***Pythium*, *Phytophthora*, and *Phytopythium* spp. Associated with Soybean in Minnesota, Their Relative Aggressiveness on Soybean and Corn, and Their Sensitivity to Seed Treatment Fungicides**

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Abstract

Pythium spp. cause seed decay, damping-off, and root rot in soybean and corn; however, their diversity and importance as pathogens in Minnesota are unknown. Our objectives were to identify the *Pythium* spp. present in Minnesota soybean fields, determine their aggressiveness on corn and soybean, and investigate their sensitivity to seed treatment fungicides. For identification, sequences obtained using internal transcribed space ITS4 and ITS1 primers were compared with reference sequences in the National Center for Biotechnology Information database. Seedling and soil samples yielded over 30 oomycete species. Aggressiveness was determined using two methods; a seed assay, which also examined temperature effects on aggressiveness, and a seedling assay. Of 21 species

evaluated, seven *Pythium* spp. were pathogenic on both soybean and corn, reducing root growth by 20% or more while two *Pythium* and one *Phytopythium* spp. were pathogenic only on soybean. Aggressiveness of many isolates increased as temperature increased from 15°C to 25°C. The sensitivity of 10 pathogenic species to azoxystrobin, ethaboxam, mefenoxam, pyraclostrobin, or trifloxystrobin was tested. EC₅₀ values for mefenoxam and ethaboxam were 10⁻² of those to strobilurin fungicides. *Pythium* spp. in Minnesota are diverse and a significant cause of seedling disease on soybean and corn. Most *Pythium* spp. isolated in this study were more sensitive to mefenoxam and ethaboxam than to strobilurin fungicides.

The oomycete *Pythium* is a major cause of seed and seedling rots in soybean (*Glycine max* (L.) Merr) and corn (*Zea mays* L.), two crops often grown in rotation in the Midwestern United States. In soybean, *Pythium* seed, seedling, and root rots are considered to be among the most important sources of yield loss (Wrather and Koenning 2009; Wrather et al. 2001, 2003). *Pythium* causes pre- and postemergence damping-off in both soybean and corn and is considered to be more damaging in cool, damp conditions (Griffin 1990; Rizvi and Yang 1996). In both soybean and corn, infection by *Pythium* is characterized by lesions on roots and pre and postemergence damping-off of seedlings (Broders et al. 2007, 2009). Plants with nonlethal infections but damaged root systems have decreased vigor and, as a result, reduced yield (Holbert et al. 1923; Rothrock et al. 2015; Sprague 1943).

In Minnesota, edaphic factors and climatic conditions combine with planting practices to favor infection by *Pythium* spp. In soybean growing areas, poorly drained clay soils are common. These soils dry slowly and the extra moisture associated with saturated conditions increases their heat capacity, slowing warmup in the spring. In flooded conditions, *Pythium* can be recovered more frequently than other soil fungi (Kirkpatrick et al. 2006). In addition, crop management practices emphasizing reduced tillage and maintenance of crop residue cover exacerbate the effect of edaphic and environmental factors and further increase the likelihood of seed and seedling infection by *Pythium* spp. This places the seed in the ground under the most “at risk” conditions. More than 20 *Pythium* spp. have been confirmed as pathogens in Ohio and Iowa (Broders et al. 2007, 2009; Dorrance et al. 2004; Ellis et al. 2012; Murillo-Williams and Pedersen 2008; Yang and Feng 2001). Although the number of *Pythium* spp. isolated and identified in the North Central states has increased dramatically, *P. irregulare*, *P. ultimum* var. *sporangiiferum*, *P. ultimum* var.

ultimum, and *P. sylvaticum* remain the most damaging as pathogens of seeds and seedlings in both corn and soybean (Broders et al. 2007, 2009; Dorrance et al. 2004; Jiang et al. 2012; Wei et al. 2011).

Research on *Pythium* spp. present in Minnesota that are pathogenic to soybean is limited and outdated. The last published survey, which was conducted in 1965 (Brown and Kennedy 1965), reported three *Pythium* spp.; *P. ultimum*, a *Pythium* sp. similar to *P. ultimum* but without sexual structures, and an unidentified *Pythium* isolate, as pathogens on soybean. Recent research conducted in neighboring North Central states has documented a much larger, more diverse community of *Pythium* spp. infecting soybean (Broders et al. 2007; Jiang et al. 2012; Marchand et al. 2014; Zitnick-Anderson and Nelson 2014). In Iowa, Zhang and Yang (2000) tested the aggressiveness of isolates collected in corn and soybean fields and found that many of the isolates were pathogenic on both corn and soybean; however, they did not identify the individual *Pythium* spp. responsible. Broders et al. (2007) identified six *Pythium* spp. pathogenic to corn and nine to soybean in Ohio. Zitnick-Anderson and Nelson (2014) reported 26 described *Pythium* spp. and three unidentified *Pythium* spp. in results from a statewide survey conducted over 2 years in North Dakota. In that survey *P. ultimum*, *P. heterothallicum*, and an unknown *Pythium* sp. made up more than 49% of the isolates. Matthiesen et al. (2015) investigated the effect of temperature on species prevalence and aggressiveness in Iowa. They found *P. torulosum* to be the predominant *Pythium* sp. present in corn at lower temperatures (13°C) but that various other *Pythium* spp. were favored by higher temperatures (18 and 20°C) indicating that the aggressiveness of *Pythium* is an interaction of species with temperature. These studies also confirmed that many of the *Pythium* spp. isolated can be pathogens of both corn and soybean (Broders et al. 2007; Hooker 1956; Johann et al. 1928; Rao et al. 1978; Robertson and Ahmad 2013; Rosso et al. 2008; Yanar et al. 1997; Zitnick-Anderson and Nelson 2014).

Management of seedling diseases caused by *Pythium* spp. has relied on the use of seed treatment fungicides metalaxyl and mefenoxam (Syngenta, Basel, Switzerland). Formerly limited to use with seed corn, the practice of treating seeds with fungicides has recently become commonplace for control of seedling diseases of soybean. For instance, in Minnesota, metalaxyl has been a common seed coating on corn for

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Accepted for publication 27 July 2016.

the last 15 to 20 years but it is only in the last decade that seed treatments on soybean have become the norm rather than the exception (D. M. Malvick, *personal communication*). The strobilurins, azoxystrobin, trifloxystrobin, and pyraclostrobin, are broad-spectrum fungicides frequently applied as seed treatments to control seed and seedling diseases and root rots caused by true fungi. Mefenoxam, the active isomer of metalaxyl, and the recently introduced fungicide, ethaboxam, are effective seed treatment compounds for controlling oomycete pathogens of both corn and soybean. Continued use of metalaxyl and mefenoxam for control of *Pythium* has resulted in insensitivity of some *Pythium* spp. to these fungicides in some cropping systems (Brantner and Windels 1998; Cohen and Coffey 1986; Cook and Zhang 1985; Weiland et al. 2014; White et al. 1988). In Minnesota considerable variability in sensitivity has been reported among isolates of *P. ultimum* var. *sporangiferum* and *P. aphanidermatum* infecting sugar beet (Brantner and Windels 1998); however, there is little information available on the sensitivity to seed treatment fungicides of *Pythium* spp. pathogenic on soybean. In Ohio, Broders et al. (2007) determined that *Pythium* spp. with globose sporangia were more sensitive than those with filamentous sporangia to strobilurins, however, the reverse was true with mefenoxam. Moorman et al. (2002) examined the mefenoxam sensitivity of several *Pythium* spp. isolated from floriculture greenhouses in Pennsylvania and found 37% of *P. irregulare* isolates were resistant to metalaxyl or mefenoxam ($EC_{50} > 100 \mu\text{g a.i./ml}$). The sensitivity to seed treatment fungicides of *Pythium* populations found in Minnesota soybean fields is unknown.

The objectives of this research were to (i) isolate and identify *Pythium* spp. associated with soybean and corn rotations in Minnesota, (ii) evaluate the pathogenicity of the *Pythium* isolates collected as the cause of seed rots, seedling rots, and damping-off of soybean and corn under a range of temperature conditions, and (iii) evaluate the isolates' sensitivity to seed treatment fungicides in an in vitro assay.

Materials and Methods

Pythium isolates were recovered from either soil or seedling samples collected from 46 soybean fields throughout Minnesota (Fig. 1). Isolates of *Pythium* spp. from seedlings in Minnesota were selected from a large collection in part based on preliminary identification done at Michigan State University (Rojas et al. 2013). Identities of all isolates from seedling and soil samples used in this study were determined at the University of Minnesota based on amplification and sequencing of COX1 and internal transcribed space ITS regions.

Collection of seedling and soil samples. During June 2011 and 2012, 25 soybean seedlings were collected from 12 production fields (six each year) located in eight counties in Minnesota (Fig. 1). Fields were chosen based on geographic distribution and disease symptoms or reduced plant stands present in those years or in previous years. Seedlings were sampled from fields at approximately 5 m intervals along a 250 m transect laid out in a W or Z pattern. Most of the seedlings had necrotic lesions on the roots, although some roots that yielded *Pythium* isolates had no symptoms. Samples were refrigerated and processed within 72 h of collection.

Soil samples were collected in eight counties from 37 different soybean production fields with a history of seedling, root and stem rots, or with a history of stand establishment problems. In the fall of 2012 and 2013 (Fig. 1), prior to harvest, 10 separate soil samples approximately 1 liter in total volume were collected within the planted row from every other row on an X transect centered on an area 7.62 by 53.4 m² in each field. The soil samples were dried in a forced air drier at 38°C, for 4 days, ground to pass through 2 mm openings in a Humboldt Soil Grinder (Humboldt Scientific, Elgin, IL), and stored at room temperature until use.

Isolation from seedlings. Seedlings were washed under running tap water for 30 min and three 0.5 to 1 cm sections were cut from each root with a sterile scalpel: one at the root tip, one in either the middle of the root or at a symptomatic area along the length of the root, and one at the hypocotyl/soil interface. Sections were blotted dry and embedded in corn meal agar (CMA) PARPB media (Jeffers and Martin 1986) and then incubated in darkness at 20°C for 1 to 7 days until hyphae emerged. Hyphae were examined microscopically and those with characteristics of *Pythium* were transferred to CMA and stored at 4°C.

Isolation from soil. *Pythium* spp. were isolated from soil samples according to the method of Dorrance et al. (2004). Three 10.2 cm pots were filled with a ground soil sample, saturated with water, and placed in a plastic tray containing water for 24 h, and allowed to drain for 24 h. Pots were then placed in a sealed plastic bag and incubated at room temperature for 2 weeks. Five seeds of soybean cultivar McCall were planted per pot and the pots were placed in a dark 13°C growth chamber where the soil was saturated with water every 24 h over a 5-day period. The seeds were then retrieved from the soil, washed, cut in half, and placed on PIBNC media selective for oomycetes (Broders et al. 2007; Schmitthenner and Bhat 1994). Colonies suspected to be *Pythium* spp. were transferred to CMA, examined under a microscope to ascertain their genus, and maintained on CMA at 4°C.

Identification of species. All isolates were identified to species using DNA sequencing techniques. Isolates of *Pythium* spp. from seedlings in Minnesota were selected from a large collection in part based on preliminary identification done at Michigan State University (Rojas et al. 2013). Identities of all isolates from seedling and soil samples used in this study were determined at the University of Minnesota based on amplification and sequencing of COX1 and ITS regions. Isolates from storage were grown on 10% clarified V8 liquid media at 20°C for up to 1 week. Hyphal mats were collected on a fine sieve, washed with deionized water, blotted dry, and frozen with liquid nitrogen. DNA was extracted with a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) after grinding with a Precellys 24 bead mill homogenizer (MoBio, Carlsbad, CA) and 50 μl of buffer was used to elute the sample. Amplification of the ITS1, ITS2, and part of the 28S region was accomplished in a 25 μl reaction with GoTaq Green Master Mix (Promega, Madison, WI) with primers ITS1 and ITS4 (White et al. 1990). Amplification of the *Cox1* gene was done with the primers OomCox1Levup and Fm85mod although OomCox1Levlo was used as an alternative reverse primer when needed (Robideau et al. 2011). Amplifications were conducted with an Eppendorf Mastercycler Pro (Eppendorf, Hauppauge, NY) using an initial denaturation step at 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 54°C

Fig. 1. Counties in Minnesota where isolates used in this study originate. The counties in gray were the sources of isolates retrieved by baiting soil collected in soybean fields. Counties in black were the sources of isolates retrieved from seedling tissue. The counties in a striped pattern were the source of multiple isolates retrieved by either of those techniques.

(ITS) or 51°C (*Cox1*) for 30 s, and 72°C for 1 min and followed by a final extension step at 72°C for 5 min and a final hold at 10°C. The product underwent electrophoresis on a gel containing 1% GenePure LE Agarose (ISC BioExpress, Kaysville, UT) which was then stained with SYBR Green I (Molecular Prober, Eugene, OR) and photographed on a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO). PCR product was purified using an UltraClean PCR Clean-Up Kit (Mo Bio Laboratories, Carlsbad, CA). Sequencing was performed bidirectionally with the amplification primers at the University of Minnesota Genomics Center. Sequences were assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI) and compared with known sequences in the National Center for Biotechnology Information (NCBI) for identification. Species identification was accomplished by comparing the ITS and *Cox1* sequence data of the unknown isolates against data in the NCBI (www.ncbi.nlm.nih.gov) database using BLAST to compare sequences to published *Pythium* and *Phytophthora* spp. data. Most isolates were very closely related (matching or within 1 to 2 bp difference) to isolates submitted and published by Robideau et al. (2011). Queries with 95% or better coverage and the fewest base pairs difference (<6 bp) were considered a species match.

Seed assay. Forty-three isolates representing the 21 most common *Pythium* and *Phytophthora* spp. isolated from these studies were tested for pathogenicity (Table 1). These isolates were chosen if they survived storage at 4°C for 1 to 2 years from the date they were isolated to the date they were revitalized for DNA extraction and if their identity was verifiable based on DNA sequences of two gene targets. Up to three isolates of each species were included in the pathogenicity assay. Isolates were grown on CMA for 1 week and then ca. 4 mm² plugs were transferred to eight petri plates containing 1% water agar plates. Five surface-sterilized seeds of the soybean cultivar McCall were placed around the perimeter of the plate equidistant from each other within 1 cm of the plate's edge prior to placement in a dark incubator either at 15, 20, or 25°C. Nine-day-old roots were scanned on a Epson V600 flatbed scanner (Epson, Long Beach, CA). After removing background debris and optimizing the contrast in Photoshop Elements 9.0 (Adobe, San Jose, CA), the images were analyzed in WinRhizo Basic 2013 (Regent Instruments Inc., Quebec City, Quebec) to determine the average total root length of the seedlings on each plate. Root lengths on inoculated plates were divided by those on uninoculated plates to determine the average root length ratio. The experiment was repeated once at each temperature. Pathogenicity

Table 1. *Pythium* spp. and isolates from this study evaluated for aggressiveness as seed and seedling pathogens and for sensitivity to fungicides^a

Species name	Isolate name	County	Isolation year	Source	Clade	Seed	Seedling	Fungicide
<i>Phytophthora boreale</i>	LSJ 1-4	Jackson	2013	Soil	Phy	x	x	
	LSJ 3-1	Jackson	2013	Soil	Phy	x		
<i>Phytophthora litorale</i>	MN7KK	Polk	2012	Seedling	Phy	x	x	x
<i>Phytophthora megacarpum</i>	LSM3 1-4	Martin	2013	Soil	Phy	x		
	LSM1 3-3 1	Martin	2013	Soil	Phy	x	x	
<i>Pythium acrogynum</i>	MNSO_1-50.1h	Waseca	2011	Seedling	E	x	x	x
<i>Pythium attrantheridium</i>	C-MNSO2_4-5	McLeod	2012	Seedling	F	x		
	MNSO_2-7.1r	Martin	2011	Seedling	F	x		
	MNSO_4-2.1r	Wilkin	2011	Seedling	F	x	x	
<i>Pythium conidiophorum</i>	MNSO_1-28.1r	Waseca	2011	Seedling	B	x	x	x
<i>Pythium heterothallicum</i>	C-MNSO2_6-22	Clay	2012	Seedling	I	x		
	PAKZ 1-4	McLeod	2013	Soil	I	x		
	MNSO_2-41.1h	Martin	2011	Seedling	I	x	x	
	Kerber 1-3	Carver	2013	Soil	B	x	x	
<i>Pythium inflatum</i>	PAKZ 1-3 1	McLeod	2013	Soil	B	x		
<i>Pythium intermedium</i>	C-MNSO2_6-1	Clay	2012	Seedling	F	x	x	x
	C-MNSO2_6-13	Clay	2012	Seedling	F	x		x
	V-MNSO2_6-44	Clay	2012	Seedling	F	x		x
<i>Pythium irregulare</i>	Kloss 8	Benton	2013	Soil	F	x	x	x
	CCNC 2-2	Isanti	2013	Soil	F	x		x
	RIL173	Ramsey	NA	Seedling	F	x		x
<i>Pythium minus</i>	MNSO_1-38.2h	Waseca	2011	Seedling	E	x	x	
<i>Pythium nagaii</i>	C-MNSO2_6-21	Clay	2012	Seedling	G	x	x	
<i>Pythium oopapillum</i>	V-MNSO2_1-63	Waseca	2012	Seedling	B	x		x
	MNSO_2-20.1h	Martin	2011	Seedling	B	x	x	x
	MNSO_5-20.1h	Redwood	2011	Seedling	B	x		x
	C-MNSO2_3-15	Clay	2012	Seedling	J	x	x	
<i>Pythium perplexum</i>	Kramer 1-3	Renville	2013	Soil	E	x	x	
<i>Pythium pleroticum</i>	Kloss 10	Benton	2013	Soil	F	x		x
<i>Pythium recalcitrans</i>	C-MNSO2_4-25	McLeod	2012	Seedling	F	x	x	x
	Kerber 8	Carver	2013	Soil	F	x		x
<i>Pythium schmittii</i>	PAKZ 2-3	McLeod	2013	Soil	E	x		
	PAKZ 2-4	McLeod	2013	Soil	E	x	x	
<i>Pythium sylvaticum</i>	MNSO_1-13.1h	Waseca	2011	Seedling	F	x		x
	MNSO_3-47.2r	Clay	2011	Seedling	F	x	x	x
<i>Pythium torulosum</i>	Zimmerman 2-3	McLeod	2013	Soil	B	x		
	C-MNSO2_2-21	Renville	2012	Seedling	B	x	x	
	MNSO_2-25.1h	Martin	2011	Seedling	B	x		
<i>Pythium ultimum</i> var. <i>sporangiferum</i>	V-MNSO2_1-59	Waseca	2012	Seedling	I	x	x	x
	V-MNSO2_3-58	Clay	2012	Seedling	I	x		x
<i>Pythium ultimum</i> var. <i>ultimum</i>	MNSO_1-20.1h	Waseca	2011	Seedling	I	x	x	x
	C-MNSO2_5-9	Redwood	2012	Seedling	I	x		x
	Clay 1-1	Clay	2013	Soil	I	x		x

^a Columns indicate isolate designation, originating county, year collected, source from soil or seedlings, clade, and use in this study. NA indicates collection date not recorded.

Seedling assay. Pathogenicity on soybean and corn seedlings was determined using an assay similar to that described by Zhang and Yang (2000). A single isolate of each of the 21 *Pythium* spp. (Table 1) that was most aggressive, i.e., resulted in the smallest root length ratios in the in vitro assay was used. Inoculum was made with subcultures grown for 6 days at 20°C on five 100 mm petri dishes containing CMA. The media from the six plates was forced through a 0.64 cm wire mesh and thoroughly mixed with 2.5 liters of vermiculite before being evenly distributed among five 10.2 cm plastic pots. Five seeds of soybean cultivar McCall were planted in each pot. Pots were then placed in flooded plastic trays without drainage holes and placed in the greenhouse with sunlight supplemented by artificial light for a 16 h photoperiod with temperatures ranging from 28°C during the day to 15°C at night. Each group of seedlings inoculated with the 21 *Pythium* spp. was paired with an uninoculated (CMA-only) set of control seedlings in a separate tray which were randomized within each tray. Trays were arranged in a randomized complete block design (RCBD) replicated five times. After 14 days, emerged seedlings were removed from pots, washed, and dried. Root dry weights were measured. Corn seedlings were inoculated in the same manner and maintained in a greenhouse where temperatures ranged from 17 to 33°C.

Sensitivity of isolates to fungicides. Twenty-two isolates representing 10 pathogenic *Pythium* spp. (Table 1) were assayed for sensitivity to five commercial fungicides commonly applied as seed treatments using the procedure of Broders et al. (2007). The fungicides were azoxystrobin (Dynasty 100FS; Syngenta Crop Protection, Greensboro, NC), ethaboxam (Intego Solo; Valent, Walnut Creek, CA), mefenoxam (Apron XL; Syngenta Crop Protection, Greensboro, NC), pyraclostrobin (Stamina; BASF, Research Triangle, NC), and trifloxystrobin (Trilex FS; Research Triangle, NC). A 4 mm × 4 mm block from a 1-week-old culture of each *Pythium* isolate grown on CMA was placed at the center of a plate containing either CMA amended with a fungicide as formulated product or unamended CMA. The fungicide was first dissolved in approximately 1 to 2 ml of water to better facilitate its mixing with the media. The sensitivity of the 22 isolates was tested at three concentrations for each fungicide including a concentration determined in preliminary experiments to be the concentration at which *Pythium* growth was inhibited approximately 50% by the respective fungicide. Sensitivity of each isolate was also assayed to all fungicides at the same two concentrations: 100 and 5 µg a.i./ml. Fungicide concentrations at which *Pythium* growth was inhibited approximately 50% by the

respective fungicide, azoxystrobin, ethaboxam, mefenoxam, pyraclostrobin, or trifloxystrobin, were, 0.5, 0.1, 1, or 250 $\mu\text{g a.i./ml}$, respectively. Plates were placed in an 18°C incubator for 48 h and then the average colony diameter was determined by measuring each colony twice at right angles centered on the midpoint of the inoculating block of CMA.

The effect of the fungicide was calculated as the ratio of the treated versus untreated colony diameter and the relative growth reduction was calculated as $1 - (\text{ratio of the treated versus untreated colony diameters})$. Three plates of each fungicide were used for each experiment and the experiment was conducted three times. Plates were considered technical replicates within days and averaged to get a single estimate for each isolate/treatment (amended with fungicide or not amended) combination for each day. Then, as the estimate of interest was the percentage comparing the colony diameter resulting from the fungicide amendment to the colony diameter of the control (unamended) value, these percentages were calculated for each isolate/fungicide treatment combination for each day by dividing the fungicide amended value by unamended value. For each isolate/treatment combination there is then one data point for each experimental unit (day), allowing the use of nonlinear least squares, which assumes independence of the data points. To find the EC_{50} value for each isolate/treatment combination, a piecewise exponential curve was then fitted with knots at the amended concentration values. For each piece, this curve was written as $y = \exp(-k \times x + b)$, where y is the percent and x is the concentration. For the first piece, b was set to zero, as the percent at concentration zero (the control) is 100%. Each piece was then parameterized instead as $y = \exp(-(\log(2) + b) \times x / \exp(\log EC_{50}) + b)$ to directly estimate the log of the EC_{50} value. The log scale was chosen because the sampling distribution of the parameter is then more normally distributed, allowing for confidence intervals to be computed directly from estimates of the standard error. The fitted values for each piece of the curve can be found analytically, but to find the confidence intervals, the nonlinear least squares function in R version 3.2.0 (R Core Team 2016) was used to obtain the standard error. Confidence intervals were calculated on the log concentration scale and then back-transformed to the original scale. Only the piece of the line that crossed the 50% line was used to find the standard errors; this did occasionally result in large standard errors as the data at the concentrations above and below 50% were both near 50%.

Statistical analysis. Data from replicated experiments conducted to determine pathogenicity on both seed and seedling were tested for normality and homogeneity of variance. The data required transformation for stabilization of variance and to normalize the distribution.

Fig. 2. Identities and frequencies of *Phytophthora*, *Phytopythium*, *Pythiogeton*, and *Pythium* spp. isolated from seedling and soil samples collected in soybean fields.

Box-Cox analysis indicated that a log transformation was adequate for transformation. The transformed data were analyzed with PROC GLM (SAS Institute, Cary, NC), and mean separation conducted comparing all means in the experiment using Fisher's least significant difference test, $P \leq 0.05$ or, if the results of inoculation were compared with an untreated control, mean separation was performed using Dunnett's test of significance at $P \leq 0.05$. The treatment values were then back-transformed for graphical and tabular presentation.

Results

Isolation and identification of *Pythium* isolates. *Pythium* isolates were recovered from either soil or soybean seedling samples collected from 49 soybean fields in Minnesota (Fig. 1). Baiting of soil samples collected from 37 fields in 15 counties in 2011 to 2013 yielded 31 *Pythium* and eight *Phytophthora* isolates. Isolation from seedling samples collected in 12 fields in eight counties in 2011 and 2012 yielded 305 *Pythium* and one *Phytophthora* isolate. Among the isolates obtained from both plant and soil samples were 31 known and affinis *Pythium* spp., and three *Phytophthora* spp. Also recovered

were 21 unknown *Pythium* isolates, one unknown *Phytophthora*, and one unknown *Pythiogeton* isolate (Fig. 2). *P. ultimum* var. *ultimum* was the species found most frequently with 67 isolates recovered (Fig. 2). *P. heterothallicum* and *P. sylvaticum* were the second and third most frequently occurring species with 61 and 33 isolates, respectively. Five isolates of the newly described species, *P. recalitrans*, were found. Forty-four isolates, from 21 different *Pythium* and *Phytophthora* spp. were chosen for further analysis of pathogenicity and fungicide sensitivity (Table 1).

Seed assay. The emerging radical from inoculated seeds exhibited visible symptoms including stunting and water soaked lesions. Species were declared pathogenic when the ratio of root length of inoculated seedlings was decreased by 20% or more compared with the root length of uninoculated seedlings. Aggressiveness of isolates differed significantly among species and was influenced by temperature. Of the 21 species tested (Table 1), seven *Pythium* spp. were pathogenic on both soybean and corn: *P. conidiophorum*, *P. intermedium*, *P. irregulare*, *P. recalitrans*, *P. sylvaticum*, *P. ultimum* var. *sporangiferum*, and *P. ultimum* var. *ultimum* (Fig. 3). Two additional

Fig. 3. Aggressiveness of *Pythium* or *Phytophthora* spp. to **A**, soybean or **B**, corn seedlings indicated by root length ratios of inoculated compared with uninoculated control seedlings at three different temperatures: 15, 20, or 25°C. Numbers in parenthesis represent the number of isolates that were tested. Columns noted with *** heading indicate comparisons significant at the 0.05 level between treated and untreated roots according to Dunnett's *t* tests. Error bars indicate standard error among three temperatures for the individual isolate.

Pythium species, *P. acrogynum*, *P. oopapillum*, and one *Phytophythium* species, *Phytophythium litorale*, were pathogenic on soybean but not on corn (Fig. 3A). *Pythium ultimum* var. *ultimum* caused the most severe root length reduction, up to 80% in soybean and 65% in corn (Fig. 3). *Pythium sylvaticum* and *P. irregulare* were the second most aggressive *Pythium* spp. infecting soybean while *P. irregulare* was the second most aggressive *Pythium* spp. infecting corn causing average reductions in root length greater than 50% and 55%, respectively (Fig. 3). In soybean, in all cases but *P. acrogynum*, which reduced root length ratio below 80% only at 25°C, this difference of 20% or greater was significantly different from the controls averaged across all temperatures tested. Averaged over all *Pythium* isolates and species the effect of infection of corn appeared to be less severe than infection of soybean.

The response to temperature of the *Pythium* isolates recovered in our survey differed among species. An increase in temperature was associated with an increase in aggressiveness for 16 of the 21 *Pythium* and *Phytophythium* spp. evaluated on soybean and for 19 of 21 species evaluated on corn (Fig. 3). In soybean inoculated with *Phytophythium litorale* a temperature increase from 15 to 25°C resulted in a 35% reduction in the ratio of inoculated to uninoculated root lengths (Fig. 3A). In corn, the most extreme responses to changes in temperature occurred with infection by *P. irregulare*, which reduced root growth by 30% when temperature was increased from 15 to 20°C (Fig. 3B).

Infection by *Pythium* spp. did not always result in reduced root growth of corn or soybean. In some cases, an increase in total root length was observed in the inoculated seedlings when compared with the uninoculated control, resulting in an inoculated to uninoculated ratio greater than one. The most dramatic example, 1.54, occurred when corn was inoculated with *P. pleroticum* and maintained at 15°C (Fig. 3B). A 28% increase in root mass was also observed when soybean was inoculated with *P. pleroticum* and maintained at 25°C (Fig. 3A). A similar but less pronounced increase in root mass occurred in corn inoculated with *P. acrogynum* and *P. inflatum*.

Seedling assay. Soybean seedlings grown for two weeks in soil infested with *Pythium* spp. in the greenhouse displayed a range of responses to inoculation with *Pythium* spp. (Fig. 4). The severity of visible symptoms varied considerably among inoculated plants and did

not appear to be correlated with the effect of inoculation on seedling growth. Inoculated seedlings had reduced root development accompanied by stunting and sometimes wilting and damping-off. Lesions and sometimes water soaking were present on the hypocotyls and cotyledons of the most severely affected seedlings. Two *Pythium* spp., *P. irregulare* and *P. ultimum* var. *sporangiferum*, decreased root mass by more than 10% when compared with the noninfested controls (Fig. 4A). In contrast, *P. minus*, *P. pleroticum*, and *P. ultimum* var. *ultimum* increased root length more than 10%. In corn, inoculation with *P. oopapillum*, *P. irregulare*, and *P. ultimum* var. *sporangiferum* caused the greatest decrease in root mass (Fig. 4B). Inoculation with *P. attrantheridium*, *P. recalcitrans*, and *P. schmittneri* resulted in the greatest increase.

Sensitivity of isolates to fungicides. Sensitivity to the fungicides azoxystrobin, ethaboxam, mefenoxam, pyraclostrobin, or trifloxystrobin, was tested on 22 isolates from 10 pathogenic species with fungicide amended media (Table 1). All isolates were sensitive to the five fungicides tested and exhibited some reduction in growth when exposed to the lowest concentration of the fungicide (data not shown); however, much greater concentrations of the strobilurin fungicides azoxystrobin and trifloxystrobin were necessary to reduce growth of any isolate than the concentration of either ethaboxam or mefenoxam. In some instances, there was a greater than 80% difference in fungicide sensitivity between species within the same fungicide treatment (Fig. 5).

The strobilurins were less effective as fungicides against the *Pythium* spp. isolated in this survey. Of the strobilurins, azoxystrobin (Fig. 5A; Table 2) was effective against more of the *Pythium* spp. tested than trifloxystrobin (Table 2). It was most effective against *P. oopapillum*, with EC₅₀ values near 4 µg a.i. ml⁻¹ (Table 2) but was less effective than pyraclostrobin against *P. irregulare* (Table 2). Pyraclostrobin had the greatest effect against *P. recalcitrans*, *P. oopapillum*, and *P. intermedium*. *P. conidiophorum* and *P. irregulare* were much more tolerant of this fungicide at concentrations lower than 100 µg a.i. ml⁻¹ (Fig. 5D). Trifloxystrobin was not an effective fungicide against the *Pythium* species tested with the exception of *P. oopapillum* which displayed some sensitivity to it (Fig. 5E, Table 2).

Fig. 4. Aggressiveness of 21 *Pythium* or *Phytophythium* spp. on **A**, soybean or **B**, corn seedlings. The data show the ratio of the root length of the inoculated seedling divided by the root length of the uninoculated control. Error bars indicate standard error.

The most effective fungicides tested were the thiazole carboxamide, ethaboxam, and the phenylamide, mefenoxam. Mefenoxam was most effective at reducing or inhibiting growth of all *Pythium* isolates except for *P. conidiophorum* which exhibited significant growth at 0.1 and 5.0 $\mu\text{g a.i. ml}^{-1}$ (Table 2; Fig. 5C). Ethaboxam was very effective against *P. intermedium*, *P. oopapillum*, and *P. sylvaticum*, with EC_{50} values below 0.3 $\mu\text{g a.i. ml}^{-1}$ (Table 2). However, *P. acrogynum* and *P. recalcitrans* were insensitive to it at concentrations of 0.5 and 5.0 $\mu\text{g a.i. ml}^{-1}$ (Fig. 5B; Table 2). One isolate of *P. recalcitrans* showed almost no sensitivity to ethaboxam (Table 2). Mefenoxam was the most effective fungicide tested as it had an EC_{50} below 0.15 $\mu\text{g a.i. ml}^{-1}$ for all but five oomycete isolates tested (Fig. 5B; Table 2). One notable exception was *P. conidiophorum* which displayed very little sensitivity at low concentrations (Fig. 5C).

While there was some variation in sensitivity to particular fungicides among individual isolates within a species, in most cases there was no significant difference in reaction among the multiple isolates within a species (data not shown). However, this was not true of all *Pythium* spp. tested; *P. recalcitrans* shows considerable variation between isolates when exposed to azoxystrobin, ethaboxam, and

pyraclostrobin (Fig. 6A). There were significant differences in sensitivity between the two isolates of *P. ultimum* var. *sporangiferum* when tested with ethaboxam, mefenoxam, and trifloxystrobin (Fig. 6B).

Discussion

This study highlights the diversity of *Pythium* spp. associated with soybean in Minnesota and their varied pathogenicity traits, environmental responses, and sensitivities to fungicides. Overall, 30 *Pythium* spp. and four *Phytopyrium* spp., a new genus combining features of *Pythium* and *Phytophthora* spp. (de Cock et al. 2015) were found in Minnesota soybean fields. This is a much greater number of *Pythium* spp. than was reported in the last study conducted in Minnesota (Brown and Kennedy 1965) when only three pathogenic species were found. This study identified nine pathogenic *Pythium* and one pathogenic *Phytopyrium* spp. *Phytopyrium* is a new genus described by Bala et al. (2010) with *Phytopyrium sindhum* as the type species. The genus is made up of species formerly assigned to *Pythium* species clade K but reassigned to *Phytopyrium* on the basis of phylogenetic analyses performed by de Cock et al. (2015). Neither the presence of *Phytopyrium* spp. nor the pathogenicity of

Fig. 5. Growth of *Pythium* and *Phytopyrium* spp. on potato dextrose agar amended at three concentrations with either **A**, azoxystrobin, **B**, ethaboxam, **C**, mefenoxam, **D**, pyraclostrobin, or **E**, trifloxystrobin. Mycelial growth on fungicide treated plates at each concentration is divided by the mycelial growth on the unamended control media to determine effect of fungicide addition on aggressiveness of the fungus. Numbers in parentheses represent multiple isolates tested. Error bars indicate standard error among three concentrations for the individual isolate and treatment.

Phytophthora litorale on soybean had been reported previously in Minnesota. Similar recent studies conducted in other states where soybean was grown in rotation with corn have also yielded high levels of diversity but the *Pythium* spp. found in those studies differed greatly among the states (Broders et al. 2007; Jiang et al. 2012; Marchand et al. 2014; Zitnick-Anderson and Nelson 2014). For example, we and Zitnick-Anderson and Nelson (2014) isolated *P. aristosporum*, *P. attrantheridium*, *P. coloratum*, *P. dissotocum*, *P. heterothallicum*, *P. intermedium*, *P. irregulare*, *P. lularium*, *P. minus*, *P. oopapillum*, *P. perplexum*, *P. sylvaticum*, and *P. ultimum* from soybean seedlings. Zitnick-Anderson and Nelson (2014) isolated 10 additional *Pythium* spp. not found in our study including *P. arrhenomanes*, *P. debaryanum*, *P. diclinum*, *P. hypogynum*, *P. inflatum*, *P. kashmirensis*, *P. nunn*, *P. orthogonum*, *P. peritum*, *P.*

rostratifingens, *P. terrestris*, *P. viniferum*, and *P. viola*. In contrast, in Minnesota we isolated an additional sixteen *Pythium* spp. and four *Phytophthora* spp. not isolated in the North Dakota study.

Of the 21 *Pythium* spp. included in pathogenicity studies seven were pathogenic on both soybean and corn, while two *Pythium* and one *Phytophthora* spp. were mildly virulent on soybean at high temperatures but not pathogenic on corn at any temperature. The *Pythium* isolates were generally also more aggressive on soybean than on corn, which is possibly the result of sampling and isolation methods. Because these two crops are often planted in rotation in Minnesota the ability to infect both crops is a concern because the soilborne inoculum population of *Pythium* spp. will likely increase when either crop is grown (Martin and Loper 1999). Zhang et al. (1998) studied the diversity of *Pythium* spp. present under three

Table 2. EC₅₀ values of *Phytophthora* and *Pythium* spp. in response to seed treatment fungicides ethaboxam, mefenoxam, azoystrobin, pyraclostrobin, and trifloxystrobin measured in µg ml^{-1a}

Species	Isolate	Ethaboxam			Mefenoxam					
		EC ₅₀	CI lower	CI upper	EC ₅₀	CI lower	CI upper			
<i>Phytophthora litorale</i>	MN7KK	0.266	0.165	0.429	0.104	0.003	3.791			
<i>Pythium acrogynum</i>	1-50.1h	>100	NA	NA	0.043	0.030	0.060			
<i>Pythium conidiophorum</i>	1-28.1r	0.402	0.274	0.589	4.002	2.619	6.115			
<i>Pythium intermedium</i>	C6-1	0.182	0.142	0.233	0.084	0.064	0.109			
<i>Pythium intermedium</i>	C6-13	0.208	0.124	0.350	0.086	0.080	0.094			
<i>Pythium intermedium</i>	V6-44	0.136	0.119	0.155	0.133	0.017	1.018			
<i>Pythium irregulare</i>	Kloss 8	0.197	0.147	0.264	0.073	0.051	0.106			
<i>Pythium irregulare</i>	CCNC 2-2	0.344	0.322	0.368	0.280	0.133	0.592			
<i>Pythium irregulare</i>	RIL173	0.438	0.206	0.933	0.076	0.040	0.143			
<i>Pythium oopapillum</i>	V1-63	0.142	0.089	0.227	0.084	0.066	0.107			
<i>Pythium oopapillum</i>	2-20.1h	0.274	0.242	0.310	1.611	1.359	1.911			
<i>Pythium oopapillum</i>	5-20.1h	0.175	0.141	0.218	0.091	0.071	0.116			
<i>Pythium recalcitrans</i>	Kloss 10	>100	NA	NA	0.492	0.128	1.889			
<i>Pythium recalcitrans</i>	C4-25	2.006	1.842	2.186	0.072	0.060	0.087			
<i>Pythium recalcitrans</i>	Kerber 8	2.016	1.833	2.216	0.086	0.061	0.120			
<i>Pythium sylvaticum</i>	1-13.1h	0.080	0.064	0.100	0.057	0.026	0.126			
<i>Pythium sylvaticum</i>	3-47.2r	0.108	0.051	0.231	0.044	0.024	0.081			
<i>Pythium ultimum</i> var. <i>sporangiferum</i>	V1-59	0.622	0.423	0.916	0.036	0.033	0.039			
<i>Pythium ultimum</i> var. <i>sporangiferum</i>	V3-58	0.198	0.174	0.226	1.132	0.682	1.880			
<i>Pythium ultimum</i> var. <i>ultimum</i>	1-20.1h	0.775	0.615	0.978	0.030	0.020	0.044			
<i>Pythium ultimum</i> var. <i>ultimum</i>	C5-9	0.662	0.367	1.195	0.026	0.014	0.051			
<i>Pythium ultimum</i> var. <i>ultimum</i>	Clay 1-1	0.655	0.523	0.820	0.020	0.011	0.038			

Species	Isolate	Azoystrobin			Pyraclostrobin			Trifloxystrobin		
		EC ₅₀	CI lower	CI upper	EC ₅₀	CI lower	CI upper	EC ₅₀	CI lower	CI upper
<i>Phytophthora litorale</i>	MN7KK	25.305	5.525	115.897	3.284	1.266	8.518	>250	NA	NA
<i>Pythium acrogynum</i>	1-50.1h	43.050	22.871	81.035	23.667	17.447	32.106	>250	NA	NA
<i>Pythium conidiophorum</i>	1-28.1r	43.786	38.712	49.524	14.624	8.514	25.119	>250	NA	NA
<i>Pythium intermedium</i>	C6-1	29.373	14.249	60.549	0.884	0.522	1.496	>250	NA	NA
<i>Pythium intermedium</i>	C6-13	31.515	22.240	44.658	0.737	0.142	3.832	>250	NA	NA
<i>Pythium intermedium</i>	V6-44	32.949	15.256	71.161	1.189	0.620	2.280	>250	NA	NA
<i>Pythium irregulare</i>	Kloss 8	204.411	162.035	257.870	24.231	16.319	35.979	>250	NA	NA
<i>Pythium irregulare</i>	CCNC 2-2	171.433	135.391	217.068	29.149	21.231	40.021	>250	NA	NA
<i>Pythium irregulare</i>	RIL173	132.848	57.875	304.943	19.397	10.998	34.208	>250	NA	NA
<i>Pythium oopapillum</i>	V1-63	4.166	3.512	4.942	0.697	0.523	0.928	3.899	3.340	4.551
<i>Pythium oopapillum</i>	2-20.1h	3.699	3.284	4.168	0.560	0.346	0.909	3.119	2.764	3.520
<i>Pythium oopapillum</i>	5-20.1h	4.005	3.328	4.820	0.540	0.492	0.592	2.715	1.083	6.808
<i>Pythium recalcitrans</i>	Kloss 10	2.380	1.821	3.113	0.208	0.163	0.267	>250	NA	NA
<i>Pythium recalcitrans</i>	C4-25	16.382	4.648	57.741	0.528	0.499	0.559	>250	NA	NA
<i>Pythium recalcitrans</i>	Kerber 8	75.950	55.785	103.404	0.759	0.481	1.197	>250	NA	NA
<i>Pythium sylvaticum</i>	1-13.1h	59.529	41.903	84.569	3.080	1.246	7.614	>250	NA	NA
<i>Pythium sylvaticum</i>	3-47.2r	6.452	0.001		0.820	0.661	1.017	>250	NA	NA
<i>Pythium ultimum</i> var. <i>sporangiferum</i>	V1-59	44.940	30.375	66.487	14.532	8.406	25.122	>250	NA	NA
<i>Pythium ultimum</i> var. <i>sporangiferum</i>	V3-58	188.827	140.987	252.899	0.993	0.909	1.084	>250	NA	NA
<i>Pythium ultimum</i> var. <i>ultimum</i>	1-20.1h	4.890	2.986	8.007	2.440	1.622	3.668	>250	NA	NA
<i>Pythium ultimum</i> var. <i>ultimum</i>	C5-9	4.014	2.478	6.504	1.180	0.232	5.992	>250	NA	NA
<i>Pythium ultimum</i> var. <i>ultimum</i>	Clay 1-1	5.000	4.557	5.486	2.068	0.845	5.065	>250	NA	NA

^a Upper and lower limits of the confidence intervals (CI) were computed from estimates of the standard error.

different crop sequences: continuous corn, a corn–soybean rotation, and continuous soybean. The authors found a greater proliferation of *Pythium* spp. in the corn–soybean rotation and continuous soybean fields than in the continuous corn fields. They suggested that the use of soybean in a rotation may increase virulent *Pythium* isolates.

Pythium spp. have different optimal temperatures for infecting soybean (Thomson et al. 1971). In this study the range of temperatures chosen for the seed assay was meant to simulate soil temperatures in Minnesota in spring. Of *Pythium* spp. tested in our seed pathogenicity assay, most were more aggressive at higher than lower temperatures on both soybean and corn. A possible explanation is that there was no competition from other organisms in the seed assay. *Pythium* spp. may be tolerant of conditions other organisms find unfavorable, favoring their growth in cool soils with a high moisture content and poor gas exchange (Hendrix and Campbell 1973). Without competition from other microorganisms, *Pythium* spp. could also proliferate freely in warmer temperatures. Hardman and Dick (1987) isolated *Pythium* during two consecutive springs to study population densities. They reported that 50% of the variation in the number of *Pythium* isolates collected was attributable to temperature such that higher temperatures resulted in higher recoveries of *Pythium*.

Not all *Pythium* spp. isolated were pathogenic on soybean or corn. For instance, *P. heterothallicum*, the second most commonly isolated species, had a limited effect on both soybean and corn roots under the experimental conditions applied in this study while *P. acrogynum* was pathogenic on soybean only at 25°C and inoculation of corn in the seed assay resulted in an increase in root length when compared with the uninoculated controls.

Several of the *Pythium* spp. isolated appeared to have a beneficial effect on growth of inoculated soybean and corn, increasing, rather than decreasing, root growth and above-ground biomass. This positive growth response was observed in soybean inoculated with *P. pleroticum* and corn inoculated with *P. acrogynum* and

P. inflatum. The isolates may stimulate plant growth and root proliferation by production of growth stimulating compounds, auxin, cytokinins or gibberellic acid, or by contributing to nutrient mobilization (Blok 1973; Pegg and Selman 1959).

Changes in comparative root mass in the seedling assay did not always correspond with comparative changes in root length and aggressiveness observed in the seed assay. Inoculation with *P. ultimum* var. *sporangiferum* and *P. irregulare* resulted in decreased root growth in the seed assay and in decreased root weights in soybean when compared with uninoculated controls but other species pathogenic in the seed assay did not decrease root mass in the seedling assay. In corn, *P. oopapillum*, and to a lesser extent, *P. ultimum* var. *sporangiferum* were pathogenic in both assays but other pathogenic species did not exhibit the same effect. This may be due, in part, to the inoculum densities present in both assays. It is probable that there are fewer propagative units present per unit volume in the seedling assay than the seed assay, leading to fewer signs of pathogenicity and possibly hormetic effects at low inoculum concentrations (Mattson 2008). For example, inoculation of corn with *P. recalcitrans* resulted in significantly decreased root length in the seed assay but increased root mass in the seedling assay.

This is a first report of *P. recalcitrans* on soybean in Minnesota and a first report of *P. recalcitrans* as a pathogen of soybean and corn. *P. recalcitrans* is a newly described species that was first collected from grape vine roots (*Vitis vinifera*) in South Africa and common beet (*Beta vulgaris*) in Spain (Moralejo et al. 2008). It has since been reported to be pathogenic on several other crop plants including carrot (*Daucus carota*) and sugar beet (*Beta vulgaris*) (Lu et al. 2013; Nechwatal et al. 2012). In 2012 and 2013 it was found on soybean in Wisconsin, but its pathogenicity on soybean was not determined (Wisconsin Department of Agriculture 2013). The seed assay conducted with the *P. recalcitrans* isolates from Minnesota determined that it is pathogenic to both soybean and corn.

The five fungicides tested varied widely in their efficacy against *Pythium* and *Phytophthora* spp. Two of the strobilurin fungicides, azoxystrobin and trifloxystrobin, were not effective against either *Pythium* or *Phytophthora* spp. at the rates tested in this study. Increasing trifloxystrobin to 250 $\mu\text{g ml}^{-1}$ did not result in inhibition greater than 50% for the species tested with the exception of *P. oopapillum*. This may be due, in part, to alternative oxidase respiratory pathways that allow oomycetes to continue to produce ATP in the presence of the fungicides (Broders et al. 2007; Wood and Holloman 2003). Some studies have attempted to ameliorate this by introducing salicylhydroxamic acid (SHAM) in their media. It was decided, in this study, to forgo the SHAM amendment in order to evaluate the relative effectiveness of the compounds as they would be utilized in seed treatment. Initial fungicide concentrations were chosen to maximize the range of interspecific variation.

Of the fungicides tested, mefenoxam was the most effective against the *Pythium* isolates followed by ethaboxam. Metalaxyl, whose active isomer is mefenoxam, has been used in the United States and in Minnesota as a seed treatment fungicide on corn for decades; however, its use as a seed treatment for soybean is relatively recent. Mefenoxam at 100 $\mu\text{g/ml}$ completely inhibited the growth of most pathogenic *Pythium* and *Phytophthora* spp. including *P. ultimum*, one of the most aggressive species on both corn and soybean.

Ethaboxam is a newly released fungicide that is marketed specifically for its effect on oomycetes (Kim et al. 2004). In this study, it was effective against the most aggressive *Pythium* spp. including *P. ultimum*, *P. irregulare*, and *P. sylvaticum*, inhibiting growth at high and medium concentration levels (100 and 5 $\mu\text{g/ml}$) by at least 97%. It may be particularly effective due to its multiple modes of action (Uchida et al. 2005). Only one case of total insensitivity to the fungicide was seen in this study. *Pythium acrogynum* did not display any sensitivity at rates of 0.0, 0.5, or 5 $\mu\text{g/ml}$. Treatment with 100 $\mu\text{g/ml}$ decreased growth by only 24%. However, only one isolate of *P. acrogynum* was available for testing and it is unknown if these results are representative of the species or are unique to this isolate.

Some fungicides were more effective against a single *Pythium* spp. than others. *P. recalcitrans*, which appeared to be relatively

Fig. 6. Intraspecific variation in **A**, three isolates of *Pythium recalcitrans* and **B**, two isolates of *P. ultimum* var. *sporangiferum* on media amended with fungicides at a concentration of 5 $\mu\text{g ml}^{-1}$.

insensitive to ethaboxam, was the most sensitive of the pathogenic species to pyraclostrobin. *P. oopapillum* was least sensitive to mefenoxam but the most sensitive to trifloxystrobin. The results suggest that it is important to recognize the differences in sensitivity among *Pythium* spp. when considering preventative treatment options. These represent possible genetic sources of fungicide resistance within their populations. Fungicide resistance is a significant concern for growers and fungicide manufacturers; however, fungicides used as seed treatments are thought to be at less risk for the development of resistance because of the low quantities used, the limited amount of time that they are active in the soil, their localized area of effect, and large background inoculum populations present in the soil that are sensitive to fungicides and would swamp resistant isolates (Brantner and Windels 1998).

Understanding the diversity, pathogenicity, and fungicide sensitivity of *Pythium* and *Phytophthora* spp. present in Minnesota soybean fields will enable producers and consultants to make more informed decisions for controlling seedling diseases caused by these organisms. Mefenoxam and ethaboxam appear to remain effective management tools in Minnesota, although decreased sensitivity or resistance has been found in other states in the North Central soybean growing region and in other cropping situations (Broders et al. 2007; Moorman and Kim 2004; Moorman et al. 2002). Another important implication of these results is the diversity of *Pythium* spp. and their importance as causes of soybean disease. Accurate identification of *Pythium* spp. prevalent in Minnesota is essential for soybean breeding programs. In general, there is little information available about sources of resistance to *Pythium* spp. Resistance to *Pythium* spp. has been identified in later maturity group (MG) soybean lines, Archer and PI 424354 (Bates et al. 2008; Rosso et al. 2008; Rupe et al. 2011). However, these lines are from later MGs, making introgression of resistance into the early MG soybean lines difficult. In addition, the resistance identified is thought to be limited to *P. irregulare* and possibly a small number of other *Pythium* spp. It is most likely not effective against all pathogenic *Pythium* spp. With the availability of molecular tools such as association mapping and the species composition information provided by this survey, breeding efforts can focus on breeding for the most commonly occurring, damaging species prevalent in Minnesota soils.

Acknowledgments

We thank the research group of M. Chilvers at Michigan State University for help with selection of *Pythium* isolates from seedlings that were used in this study. This was part of a large collaborative effort that will be published separately. Funding for this research was provided by the Minnesota Soybean Research and Promotion Council, The North Central Soybean Research Program, the USDA National Institute of Food and Agriculture, and The Minnesota Agricultural Experiment Station.

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