Characterization of *Pythium* Species Associated With Greenhouse Floriculture Crops in Michigan

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Abstract

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Michigan ranks third in the United States for the wholesale value of floriculture products, with an estimated value of \$375.7 million. Seedling damping-off and root and crown rot are commonly caused by *Pythium* spp. and are important problems for greenhouse growers. *Pythium* spp. associated with Michigan's floriculture crops were characterized as a means to improve current management strategies. During 2011 and 2012, potted poinsettias with root rot symptoms were sampled from nine greenhouses located in Kent, Kalamazoo, and Wayne counties. In 2013, from the same three counties, symptomatic geranium and snapdragon bedding plants were sampled from 12 greenhouses. Additionally, symptomatic hibiscus and lantana plants were sampled at one greenhouse facility. Isolates were confirmed to be *Pythium* spp. via morphology and sequencing of the

ITS region. A total of 287 *Pythium* spp. isolates were obtained from poinsettias and 726 isolates from geranium, snapdragon, hibiscus, and lantana. Seven *Pythium* spp., and a group of isolates determined as *Pythium* sp. 1 were identified. The most prevalent species were *P. irregulare*, *P. ultimum*, and *P. aphanidermatum*. A subset of isolates was chosen for pathogenicity and mefenoxam sensitivity testing. Six of the species were virulent to germinating geranium seeds. Most *P. ultimum* and *P. cylindrosporum* isolates tested were intermediate to highly resistant to mefenoxam, whereas most *P. aphanidermatum* isolates were sensitive. This study suggests that *Pythium* spp. recovered from Michigan greenhouses may vary depending on the host, and that mefenoxam may not be effective to control *P. ultimum* or *P. cylindrosporum*.

Michigan ranks third in the United States for the wholesale value of floriculture products, with an estimated wholesale value of \$375.7 million, and leads the nation in value of sales for nine floriculture crops (USDA 2013a). In Michigan, floriculture ranks fourth in cash receipts (USDA 2013b). Growers of floriculture crops must meet market demands for quality, and limiting disease is critical to optimize plant growth and flowering. Root rot caused by *Pythium* spp. is considered to be an important problem for greenhouse-grown crops (Daughtrey and Benson 2005). *Pythium* spp. associated with ornamentals may cause seed rot, root rot, seedling damping-off, black leg, and rot of lower stems. Seeds and seedlings tend to be more affected than mature plants (Martin and Loper 1999; Moorman et al. 2002) and exhibit reduced plant vigor that subsequently affects plant growth, marketability, and overall production (Garzón et al. 2011). *Pythium* spp. infect a wide variety of ornamental plants (Kucharek and Mitchell 2000; Stephens and Powell 1982). Moorman et al. (2002) surveyed ornamental crops for *Pythium* spp. in Pennsylvania greenhouses and detected *P. irregulare*, *P. aphanidermatum*, and *P. ultimum* most frequently (45, 29, and 10%, respectively). Geranium and poinsettia were common hosts for all three species; snapdragons, begonias, and chrysanthemum were often infected by *P. irregulare* and *P. ultimum* (Moorman et al. 2002). Earlier studies of *Pythium* spp. associated with bedding plants in Ohio (Stephens and Powell 1982) found that *P. aphanidermatum*, *P. ultimum*, *P. spinosum*, and *P. debaryanum* caused damping-off of impatiens, celosia, and vinca; *P. ultimum* was the most prevalent species.

Traditionally, classification and identification of *Pythium* spp. have been based on morphological features of the reproductive structures (Uzuhashi et al. 2010; Van der Plaats-Niterink 1981). However, microscopic characteristics can appear similar among species, and reproductive structures may not form in culture making

identification laborious and prone to mistakes (Lee and Moorman 2008; Lévesque and De Cock 2004; Martin 2000; Schroeder et al. 2013; Uzuhashi et al. 2010). Molecular methods such as sequencing the ribosomal DNA internal transcribed spacer (ITS) region, the large nuclear ribosomal subunit (LSU), and the cytochrome oxidase (*cox I* and *II*) region can now be used to accurately identify *Pythium* spp. (Lévesque and De Cock 2004; Martin 2000; Uzuhashi et al. 2010).

The ITS region is the most commonly used marker for identifying oomycetes at the species level (Robideau et al. 2011; Weiland 2011). The ITS region, as an identification marker, has been used to elucidate *Pythium* spp. from forest nursery soils in Oregon and Washington (Weiland 2011), corn and soybean plants in Ohio (Broders et al. 2007), wheat soils in Washington (Paulitz and Adams 2003), and ornamental crops in Pennsylvania (Moorman et al. 2002). Phenotypic characterization of *Pythium* spp. populations has been based on pathogenicity, virulence, and sensitivity to the commonly used fungicide such as mefenoxam (Al-Sa'di et al. 2007; Broders et al. 2007; Moorman et al. 2002; Weiland et al. 2013).

The objectives of this study were to identify the *Pythium* spp. associated with greenhouse floral crops in Michigan and characterize the population for pathogenicity and fungicide sensitivity.

Materials and Methods

Isolate collection. Potted poinsettias (*Euphorbia pulcherrima*) exhibiting root rot symptoms such as stunting and wilting were randomly sampled from nine greenhouses located in Kent (3), Kalamazoo (3), and Wayne (3) counties during the months of October and November of 2011 and 2012. Root rot symptomatic geranium (*Pelargonium × hortorum*) and snapdragon (*Antirrhinum majus*) bedding plants were randomly sampled from 12 greenhouses located in Kent (4), Kalamazoo (5), and Wayne (3) counties during the months of March and April in 2012. In 2013, hibiscus (*Hibiscus* sp.) and lantana (*Lantana camara*) plants with root rot symptoms were also sampled from one location in Kent County. Roots of symptomatic plants were washed under running tap water, cut (0.8 cm length), air-dried, and four root pieces were placed on three plates per sample of corn meal agar (CMA) amended with ampicillin (0.25 mg/liter), rifampicin (0.01 g/liter), pentachloronitrobenzene (0.1 g/liter), and benomyl (0.05 g/liter) (Jeffers

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and Martin 1986). Suspected isolates of *Pythium* spp. were initially confirmed via microscopic observation of oogonia and sporangia following the Van der Plaats-Niterink identification key (1981), and confirmed *Pythium* isolates were transferred individually and purified using amended CMA. After 48 h, an actively growing hyphal tip of each isolate was transferred to CMA to establish a single culture. For long-term storage, three small agar blocks (0.49 cm²) from a 1-week-old culture were placed in 1.5-ml microtubes containing sterile distilled water and hemp seeds, and stored at 20°C (Quesada-Ocampo et al. 2009).

***Pythium* spp. identification.** Plugs (0.49 cm²) of actively growing mycelia from 700 pure cultures were placed into petri dishes containing 20 ml of V8 broth (163 ml of filtered V8 juice, 3 g of CaCO₃, 1 liter of distilled water, 0.25 ml/liter of ampicillin, and 0.01 g/liter of rifampicin) (Quesada-Ocampo et al. 2011). Plates were incubated for 1 week at 20°C in the dark. Mycelia were harvested with sterile tongue depressors, placed in 1.5 ml microtubes, stored at -20°C, and lyophilized. DNA extractions were performed in the Research Technology Support Facility (RTSF) at Michigan State University, East Lansing, MI using the Autogen 850 robot. DNA was quantified with a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE).

Amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA was performed with the ITS4 and ITS5 (White et al. 1990) primers, and additional amplification of the mitochondrially-encoded cytochrome oxidase II (*cox II*) gene was performed with the FM58 and FM66 primers (Martin 2000) for a group of isolates identified as *Pythium* sp. 1 with the ITS sequence. Reactions consisted of 2 mM MgCl₂, 1× Buffer, 0.2 μM dNTPs, 0.2 μM of each set of primers, 1 U of Taq polymerase, and 1 μl of DNA (50 ng) in a 25 μl reaction volume. Amplifications were performed on a Mastercycler thermal cycler (Eppendorf, Westbury, NY) with initial denaturation at 96°C for 3 min, followed by 35 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Amplicons were visualized on 1% agarose gel using Quantity One Software (Biorad, Hercules, CA) before sequenced. Resulting sequences were assembled in CLC Main Workbench 6 (CLCbio, Aarhus, Denmark) and compared with a local library, built with curated sequences from Robideau et al. (2011) and with sequences deposited in GenBank.

Pathogenicity and virulence assay. A subset of 202 representative isolates from all those collected were randomly selected, based on greenhouse location sampled and host of origin. These isolates were tested for pathogenicity using an in vitro petri dish assay similar to that used to evaluate *Pythium* spp. from soybean and corn seed (Broders et al. 2007). Isolates of *P. aphanidermatum* (50), *P. irregulare* (54), *P. ultimum* (52), *P. cylindrosporum* (10), *P. segnitium* (18), *Pythium* sp. 1 (14), *P. dissotocum* (1), and *P. sylvaticum* (1) were grown for 4 days on CMA and a 5-mm plug of actively growing mycelia was transferred to the center of a 8-cm plate of 2% water agar. The isolates colonized the plate for 3 days, then 10 'Pinto Premium White' geranium seeds were disinfested for 1 min in a 1% sodium hypochlorite solution and placed on the plate 2 cm from the edge and equidistantly from each other. The control consisted of geranium seeds placed onto noninfested water agar plates. Plates were incubated at 20°C in the dark for 7 days. Pathogenicity was evaluated using a virulence scale from 0 to 3 as follows: 0 = 100% germination with a healthy appearance; 1 = 99 to 70% germination, small brown lesions on the radicle or hypocotyl; 2 = 69 to 30% germination, large brown lesions on the hypocotyl and radicle; 3 = 30 to 0% germination, coalesced lesions covering the hypocotyl and radicle (Broders et al. 2007). The experiment was arranged as a complete randomized design with three replicate plates per isolate. Each experiment was conducted twice (trials). Virulence data were analyzed in SAS 9.3 (SAS Institute Inc., Cary, NC), with the proc glimmix procedure using a multinomial distribution. This procedure is used for categorical, non-normally distributed response variables (Gbur et al. 2012; O'Connell et al. 2008), as the ones evaluated in the virulence data. Trials were considered random variables. *Pythium* spp. and isolates were considered fixed variables. If ANOVA was significant for main effects, pair wise comparisons were performed by contrasts.

Seed germination (%) data were analyzed in SAS 9.3 (SAS Institute Inc., Cary, NC) with the proc mixed procedure. Trials were considered random variables. *Pythium* spp., and isolates were considered fixed variables. There were no significant differences among the trials, so data were pooled and analyzed together. If ANOVA was significant for main effects or interaction terms, treatment means (*Pythium* spp. tested) were compared using least square means test at *P* = 0.05.

A subset of 60 representative *Pythium* spp. isolates corresponding to all the species found and tested using the in vitro petri dish assay were randomly selected for an additional virulence assay in the greenhouse. The selection of these isolates was based on greenhouse location and host of origin. Inoculum was produced by adding five plugs (1 cm diameter) of actively growing mycelia in CMA to a flask containing 20 g of millet seeds mixed with asparagine (0.016 g) and water (15 ml) that had been autoclaved twice (Quesada-Ocampo et al. 2009). The inoculated millet seeds were incubated at 20°C under constant fluorescent light for 10 days, and flasks were shaken every other day to disperse the inoculum evenly. Inoculum of each isolate (3 g) was transferred to the top of 42 g of soilless media (Suremix MI Grower Products Inc., Galesburg, MI), contained in 5.5 × 7.0 × 7.0 cm plastic pots, where 4-week-old geranium 'Pinto Premium White' seedlings were transplanted. Controls consisted of plants transplanted into pots containing uninfested soilless media. Each inoculated and control plant was placed on a saucer and grown on a raised plant bench in the MSU Plant Sciences Research Greenhouses for 4 weeks. The mean air temperature ranged from 20.0 to 26.4°C, with a photoperiod of 14 h per day. Plants were irrigated from the bottom as needed or every second day. After 4 weeks, plants were removed from the pots, and roots were washed with running tap water. Plants were visually assessed for the presence of lesions on the roots, lower stems, and the root area was estimated using the image analysis software Asses 2.0 (Lamari 2008). The tissue area infected (%) was calculated following a scale from 31 to 101 color hue, obtaining the lowest area infected (%) for the control uninoculated plants as 65% and the highest as 100%. The relative tissue area (%) infected caused by each isolate tested was calculated by subtracting the area infected (%) of the control uninoculated plants from the area infected (%) of the inoculated plants, and dividing it by the area infected (%) of the control uninoculated plants. To estimate the root area of each plant, 1 cm² of a real size ruler scale was calibrated in Asses 2.0, and the root area was measured. The experiment was arranged as a complete randomized designed, with three replicate plants per isolate. Each experiment (trial) was conducted twice. To estimate differences in the root area, and the relative tissue area (%) infected data analysis was performed with SAS 9.3 (SAS Institute Inc.) using a proc mixed procedure. Trials were considered random variables. *Pythium* spp. and isolates were considered fixed variables. There were no significant differences between the trials so data were pooled and analyzed together. If ANOVA was significant for main effects or interaction terms, treatment means were compared using least square means test at *P* = 0.05.

To confirm that the symptoms observed were caused by the *Pythium* species used for inoculum, reisolation of the pathogen from symptomatic plant tissue was achieved by placing four pieces of 0.8-cm washed roots on amended CMA. The isolates were identified as previously described.

Mefenoxam sensitivity test. The subset of 202 *Pythium* spp. isolates used in the pathogenicity test was grown for 3 days on CMA and a 5-mm plug of actively growing mycelia was transferred to the center of an 8-cm plate of CMA amended with mefenoxam at 0, 10, and 100 μg a.i./ml (Ridomill gold, Syngenta Crop Protection Inc, Greensboro, NC). Isolates of *P. irregulare*, *P. ultimum*, *P. aphanidermatum*, *P. cylindrosporum*, *P. dissotocum*, and *P. sylvaticum* were incubated for 2 days, and *P. segnitium* and *Pythium* sp. 1 isolates were incubated for 4 days at 20°C under fluorescent light. The relative growth rate of an isolate (%) at each fungicide concentration was calculated by measuring in two perpendicular directions and averaging the result of the culture diameter of radial hyphal growth, then dividing it by the radial growth on the nonamended media and multiplying by 100 (Olson and Benson 2011; Broders et al. 2007; Moorman et al. 2002). Isolates were characterized as follows: i) sensitive if culture diameter <30% of

growth on a nonamended plate, ii) intermediate if culture diameter >30% but <50% of growth on the nonamended plate, and iii) resistant if culture diameter was >50% of growth on the nonamended plate (Olson and Benson 2011). The experiment was arranged as a randomized design, with three replicate plates per isolate at each concentration. Each experiment was conducted twice. The culture diameter (%) data obtained at each concentration was analyzed with SAS 9.3 (SAS Institute Inc.), using a proc mixed procedure. The culture diameter was log transformed. The random and fixed variables were the same as in the pathogenicity assay. If ANOVA was significant for main effects or interaction terms, treatment means were compared using least square means test at $P = 0.05$.

To determine the effective concentration causing 50% inhibition of mycelial growth (EC_{50}) of mefenoxam, a group of resistant (29) and susceptible (28) *Pythium* spp. isolates representing different greenhouses (location) and hosts were randomly selected. Culture plugs of actively growing *Pythium* spp. were transferred to CMA plates amended at 0.1, 0.3, 0.5, and 1 μg a.i./ml; and 100, 200, 300, 500 μg a.i./ml for susceptible and resistant isolates, respectively. Plates were incubated at 20°C under fluorescent light, and culture diameter of radial hyphal growth was measured as described

above. Growth inhibition (%) was calculated, and the EC_{50} was obtained by the regression analysis of the $\log_{10}$ of the concentrations against the probit of the inhibition percentage. The regression equations were used to interpolate the EC_{50} value for each isolate (Lu et al. 2012; Olson and Benson 2011). The experiment was arranged as a complete randomized design, with three replicate plates per isolate at each concentration. Each experiment was conducted twice.

Results

***Pythium* spp. collection and identification.** A total of 287 isolates were obtained from poinsettia collected during the fall of 2011 and 2012 from greenhouses in Kent, Kalamazoo, and Wayne counties. From the spring sampling conducted in 2012 and 2013, a total of 634 isolates were obtained from geranium, 54 from snapdragon, 25 from hibiscus, and 13 from lantana (Table 1). In all, seven *Pythium* spp. were identified by sequencing the ITS region and subsequent local BLAST analysis with the curated database containing sequences from Robideau et al. (2011). Sequences were identified to species based on an identity higher than 98% to ITS sequences deposited in the curated database and to ITS sequences deposited in GenBank. The most common species identified included *P. irregulare* (50%);

Table 1. Total number of *Pythium* spp. isolates obtained from symptomatic host plants sampled in different greenhouses in Kalamazoo, Kent, and Wayne counties during fall of 2011 and 2012 and spring of 2012 and 2013

Area	Greenhouse	Plants sampled ^x	Fall 2011-2012		Plants sampled ^x	Spring 2012-2013							
			Poinsettia			Geranium		Snapdragon		Hibiscus		Lantana	
			Plants (+) ^y	Isolates ^z		Plants (+) ^y	Isolates ^z	Plants (+) ^y	Isolates ^z	Plants (+) ^y	Isolates ^z	Plants (+) ^y	Isolates ^z
Kalamazoo	1	79	31	71	30	6	7	...	...	...	...	...	...
	2	80	34	80	30	2	3	...	...	...	...	...	...
	3	...	...	...	52	22	53	2	2	...	...	...	...
	4	...	...	...	65	27	52	...	...	...	...	...	...
	5	16	2	3	155	96	223	...	...	...	...	...	...
Kent	1	36	6	18	50	11	22	...	...	11	25	5	13
	2	30	5	6	50	13	25	6	11	...	...	...	...
	3	33	19	42	88	48	121	16	34	...	...	...	...
	4	...	...	...	30	7	14	...	...	...	...	...	...
Wayne	1	20	15	29	30	3	5	5	7	...	...	...	...
	2	20	1	2	74	20	49	...	...	...	...	...	...
	3	...	...	...	70	25	60	...	...	...	...	...	...
	4	19	17	36	...	...	...	...	...	...	...	...	...
Totals		333	130	287	724	280	634	29	54	11	25	5	13

^x Total number of plants sampled at each greenhouse facility during fall of 2011 and 2012 and during spring of 2012 and 2013.

^y Total number of symptomatic host plant (poinsettia, geranium, snapdragon, hibiscus, and lantana) confirmed to be infected by *Pythium* spp. at the greenhouse sampled.

^z A total of three isolations were done per plant sample. Roots of symptomatic plants were washed under running tap water, cut, air-dried, and placed on three plates per sample of corn meal agar (CMA) amended with ampicillin (0.25 mg/liter), rifampicin (0.01 g/liter), pentachloronitrobenzene (0.1 g/liter), and benomyl (0.05 g/liter).

Fig. 1. Number of isolates of *Pythium* spp. recovered from greenhouse floral crops in Michigan during fall of 2011 and 2012 and spring of 2012 and 2013.

with sequences having an ITS identity with *P. irregulare* voucher BR900 (GenBank Accession No. HQ643595), and with the *P. irregulare* ex-type strain CBS 250.28 (AY598702) (Lévesque and De Cock 2004); *P. ultimum* (25%): Sequences having an ITS identity with *P. ultimum* voucher CBS72994 (HQ643869), and with *P. ultimum* CBS 398.51 (AY598657) (Lévesque and De Cock 2004); and *P. aphanidermatum* (18%): Sequences having an ITS identity with *P. aphanidermatum* voucher CBS28779 (HQ643439) and with *P. aphanidermatum* strain CBS 118.80 (AY598622) (Lévesque and De Cock 2004) (Fig. 1). Additional species were also identified but were found in a lower proportion and included *P. cylindrosporum* (identity >98% to HQ643516 and to AY598643), *P. segnitium* (identity >98% to HQ643772), *P. sylvaticum* (identity >98% to HQ643849 and to AY598645), and *P. dissotocum* (identity >98% to HQ643423 and to AY598634). A group of isolates was determined as unidentified *Pythium* sp. 1, obtaining an identity >98% to the ITS region with *Pythium* sp. AL-2010 voucher P19400 (HQ261739) (Robideau et al. 2011) sequences deposited in the curated database, and with *Pythium* sp. UZ382 (AB468808) (Uzuhashi et al. 2010) sequence. The same identity results were obtained with the sequences of the *cox II* for the *Pythium* sp. 1 isolates. In this manuscript, these isolates are referred to as *Pythium* sp. 1. The species most frequently isolated from poinsettia included *P. ultimum* (57%), *P. aphanidermatum* (22%), and *P. irregulare* (18%). *Pythium* spp. commonly associated with geranium included *P. irregulare* (68%) and *P. aphanidermatum* (18%); *P. ultimum*, *P. cylindrosporum*, *P. segnitium*, *P. dissotocum*, and *Pythium* sp. 1 were also detected but in low (5%) proportions (Fig. 1). Most of the isolates (97%) recovered from snapdragon were identified as *P. irregulare*. *Pythium* spp. isolated from hibiscus included *P. segnitium* (60%) and *P. irregulare* (40%). Most of the lantana (83%) sampled were infected by *P. irregulare*.

Pathogenicity and virulence test. All of the *Pythium* spp. tested were pathogenic to germinating geranium seeds, and caused disease symptoms on geranium seedlings. Virulence varied significantly among the species ($P < 0.001$) but not among the isolates within each species with the exception of the *P. segnitium* isolates (Table 3). *P. aphanidermatum*, *P. irregulare*, *P. ultimum*, *P. cylindrosporum*, *P. dissotocum*, *P. sylvaticum*, and the *Pythium* sp. 1 were highly virulent (average virulence 2.95) inhibiting germination of more than 85% of the geranium seeds (Table 2); all seedling tissue was covered with coalesced lesions. Isolates of *P. segnitium* exhibited low virulence (average virulence 0.64) and inhibited germination of only 4.1% of the seeds with small lesions on the seedling. Seed germination (%)

was significantly reduced by all *Pythium* spp., with the exception of *P. segnitium*.

All of the *Pythium* spp. used to inoculate geranium plants were virulent to 4-week-old seedlings. The root area of the inoculated plants were significantly different from the control uninoculated plants. Also, there were significant differences among the root area of plants inoculated with various *Pythium* spp. ($P < 0.001$). *P. aphanidermatum*, *P. irregulare*, *P. ultimum*, *P. cylindrosporum*, and *P. dissotocum* had the lowest root area (average 0.44 cm²) whereas plants inoculated with *P. sylvaticum*, *P. segnitium*, or *Pythium* sp. 1 had root area ranging from 0.8 to 1.4 cm² (Table 2). There were significant differences in the relative percentage of root area infected among all the *Pythium* spp. tested (Table 2). *P. aphanidermatum*, *P. ultimum*, *P. irregulare*, *P. dissotocum*, and *P. cylindrosporum* were most virulent on geranium, causing large brown lesions on the roots, chlorosis of lower leaves, plant wilting and in some instances, plant death. Inoculation of geranium with isolates from *Pythium* sp. 1, *P. sylvaticum*, and *P. segnitium* had the lowest percentage of area

Table 3. Virulence of 10 *P. segnitium* isolates on geranium seeds

Virulence in geranium seeds ^y		
Isolate	Virulence ^z	% of seed germination
37.24B	0 a	100 b
37.25C	0 a	100 b
37.28C	0 a	63.3 a
37.24A	0.3 a	96.6 b
37.24C	0.5 a	98.5 b
37.18C	0.6 a	66.7 a
37.28B	0.8 ab	100 b
37.26A	1.0 b	63.3 a
37.28A	1.6 b	93.3 b
37.21C	2.3 b	100 b

^y Values in a column followed by the same letter are not significantly different according to least square means significant difference ($P < 0.05$). Average virulence of geranium seeds were analyzed using a proc glimmix procedure and compared by contrasts; average virulence of seedlings were analyzed using a proc mixed procedure with SAS 9.3 (SAS Institute Inc., Cary, NC).

^z A virulence scale from 0 to 3 was established based on: 0 = 100% germination with a healthy appearance; 1 = 99 to 70% germination, small brown lesions on the radicle or hypocotyl; 2 = 69 to 30% germination, large brown lesions on the hypocotyl and radicle; 3 = 30 to 0% germination, coalesced lesions covering the hypocotyl and radicle (Broders et al. 2007).

Table 2. Virulence of seven *Pythium* species on geranium seeds and seedlings

Species	Virulence in geranium seeds ^y					Virulence in geranium seedlings ^y				
	N ^w	Average virulence ^x	SE ^y	% of seed germination	SE ^y	N ^w	Root area (cm ²)	SE ^y	% of area infected ^z	SE ^y
Control (media non infested)	3	0 a	0	99.1 c	0.11	3	4.2 c	0.20		
<i>P. segnitium</i>	18	0.6 a	0.86	95.9 c	0.03	6	1.0 b	0.12	14.1 a	1.9
<i>Pythium</i> sp. 1	14	2.8 b	0.44	14.9 b	0.03	5	0.8 ab	0.13	21.8 a	2.2
<i>P. aphanidermatum</i>	50	2.9 b	0.15	0.9 a	0.02	10	0.4 a	0.09	42.5 b	1.5
<i>P. ultimum</i>	52	3.0 b	0	6.5 a	0.02	14	0.5 a	0.08	42.2 b	1.2
<i>P. cylindrosporum</i>	10	3.0 b	0	3.1 a	0.04	7	0.4 a	0.11	38.7 b	1.8
<i>P. irregulare</i>	54	3.0 b	0	2.4 a	0.02	13	0.4 a	0.08	40.7 b	1.3
<i>P. dissotocum</i>	1	3.0 b	0	1.5 a	0.12	1	0.5 a	0.30	40.5 b	4.8
<i>P. sylvaticum</i>	1	3.0 b	0	1.5 a	0.12	1	1.4 b	0.30	21.0 a	4.8

^y Values in a column followed by the same letter are not significantly different according to least square means significant difference ($P < 0.05$). Average virulence of geranium seeds were analyzed using a proc glimmix procedure and compared by contrasts; average virulence of seedlings were analyzed using a proc mixed procedure with SAS 9.3 (SAS Institute Inc., Cary, NC).

^w Number of isolates analyzed.

^x A virulence scale from 0 to 3 was established based on: 0 = 100% germination with a healthy appearance; 1 = 99 to 70% germination, small brown lesions on the radicle or hypocotyl; 2 = 69 to 30% germination, large brown lesions on the hypocotyl and radicle; 3 = 30 to 0% germination, coalesced lesions covering the hypocotyl and radicle (Broders et al. 2007).

^y Standard error.

^z The percentage of tissue area infected was calculated using Assess 2.0 (Lamari 2008), following a scale from 31 to 101 color hue, obtaining the lowest area infected (%) for the control uninoculated plants as 65% and the highest as 100%. The relative (%) of tissue area infected caused by each isolate tested was calculated by subtracting the percentage of area infected of the control uninoculated plants from the percentage of area infected of the inoculated plants and dividing it by the (%) of area infected of the control uninoculated plants.

infected (Table 2) with only small brown root lesions; the plant foliage remained symptomless. All of the species used as inoculum were successfully reisolated from the infected tissue, and identified by sequencing the ITS region, obtaining an identity greater than 98% to ITS sequences deposited in the curated database for each isolate.

Mefenoxam sensitivity test. The mycelial growth (%) at 10 and 100 ppm of mefenoxam was significantly different ($P < 0.001$) than the growth on control nonamended plates for all the *Pythium* spp. tested (Fig. 2). The species with the greatest mycelial growth of 97.6 and 90.2% on media amended with fungicide at 10 and 100 ppm, respectively, was *P. cylindrosporum*. The radial mycelial growth for *P. ultimum*, *P. irregulare*, and *P. segnitium* ranged from 46 to 64% at 10 ppm, and from 29 to 50% at 100 ppm. The lowest radial mycelial growth (<19% at 10 or 100 ppm) was observed with *P. aphanidermatum*, *P. sylvaticum*, *P. dissotocum*, and *Pythium* sp. 1. (Fig. 2). The most mefenoxam resistant species was *P. cylindrosporum* and all 10 isolates tested were resistant. More than half (62%) of the 53 *P. ultimum* isolates tested were resistant to mefenoxam; the majority of the resistant *P. ultimum* isolates were obtained from poinsettia in two greenhouses in Kalamazoo County and one greenhouse in Wayne County. For *P. irregulare*, 43% of the 55 isolates tested were resistant to mefenoxam; 50% of the resistant isolates were obtained from geranium collected from greenhouses in Kent (2), Kalamazoo (3), and Wayne (2) counties. Most (84%) of the 50 *P. aphanidermatum* isolates tested were sensitive to mefenoxam; intermediate and resistant isolates were recovered from geranium at one greenhouse in Kalamazoo County and one in Wayne County where *P. ultimum* resistant isolates were also recovered. *P. segnitium* isolates (18) recovered from hibiscus at one greenhouse in Kent County were mostly (67%) sensitive to mefenoxam; one isolate was intermediate and five were resistant to mefenoxam. Isolates of *P. dissotocum* (1), *P. sylvaticum* (1), and *Pythium* sp. 1 (14) were sensitive to mefenoxam.

The EC_{50} values for the resistant isolates of *Pythium* spp. varied among the species. *P. ultimum* ranged from 197 to >500 $\mu\text{g a.i./ml}$; *P. cylindrosporum* and *P. irregulare* from 190 to 500 $\mu\text{g a.i./ml}$. Only a few *P. aphanidermatum* isolates were resistant and ranged from 197 to 396 $\mu\text{g a.i./ml}$. The EC_{50} for resistant isolates of *P. segnitium* ranged from 130 to 256 $\mu\text{g a.i./ml}$ (Table 4). For the sensitive isolates, the

EC_{50} ranged from 0.3 $\mu\text{g a.i./ml}$ up to 1 $\mu\text{g a.i./ml}$ for all of the *Pythium* spp. isolates. Exceptions were noted for two *P. ultimum* isolates ($EC_{50} = 2.25, 3.63 \mu\text{g a.i./ml}$) (Table 4).

Discussion

Disease caused by *Pythium* spp. is not a new problem for greenhouse growers but remains a challenge with relatively few effective control options. This study was conducted to identify and characterize the *Pythium* spp. infecting greenhouse floriculture crops in Michigan as a means to develop improved and effective management strategies for the state's industry.

Seven *Pythium* spp. and a group of isolates with similar morphological and molecular characters that have not been described were recovered from floriculture crops over the course of the 3-year study; *P. irregulare* and *P. ultimum* were most frequently isolated. In a similar study conducted in Pennsylvania more than a decade ago, *P. irregulare* and *P. aphanidermatum* were the most common species detected (Moorman et al. 2002). Another study conducted in Ohio more than 30 years ago showed that *P. ultimum* was the species most frequently associated with seedling disease in bedding plants (Stephens and Powell 1982).

In this study, *P. irregulare* had the broadest host range and was isolated from geranium, poinsettia, snapdragon, hibiscus, and lantana. *P. irregulare* infecting these crops have been previously reported with the exception of lantana: infected geranium has been reported in Michigan (Garzón et al. 2007), poinsettia in California (Tompkins and Middleton 1950), snapdragon in Pennsylvania (Moorman et al. 2002), and hibiscus in Australia (Shivas 1989).

Most of the *P. ultimum* isolates (89%) were obtained from poinsettia sampled across different greenhouses in three Michigan counties. Historically, *P. ultimum* has been associated with poinsettia and other ornamental crops, (Bateman and Dimock 1959; Benson et al. 2002; Hausbeck et al. 1989; Kaye et al. 1984; Scheffer and Haney 1956), but more recent studies did not identify *P. ultimum* as a significant pathogen of greenhouse ornamentals (Moorman and Kim 2004; Moorman et al. 2002). One possible explanation for the prevalence of *P. ultimum* in Michigan may be the use of a different source of soilless media compared with other greenhouses in the country. Although soilless media comprised of sphagnum peat moss along with vermiculite and/or perlite, is thought to be superior to field

Fig. 2. Percentage of mycelial growth on media amended with mefenoxam at 10 and 100 $\mu\text{g/ml}$ compared with mycelial growth on nonamended media for *Pythium* spp. recovered from greenhouse floral crops in Michigan. Bars followed by the same letter are not significantly different according to least square means significant difference ($P < 0.05$).

soil in preventing the introduction of pathogens to greenhouses, soilborne pathogens may be present in soilless media (Daughtrey and Benson 2005) and serve as a source of inoculum (Parke and Grünwald 2012). *P. ultimum* may also be introduced to Michigan greenhouses via irrigation water. Most of the greenhouses sampled in this study use well water, and two greenhouses in Wayne County use city water as irrigation sources. Plant pathogens can enter the water sources of greenhouse facilities at several points of the distribution path, and repeatedly infect plants (Hong and Moorman 2005).

P. aphanidermatum was frequently isolated from both poinsettia and geranium. The association of *P. aphanidermatum* with poinsettia has been documented (Benson et al. 2002; Bolton 1978), and this species was the most prevalent *Pythium* spp. recovered from poinsettia in Pennsylvania (Moorman et al. 2002). *P. cylindrosporum* isolates were not frequently detected (2%) in this study, but were recovered from geranium and poinsettia; *P. cylindrosporum* had been previously isolated from snapdragon bedding plants (Moorman et al. 2002). In our study, *P. segnitium* was obtained from hibiscus and geranium. This *Pythium* sp. was the second most prevalent species isolated from the recent study of *Pythium* spp. in floricultural greenhouses in Long Island, NY (Sain 2014) but has not been previously documented on ornamental plants. It is possible that *P. segnitium* is a recent introduction to greenhouse facilities in Michigan. *P. segnitium* has been previously isolated from soil samples in Canary Islands (Paul 2002) and from bean in Rwanda (Nzungize Rusagara 2012). To the authors' knowledge, the species *P. dissotocum*, and *P. sylvaticum* isolated from geranium and snapdragon respectively, have not been previously reported in ornamental bedding plants. *P. dissotocum* and *P. sylvaticum* have been isolated from lettuce in Arizona (Stanghellini and Kronland 1986), alfalfa in Missouri (Larkin et al. 1995), corn and soybean in Ohio (Broders et al. 2007), Douglas-Fir seedling in Oregon (Weiland 2011), and carrot in Michigan (Lu et al. 2012).

Interestingly, most of the isolates (76%) designated as *Pythium* sp. 1 were recovered from geranium at the same greenhouse facility. In order to identify the species of these isolates, additional morphological and genetic studies are needed. Although ITS is the most common region used for identification of oomycetes to the species level (Robideau et al. 2011), for some organisms the ITS region cannot designate species due to the low level of sequence divergence among closely related species. In this case, the examination of more than two loci will be required to designate species (Robideau et al. 2011; Schroeder et al. 2013; Villa et al. 2006) and to determine the phylogenetic relatedness of the *Pythium* sp. 1 with the *Pythium* species currently identified.

Pathogenicity testing on geranium seeds placed in petri plates revealed that all of the species except *P. segnitium* were pathogenic and highly virulent resulting in inhibition of seed germination and causing lesions. The high virulence of the *Pythium* spp. isolates

tested confirms that geranium is a likely host and susceptible to *Pythium* spp. Pathogenicity and virulence studies of *Pythium* spp. obtained from different hosts indicate a wide range of virulence (low, moderate, and high) among the species and isolates infecting corn and soybean seeds (Broders et al. 2007) and Douglas-fir (Weiland et al. 2013). These results contrast with our study where all the species tested, with the exception of *P. segnitium*, were highly virulent to geranium seeds. In this study, *P. segnitium* isolates were determined as avirulent to geranium seeds. The *P. segnitium* isolates in our study were primarily recovered from hibiscus exhibiting root rot symptoms, suggesting that the pathogenicity and virulence of this *Pythium* sp. may be restricted to hibiscus.

The pathogenicity test conducted on geranium plants corroborated the results of the geranium seed bioassay where all the species infected 4-week-old geranium seedlings. A high degree of virulence was observed in several species including *P. aphanidermatum*, *P. ultimum*, *P. irregulare*, *P. dissotocum*, and *P. cylindrosporum*. The most prevalent species found in this study were *P. irregulare* and *P. ultimum*, and they were the most virulent species as determined by the seed bioassay and the seedling inoculation test. It is clear that these species are a significant risk to ornamental crop production. The *P. segnitium*, *P. sylvaticum*, and isolates of the *Pythium* sp. 1 were less virulent with reduced root area infected than *P. ultimum*, *P. irregulare*, and *P. aphanidermatum*. The relatively low virulence of these species on the geranium seedlings used in this study may be explained by possible host preference of these pathogens. *P. sylvaticum* has been predominantly found on alfalfa (Altier and Thies 1995), corn, and soybean (Broders et al. 2007). The isolates included in the *Pythium* sp. 1 may be potentially pathogenic, but with low virulence of geranium seedlings.

Of the 202 *Pythium* spp. isolates tested for mefenoxam sensitivity in this study, 39% were resistant to mefenoxam at 10 and 100 µg a.i./ml. The first report of *Pythium* isolates resistant to metalaxyl, an analog of mefenoxam, in the United States (Sanders 1984), reported that metalaxyl was unable to control *Pythium* turf blight in Pennsylvania, with more than 60% of *P. aphanidermatum* isolates found to be resistant. Also, resistance to metalaxyl was reported in *P. irregulare* and *P. torulosum* isolates from wheat (Cook and Zhang 1985) and *P. dissotocum* and *P. sulcatum* isolates from carrot (White et al. 1988). Mefenoxam resistance was found on 4% of *P. ultimum* isolates from potato collected in Minnesota, Washington, and Idaho (Taylor et al. 2002); and 5% of *Pythium* spp. isolates from carrot in Michigan (Lu et al. 2012). Specifically in ornamentals, 32% of *Pythium* isolates collected from ornamental crops in Pennsylvania were found to be resistant to mefenoxam (Garzón et al. 2011; Moorman et al. 2002; Moorman and Kim 2004).

The ornamental industry is segmented among various types of producers and infected plant material including cuttings, prefinished and finished plants infected with fungicide resistant *Pythium* spp. isolates

Table 4. Distribution of EC₅₀ values of mefenoxam for *Pythium* spp. isolates recovered from greenhouse floral crops in Michigan during the fall of 2011 and 2012 and spring of 2012 and 2013

Species	N	Distribution of isolates based on EC ₅₀ values (µg/ml) ^z									
		Sensitive					Resistant				
		0.3-0.5	0.5-0.7	0.7-0.9	0.9-1.0	>1	100-200	200-300	300-400	400-500	>500
<i>P. irregulare</i>	13	1	4	1	...	...	1	2	2	1	1
<i>P. ultimum</i>	14	4	...	...	...	2	...	2	1	2	3
<i>P. aphanidermatum</i>	10	0	2	4	1	...	1	...	2	...	...
<i>Pythium</i> sp. 1	5	4	1	...	...	...	...	...	...	...	...
<i>P. cylindrosporum</i>	7	...	...	...	...	...	1	2	3	...	1
<i>P. segnitium</i>	6	...	...	...	2	1	2	1	...	...	...
<i>P. sylvaticum</i>	1	1	...	...	...	...	...	...	...	...	...
<i>P. dissotocum</i>	1	...	1	...	...	...	...	...	...	...	...

^z Number of isolates distributed across a range of EC₅₀ values (µg/ml) of mefenoxam. Hyphal growth was measured from CMA plates amended at 0.1, 0.3, 0.5, and 1 µg a.i./ml; and 100, 200, 300, and 500 µg a.i./ml for susceptible and resistant isolates, respectively. Growth inhibition (%) was calculated, and the EC₅₀ was obtained by the regression analysis of the log₁₀ of the concentrations against the probit of the inhibition percentage. The regression equations were used to interpolate the EC₅₀ value for each isolate.

may be transported and shared among greenhouse facilities within the state and country. The movement of infected plant material can increase the probability of introducing resistant isolates to greenhouses. Early symptoms of *Pythium* infection can go unnoticed until the disease becomes severe and aboveground symptoms become evident.

The range of EC₅₀ values of mefenoxam (0.3 to 1.0 µg a.i./ml) observed in our study for the sensitive isolates are consistent with EC₅₀ values previously reported. Taylor et al. (2002) reported EC₅₀ values of mefenoxam in the range of 0.1 to 1.0 µg a.i./ml for *P. ultimum* isolates from potato collected in Minnesota, North Dakota, Washington, and Oregon. The *P. irregulare*, *P. sylvaticum*, and *P. ultimum* isolates from Douglas-fir in Oregon had average EC₅₀ values of 0.20, 0.06, and 0.06 µg a.i./ml, respectively (Weiland et al. 2014). The resistant *P. ultimum*, *P. irregulare*, and *P. cylindrosporum* isolates had EC₅₀ values ranging from 100 to 500 µg a.i./ml. For oomycetes, high EC₅₀ values of mefenoxam have been reported (Hwang and Benson 2005; Olson and Benson 2011). *Phytophthora* spp. infecting ornamental crops in North Carolina were characterized (Olson and Benson 2011) with the EC₅₀ values of mefenoxam for resistant isolates of *Phytophthora nicotianae* ranging from 246 to 435 µg a.i./ml; *Phytophthora drechsleri* isolates had EC₅₀ values over 700 µg a.i./ml.

P. cylindrosporum and *P. ultimum* had the greatest number of mefenoxam-resistant isolates. Most of these isolates were recovered from poinsettia from two greenhouse facilities sampled in Kalamazoo and one greenhouse in Wayne County. Resistant *Pythium* spp. may be established at these locations and an improved management that combines the use of different fungicides and biological controls to control *Pythium* root rot could be helpful (Harman 2000; Yuan and Crawford 1995). The highest EC₅₀ values (>500 µg a.i./ml) obtained for isolates within *P. irregulare* (1), *P. ultimum* (3), and *P. cylindrosporum* (1) were collected from poinsettia in two locations in Kalamazoo and one in Wayne County. The high percentage of mefenoxam resistant *P. ultimum* isolates found in this study suggests that mefenoxam may not be effective to control this pathogen in Michigan greenhouses. Other chemistries that include azoxystrobin, fosetyl-aluminum, and etridiazole, which have been used to protect plants from *Pythium* spp. (Cohen and Coffey 1986; Moorman and Kim 2004), and biological control agents containing *Trichoderma harzianum* (Sivan et al. 1984), *T. virens* (Howell 2002), *Bacillus subtilis* (Jacobsen et al. 2004), *Streptomyces griseoviridis* (Paulitz and Bélanger 2001), and *S. lydicus* (Yuan and Crawford 1995), could be implemented to manage *Pythium* root rot.

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