Altier and Thies 1995). Control plates were used to calculate the expected seed germination percentage for each trial, and to differentiate pathogen-killed seed from seeds on the inoculated plates that failed to germinate for other reasons. Calculated killed-seed values were corrected to zero if negative. An Average Severity Index (ASI) modified from Altier and Thies (1995) was calculated using the following formula:

$$\text{ASI} = \frac{\{[(\text{number in class 5} - \text{number of ungerminated seeds in control}) \times 5] + (\text{number in class 4} \times 4) + (\text{number in class 3} \times 3) + (\text{number in class 2} \times 2) + \text{number in class 1}\}}{\text{expected number of germinated seeds}}$$

Pathogenicity was defined as an ASI ≥ 3.0 .

The culture plate method was also used for testing pathogenicity and aggressiveness of seven *Fusarium* isolates of different species baited from soil. The assay was done as described above except that the

Fusarium strains were cultured on PDA for production of culture plugs, then cultured on WA for 7 days, and seeds were incubated in a lighted growth chamber at 24°C for 7 days. Symptoms were rated on the same 1 to 5 scale as for the *Pythium* plate assay. Disease severity data were compared using the Kruskal-Wallis test and Dunn's multiple comparison test by GraphPad Prism7 analysis software (GraphPad Software, La Jolla, CA).

An infested soil assay was developed to evaluate seed root and seedling damping-off caused by nine *Pythium* strains and to compare results to the culture plate method. To prepare the soil, 250 cm³ of a peat-based potting mix (Sungro LC8, SunGrow Horticultural Distribution, Agawam, MA) was mixed with 5 g corn meal, and autoclaved for 20 min. Each strain of *Pythium* was cultured on a 15 × 100 mm CMA plate at 21°C for 7 days, homogenized in a blender in a total of 1 liter sterile water, and 125 ml of homogenate added to the soil mix. Approximately 50 cm³ of soil mix was placed in a 3 × 6 cm pot. Twenty five alfalfa seeds were placed in each pot, covered with uninfested potting mix, and placed in a lighted growth chamber at 21°C. Controls consisted of pots of 25 seeds in commercial-amended soil treated with homogenate made using an uninoculated CMA plate. The experiment was arranged as a randomized complete block with four replicates per isolate with each replicate as a block. After 5 days, percent seed germination was assessed. Percentages were arcsine square root transformed before analysis by one-way ANOVA. Mean separation was

Table 1. Frequency of *Pythium* and *Fusarium* species isolated from five Minnesota field soil samples with a high incidence of damping-off

Species name	Location					Total
	Becker	Fulda	Parkers Prairie	St. Hilaire	Waseca	
<i>Pythium acrogynum</i>					1	1
<i>Pythium attrantheridium</i>		2	1	5	1	9
<i>Pythium carolinianum</i>					1	1
<i>Pythium heterothallicum</i>	1	2		13	12	28
<i>Pythium irregulare</i>	2		1			3
<i>Pythium pleroticum</i>		1				1
<i>Pythium schmitthenneri</i>					2	2
<i>Pythium sylvaticum</i>	20	5	8	3		36
<i>Pythium ultimum</i> var. <i>ultimum</i>					2	2
<i>Fusarium incarnatum</i> <i>equiseti</i>		1				1
<i>Fusarium oxysporum</i>		2	2	6	4	14
<i>Fusarium redolens</i>				1		1
<i>Fusarium solani</i>	2	2	2	4	9	18
<i>Fusarium tricinctum</i>				1	5	6

Fig. 1. Pathogenicity of *Pythium* species baited from field soil with high levels of seed rot and damping off of alfalfa. Average disease severity of each strain was assayed in a culture plate assay with 25 seeds in each plate with three replicate plates for each strain. After 5 days incubation at 21°C, each seed was scored on a 1 to 5 scale: 1 = the primary root free of necrosis; 2 = the primary root tip necrotic, but firm; 3 = the primary root tip soft and rotted; 4 = the seed germinated with an emerged, rotted radicle; 5 = seeds ungerminated and rotted. Data are the average disease severity and standard deviation per replicate averaged over two experiments ($n = 6$). Controls lacking the pathogen had an average disease severity score of 1.

tested by Tukey's multiple comparison test using GraphPad Prism7 analysis software.

Fungicide efficacy and sensitivity. Using the culture plate method, 17 pathogenic *Pythium* strains were tested against the fungicides Apron XL (active ingredient mefenoxam) and Stamina (active ingredient pyraclostrobin) applied as commercial seed coatings. Treated 'Vernal' alfalfa seeds were obtained from Summit Seed Coatings (Caldwell, ID). The seed coatings were commercially prepared in a Rotostat seed-coating machine and consisted of calcium carbonate at 0.34 g/g of alfalfa seed with fungicides at the recommended rates: Apron XL at 0.64 fluid ounces per 100 pounds of seed (18.9 ml per 45.4 kg) or Stamina at 3.1 fluid ounces per 100 pounds of seed (92 ml per 45.4 kg). Seeds coated with calcium carbonate without fungicide were used as controls. Seeds and seedlings were rated for disease severity using the same 1 to 5 scale used in the pathogenicity assays. Three replicate plates of each strain, 25 seeds/plate, were tested in each trial, and each trial was repeated. The percentage of protected plants (% P) was calculated as: %

$P = [(number\ in\ class\ 1 + number\ in\ class\ 2) / number\ of\ expected\ germinated\ seeds] \times 100$.

Ten strains were assessed for sensitivity to pyraclostrobin (BASF Corp., Research Triangle Park, NC) in the presence of salicylhydroxamic acid (SHAM; Aldrich Chem. Co., Milwaukee, WI), which blocks the alternative oxidase pathway. CMA was amended with 1.0 µg/ml, 0.1 µg/ml, or 0.01 µg/ml pyraclostrobin with 50 µg/ml or 100 µg/ml SHAM (dissolved in methanol). Two controls were used for comparison with the fungicide: CMA amended with SHAM; or CMA with 2 ml/liter methanol. A 3.5-mm diameter plug from the edge of a 2- to 3-day-old colony of *Pythium* on CMA was transferred to the center of WA plates amended with fungicide and control plates. The experiment was arranged as a randomized complete block. There were three replications of each fungicide concentration and control and the experiment was done twice. Colony growth was determined as the average of two diameters measured at right angles to each other after 48 h incubation at 21°C in the dark. Growth reduction and fungicide concentration causing 50% relative

Fig. 2. Percent germination of alfalfa seeds in soil infested with *Pythium* species baited from soil with high levels of seed rot and damping off of alfalfa. Sterilized potting mix was infested with a mycelial homogenate and planted with 25 alfalfa seeds in each pot. Percent germination compared with uninfested soil was determined after 5 days incubation at 21°C. Data are average percent germination and standard deviation in each pot ($n = 12$). No germination was observed for soil infested with *P. irregulare* and *P. ultimum* var. *ultimum*.

Fig. 3. Pathogenicity on alfalfa of *Pythium* and *Phytophthora* species from Minnesota pathogenic on corn and soybean. Average disease severity of each strain was assayed in a culture plate assay with 25 seeds in each plate with three replicate plates for each strain. After 5 days incubation at 21°C, each seed was scored on a 1 to 5 scale as described for Fig. 1. Data are the average disease severity and standard deviation per replicate. Controls lacking the pathogen had an average disease severity score of 1.

reduction of mycelial growth (EC₅₀) was determined following the method of Taylor et al. (2002). Three strains were tested for sensitivity to pyraclostrobin in the absence of SHAM using CMA amended with 10, 20, or 40 µg/ml pyraclostrobin.

The same 10 *Pythium* strains were tested for sensitivity to Apron XL (Syngenta Agrochemical Company, Basel, Switzerland). CMA was amended with the commercial fungicide preparation so that media contained mefenoxam at 1.0 µg/ml, 0.1 µg/ml, or 0.01 µg/ml and growth was compared with CMA controls without fungicide. Plates were inoculated, incubated, and colony growth determined as above.

Results

Isolation and identification of seed rot and damping-off pathogens. A total of 149 isolates were baited from soil samples obtained from five alfalfa fields in Minnesota with high levels of alfalfa seed rot and seedling damping-off. Of these, 93 isolates (62%) were identified as *Pythium* using ITS and/or COI sequence similarity to accessions in the NCBI database and 76 isolates were identified to species. The remaining isolates could not be identified unambiguously to species. *Pythium sylvaticum* was most frequently isolated, followed in abundance by *P. heterothallicum*, *P. attrantheridium*, *P. irregulare*, *P. ultimum* var. *ultimum*, *P. schmitthenneri*, *P. acrogynum*, *P. carolinianum*, and *P. pleroticum* (Table 1). The incubation temperature at which baiting was done influenced the species recovered. *P. ultimum* var. *ultimum*, *P. schmitthenneri*, and *P. pleroticum* were recovered only from low temperature incubation. The single *P. carolinianum* strain was isolated from incubation at the higher temperature. All other species were isolated at both incubation temperatures.

Fig. 4. Pathogenicity of *Fusarium* species baited from soil with high levels of seed rot and damping off of alfalfa. Average disease severity of each strain was assayed in a culture plate assay with 25 seeds in each plate with three replicate plates for each strain. After 7 days incubation at 24°C, each seed was scored on a 1 to 5 scale as described in Fig. 1. Data are the average disease severity per replicate. Controls lacking the pathogen had an average disease severity score of 1. Bars with a different letter are significantly different at $P < 0.05$.

Non-*Pythium* isolates accounted for 38% of the organisms; 43 of the 57 isolates were identified by ITS sequence as *Fusarium*. Multi-locus sequence typing with ITS and EF1- α sequences identified five species: *F. solani* was the most frequently isolated followed by *F. oxysporum*, *F. tricinatum*, *F. redolens*, and *F. incarnatum-equiseti* (Table 1). Soil source influenced the number of total species identified. The Waseca soil sample had the highest number of species with six *Pythium* spp. and three *Fusarium* spp. while the Becker soil had the lowest number of species with three *Pythium* spp. and one *Fusarium* spp.

Pathogenicity assays. Average disease severity (ASI) ratings ≥ 3 in the culture plate assay indicated that eight strains, *P. ultimum* var. *ultimum*, *P. irregulare*, and six *P. sylvaticum* strains, were pathogenic on alfalfa (Fig. 1). The *P. ultimum* var. *ultimum* and *P. irregulare* strains were significantly more pathogenic ($P < 0.001$) than *P. sylvaticum* strains and were very aggressive seed rot pathogens while the *P. sylvaticum* caused both seed rot and root rot of seedlings. One strain of *P. attrantheridium* (WAS3) and *P. heterothallicum* (WAS131) caused moderate amounts of seed rot and root rot of seedlings (ASI > 1.5). Ten strains, four *P. heterothallicum*, two *P. attrantheridium*, and one strain each of *P. carolinianum*, *P. schmitthenneri*, *P. pleroticum*, and *P. acrogynum*, caused low amounts of seed rot (Fig. 1).

The results of the plate assays were compared with those of with nine strains in growth chamber experiments in which seedlings were grown in *Pythium*-infested potting medium. The *P. irregulare* (BEC56) and *P. ultimum* var. *ultimum* (WAS53) strains caused severe seed rot, with no germinated seeds observed (Fig. 2), which was also observed in the culture plate assay. The *P. sylvaticum* strains and *P. attrantheridium* STH80 caused a moderate amount of seed rot, reducing germination by 17 to 49% compared with the control treatment. Although the three *P. sylvaticum* strains tested had a similar disease phenotype in the plate assay, strain BEC124 caused significantly more seed rot than strain BEC58 in the soil assay ($P = 0.0332$). The *P. attrantheridium* WAS3 and *P. heterothallicum* strains were not significantly different from the control treatment. A low amount of seed rot caused by *P. attrantheridium* and *P. heterothallicum* strains was observed in the culture plate assay.

Pythium and *Phytophthora* strains from Minnesota soils isolated by baiting using soybean or isolated from infected soybean seedlings (Radmer et al. 2017) were tested using the culture plate assay to determine their ability to cause disease in alfalfa. Seven of the 21 strains assayed were found to be aggressive alfalfa pathogens with an ASI ≥ 3.0 (Fig. 3). These seven strains were also the most aggressive on both soybean and corn in seedling assays (Radmer et al. 2017). The *P. ultimum* var. *ultimum*, *P. irregulare*, and *P. ultimum* var. *sporangiferum* strains were found to be alfalfa seed rot pathogens. The *P. conidiophorum* and *P. sylvaticum* strains caused seed rot and root rot of alfalfa seedlings while the *P. intermedium*, *P. recalcitrans*, and *P. oopapillum* strains primarily caused alfalfa seedling root rot. The other species assayed caused low amounts of seed rot (Fig. 3). This is the first observation of *P. intermedium*, *P. recalcitrans*, *P. oopapillum*, *Phytophthora boreale*, and *P. megacarpum* causing disease on alfalfa.

The culture plate assay was used to evaluate pathogenicity and aggressiveness of *Fusarium* strains isolated by baiting from soil samples. Seed colonization and seed rot and root stunting and root necrosis were observed. *Fusarium solani*, *F. redolens*, *F. tricinatum*, and *F. oxysporum* 87 caused low amounts of seed rot (Fig. 4) and at 5 days after plating were sporulating on infected seeds, but did not cause root necrosis. In contrast, *F. oxysporum* 113 and 184 as well as *F. incarnatum-equiseti* 180 were significantly different ($P < 0.001$) than the control and other strains and caused seed rot, root stunting, and root necrosis.

Fungicide efficacy and sensitivity of *Pythium* strains to fungicides. Fungicide efficacy toward 17 *Pythium* strains pathogenic on alfalfa was tested using alfalfa seeds with a commercial application of Stamina (pyraclostrobin) or Apron XL (mefenoxam) at the recommended rates. Apron XL was effective against seven strains when used as a seed treatment but for 10 strains there were fewer than 75% protected seeds (Fig. 5). The two most aggressive seed rot pathogens in the culture plate assay, *P. irregulare* (BEC56) and *P. ultimum* var. *ultimum* (WAS53), had the lowest percentage of protected seeds. Efficacy of Stamina as a seed treatment in the in vitro assay varied from 0 to 79% protected seeds.

Fungicide sensitivity was tested by measuring mycelial growth inhibition on CMA amended with Apron XL. EC₅₀ values were between 0.043 and 0.132 µg/ml mefenoxam (Table 2). We observed reduced density of mycelium at higher fungicide concentrations, a reduction in growth that was not fully captured by radial growth. EC₅₀ values for the active ingredient in Stamina, pyraclostrobin, in the presence of 50 µg/ml SHAM to inhibit the alternative oxidase pathway ranged from 0.448 to 0.885 µg/ml pyraclostrobin (Table 2). Growth inhibition by 50 µg/ml SHAM ranged from 74 to 84%. Similar EC₅₀ values were obtained when 100 µg/ml SHAM was added to the medium to obtain 36 to 51% growth reduction (*data not shown*). When SHAM was omitted from the medium, the EC₅₀ values for the three strains tested—*P. ultimum* var. *ultimum* (WAS53), *P. sylvaticum* (FUL188), and *P. sylvaticum* (PAR190)—were 15.8 µg/ml, 20.9 µg/ml, and 24.1 µg/ml, respectively, indicating that these *Pythium* strains utilize the alternative oxidase pathway as a means of tolerating pyraclostrobin.

Discussion

Globally, 15 species of *Pythium* have been reported to cause seed rot and damping-off of alfalfa (Malvick and Grau 2015). However, information on *Pythium* spp. diversity on alfalfa in the midwestern United States, specifically Minnesota, is limited. This study identified highly aggressive *P. irregulare*, *P. ultimum* var. *ultimum*, and *P. sylvaticum* strains from field soils with high levels of damping-off that caused seed rot and root rot of alfalfa. Additionally, highly aggressive *F. oxysporum* and *F. incarnatum-equiseti* strains causing alfalfa seed rot and root necrosis were isolated from these field soils. Aggressive alfalfa pathogens were also identified in a set of *Pythium* spp. previously

identified as pathogens of corn and soybean. Specifically, *P. conidio-*
phorum, *P. intermedium*, and *P. recalcitrans* isolated from soybean or
baited from soil of soybean fields (Radmer et al. 2017) were demon-
strated to cause disease in alfalfa for the first time. These strains, as well
as soybean/corn strains of *P. ultimum* var. *ultimum*, *P. irregulare*, *P.*
ultimum var. *sporangiiferum*, and *P. sylvaticum* were the most aggres-
sive against alfalfa in our studies and against soybean in a previous
study (Radmer et al. 2017). Additionally, strains of *Phytophthora bor-*
eale and *P. megacarpum*, which infect corn and soybean, were found
to cause alfalfa seed rot in a culture plate assay. An extensive survey for
oomycetes associated with soybean seedlings in 11 states within the
United States and in Ontario, Canada, found *Pythium* spp. to be the
most prevalent, with *P. sylvaticum*, *P. heterothallicum*, *P. ultimum*,
P. oopapillum, *P. dissotocum*, and *P. irregulare* to be the most fre-
quently isolated across locations (Alejandro Rojas et al. 2017). These
species tended to be the most aggressive in seed rot and root rot assays.
We found that all of these species, except *P. dissotocum*, which was
not part of our study, to cause seed rot and damping-off of alfalfa.
The wide distribution of highly aggressive, broad-host-range strains
of *Pythium* indicates that crop rotation may have limited effectiveness
for reducing pathogen populations that can cause significant seed rot
and seedling root rot of the most important crop plants in the midwest-
ern United States.

Nine *Pythium* spp. were isolated from five alfalfa field soils using a
baiting technique. Three of these, *P. irregulare*, *P. ultimum* var. *ultimum*,
and *P. sylvaticum*, were highly aggressive on alfalfa in culture plate as-
says. Pathogenicity of these strains was confirmed using an artificially
infested soil assay. The *P. irregulare* and *P. ultimum* var. *ultimum* strains

Fig. 5. Percentage of plants protected from pathogenic *Pythium* species by Apron XL and Stamina seed treatments. Each strain was assayed in a culture plate assay with 25 treated seeds in each plate with three replicate plates for each strain. Control plates were without pathogen inoculation. After 5 days incubation at 21°C, each seed was scored on a 1 to 5 scale as described for Fig. 1. Protected plants had a score of 1 or 2. No protected plants were observed for seeds lacking fungicide treatments. Data are the average percentage of protected plants and standard deviation per replicate.

Table 2. Concentrations of mefenoxam and pyraclostrobin that reduced growth of pathogenic *Pythium* strains by 50% (EC₅₀)

Strain	Species	Mefenoxam EC ₅₀ (µg/ml) ^a	Pyraclostrobin EC ₅₀ (µg/ml) ^b	% Growth inhibition by SHAM ^c
BEC56	<i>Pythium irregulare</i>	0.127	0.890	75
BEC58	<i>Pythium sylvaticum</i>	0.132	0.652	79
BEC63	<i>Pythium sylvaticum</i>	0.112	0.541	84
FUL179	<i>Pythium sylvaticum</i>	0.070	0.491	78
FUL188	<i>Pythium sylvaticum</i>	0.075	0.448	78
PAR164	<i>Pythium sylvaticum</i>	0.073	0.499	78
PAR190	<i>Pythium sylvaticum</i>	0.077	0.506	82
STH33	<i>Pythium sylvaticum</i>	0.063	0.501	74
WAS126	<i>Pythium ultimum</i> var. <i>ultimum</i>	0.052	0.532	77
WAS53	<i>Pythium ultimum</i> var. <i>ultimum</i>	0.043	0.496	78

^a Apron XL was added to CMA to achieve mefenoxam concentrations of 1.0 µg/ml, 0.1 µg/ml, 0.01 µg/ml, and 0 µg/ml.

^b Pyraclostrobin was added to CMA at 1.0 µg/ml, 0.1 µg/ml, 0.01 µg/ml, and 0 µg/ml. Salicylhydroxamic acid (SHAM) was added at 50 µg/ml to inhibit the action of the alternative oxidase pathway.

^c Growth inhibition on CMA with SHAM at 50 µg/ml compared with CMA amended with 2 ml/liter methanol.

primarily caused seed rot, while the *P. sylvaticum* strains caused seed rot and seedling root rot. Stelfox and Williams (1980) noted that *P. sylvaticum* is moderately virulent to alfalfa in the cotyledon stage, and the current study demonstrated that it is a significant pathogen in vitro and when inoculated into soil. The culture plate assay has the advantage of discriminating aggressiveness of isolates and the tissue attacked. It was used previously to characterize alfalfa cultivars for resistance to *Pythium* spp. (Altier and Thies 1995). A similar assay was used to evaluate pathogenicity of *Pythium* strains on corn and soybean (Broders et al. 2007a; Zhang and Yang 2000). Not all of the *Pythium* spp. isolated by the baiting technique were aggressive pathogens on alfalfa. Interactions of *Pythium* spp. with plants range across a spectrum from beneficial to pathogenic (McLeod et al. 2009). Some *Pythium* spp. are saprophytes (Uzuhashi et al. 2010), while others act as competitors and mycoparasites, including on other *Pythium* spp.

We adapted the culture plate assay for evaluating pathogenicity of the five *Fusarium* spp. isolated by baiting from Minnesota field soils with high levels of seed rot and damping-off of alfalfa. *Fusarium* spp. were recognized early as part of the seed rot and damping-off disease complex disease complex on alfalfa (Hancock 1983; Schmitthenner 1964), but information on pathogenicity and aggressiveness of individual strains has been lacking. The most aggressive strain identified in this study was the *F. incarnatum-equiseti* strain isolated from the Fulda field soil, which caused seed rot and root necrosis of seedlings. Previously, *F. equiseti* was identified as an infrequent member of the alfalfa crown rot complex (Pegg and Parry 1983). Recently, this species was clustered with morphologically similar *F. incarnatum* to form the *F. incarnatum-equiseti* species complex (O'Donnell et al. 2012). *F. oxysporum* causes crown rot of alfalfa (Ariss et al. 2007) and vascular wilt of alfalfa (Leath et al. 1988). We believe this is the first observation of seed rot and seedling root rot of alfalfa caused by *F. incarnatum-equiseti* and *F. oxysporum*. The results of our experiments suggest that *Fusarium* spp. have been overlooked as causal agents of seed rot and seedling root rot of alfalfa.

The culture plate assay was useful for evaluating efficacy of seed treatments. A similar assay was used to evaluate fungicide seed treatments on corn seeds challenged with *Fusarium* spp. (Broders et al. 2007b; Munkvold and O'Mara 2002). Alfalfa seeds commercially treated with the recommended rates of Apron XL or Stamina were tested with each of the *Pythium* strains pathogenic on alfalfa. For six out of 10 strains tested with the Apron XL treated seeds, the percentage of protected plants was <75%, indicating that the seed treatment was not highly effective in reducing disease symptoms. The seed treatment was least effective against the more aggressive seed rot strains, *P. ultimum* var. *ultimum* (WAS53) and *P. irregulare* (BEC56), and more effective against the *P. sylvaticum* strains causing both seed rot and root rot (Fig. 5). However, we observed all strains to have low EC₅₀ values in the mefenoxam-amended medium, indicating that they are sensitive to Apron XL. The results of the plate assay suggest that highly aggressive seed rot strains escape control by the seed treatment, perhaps by rapid colonization of the seed interior, which would impact seed germination and seedling establishment. Fibrous feeder roots of mature alfalfa plants are vulnerable to attack by *Pythium* during periods of wet cool weather. Breeding for resistance to *Pythium* diseases could enhance initial stand establishment and mature plant productivity.

The phenylamide fungicides, including metalaxyl and mefenoxam, were introduced in the 1970s and were used widely for management of diseases caused by oomycetes. These fungicides have a very specific mode of action, inhibiting pathogen ribosomal RNA polymerases, making them at high risk for development of resistance in pathogen populations. Early studies on the effect of metalaxyl on *Pythium* pathogens of alfalfa found strains that varied in sensitivity to the fungicide (Hwang 1988; Larkin et al. 1995). Seed treatments with metalaxyl were found to provide incomplete protection against infection by *Pythium* spp. (Edmisten et al. 1988), while drenches significantly increased protection (Larkin et al. 1995; 1996). It is not clear if repeated use of phenylamide fungicide seed treatments have increased the populations of *Pythium* spp. with tolerance to the fungicides. The EC₅₀ values for mefenoxam vary widely for *Pythium* strains isolated from soybean (Radmer et al. 2017) and potato (Taylor et al. 2002), although most isolates are considered sensitive.

The QoI fungicide Stamina has recently been labeled for use on alfalfa seed. These fungicides act by blocking electron transport at the mitochondrial cytochrome oxidase bc₁ complex thereby inhibiting respiration (Venancio et al. 2003). The Stamina seed coating was ineffective against most of the strains in the plate assay. However, the in vitro growth assays of *Pythium* strains in pyraclostrobin-amended media found that alfalfa strains are sensitive if cultured in the presence of SHAM, which inhibits the alternative oxidase pathway (Table 2). In the absence of SHAM, we found strains to have high EC₅₀ values approximately 40 fold higher than in the presence of SHAM. This indicates that *Pythium* strains can tolerate QoI fungicides by use of the alternative oxidase pathway. We assume the seed treatment was ineffective due to the activity of the alternative oxidase pathway. High EC₅₀ values for *Pythium* spp. have been reported in the presence of SHAM (Matthiesen et al. 2016), suggesting that insensitivity to QoI fungicides occurs in nature. Insensitivity to the QoI fungicides is seen in some pathogens as a result of mutations at the target-binding site (Bartlett et al. 2002). The mechanism of insensitivity in *Pythium* spp. to QoI fungicides has not been investigated.

Stamina is labeled for use on alfalfa seed for managing Aphanomyces root rot and Phytophthora root rot as well as *Fusarium* spp. and *Rhizoctonia solani*. From the results of this study, Stamina would likely provide little control of seed rot and root rot caused by *Pythium* spp. when used on alfalfa seeds. Our results also suggest that Apron XL seed treatments may not be effective in controlling all strains of *Pythium* attacking alfalfa and that *Fusarium* spp. may be contributing to seed rot and damping-off. The effect of other fungicides on the *Pythium* and *Fusarium* pathogens causing seed rot and damping-off of alfalfa warrants further investigation. The culture plate assay used and pathogens isolated in this study provide resources to screen candidate germplasm for resistance to seed rot and damping-off of alfalfa and for developing resistant cultivars.

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Literature Cited

- Alejandro Rojas, J. A., Jacobs, J. L., Napieralski, S., Karaj, B., Bradley, C. A., Chase, T., Esker, P. D., Giesler, L. J., Jardine, D. J., Malvick, D. K., Markell, S. G., Nelson, B. D., Robertson, A. E., Rupe, J. C., Smith, D. L., Sweets, L. E., Tenuta, A. U., Wise, K. A., and Chilvers, M. I. 2017. Oomycete species associated with soybean seedlings in North America-Part I: Identification and pathogenicity characterization. *Phytopathology* 107:280-292.
- Altier, N. A., and Thies, J. A. 1995. Identification of resistance to *Pythium* seedling diseases in alfalfa using a culture plate method. *Plant Dis.* 79:341-346.
- Ariss, J. J., Rhodes, L. H., Sulc, R. M., and Hammond, R. B. 2007. Potato leafhopper injury and *Fusarium* crown rot effects on three alfalfa populations. *Crop Sci.* 47:1661-1671.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. Review: The strobilurin fungicides. *Pest Manag. Sci.* 58:649-662.
- Brantner, J. R., and Windels, C. E. 1998. Variability in sensitivity to metalaxyl in vitro, pathogenicity, and control of *Pythium* spp. on sugar beet. *Plant Dis.* 82: 896-899.
- Broders, K. D., Lipps, P. E., Paul, P. A., and Dorrance, A. E. 2007a. Characterization of *Pythium* spp. associated with corn and soybean seed and seedling disease in Ohio. *Plant Dis.* 91:727-735.
- Broders, K. D., Lipps, P. E., Paul, P. A., and Dorrance, A. E. 2007b. Evaluation of *Fusarium graminearum* associated with corn and soybean seed and seedling disease in Ohio. *Plant Dis.* 91:1155-1160.
- Cook, R. H., and Zhang, B. X. 1985. Degrees of sensitivity to metalaxyl within the *Pythium* spp. pathogenic to wheat in the Pacific Northwest. *Plant Dis.* 69: 686-688.
- Edmisten, K. L., Wolf, D. D., and Stromberg, E. L. 1988. Compatibility of metalaxyl with *Rhizobium meliloti* on alfalfa seed to control *Pythium* damping off. *Crop Sci.* 28:568-570.
- Grau, C. R., Arny, D. C., and Nygaard, S. L. 1989. Assessment of alfalfa reaction to Anthracnose in controlled and field environments. *Plant Dis.* 73:167-170.
- Hancock, J. G. 1983. Seedling diseases of alfalfa in California. *Plant Dis.* 67: 1203-1208.
- Hancock, J. G. 1985. Fungal infection of feeder rootlets of alfalfa. *Phytopathology* 75:1112-1120.

- Hwang, S. F. 1988. Effects of VA mycorrhizae and metalaxyl on growth of alfalfa seedlings in soils from fields with "alfalfa sickness" in Alberta. *Plant Dis.* 72: 448-452.
- Jones, C. R., and Samac, D. A. 1996. Biological control of fungi causing alfalfa seedling damping-off with a disease-suppressive strain of *Streptomyces*. *Biol. Control* 7:196-204.
- Larkin, R. P., English, J. T., and Mihail, J. D. 1995. Effects of infection by *Pythium* spp. on root system morphology of alfalfa seedlings. *Phytopathology* 85: 430-435.
- Larkin, R. P., English, J. T., and Mihail, J. D. 1996. The relationship of infection by *Pythium* spp. to root system morphology of alfalfa seedlings in the field. *Plant Dis.* 80:281-285.
- Leath, K. T., Erwin, D. C., and Griffin, G. D. 1988. Diseases and nematodes. Pages 621-670 in: *Alfalfa and Alfalfa Improvement*. A. A. Hanson, D. K. Barnes, and R. R. Hill, eds. ASA-CSSA-SSSA, Madison, WI.
- Malvick, D. K., and Grau, C. R. 2015. *Pythium* seed and root rot. Pages 8-9 in: *Compendium of Alfalfa Diseases and Pests*, 3rd Ed. D. A. Samac, L. H. Rhodes, and W. O. Lamp, eds. American Phytopathological Society, St. Paul, MN.
- Matsumoto, C., Kageyama, K., Suga, H., and Hyakumachi, M. 1999. Phylogenetic relationships of *Pythium* species based on ITS and 5.8S sequences of ribosomal DNA. *Mycoscience* 40:321-331.
- Matthiesen, R. L., Ahmad, A. A., and Robertson, A. E. 2016. Temperature affects aggressiveness and fungicide sensitivity of four *Pythium* spp. that cause soybean and corn damping off in Iowa. *Plant Dis.* 100:583-591.
- McLeod, A., Botha, W. J., Meitz, J. C., Spies, C. F. J., Tewoldemedhin, Y. T., and Mostert, L. 2009. Morphological and phylogenetic analyses of *Pythium* species in South Africa. *Mycol. Res.* 113:933-951.
- Munkvold, G. P., and O'Mara, J. K. 2002. Laboratory and growth chamber evaluation of fungicidal seed treatments for maize seedling blight caused by *Fusarium* species. *Plant Dis.* 86:143-150.
- O'Donnell, K., Humber, R. A., Geiser, D. M., Kang, S., Park, B., Robert, V. A. R. G., Crous, P. W., Johnson, P. R., Aoki, T., Rooney, A. P., and Rehner, S. A. 2012. Phylogenetic diversity of insecticulous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and *Fusarium* MLST. *Mycologia* 104:427-445.
- O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 95:2044-2049.
- O'Donnell, K., Ward, T. J., Robert, V. A. R. G., Crous, P. W., Geiser, D. M., and Kang, S. 2015. DNA sequence-based identification of *Fusarium*: Current status and future directions. *Phytoparasitica* 43:583-595.
- Parke, J. L., Rand, R. E., Joy, A. E., and King, E. B. 1991. Biological control of *Pythium* damping-off and *Aphanomyces* root rot of peas by application of *Pseudomonas cepacia* or *P. fluorescens* to seed. *Plant Dis.* 75:987-992.
- Pegg, G. F., and Parry, D. W. 1983. Infection of lucerne (*Medicago sativa*) by *Fusarium* species. *Ann. Appl. Bot.* 103:45-55.
- Radmer, L., Anderson, G., Malvick, D. M., Kurle, J. E., Rendahl, A., and Mallik, A. 2017. *Pythium*, *Phytophthora*, and *Phytophthora* spp. associated with soybean in Minnesota, their relative aggressiveness on soybean and corn, and their sensitivity to seed treatment fungicides. *Plant Dis.* 101:62-72.
- Robideau, G. P., de Cock, A. W. A. M., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D. W., Désaulniers, N., Eggertson, Q. A., Gachon, C. M. M., Hu, C. H., Küpper, F. C., Rintoul, T. L., Sarhan, E., Verstappen, E. C. P., Zhang, Y., Bonants, P. J. M., Ristaino, J. B., and Lévesque, C. A. 2011. DNA barcoding of oomycetes with cytochrome *c* oxidase subunit 1 and internal transcribed spacer. *Mol. Ecol. Resour.* 11:1002-1011.
- Schmitthenner, A. F. 1964. Prevalence and virulence of *Phytophthora*, *Aphanomyces*, *Pythium*, *Rhizoctonia*, and *Fusarium* isolated from diseased alfalfa seedlings. *Phytopathology* 54:1012-1018.
- Schroeder, K. L., Martin, F. N., de Cock, A. W. A. M., Lévesque, C. A., Spies, C. F. J., Okubara, P. A., and Paulitz, T. C. 2013. Molecular detection and quantification of *Pythium* species: evolving taxonomy, new tools, and challenges. *Plant Dis.* 97:4-20.
- Stelfox, D., and Williams, J. R. 1980. *Pythium* species in alfalfa fields in central Alberta. *Can. Plant Dis. Surv.* 60:35-36.
- Taylor, R. J., Salas, B., Secor, G. A., Rivera, V., and Gudmestad, N. C. 2002. Sensitivity of North American isolates of *Phytophthora erythroseptica* and *Pythium ultimum* to mefenoxam (metalaxyl). *Plant Dis.* 86:797-802.
- Uzuhashi, S., Tojo, M., and Kakishima, M. 2010. Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience* 51:337-365.
- Venancio, W. S., Rodrigues, M. A. T., Begliomini, E., and Souza, N. L. 2003. Physiological effects of strobilurin fungicides on plants. *Agr. Sci. Eng.* 9:59-68.
- Zhang, B. Q., and Yang, X. B. 2000. Pathogenicity of *Pythium* populations from corn-soybean rotation fields. *Plant Dis.* 84:94-99.