Characterization of *Pythium* spp. Associated with Corn and Soybean Seed and Seedling Disease in Ohio

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ABSTRACT

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Cool, moist conditions in combination with minimum tillage, earlier planting, and recent shifts in commercial fungicide seed-treatment active ingredients have led to an increase in corn (*Zea mays*) and soybean (*Glycine max*) seedling establishment problems. This situation resulted in an investigation of *Pythium* spp. associated with seed and seedling diseases. Samples of diseased corn and soybean seedlings were collected from 42 production fields in Ohio. All isolates of *Pythium* recovered were identified to species using morphological and molecular techniques and evaluated in an in vitro pathogenicity assay on both corn and soybean seed, and a subset of the isolates was tested for sensitivity to fungicides currently used as seed treatments. Eleven species and two distinct morphological groups of *Pythium* were identified, of which six species were moderately to highly pathogenic on corn seed and nine species were highly pathogenic on soybean seed. There was significant variation ($P < 0.05$) in sensitivity to mefenoxam, azoxystrobin, trifloxystrobin, and captan both across and within species. Multiple species of *Pythium* had the capacity to reduce germination of both corn and soybean seed. Results indicated that mefenoxam, azoxystrobin, trifloxystrobin, or captan, when used individually, may not inhibit all pathogenic species of *Pythium* found in Ohio soils.

Additional keywords: ITS, SHAM

Infection of corn (*Zea mays* L.) or soybean (*Glycine max* (L.) Merr.) seed or seedlings by *Pythium* spp. can result in pre- or post-emergence damping-off, but some plants, given more favorable growing conditions, may continue to grow despite the presence of root infection (8). Although plants may survive the initial infection, nonlethal wounds caused by the pathogen may lead to reduced root and shoot growth, resulting in reduced plant vigor and yield, especially under periods of stress conditions. A recent shift in planting dates for corn has occurred, starting on average 8 days earlier and finishing 16 days earlier than during the 10 years previous (33). Earlier planting means seed are more likely to be placed under environmental conditions unfavorable for rapid germination, thus increasing the level of stress on the seed and, more importantly, the length of time fungicide seed treatments must provide adequate protection. The combination of these factors may have contributed to the increased number of emergence problems recently experienced by growers.

Pythium spp. frequently are associated with seed and seedling diseases, and commonly have been isolated from corn (30) and soybean (5,11,32) early in the season when the soils are predominantly moist and cool. In some cases, the increased prevalence of seedling disease such as damping-off and the relative abundance of pathogenic *Pythium* spp. have been associated with the increase in reduced tillage practices (29). Reduced tillage leaves more of the soil covered with crop debris and prevents rapid drying and warming of the soil in spring, slowing the germination and growth of young seedlings (12,39). This contributes to an increase in the *Pythium* populations in the soil (29). Several *Pythium* spp. may be isolated from a single piece of root tissue or from the soil surrounding the roots. This makes it difficult to attribute the disease causal agent to an individual species, because both pathogens and saprophytes may be recovered. Species of *Pythium* also are known to vary in pathogenicity on soybean (9,43), corn (7,30), and wheat (14). This complicates the development of effective seedling disease management strategies because, in many cases, multiple species must be managed. To achieve adequate control, each crop must have appropriate genes for resistance or fungicides must have activity against multiple species of *Pythium*.

One of the major challenges of managing diseases caused by more than one spe-

cies of *Pythium* is the problem of properly identifying all of the species associated with the disease. Difficulty in correctly identifying *Pythium* spp. hampers attempts to determine how many different species are present in a field, which species are the most abundant, and which are the most pathogenic. Earlier studies in Ohio isolated and identified *Pythium catenulatum* Matthews, *P. irregulare* Buisman, *P. paroecandrum* Drechs., *P. splendens* H. Braun, and *P. torulosum* Coker & F. Patterson from soybean seedlings using a soil baiting technique (9); and *P. arrhenomanes* Drechs., *P. dissotocum* Drechs., *P. graminicola* Subramanian, *P. ultimum* Trow, and *P. torulosum* from corn seedlings (21,30).

Currently, seedling diseases and seed rots are managed by planting seed treated with a combination of fungicides. Metalaxyl or mefenoxam were applied to seed predominantly for the management of *Pythium* and other oomycetes, whereas captan, a broader-spectrum fungicide, was used to manage *Pythium*, *Fusarium*, and *Rhizoctonia* spp. Newer fungicide seed treatments such as the strobilurins, azoxystrobin and trifloxystrobin, with activity against a broad spectrum of pathogens, recently were introduced as companion fungicides to improve efficacy. In addition, fludioxonil, with activity against *Fusarium* spp., also was added to some seed-treatment products. In many commercial seed treatments, captan has been replaced by one of the strobilurins, fludioxonil, or both. In addition, the continued use of metalaxyl and mefenoxam for the control of *Pythium* spp. has led to increased insensitivity of several *Pythium* spp. in some crop systems (4,6,26,37). Strobilurin fungicides differ in their mode of action from metalaxyl and mefenoxam; however, like metalaxyl and mefenoxam the activity of the strobilurins is site specific (42). Thus, the continued use of these fungicides may lead to reduced efficacy due to increased insensitivity of the *Pythium* population. Currently, little is known about the level of sensitivity of *Pythium* spp. to the strobilurin fungicides. However, there have been several reports of other fungi, such as *Fusarium* spp. (13) and *Alternaria alternata* (24,31), with decreased sensitivity to the different strobilurin fungicides, and that risk may be associated with target-site mutations (3,36). It also is proposed that some soilborne pathogens may constitutively express their cytochrome and alter-

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native oxidase respiratory pathways (15,16), enabling them to continue production of ATP and, hence, appear resistant to strobilurin fungicides.

The objectives of this study were to (i) isolate and identify *Pythium* spp. associated with corn and soybean seed and seedling diseases in Ohio, (ii) determine the pathogenicity of isolates on both corn and soybean seed, and (iii) determine the level of sensitivity of the *Pythium* spp. to mefenoxam, trifloxystrobin, azoxystrobin, and captan.

MATERIALS AND METHODS

Isolation and identification. Diseased soybean and corn seedlings were collected from fields with reports of emergence problems during the spring of 2004 and 2005. Sections of root or shoot tissue from diseased seedlings were placed between two paper towels and pressed using a rolling pin to remove excess water, and then tissue was placed onto water agar (WA) (20 g of agar per liter). Isolates with characteristic coenocytic hyphae, typical of oomycetes, were transferred as hyphal tips to a dilute V8 media containing pentachloronitrobenzene, iprodione, benlate, neomycin sulfate, and chloramphenicol (PIBNC; 34) that is selective for Oomycetes. A single hyphal tip from the PIBNC culture of each isolate then was transferred to potato-carrot agar (PCA) (38). For long-term storage, each isolate was transferred to a slant vial containing 15 ml of PCA, allowed to colonize the agar for 10 days, covered with sterile mineral oil, and then stored at 10°C. For identification, isolates were cultured on PCA and in water-grassblade cultures as described by Waterhouse (40). Isolates were identified by observing morphological characteristics of oogonia, antheridia, and sporangia using the standard keys by van der Plaats-Niterink (38), Waterhouse (40), and Middleton (23). Isolates which did not produce all the reproductive structures necessary for conclusive species identification were grouped according to the similarity in structures available and the mycelial growth pattern on PCA.

DNA extraction, polymerase chain reaction, and sequence analysis. The internal transcribed spacer (ITS) sequence from at least one representative isolate of each putative species and each unidentified group of isolates was sequenced to either confirm identification or assist in further identification. Each isolate was grown on PCA for 4 days and, from these cultures, five 10-mm plugs were placed in 50 ml of dilute V8 broth (34) in a 125-ml Erlenmeyer flask for the production of mycelia. V8 broth cultures were incubated at 22°C in the dark for 7 days. Mycelium was collected on filter paper using a Buchner funnel, macerated using liquid nitrogen, collected in a 1.5-ml microcentrifuge tube, and stored at -20°C until used to extract DNA. The DNeasy plant minikit by Qiagen

(Qiagen Inc., Valencia, CA) was used to extract DNA according to the manufacturer's instructions. The DNA product was suspended in 100 µl of sterile Tris-EDTA buffer and kept at -20°C until ready for polymerase chain reaction (PCR).

The universal ITS primers ITS1 (TCC GTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used to amplify a portion of the 18S region, all of ITS1, the 5.8S region, ITS2, and a portion of the 28S region of ribosomal DNA (41). A 50-µl reaction consisting of 10 µl of 5× Green GoTaq Reaction buffer (Promega Corp., Madison, WI), 3 µl of 25 mM MgCl₂, 2 µl containing 4 mM each dNTP, 0.5 µl of Taq polymerase, 5 µl each of a 5-µM concentration of primers ITS1 (5'-tcgtaggtgaacctgctgg-3') and ITS4 (5'-tctctccgcttattgatatgc-3'), 6 µl of DNA at 1:10 dilution (one part DNA in Qiagen buffer AE to nine parts deionized filter-sterilized H₂O), and 24.5 µl of de-ionized, filter-sterilized water was carried out in a 0.2-ml thin-walled microcentrifuge tube. PCR parameters according to Moorman et al. (25) were 94°C for 5 min; followed by 34 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min; and completed with 72°C for 5 min followed by 4°C. PCR products were purified using Qiaquick spin columns (Qiagen, Inc.). For sequencing, 3 µl of ITS1 or ITS4 at 2 pmoles/µl were added to 5 µl of purified DNA (20 ng/µl) product. Sequencing was done at the Molecular and Cellular Imaging Center at the Ohio Agricultural Research and Development Center (OARDC, Wooster). The basecalling software PHRED (University of Washington, Seattle) was used to convert the chromatogram data into sequences, which were aligned and edited using Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI). Sequence data was compared with known sequences deposited in the National Center for Biotechnology Information (NCBI) nonredundant database to confirm morphological identification and assist with identification of isolates which did not produce all morphological traits.

To better understand the diversity of *Pythium* spp. isolated from diseased corn and soybean in Ohio, a phylogenetic tree was constructed using the ITS1-5.8S-ITS2 sequence of at least one isolate of each species and each unassigned morphological group, similar to Lévesque and De Cock (20). The phylogenetic and molecular analyses were completed using *MEGA* version 3.1 (19). An alignment using ClustalW and an unweighted pair group method with arithmetic means analysis with all the ITS sequences and several ITS sequences from GenBank was performed. A bootstrap 50% majority-rule consensus tree generated using 1,000 replications also was included.

Using a BLAST search, *P. ostracodes* Drechs. and *P. boreale* R. L. Duanwere

sequences were included from GenBank, as well as the ITS sequence of an isolate of *P. chamaeophyon* Sideris recovered from a wheat seedling in Ohio. These species were included because of their morphological and sequence similarity to the isolates in morphological group 3 and group 6. ITS sequences from GenBank of *Phytophthora sojae* Kaufmann & Gerdemann, *P. cinnamomi* Rands, and *P. infestans* (Mont.) de Bary also were included because of their previously demonstrated sequence similarity to *Pythium* spp. with proliferating sporangia (20). The ITS sequence of *Aphanomyces euteiches* Drechs. was used as the outgroup.

Pathogenicity assays. All of the isolates collected were tested for pathogenicity on both corn and soybean seed. A petri dish assay, similar to that used previously to evaluate pathogenicity and aggressiveness of *Pythium* and *Rhizoctonia* spp. on soybean (9,28,43) and *Pythium* spp. on alfalfa (2) and corn (9), was used. Each isolate was grown on PCA for 4 days and a 3-mm plug was transferred to the center of an 8-cm-diameter petri plate containing 15 ml of 2% WA. The isolates were allowed to colonize the plate for 72 h. Ten seeds of either corn hybrid LG2540 (LG Seeds, Elmwood, IL) or soybean cv. Sloan were surface disinfested in a 0.525% sodium hypochlorite solution and then equally spaced around the plate, approximately 1 cm from the edge. Plates were incubated at 22°C in the dark for 7 days, then healthy seeds were counted. Plates were scored by counting the number of seeds that successfully germinated on inoculated plates compared with the number that germinated on the noninoculated plates. Seeds were considered to have successfully germinated if the radicle was >1 cm long and was not visibly colonized by the pathogen. Each isolate was scored for pathogenicity based on the following rating scale: 0 = 100% germination with no symptoms of infection; 1 = 70 to 99% germination with lesion formation on the roots; 2 = 30 to 69% germination with coalesced lesions; and 3 = 0 to 29% germination, where all seed tissues were colonized. Isolates were arranged in a randomized complete block design with replicates as individual blocks. There were three replicate plates for each isolate in each experiment and the experiment was conducted three times.

Because pathogenicity was evaluated using an ordinal rating scale, the data was analyzed using a nonparametric approach as described by Shah and Madden (35). In separate analyses using PROC MIXED of SAS (SAS Institute, Cary, NC), species and isolates were treated as fixed effects and the relative pathogenicity among species and isolates within species were compared using contrasts.

In addition to the in vitro pathogenicity assay, a greenhouse assay was used to

complete Koch's postulates on species that were not previously reported as pathogens of corn or soybean. One isolate each of *Pythium attrantheridium* Allain-Boulé & Lévesque, *P. inflatum* Matthews, *P. dissotocum*, an isolate of each mating type of *P. sylvaticum* W. A. Campbell & J. W. Hendrix, and morphological group 3 and group 6 were evaluated in this greenhouse assay. Inoculum was produced using a modified protocol from Kirkpatrick et al. (18) by adding 10 5-mm plugs of each isolate to a previously sterilized 500-ml flask containing 237.5 g of sand, 12.5 g of cornmeal, and 80 ml of deionized H₂O. Isolates were allowed to colonize the medium for 9 days, and flasks were shaken every other day to evenly disperse inoculum. Inoculum of each isolate then was transferred to three 500-ml styrofoam cups, and 10 corn (LG2540) and 10 soybean (Sloan) seeds were planted in each of three cups. Seeds were allowed to germinate and grow for 14 days, at which time seedlings were scored for the presence of lesions. Seedlings with lesions on either the seed, root, or shoot were plated on WA, and isolates were identified in the same manner as described previously.

Fungicide sensitivity. A subset, consisting of 58 *Pythium* isolates, representing all the species and collection sites, was used to assess baseline sensitivity to mefenoxam (ApronXL; Syngenta Crop Protection Inc., Greensboro NC), trifloxystrobin (Trilex; Gustafson LLC Plant, TX), azoxystrobin (Dynasty; Syngenta Crop Protection Inc.), and captan (Captan 400; Gustafson LLC). All fungicides were dissolved in deionized H₂O to achieve the proper concentration of active ingredients and added to PCA after autoclaving, when the media had cooled to approximately 50°C. Commercial-grade mefenoxam was evaluated at 5 and 100 µg a.i./ml, and nonamended PCA was used for the control. Trifloxystrobin and azoxystrobin were evaluated at 1, 10, and 100 µg a.i./ml of commercial-grade product. For the strobilurin fungicides, each concentration also was amended with salicylhydroxamic acid (SHAM) at 50 µg/ml to inhibit the action of the alternative oxidase respiratory pathway. Two controls were included for comparison with the strobilurin fungicides; the first was nonamended PCA and the second was PCA amended with SHAM at 50 µg/ml. A preliminary experiment was completed to determine the amount of SHAM that would provide 20 to 40% inhibition of mycelial growth of all species. SHAM was dissolved in a 1:1 concentration of dimethylsulfoxide and methanol; amended to PCA at 0, 25, 50, 75, and 100 µg/ml; and evaluated in the same manner as the fungicides. Captan was evaluated at 50, 100, 200, and 400 µg a.i./ml of commercial-grade product, and nonamended PCA was used as the control.

For each isolate, a 3-mm-diameter plug from the edge of a 3- to 4-day-old colony

was transferred to the center of each plate for each concentration of the fungicide evaluated. The colony diameter was measured in two places after 48 h for the fast-growing species and 72 h for the slow-growing species. The percent growth inhibition was calculated for each concentration by dividing the average colony diameter of the isolate, minus the 3 mm for the agar plug, by the average colony diameter of the nonfungicide-amended media, multiplied by 100. For the strobilurin fungicides, the colony diameter at each concentration was compared with the colony diameter on PCA amended with SHAM at 50 µg/ml alone to calculate the percent mycelial growth of the control. The experimental design was a randomized complete block design with each replication separated over time, with at least 24 h between each replicate. Each isolate was evaluated at all fungicide concentrations and each isolate by concentration combination was each replication. Variation in sensitivity across species and among isolates of a species to the four different fungicides, as well as the effect of fungicide concentration and species on the efficacy of the four fungicides, were analyzed using PROC GLM of SAS (SAS Institute).

RESULTS

***Pythium* spp. isolation and identification.** In all, 19 isolates of *Pythium* were recovered from 10 locations in 8 counties during 2004, and 105 isolates from 30 locations in 15 counties in Ohio and 2 locations in northeast Indiana during 2005. Of the 32 locations sampled during 2005, 4 were sampled extensively, providing 45 isolates. The other 60 isolates were recovered from diseased corn and soybean tissue from the remaining 28 locations. In total, 11 species (*P. attrantheridium*, *P. dissotocum*, *P. echinulatum* Matthews, *P. graminicola*, *P. inflatum*, *P. irregulare*, *P. heli-*

coides Drechs., *P. sylvaticum*, *P. torulosum*, *P. ultimum* var. *ultimum*, and *P. ultimum* Trow var. *sporangiferum* Drechs.) were identified by their morphological characteristics and confirmed by sequence analysis (Fig. 1). Two distinct morphological groups, designated group 3 and group 6, were identified but could not be assigned to species based on morphological characteristics or sequence data. Over the 2-year period, *P. dissotocum* and *P. sylvaticum* were the species most frequently recovered from both corn and soybean (Fig. 1), representing 23 and 20% (29 and 24 isolates) of the isolate collection, respectively.

Only one mating type of the heterothallic species *P. attrantheridium* was recovered. Isolates of this species on PCA had a chrysanthemum-like growth pattern, with main hyphae 3 to 4 µm wide, abundant intercalary and terminal hyphal swellings ranging from 18 to 24 µm in diameter, and bean-shaped appressoria, which sometimes occurred in chains. No oogonia, antheridia, or sporangia could be induced. The sequence data and the morphological traits indicated that all isolates in this group were of the negative mating strain (1).

Isolates of both morphological group 3 and group 6 possessed terminal, globose, internally proliferating sporangia. The sequence data suggested the isolates of morphological group 3 and group 6 were most closely related to other species with proliferating sporangia, such as *P. ostra-codes* and *P. boreale*.

Analysis of the ITS sequence separated the different isolates recovered from Ohio soils into three major phylogenetic clades (Fig. 2). Clade A consisted of *P. irregulare*, *P. sylvaticum*, *P. attrantheridium*, *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiferum*, and *P. echinulatum*. Clade B was composed of *P. graminicola*, *P. dissotocum*, *P. inflatum*, and *P. torulosum*. The ITS sequences of the three *Phytophthora*

Fig. 1. Number of isolates of each *Pythium* sp. recovered in Ohio from either corn or soybean diseased root or shoot tissue during 2005 (105 isolates) or baited from soil during 2004 (19 isolates).

spp., *Phytophthora sojae*, *P. cinnamomi*, and *P. infestans* imported from GenBank, formed Clade C. Group 3 and group 6 were included in clade D, along with *Pythium chameahyphon*, *P. helicoides*, *P. ostracones*, and *P. boreale*.

Pathogenicity assays. Isolates with a pathogenicity score from 0 to 1, 1 to 2, or 2 to 3 were considered to have a low, moderate, or high level of pathogenicity, respectively. All species of *Pythium* reduced germination of soybean more than corn

(Fig. 3), and all species of *Pythium* were significantly ($P < 0.001$) more pathogenic on both corn and soybean than the control. However, there also was a significant ($P < 0.001$) amount of variation among all species of *Pythium*. *P. graminicola*, *P. ultimum* var. *ultimum*, and *P. ultimum* var. *sporangiiferum* were highly pathogenic on both corn and soybean. *P. sylvaticum* isolates were highly pathogenic on soybean and moderately pathogenic on corn. *P. dissotocum* and morphological group 3 and group 6 were highly pathogenic on soybean, but had only a low level of pathogenicity on corn. *P. inflatum*, *P. torulosum*, *P. echinulatum*, and *P. attrantheridium* had lower pathogenicity scores on corn and soybean, but all were able to cause lesions on seedling roots. There was significant variation ($P < 0.001$) among isolates of *P. irregulare* for severity scores and for inhibition of seed germination. The isolates causing disease were highly pathogenic on soybean and moderately pathogenic on corn, whereas other isolates had low pathogenicity scores on both corn and soybean.

P. attrantheridium was able to infect both corn and soybean seedlings. Brown lesions developed on seedling roots as well as lesions on the root tips that restricted root growth. This species was recovered successfully from the diseased tissue upon reisolation. This is the first report of *P. attrantheridium* as a pathogen of both corn and soybean seedlings. In all, seven isolates of this species were recovered from six locations; six isolates were from diseased corn seedlings and one was from diseased soybean. *P. inflatum* was isolated from lesions on root tissue following inoculation of corn and soybean in the greenhouse assay. *P. inflatum* did not inhibit germination; however, small brown lesions were observed at the root tips of all plants and elongated lesions occasionally formed on the roots. This is also the first report of *P. inflatum* as a pathogen of both corn and soybean seedlings in the United States. Seven isolates of *P. inflatum* were recovered from diseased corn tissue from four different locations. Both mating types of *P. sylvaticum* were recovered from both seed and roots of diseased corn and soybean tissue in the greenhouse assay. *P. sylvaticum* was able to infect both seed and seedlings and had a negative impact on germination. In addition, *P. dissotocum* was isolated from root lesions of corn and from root lesions and seed of soybean. This is the first report of *P. dissotocum* as a seed and root pathogen of soybean. The isolates of morphological group 3 and group 6 also were able to cause lesions on both corn and soybean seedlings, and were reisolated from those lesions.

Fungicide sensitivity. There was a significant ($P < 0.0001$) main effect of species and fungicide concentration and species–concentration interaction on the efficacy of mefenoxam, azoxystrobin, and

Fig. 2. Phylogeny of *Pythium* spp. based on internal transcribed spacer (ITS)1-5.8S-ITS2 sequence of nuclear rDNA. Numbers within the tree represent the bootstrap values (1,000 replications). Species with an isolate number in front of the species name were recovered from diseased corn or soybean tissue in Ohio, with the exception of *Pythium chameahyphon*, which was recovered from wheat seedlings in Ohio. Species names followed by an (*) indicates that gene sequence data was imported from GenBank.

Fig. 3. Mean pathogenicity of 12 species of *Pythium* on corn and soybean seed. A petri plate assay was used to determine pathogenicity using a 0-to-3 rating scale based on percent germination compared with the control. The rating was 0 = 100% germination with no symptom of infection, 1 = 70 to 99% germination with lesion formation on roots, 2 = 30 to 69% germination with coalesced lesions and reduced root growth, and 3 = 0 to 29% germination with colonization of all seed.

captan on the mycelial growth of *Pythium* spp. (Table 1). There was a significant ($P < 0.0001$) main effect of species for trifloxystrobin on *Pythium* spp. growth but not concentration. From the subset of 58 *Pythium* isolates evaluated, the sensitivity to mefenoxam, trifloxystrobin, azoxystrobin, and captan varied across species (Table 2) and also among isolates within a species (Tables 3 and 4). Sensitivity to mefenoxam ranged from 0 to 47% growth of the con-

trol at 100 µg/ml. Species with filamentous sporangia (*P. graminicola*, *P. dissotocum*, *P. inflatum*, and *P. torulosum*) and species with proliferating sporangia (morphological group 3 and group 6) were significantly ($P < 0.05$) less sensitive to mefenoxam than species with globose sporangia (*P. irregulare*, *P. ultimum* var. *ultimum*, *P. sylvaticum*, *P. attrantheridium*, and *P. echinulatum*) (Fig. 4). In addition, *P. irregulare*, *P. ultimum* var. *ultimum*, *P. sylvati-*

cum, *P. attrantheridium*, group 3, and group 6 were significantly ($P < 0.05$) less sensitive to azoxystrobin (Fig. 5) and trifloxystrobin (Fig. 6) than *P. graminicola*, *P. dissotocum*, *P. inflatum*, and *P. torulosum*. Sensitivity to azoxystrobin and trifloxystrobin ranged from 0 to 28 and 0 to 87% growth of the control at 100 µg/ml, respectively. Sensitivity to captan ranged from 0 to 8% growth of the control at 400 µg/ml, and *P. inflatum*, *P. torulosum*, and *P.*

Table 1. *F* values and *P* statistics for effects of *Pythium* spp., fungicide concentration, and experiment on mycelial growth in plate assays

Source	Mefenoxam			Azoxystrobin			Trifloxystrobin			Captan		
	df	<i>F</i> value	<i>P</i>	df	<i>F</i> value	<i>P</i>	df	<i>F</i> value	<i>P</i>	df	<i>F</i> value	<i>P</i>
Species (S) ^x	11	124.36	<0.0001	11	82.50	<0.0001	11	469.41	<0.0001	11	96.68	<0.0001
Concentration (C) ^y	1	79.84	<0.0001	2	78.10	<0.0001	2	2.73	0.0600	3	251.61	<0.0001
S × C	11	7.08	<0.0001	22	7.94	<0.0001	22	1.16	0.2848	33	14.64	<0.0001
Experiment (E) ^z	2	0.07	0.9282	2	0.24	0.7871	2	0.08	0.9225	2	1.52	0.2200
S × E	22	0.16	1.0000	22	0.14	1.0000	22	0.94	0.5429	22	0.67	0.8722
C × E	2	0.66	0.5177	4	0.07	0.9922	4	0.82	0.5133	6	0.35	0.9105
S × C × E	22	0.40	0.9933	44	0.07	1.0000	44	0.47	0.9985	66	0.14	1.0000

^x In all, 60 isolates representing nine different species and two distinct morphological groups were used to evaluate the efficacy of all four fungicides.

^y Mefenoxam was evaluated at 5 and 100 µg a.i./ml; azoxystrobin and trifloxystrobin were evaluated at 1, 10, and 100 µg a.i./ml; and captan was evaluated at 50, 100, 200, and 400 µg a.i./ml.

^z The experimental design was a complete randomized block design with three replications separated over time, with at least 24 h between replications.

Table 2. Growth of 12 species of *Pythium* evaluated on media amended with the active ingredients of four different fungicide seed treatments

Species ^z	Fungicide ^x							
	Mefenoxam		Azoxystrobin ^y		Trifloxystrobin ^y		Captan	
	5 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	50 µg/ml	200 µg/ml
Group 6 (2)	54.7 a	47.0 a	34.2 a	19.1 bc	65.9 bc	67.3 c	12.1 c	1.4 b
Group 3 (4)	32.1 b	18.3 b	36.4 a	24.0 ab	53.7 cd	55.8 d	25.6 bc	3.6 b
<i>Pythium graminicola</i> (2)	27.5 bc	16.0 b	0.0 e	0.0 d	39.1 e	36.3 e	18.0 ab	4.2 b
<i>P. dissotocum</i> (9)	25.1 cd	12.6 b	1.8 e	0.5 d	34.7 ef	33.8 ef	40.9 ab	14.5 a
<i>P. inflatum</i> (5)	19.6 d	4.7 c	0.0 e	0.0 d	22.4 f	19.6 g	48.2 a	20.5 a
<i>P. torulosum</i> (5)	12.0 e	1.9 c	0.0 e	0.0 d	1.1 g	0.0 h	43.8 a	16.4 a
<i>P. helicoide</i> s (1)	4.8 f	0.8 c	23.5 c	26.3 a	70.0 ab	75.6 bc	11.4 c	0.9 b
<i>P. irregulare</i> (4)	3.5 f	1.4 c	34.2 a	26.8 a	83.1 a	82.0 ab	20.2 c	1.5 b
<i>P. sylvaticum</i> (11)	3.4 f	1.4 c	23.5 c	18.3 c	73.5 ab	74.8 bc	13.6 c	2.3 b
<i>P. ultimum</i> (8)	2.5 f	0.7 c	27.5 b	23.1 abc	72.0 ab	73.5 bc	13.1 c	1.2 b
<i>P. attrantheridium</i> (4)	1.9 f	0.0 c	12.2 d	1.1 d	80.8 a	87.6 a	11.2 c	2.3 b
<i>P. echinulatum</i> (3)	0.0 f	0.0 c	0.0 e	0.0 d	23.7 f	25.4 fg	0.5 c	0.0 b

^x Values are the mean percent growth of the control for a species. Values in a column followed by the same letter are not significantly different according to Fisher's protected least significant difference ($P < 0.05$).

^y Salicylhydroxamic acid (SHAM) was added to all concentrations of azoxystrobin and trifloxystrobin at 50 µg/ml to inhibit the action of the alternative oxidase pathway; SHAM at 50 µg/ml also was added to the control.

^z Values in parenthesis represent the number of isolates of each species evaluated on all four fungicides.

Table 3. Growth of nine isolates of *Pythium dissotocum* on media amended with four fungicides used as seed treatments

Isolate	Fungicide ^y							
	Mefenoxam		Azoxystrobin ^z		Trifloxystrobin ^z		Captan	
	5 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	50 µg/ml	200 µg/ml
347	46.1 a	25.2 a	0.0 b	0.0 a	35.5 a	33.3 ab	42.6 bc	14.7 bc
K104R	41.9 a	12.6 bc	0.0 b	0.0 a	31.1 ab	27.7 abc	33.1 de	13.1 bc
K23R	39.7 a	18.2 b	0.0 b	0.0 a	34.0 ab	36.1 a	31.7 e	12.9 bc
K4R	26.4 b	10.9 cd	2.6 a	0.0 a	34.7 a	31.8 ab	37.4 cde	13.0 bc
156	17.2 c	14.6 bc	0.0 b	0.0 a	22.6 b	22.6 bc	49.6 ab	22.6 a
237	16.2 c	11.8 c	0.0 b	0.0 a	34.5 a	30.8 abc	34.2 de	13.4 bc
K65R	13.0 c	5.4 de	0.0 b	0.0 a	30.7 ab	29.9 abc	49.9 ab	19.3 ab
226	12.7 c	4.7 e	0.0 b	0.0 a	29.2 ab	18.5 c	54.7 a	23.7 a
115	12.4 c	10.5 cde	2.0 ab	0.0 a	31.6 ab	31.6 abc	40.2 cd	11.5 c
Mean	25.1	12.8	0.5	0.0	31.5	29.1	41.62	16.06

^y Values are the mean percent growth of the control averaged across three replications for each isolate. Values in a column followed by the same letter are not significantly different according to Fisher's protected least significant difference ($P < 0.05$).

^z Salicylhydroxamic acid was added to all concentrations of azoxystrobin, trifloxystrobin, and a nonfungicide-amended control at 50 µg/ml to inhibit the action of the alternative oxidase pathway.

dissotocum were significantly ($P < 0.05$) less sensitive than the other nine species evaluated (Fig. 7). There was a significant ($P < 0.001$) effect of concentration on the efficacy of SHAM and there also was significant ($P < 0.001$) variation in the sensitivity among species of *Pythium* (Fig. 8). Sensitivity to SHAM ranged from 49 to 89 and 8 to 53% growth of the control at 50 and 100 µg/ml, respectively. Based on these results, 50 µg/ml was the rate of SHAM incorporated to inhibit the alternative oxidase in media amended with azoxystrobin or trifloxystrobin.

DISCUSSION

In Ohio, the most common practice to manage pre- and post-emergence damping-off is through the application of fungicide seed treatments. However, a diverse range of *Pythium* spp. have been known to cause both seed and root rot of corn and soybean (30,43) and this investigation confirms that a variety of *Pythium* spp. have the capacity to reduce germination and cause lesions on roots of corn and soybean seed and seedlings in Ohio. Many species of *Pythium* from other states and in previous studies in Ohio have been reported as pathogens of corn and soybean, but this is the first report of both *P. attrantheridium* and *P. inflatum* as pathogens of corn and soybean.

The petri plate pathogenicity assay is an effective method for determining the pathogenic capability of species of *Pythium*. Those species considered moderately to highly pathogenic may be the primary pathogens, and may have the capacity to cause large reductions in stand by themselves. Similar plate assays were used previously to identify resistance to *Pythium* spp. in alfalfa (2), as well as evaluate fungicide seed treatments on corn seedlings for the control of *Fusarium* spp. (27). Therefore, the petri plate method may prove useful in the future for evaluating different corn and soybean lines for resistance to multiple species of *Pythium*. The

greenhouse assay also demonstrated the wide range of pathogenicity among these species of *Pythium*. Some species, such as *P. inflatum* and *P. attrantheridium*, caused lesions only on the roots, whereas other species, such as *P. sylvaticum* and *P. dissotocum*, were able to infect the entire root system, mesocotyl, coleoptile, and hypocotyl, and penetrate the seed coat. However, even species with low levels of pathogenicity had the ability to cause lesions, most generally on the root tip. These less-pathogenic species may not be able to significantly reduce germination or plant stand by themselves, but they may reduce overall size and function of the roots and potentially provide an infection court for other soilborne pathogens such as *Fusarium*, *Rhizoctonia*, and other *Pythium* spp. *P. irregulare* has been associated with *Fusarium acuminatum* and *Phoma ter-*

restris in causing red root rot of corn (22) and *Pythium ultimum*, along with *Rhizoctonia* and *Fusarium* spp., compose a disease complex causing preemergence damping-off of the soybean cv. Essex reported in Virginia (11).

There was variation in pathogenicity among isolates of a species, specifically *P. irregulare*, which consisted of highly pathogenic isolates and less-pathogenic isolates. Recently *P. irregulare* was reported to be composed of two cryptic species, which may account for differences in pathogenicity among isolates in our study (10). *P. irregulare* isolates K34R, K52R, and K90R, which were moderately to highly pathogenic on both corn and soybean, were used for sequence analysis and, when aligned with several isolates from the study by Garzon et al. (10), were grouped with two different isolates of *P.*

Fig. 4. Growth of 12 *Pythium* spp. on media amended with mefenoxam at 5 and 100 µg/ml. The mycelial growth of each isolate at each concentration was divided by the growth on the nonfungicide-amended control to determine the percent mycelial growth.

Table 4. Growth of 11 isolates of *Pythium sylvaticum* on media amended with four fungicides used as seed treatments

Isolate	Fungicide ^y							
	Mefenoxam		Azoxystrobin ^z		Trifloxystrobin ^z		Captan	
	5 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	50 µg/ml	200 µg/ml
118	12.4 a	8.0 a	18.4 b	17.3 bc	69.3 b	70.6 a	7.4 c	0.6 bc
134	9.7 ab	4.0 ab	16.8 b	14.4 c	74.3 ab	78.2 a	20.6 a	2.9 abc
46	4.8 bc	0.0 b	22.6 ab	17.4 bc	73.4 ab	71.6 a	22.7 a	7.3 a
210	2.2 c	0.8 b	17.9 b	26.3 a	76.4 ab	78.3 a	2.3 c	1.2 bc
199	1.7 c	0.0 b	27.6 ab	20.2 abc	73.5 ab	75.5 a	1.7 c	0.5 bc
K11R	1.6 c	0.8 b	35.6 a	23.4 ab	79.9 a	78.7 a	23.0 a	3.9 abc
K75R	0.8 c	0.0 b	27.2 ab	24.8 ab	74.0 a	75.2 a	18.4 a	1.2 bc
329	0.8 c	0.8 b	19.7 ab	13.7 c	75.5 ab	78.7 a	8.2 bc	2.0 bc
168	0.0 c	0.0 b	18.1 b	13.9 c	67.5 b	67.0 a	5.8 c	0.6 bc
362	0.0 c	0.0 b	29.9 ab	20.9 abc	73.4 ab	73.5 a	6.8 c	0.0 c
360	0.0 c	0.8 b	25.0 ab	16.4 bc	70.7 ab	71.8 a	16.5 ab	5.1 ab
Mean	3.1	1.4	23.5	19.0	73.5	74.5	12.1	2.3

^y Values are the mean percent growth of the control averaged across three replications for each isolate. Values in a column followed by the same letter are not significantly different, according to Fisher's protected least significant difference ($P < 0.05$).

^z Salicylhydroxamic acid was added to all concentrations of azoxystrobin, trifloxystrobin, and nonfungicide-amended control at 50 µg/ml to inhibit the action of the alternative oxidase pathway.

irregulare sensu stricto. It is difficult to determine the role of genetic diversity on pathogenicity within this species, because only three isolates were sequenced in this study; however, genetic diversity may in part explain the significant variation in pathogenicity among isolates of *P. irregulare*.

The results of this study indicate that *P. sylvaticum* and *P. dissotocum* may be two of the more important seed and seedling pathogens associated with corn and soybean in Ohio. This is based on the frequency of recovery, the number of locations where these species were recovered, the high level of pathogenicity of *P. sylvaticum* on both corn and soybean, and the insensitivity of *P. dissotocum* to mefenoxam and *P. sylvaticum* to azoxystrobin. However, the frequency at which these two species were recovered may have more to do with their ability to grow well on the media used to make isolations. *P. sylvaticum* grows very rapidly and may have been preferentially isolated from diseased tissue. Regardless, *P. sylvaticum* is highly pathogenic on both corn and soybean seed, indicating its importance in reducing germination. *P. dissotocum* also appears to be important because of its relative insensitivity to mefenoxam, the most frequently used seed-treatment fungicide. In the past, *P. dissotocum* was not reported as being of great importance as a seed and seedling pathogen of corn and soybean in Ohio, although it was present. The continued use of mefenoxam as a seed treatment possibly may select for species with decreased sensitivity, such as *P. dissotocum*, *P. inflatum*, *P. torulosum*, and *P. graminicola*, causing a shift in species composition in corn and soybean fields, especially in those fields with persistent problems of substantial stand loss.

The phylogenetic analysis divided the species of *Pythium* recovered from Ohio into three different clades corresponding to sporangia morphology, which agrees with previous phylogenetic trees generated by Lévesque and De Cock (20). The phylogenetic tree also suggests that morphological group 3 and group 6 are more closely related to *Phytophthora* spp. than species of *Pythium* possessing filamentous or globose sporangia. Both morphological group 3 and group 6 isolates produced large (25 to 35 µm) terminal sporangia that proliferate internally, but both produced zoospores in a vesicle characteristic of *Pythium* spp., which distinguishes them from *Phytophthora* spp. Most importantly, the phylogenetic analysis demonstrates the wide range of genetic diversity among species of *Pythium* present in Ohio soybean and corn fields. It also is interesting to point out that there were species of *Pythium* from all three of the major clades that had a moderate to high level of pathogenicity on both corn and soybean.

None of the four fungicides evaluated had the capacity to inhibit the growth of all

the *Pythium* spp. in this study. In addition, all species of *Pythium* did not respond to each of the four fungicides in the same manner, which agrees with an earlier study by Kato et al. (17). They evaluated the sensitivity of several *Pythium* spp. and other oomycetes to hymexazol and metalaxyl and found that different species of *Pythium* had a range of sensitivities to both metalaxyl and hymexazol. In this study, there appears to be an association with sporangia morphology and response to fungicides. Species with globose spo-

rangia (clade A) were less sensitive to the strobilurins than species with filamentous sporangia (clade B). However, in contrast, species with filamentous sporangia were significantly less sensitive to mefenoxam than species with globose sporangia. Morphological group 3 and group 6, with proliferating sporangia, also responded differently than species with filamentous or globose sporangia and had increased levels of insensitivity to both mefenoxam and strobilurin fungicides. Conversely, the lowest level of variability among species

Fig. 5. Growth of 12 *Pythium* spp. on media amended with azoxystrobin at 1, 10, and 100 µg/ml. All concentrations, including the control, contained salicylhydroxamic acid at 50 µg/ml to inhibit the activity of the alternative oxidase pathway. The mycelial growth of each isolate at each concentration was divided by the growth on the nonfungicide-amended control to determine the percent mycelial growth.

Fig. 6. Growth of 12 *Pythium* spp. on media amended with trifloxystrobin at 1, 10, and 100 µg/ml. All concentrations, including the control, contained salicylhydroxamic acid at 50 µg/ml to inhibit the activity of the alternative oxidase pathway. The mycelial growth of each isolate at each concentration was divided by the growth on the nonfungicide-amended control to determine the percent mycelial growth.

of *Pythium* to a fungicide was with captan at 200 µg/ml. Minimum and maximum amounts of active ingredient of captan applied per seed when using the range of the recommended dose on the label was calculated to be 100 and 200 µg/ml, respectively. Mycelial growth was only 7 and 2.7% of the control for morphological group 3 and group 6, respectively, at 100 µg/ml. In addition, only *P. dissotocum*, *P. inflatum*, and *P. torulosum* had mycelial growth greater than 10% of the control with captan at 200 µg/ml.

It is difficult to determine whether this shift from older, broad-spectrum mode of

action fungicides to new, site-specific mode of action fungicides has led to an increase in pre- and post-emergence damping-off caused by species resistant or insensitive to these newer fungicide chemistries. In any case, the results from this study suggest that soybean and corn being planted in areas with a history of emergence problems caused by *Pythium* spp. should use a combination of fungicides to provide protection against several different species of *Pythium*, because these different species may be insensitive to one or more fungicides. It is this diversity which makes it difficult to manage all species of *Py-*

thium associated with corn and soybean seedling disease with a single seed-treatment fungicide, and integrated management strategies such as improving drainage and planting only after soil temperatures have reached 10°C may need to be used along with multiple fungicide chemistries in areas with a history of substantial stand loss.

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Fig. 7. Growth of 12 *Pythium* spp. on media amended with captan at 50, 100, 200, and 400 µg/ml. The mycelial growth of each isolate at each concentration was divided by the growth on the nonfungicide-amended control to determine the percent mycelial growth.

Fig. 8. Growth of 10 isolates of *Pythium* on salicylhydroxamic acid (SHAM) amended to potato-carrot agar at 25, 50, 75, and 100 µg/ml. The mycelial growth of each isolate at each concentration was divided by the growth on the nonamended control to determine the percent mycelial growth.

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