

Oomycete Species Associated with Soybean Seedlings in North America—Part I: Identification and Pathogenicity Characterization

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ABSTRACT

Oomycete pathogens are commonly associated with soybean root rot and have been estimated to reduce soybean yields in the United States by 1.5 million tons on an annual basis. Limited information exists regarding the frequency and diversity of oomycete species across the major soybean-producing regions in North America. A survey was conducted across 11 major soybean-producing states in the United States and the province of Ontario, Canada. In 2011, 2,378 oomycete cultures were isolated from soybean seedling roots on a semiselective medium (CMA-PARPB) and were identified by sequencing of the internal transcribed spacer region of rDNA. Sequence results distinguished a total of 51 *Pythium* spp., three *Phytophthora* spp., three *Phytophytum* spp., and one *Aphanomyces* sp. in 2011, with *Pythium sylvaticum* (16%) and *P. oopapillum* (13%) being the most prevalent. In 2012, the survey was repeated, but, due to drought conditions across the sampling area, fewer total isolates ($n = 1,038$) were collected. Additionally, in 2012, a second

semiselective medium (V8-RPBH) was included, which increased the *Phytophthora* spp. isolated from 0.7 to 7% of the total isolates. In 2012, 54 *Pythium* spp., seven *Phytophthora* spp., six *Phytophytum* spp., and one *Pythiogeton* sp. were recovered, with *P. sylvaticum* (14%) and *P. heterothallicum* (12%) being recovered most frequently. Pathogenicity and virulence were evaluated with representative isolates of each of the 84 species on soybean cv. Sloan. A seed-rot assay identified 13 and 11 pathogenic species, respectively, at 13 and 20°C. A seedling-root assay conducted at 20°C identified 43 species as pathogenic, having a significantly detrimental effect on the seedling roots as compared with the noninoculated control. A total of 15 species were pathogenic in both the seed and seedling assays. This study provides a comprehensive characterization of oomycete species present in soybean seedling roots in the major production areas in the United States and Ontario, Canada and provides a basis for disease management and breeding programs.

Soybean (*Glycine max* [L.] Merr.) is second only to corn (*Zea mays*) in the United States in importance for feed and industrial uses. Poor crop establishment and plant stand due to seed and seedling diseases greatly reduces the soybean crop yield potential in many areas. In 2009, soybean yield loss as a result of seedling diseases in the United States was estimated to be 1.51 million tons (Koenning and Wrather 2010). Many factors may influence plant stand and root health, such as seed quality, edaphic, and environmental conditions (e.g., soil type, soil moisture, precipitation, and temperature), soil microorganisms, and especially diseases (Broders et al. 2009). Soilborne seed and root diseases are attributed to many pathogens, including *Fusarium* and *Rhizoctonia*

species from the kingdom Fungi and the oomycetes *Pythium* and *Phytophthora* from the kingdom Stramenopila (Anderson 1987; Kaufmann and Gerdemann 1958; Rizvi and Yang 1996). In the United States, there has been an increase in soybean yield loss caused by the oomycetes *Phytophthora* and *Pythium* (Koenning and Wrather 2010; Wrather and Koenning 2009). This increased incidence of oomycete-related diseases could be due to lack of material resistant to *Pythium* spp., pathotypes of *Phytophthora sojae* able to overcome existing *Rps* resistance genes, changes in precipitation patterns, and cultural practices used by growers, such as earlier planting dates and greater rainfall in spring and early summer, in conjunction with minimum tillage practices (Dorrance et al. 2016; Melillo et al. 2014).

Conducive environmental conditions for root and seed rot are generally considered to be moist soils, low temperatures that result in delayed seed germination, and plant stress (Leopold and Musgrave 1979) and free moisture, ideal for oospore germination, zoospore production, and subsequent plant infection (Broders et al. 2007; Martin and Loper 1999). Seedling and root-rot diseases can impact yield through plant stand loss, but they are also capable of

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causing sublethal infections that impact plant health and yield (Kirkpatrick et al. 2006; Schluß and Lockwood 1981). *Phytophthora* and *Pythium* spp. are two of the most widely recognized genera of seedling pathogens of soybean. Although *Phytophthora sojae* is a common root and stem rot pathogen of soybean, it can also cause damping off of seedlings (Tyler 2007). Several *Pythium* spp. are reported to have a major impact at the seed and seedling stage in soybean and other field crops (Broders et al. 2007; Zitnick-Anderson and Nelson 2015). According to a host-fungal database, 16 *Pythium* spp. have been confirmed as plant pathogens having an association with soybean (Farr and Rossman 2013). However, studies of oomycete species associated with soybean root rot are often limited or restricted to individual states (Broders et al. 2009; Jiang et al. 2012; Rizvi and Yang 1996; Zitnick-Anderson and Nelson 2015). Nonetheless, information provided by these studies has been extremely valuable in identifying common causal agents of root rot in soybean, including *Pythium ultimum*, *P. irregularare*, and *P. sylvaticum*.

In the United States, soybean production acreage is concentrated in the Midwest and within limited regions of the South. In Canada, Ontario is the major soybean-producing province, followed by Manitoba and Quebec. Thus, soybean production occurs across a large area of North America. These areas encompass a vast diversity of environmental and edaphic conditions that could affect oomycete species composition. In addition, cultural practices, such as crop rotation and soybean cultivar selection, can potentially affect the oomycete communities present in a given area. Broders et al. (2009) conducted an extensive *Pythium* community survey in Ohio and reported an association of pH, calcium, and field capacity with five *Pythium* communities designated, based on species composition. Zitnick-Anderson et al. (2014) studied the effect of soil properties on *Pythium* communities from soybean roots in North Dakota, finding that levels of zinc were associated with increasing abundance of *P. ultimum* and cation exchange capacity correlated with specific species, such as *P. kashmirensis*, *P. heterothallicum*, and *P. irregularare*, increasing their frequency.

To gain a better understanding of the diversity of oomycete species causing soybean seedling diseases in the United States and Ontario, Canada, an extensive survey was conducted in 2011 and 2012. The survey included 11 of the 31 reported soybean-producing states in the United States and in Ontario. These states constitute the soybean belt and they produce 77% of the total soybeans produced in the United States (United States Department of Agriculture (USDA) National Agricultural Statistics Service). The objectives of this study were to determine the diversity and frequency of oomycete species associated with diseased soybean seedlings across the major soybean production area of North America and then, using a classic culture-based survey, characterize these species to determine the key pathogenic oomycete species responsible for seed rot and for root rot. The knowledge gained will inform future efforts toward oomycete management through improved diagnostics, screening of soybean breeding material, and improved chemical management approaches.

MATERIALS AND METHODS

Sample collection and isolation. In 2011 and 2012, a survey was conducted across 11 states, covering the primary United States soybean production area, and Ontario, Canada (Fig. 1; Supplementary Table S1). A total of 64 and 61 fields, respectively, were sampled in 2011 and 2012. Approximately six fields were sampled per year in each participating state and those fields were selected based on field history of seedling diseases and plant stand issues. Collaborators followed a standard sampling procedure that specified collection of 50 symptomatic soybean seedlings from a W-shaped transect across each field. Due to crop rotation practices, diseased soybean fields sampled in 2011 were different from the fields sampled in 2012. Seedling samples from the field were

transported to the laboratory in coolers and were refrigerated; all plant samples were processed within 24 h postcollection. Seedlings were prepared for isolation by washing them under running tap water for 30 min, until all visible soil was removed. Seedlings were patted dry with sterile paper towels to remove excess water and 1-cm root sections of symptomatic tissue were removed, using a sterile scalpel. Sections from all 50 plants per field were placed onto a semiselective medium, corn meal agar (CMA-PARPB) amended with pentachloronitrobenzene (PCNB) (50 mg/liter), ampicillin (250 mg/liter), rifampicin (10 mg/liter), pimaricin (5 mg/liter), and benomyl (10 mg/liter) (Jeffers 1986). For 2012, an additional semiselective medium was included to increase the recovery of *Phytophthora* spp., i.e., 4% V8 medium (V8-RPBH) that contained calcium carbonate (CaCO_3 , 0.6 g/liter), sucrose (1 g/liter), yeast extract (0.2 g/liter) amended with rifampicin (10 mg/liter), PCNB (20 mg/liter), benomyl (10 mg/liter), and hymexazol (20 mg/liter) (Dorrance et al. 2008). Half of the 50 seedlings per field were plated on the CMA-PARPB medium and half were plated onto the V8-RPBH medium. Culture plates were incubated for 7 days at room temperature (20°C) and were checked daily for hyphal growth and morphology consistent with oomycetes. If oomycete mycelial growth was observed, cultures were transferred to fresh CMA-PARPB or V8-RPBH medium by hyphal tipping. Pure isolates were shipped to Michigan State University for identification and characterization.

Isolate storage and DNA extraction. Isolates shipped to Michigan State University were transferred to CMA-PARPB medium and 5-mm plugs were taken from fresh cultures and were transferred to potato carrot agar slants and hemp seed vials for long-term storage (Erwin and Ribeiro 1996; van der Plaats-Niterink 1981). Three to five 5-mm plugs from fresh cultures were transferred into 50 ml of a 10% V8 broth amended with ampicillin (100 mg/liter) in 125-ml Erlenmeyer flasks and were incubated for 7 to 10 days at room temperature, without agitation. Mycelia were harvested from broth cultures, were lyophilized overnight, and were ground for DNA extraction. For DNA extraction, 100 mg of ground mycelia were resuspended in 800 μl cetyltrimethylammonium bromide lysis buffer (AutoGen AG00121, AutoGen Inc.) and were incubated for 1 h at 65°C. A phenol-chloroform automated DNA extraction was performed using the AutoGen 850 system (AutoGen Inc.). DNA was resuspended in 200 μl Tris-EDTA (TE) buffer, was incubated on an orbital shaker for 1 h, was then transferred to 1.5-ml tubes, and was stored at -20°C.

Identification of isolates. Isolates were identified using internal transcribed spacers (ITS) 1 and 2 of rDNA, by amplification with primers ITS6 and ITS4 (Cooke et al. 2000). The polymerase chain reaction (PCR) amplifications consisted of a final concentration of 1× DreamTaq buffer, 2 mM MgCl_2 , 0.2 mM dNTPs, 0.2 μM ITS6, and 0.2 μM ITS4, 4 μg of bovine serum albumin per milliliter, 1 U of DreamTaq polymerase (Thermo Scientific), and 1 μl of DNA. The amplification program consisted of 95°C for 2 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Amplicons were purified by adding 5 μl of a mixture of 3 U of exonuclease I and 0.5 U of FastAP thermosensitive alkaline phosphatase (Thermo Scientific). Samples were incubated for 45 min at 37°C, and enzymes were inactivated by incubation at 85°C for 10 min. Amplicons were Sanger-sequenced in both directions, and consensus sequences were queried against a curated database of oomycete ITS sequences (Robideau et al. 2011) by using the BLASTn search algorithm for identification (Altschul et al. 1990). Samples with a bitscore higher than 1,000 and identity higher than 97% were assigned to a taxonomic designation based on the BLAST output. Sequences were deposited in GenBank under accession codes KU208091 to KU211502.

Seed pathogenicity assay. When available, three isolates of each identified species, which were arbitrarily selected, were evaluated for pathogenicity and virulence on soybean seeds. For a limited number of species, isolates obtained from corn were

substituted, in order to screen three isolates per species for pathogenicity as indicated in Supplementary File 1. A total of 207 isolates representing 84 oomycete species were characterized. A petri-dish seed-pathogenicity assay was conducted as reported by Broders et al. (2007) with the following modifications: isolates were grown on CMA for 4 to 7 days, and a 5-mm plug was transferred from this CMA active culture to the center of a 1.5% water agar plate and was incubated for 2 days. Seeds of the soybean cultivar Sloan were surface-disinfested with a 0.36% sodium hypochlorite solution for 10 min, were rinsed with sterile, distilled water three times, and were allowed to air dry in a laminar flow hood for 15 min. Ten seeds were placed at the growing edge of the colony. Plates were incubated in the dark for 7 days at 13 or 20°C. These temperatures were based on the average soil temperature at planting in northern and southern United States climates (Rojas et al. 2017). Each isolate was evaluated on three replicate plates at each temperature and the experiment was conducted three times per temperature.

Seeds were assigned a disease severity value using the following rating scale: 0 = germinated healthy seed, 1 = delayed development with minimal or no discoloration, 2 = germination with isolated lesions, 3 = germination with coalesced lesions, and 4 = no germination and seed colonized. A disease severity index (DSI) was calculated using the formula:

$$DSI = \frac{\sum (\text{severity rating} \times \text{seeds per rating})}{(\text{total seeds} \times \text{highest severity rating})} \times 100$$

Due to the large number of isolates and replicates, the species were divided into seven sets, with each set containing a control without pathogen. A linear mixed model was used to evaluate DSI as a response variable and species as a fixed effect and nesting isolates within species and experiment as a random effect. Dunnett's contrast was applied to determine species that were significantly different from the control. Temperatures were analyzed

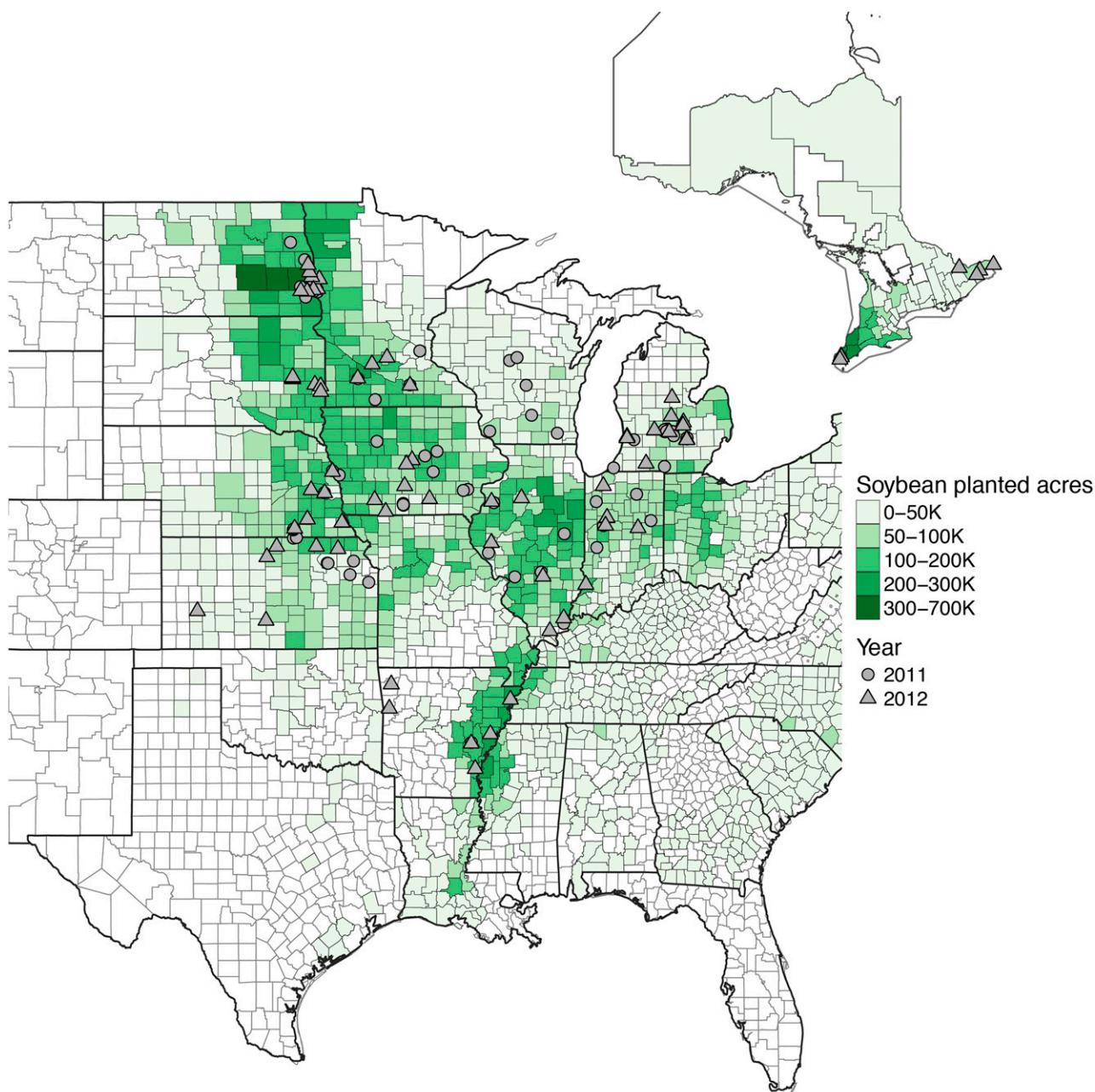


Fig. 1. Map of sampled soybean fields in 2011 and 2012 and of intensity of planted soybean acres demonstrated by color intensity at the county/parish/province level.

independently. In addition, hierarchical clustering was performed to separate species into a cluster, using DSI at 13 and 20°C. Statistical analyses were conducted in R version 3.2 (R core team 2015, Vienna, Austria), using packages ‘lme4’ and ‘lsmeans,’ and graphs were generated with the package ‘ggplot2.’

Seedling root-rot assay. The same isolates used for the seed pathogenicity assay were also evaluated in a seedling root-rot assay. Inoculum was prepared by placing 25 g of long-grain rice and 12 ml of distilled water in 125-ml flasks, and then, autoclaving for 25 min and cooling overnight (Holmes and Benson 1994). The rice grains were mixed using a sterile technique, were autoclaved for another 30 min, and were cooled overnight. Five 5-mm plugs from 4- to 7-day-old cultures of each isolate were transferred into the rice flasks and were incubated in the dark at room temperature (20 to 22°C) for 10 to 14 days. The rice inoculum was mixed regularly to ensure full colonization of rice grains and to loosen and separate grains. Seedling assays were performed in 355-ml capacity paper cups (Solo cups) with four 0.5-cm drainage holes in the bottom. Cups were layered from bottom to top with 50 ml of coarse vermiculite, 150 ml of fine vermiculite, 7 g of colonized rice, 100 ml of fine vermiculite, 6 soybean cv. Sloan seeds, and 100 ml of coarse vermiculite. The vermiculite substrate was initially moistened to water-holding capacity, and thereafter, plants were watered every other day with de-ionized water. Cups were maintained in a growth chamber (BioChambers) with a light regime of 14 h of light (250 $\mu\text{E m}^{-2}\text{s}^{-1}$) and 10 h of dark, at 98% humidity and 20°C for 14 days. Due to the large number of isolates, the isolates were grouped by species and were randomly assigned into seven sets that were used as a block. Every isolate had three cup replicates per experiment, and each experiment was conducted three times for every set. Two controls were included within every experiment, a control with noninoculated autoclaved rice and a non-rice control to account for any effects of the rice on the seedlings. At the completion of the experiment, plant roots were washed with tap water to remove debris for evaluation. Five washed plants were scanned to determine root area and root length and were placed in a drying oven at 50°C for 48 to 72 h, to establish dry weight of roots and shoots. Reisolations were made as described below.

Koch's postulates and single-strand conformation polymorphism (SSCP) confirmation. To fulfill Koch's postulates, in each seedling root-rot experiment, a single plant was arbitrarily selected from one of the three replicates for reisolation of the pathogen. Plants were washed with tap water to remove vermiculite and isolations were performed as described above. Plates were incubated at room temperature for 7 days and were checked daily for the presence of mycelia with growth characteristic of oomycetes. When hyphal growth was observed, transfers were made onto CMA-PARPB medium. Incubation time was extended 7 days for plates without any growth, after the initial incubation period.

The identity of the isolates was confirmed by SSCP (Kong et al. 2004, 2005). In order to have a positive confirmation, colony PCR was conducted on the isolate inoculated and the isolate recovered from infected root tissue, using primers ITS6 and ITS7 (Kong et al. 2004). Briefly, a small fragment of mycelia was taken from the plate, was placed into 100 μl of sterile distilled water and boiled for 5 min in a heat block at 95°C, and 1 μl of this boil prep was used for PCR. Amplification was completed as described previously, using primers ITS6 and ITS7. The PCR products were used for the SSCP analysis, following methods described by Kong et al. (2004). To ease scoring of gels, denatured PCR products from each original and recovered isolate were run side by side in the polyacrylamide gel. Isolation and SSCP confirmation were conducted for each experiment, thus each isolate had three reisolation attempts.

Root area and root length image analysis. Images of roots from the seedling root-rot assay were obtained with a flatbed scanner (Epson Perfection 4870 Photo Pro; Epson America, Inc.) at

a resolution of 300 dpi and saved as JPEG files. Every image included a photographic reference scale to calibrate measurements from pixels to centimeters. All images were analyzed with Assess 2.0 (American Phytopathological Society), using HSI color space (hue values between 0 and 121) to limit the selection to just root tissue, for determination of root area and length, using a calibrated scale.

Data analysis for root measurements. A multivariate analysis of variance (MANOVA) was performed to evaluate seedling variables measured, i.e., root dry weight, shoot dry weight, shoot/root weight ratio, root area, and root length. Prior to analysis, the variables were log₁₀ transformed to improve normality and scaled and centered to aid analysis. An initial exploration of all the response variables was conducted, using principal component analysis (PCA), and the contribution of each variable was examined. Based on contribution, a MANOVA test was utilized to examine differences among the 84 oomycete species characterized, using root dry weight, root area, and root length as response variables. Species was treated as a fixed effect, while isolates and experiment were treated as random effects. To verify significance, univariate analyses were conducted for each of the correspondent response variables using the same model. Dunnett's test was used to determine those species significantly different from the non-rice control. Data were analyzed using R version 3.2 (R core team 2015) with packages ‘FactoMineR’, ‘nlme’, ‘MASS’ and ‘lsmeans’, and graphs were generated with the package ‘ggplot2’. All data and R scripts used in the analyses shown here are deposited on github (https://github.com/Chilverslab/Rojas_Survey_Phytopath_2016) (Rojas et al. 2016).

RESULTS

Sample collection. A total of 3,418 oomycete isolates were collected during the two-year study, 2,380 isolates in 2011 and 1,038 isolates in 2012. A total of 84 oomycete species were identified (Fig. 2), using the ITS region of the rDNA. The genus *Pythium* was the most dominant across the samples, followed by *Phytophthora*, *Phytopythium*, and *Aphanomyces* spp. In 2011, the 12 most abundant species recovered, which included more than 78% of total isolates, were *P. sylvaticum* (16.3%), *P. oopapillum* (13.3%), *P. irregularare* (10.1%), *P. heterothallicum* (7.9%), *P. aff. torulosum* (4.7%), *P. spinosum* (4.6%), *P. ultimum* var. *ultimum* (4.5%), *P. aff. dissotocum* (4.4%), *P. lutarium* (4.1%), *P. paroecandrum* (2.9%), *P. attrantheridium* (2.8%), and *P. ultimum* (2.8%) (Fig. 2). In 2012, there was a shift in the frequency of species isolated; however, there were similarities between the two years. Seven of the 12 most abundant species, which is more than 70% of the isolates recovered, in 2012 were also within the top 12 species recovered in 2011. The most abundant species in 2012 were *P. sylvaticum* (14.5%), *P. heterothallicum* (12.2%), *Phytophthora sojae* (9.3%), *P. ultimum* var. *ultimum* (6.1%), *P. perplexum* (6.0%), *P. irregularare* (5.8%), *P. oopapillum* (3.2%), *P. inflatum* (2.9%), *P. attrantheridium* (2.7%), *P. intermedium* (2.7%), *P. rostratifringens* (2.6%), and *P. ultimum* (2.5%) (Fig. 2). Other genera recovered from soybean seedlings that were outside the scope of this study included members of the fungal genera *Mortierella*, *Mucor*, *Gongronella*, *Rhizoctonia*, and the mycoparasite genus *Laetisaria*.

Among the 84 species isolated in this study, only 13 species were previously reported to be associated with soybean in the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) fungal-host database (<http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm>), including the well-known pathogen *Phytophthora sojae*. The isolation frequency of *Phytophthora sojae* at the early plant-growth stage sampled was 0.5% in 2011. A second semiselective medium was included in 2012, in an attempt to increase the recovery of *Phytophthora sojae* and other *Phytophthora* species. In 2012, the recovery of *Phytophthora* spp. increased to 12% when different

methods (different medium and numbers of plants per medium) were used. The frequency of *Phytophthora* spp. increased but still remained low in comparison with genus *Pythium*. The low recovery could be attributed to recovery of fast-growing species, such as *Pythium* and *Mortierella* spp., that interfere with the isolation of *Phytophthora* spp. (Tsao and Guy 1977).

Summarizing the data by clade (Fig. 3), *Pythium* clades F (36% in 2011 and 26% in 2012) and B (23% in 2011 and 13% in 2012) were the most abundant clades isolated during this study. These clades contain known pathogenic species such as *P. sylvaticum* and *P. irregularare* in clade F and *P. oopapillum* and *P. torulosum* in clade B. With respect to *Phytophthora*, clade 7 was present at a frequency

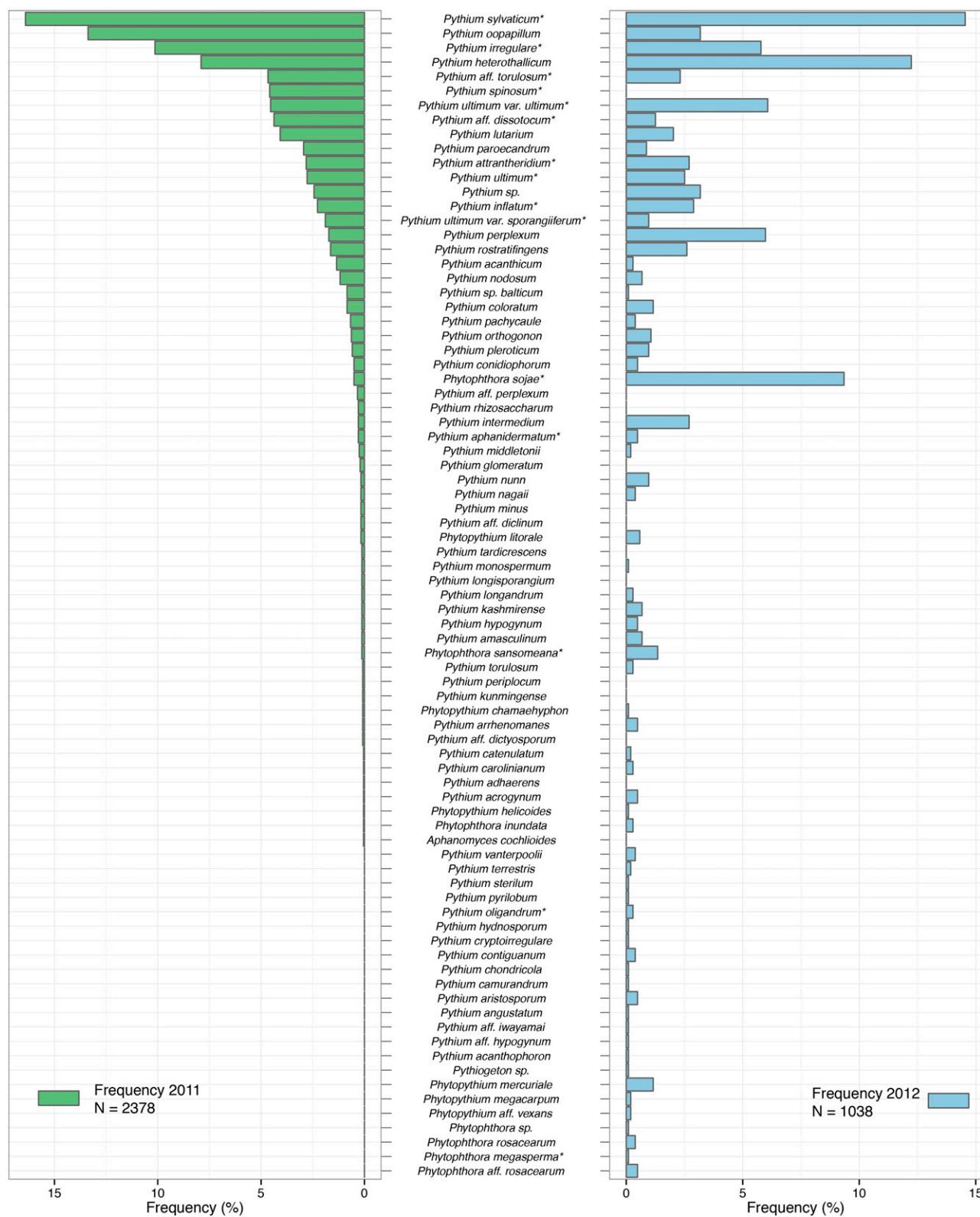


Fig. 2. Frequency at which different oomycete species were recovered from diseased soybean seedlings in 2011 and 2012. Asterisks (*) indicate species previously reported as associated with soybean in the USDA-ARS fungal-host database (<http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm>).

of 1% in 2011 and 4% in 2012, and clade 8 at <1% in 2011 and 3% in 2012. *Phytophthora* clades 7 and 8 contain the species *Phytophthora sojae* and *P. sansomeana*, respectively. The recently recognized genus *Phytopythium* was recovered at a lower frequency with respect to the other related genera and was detected at just

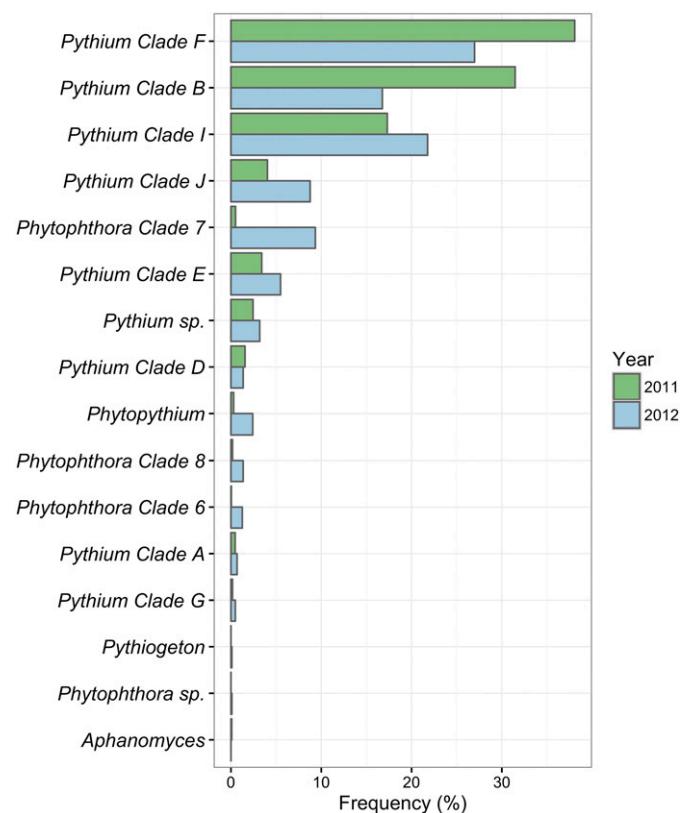


Fig. 3. Frequency of the oomycete species summarized by clade for 2011 and 2012. Oomycete genera outside of *Pythium* and *Phytophthora* were summarized by genus. Those species designated as spp. are not well-resolved, based only on the internal transcribed spacer sequence.

TABLE 1. Mean disease severity index (DSI) of soybean cv. Sloan seeds in response to multiple oomycete species as compared with the noninoculated control at 13°C or 20°C^a

Species	No. ^b	Disease index (%) 13°C ^c		Disease index (%) 20°C	
		Mean ± SE	P value	Mean ± SE	P value
<i>Phytophthora drechsleri</i>	1	13.06 ± 3.19	NS	90.28 ± 2.02	0.049
<i>Phytophthora sansomeana</i>	2	58.89 ± 10.01	NS	94.72 ± 1.31	0.004
<i>Phytopythium helicoides</i>	2	22.50 ± 1.90	NS	91.94 ± 1.61	0.004
<i>Pythium aff. diclinum</i>	3	78.70 ± 2.50	0.032	32.96 ± 5.28	NS
<i>P. aff. dictyosporum</i>	2	93.33 ± 1.14	0.006	85.14 ± 4.22	0.017
<i>P. aphanidermatum</i>	3	53.24 ± 3.82	NS	99.91 ± 0.09	<0.001
<i>P. cryptoirregulare</i>	1	99.72 ± 0.28	0.018	95.56 ± 1.00	0.021
<i>P. intermedium</i>	3	83.06 ± 4.78	0.016	53.43 ± 6.64	NS
<i>P. irregularare</i>	3	98.89 ± 0.45	0.001	80.46 ± 3.53	0.027
<i>P. kunmingense</i>	2	100.00 ± 0.00	0.002	89.31 ± 1.50	0.007
<i>P. paroecandrum</i>	3	93.98 ± 1.64	0.002	49.26 ± 4.37	NS
<i>P. spinosum</i>	3	80.56 ± 4.12	0.023	44.72 ± 7.00	NS
<i>P. sylvaticum</i>	3	99.44 ± 0.24	0.001	74.44 ± 2.64	NS
<i>P. terrestris</i>	1	99.17 ± 0.59	0.021	77.78 ± 3.42	NS
<i>P. ultimum</i>	3	99.17 ± 0.30	0.001	99.26 ± 0.32	<0.001
<i>P. ultimum</i> var. <i>sporangiferum</i>	3	96.48 ± 1.04	0.001	98.06 ± 0.72	0.001
<i>P. ultimum</i> var. <i>ultimum</i>	3	99.81 ± 0.13	0.001	99.63 ± 0.22	<0.001
Control		2.58 ± 0.31	—	8.73 ± 0.77	—

^a Only species with significant differences from the control at either temperature are represented. A seed-rot assay was used to determine pathogenicity of oomycete species using a disease severity scale of 0–4 to rate individual seeds. Data were transformed to DSI (0 = nonpathogenic; 100 = highly virulent). A total of 84 species were tested at both temperatures.

^b Number of isolates tested per species.

^c SE = standard error. P value based on Dunnett's test, significantly different from the non-rice control ($\alpha = 0.05$); NS = not significant.

1% and 3%, respectively, in 2011 and 2012 (Fig. 2). There were 91 isolates designated as *Pythium* spp. that were not resolved to species level, but these are currently under further evaluation.

Seed pathogenicity. The main goal of this assay was to determine which species were pathogenic to soybean (i.e., resulting in significant seed rot compared with a noninoculated control) at temperatures representative of planting conditions in the northern and southern United States. The seed-rot assay at 20°C identified 11 species that were pathogenic, with DSI scores ranging from 80 to a maximum score of 100 (Table 1; Supplementary Table S2). Among the species observed, *P. aphanidermatum*, *P. ultimum* sensu lato, and *P. cryptoirregulare* were the most virulent and caused severe seed rot. In addition to *Pythium* spp., *Phytophthora sansomeana*, *Phytophthora drechsleri*, and *Phytopythium helicoides* were also pathogenic on soybean seed, colonizing the seed and causing significantly reduced germination at 20°C.

Evaluation of seed rot at 13°C identified 13 *Pythium* spp. as pathogenic, with DSI scores ranging from 78 to a maximum score of 100 (Table 1). However, none of the *Phytophthora* or *Phytopythium* spp. was identified as being pathogenic at this temperature, typical of planting conditions in the Midwest. Only seven *Pythium* spp. were pathogenic at both temperatures, with *P. ultimum* sensu lato and *P. cryptoirregulare* being the most virulent. However, there were shifts in virulence observed among oomycete species at the different temperatures. For instance, *P. sansomeana*, *P. drechsleri*, and *Phytopythium helicoides* showed less virulence at 13°C than 20°C (Fig. 4; Table 1), while *P. sylvaticum*, *P. terrestris*, and *P. paroecandrum* appeared more virulent at 13 but not at 20°C.

Due to the large range of virulence responses, disease severity indices at 13 and 20°C were compared using a hierarchical clustering to group oomycete species, which resulted in three defined clusters (Supplementary Fig. S1). Cluster A represents all species that did not have a negative effect on seed germination. Cluster B contains species with virulence that were not significantly different from the control but still caused reduced seed health, expressed as DSI. Cluster C includes highly virulent species that were significantly different from the control and two species that were not significantly different from the control, *P. lutarium* and *P. coloratum*, which had DSI scores at 13°C of 62.7 and 49.3 and at 20°C of 71.7 and 49.6, respectively.

Seedling root-rot assay. Five parameters were measured to determine which species were detrimental to growth of soybean seedlings: root dry weight, shoot dry weight, shoot/root ratio, root area, and root length. Using PCA, the five parameters were evaluated for their contribution in the discrimination of the different species. All of the parameters measured showed differences between the inoculated treatments and the noninoculated controls (Supplementary Figs. S2 and S3). The analysis showed that root area, root length, and weight per root had the greatest contribution in separating the species in PCA 1, explaining 67.7% of the variability observed in the data. Shoot dry weight and shoot/root ratio contributed only 13 and 18% of the variability, respectively. Therefore, shoot dry weight and shoot/root ratio were not used in further analyses. The other three parameters had high correlation values (weight per root, $r^2=0.955$, $P \leq 0.001$; root length, $r^2 = 0.934$, $P \leq 0.001$; root area, $r^2 = 0.921$, $P \leq 0.001$) with the first dimension of the PCA, while the shoot dry weight correlation was lower ($r^2 = 0.730$).

Based on the PCA results, a MANOVA analysis was conducted, using the three parameters: root area, root length, and weight per root (Supplementary Fig. S4). Dunnett's test identified 43 oomycete species as significantly different from the non-rice control. In addition, the noninoculated control was not significantly different from the non-rice control. These parameters had a negative effect on the combined parameters measured on the soybean seedlings

(Table 2). In order to determine the contribution of each variable and further explore the results obtained in the MANOVA analysis, univariate analyses were performed on the three parameters, root dry weight, root length, and root area. The univariate analysis for root area showed similar results to the MANOVA analysis, resulting in 43 species with a significant effect on seedlings when compared with the non-rice control. A total of 21 oomycete species were determined to have a significant detrimental effect on seedlings across the multivariate and univariate analyses (Table 2).

Of the 21 pathogenic species across the multivariate and univariate analyses, the *Phytophthora* spp. *Phytophthora sojae*, *Phytophthora sansomeana*, and *Phytophthora drechsleri* were the most virulent, causing significant root reduction or death of radicles. The remaining pathogenic species belonged in the genus *Pythium*, and most species were within clade F (*P. cryptotrichum*, *P. irregularare*, *P. sylvaticum*, *P. atrantheridium*, *P. intermedium*, and *P. kumingense*) and clade B (*P. aff. dissotocum*, *P. aff. torulosum*, *P. aff. diclinum*, *P. aff. dictyosporum*, *P. lutarium*, and *P. oopapillum*). Clades with lower numbers of species found during this study were also designated as pathogenic based on our analysis, like *Pythium* clade I (*P. heterothallicum*, *P. ultimum* sensu lato), clade D (*P. periplocum*), and *Aphanomyces cladogamus*. All of the *Phytophthora*, *Pythium*, and *Aphanomyces* isolates evaluated caused a considerable reduction in root development and, in some cases, death of the radicle as well (Supplementary Fig. S5).

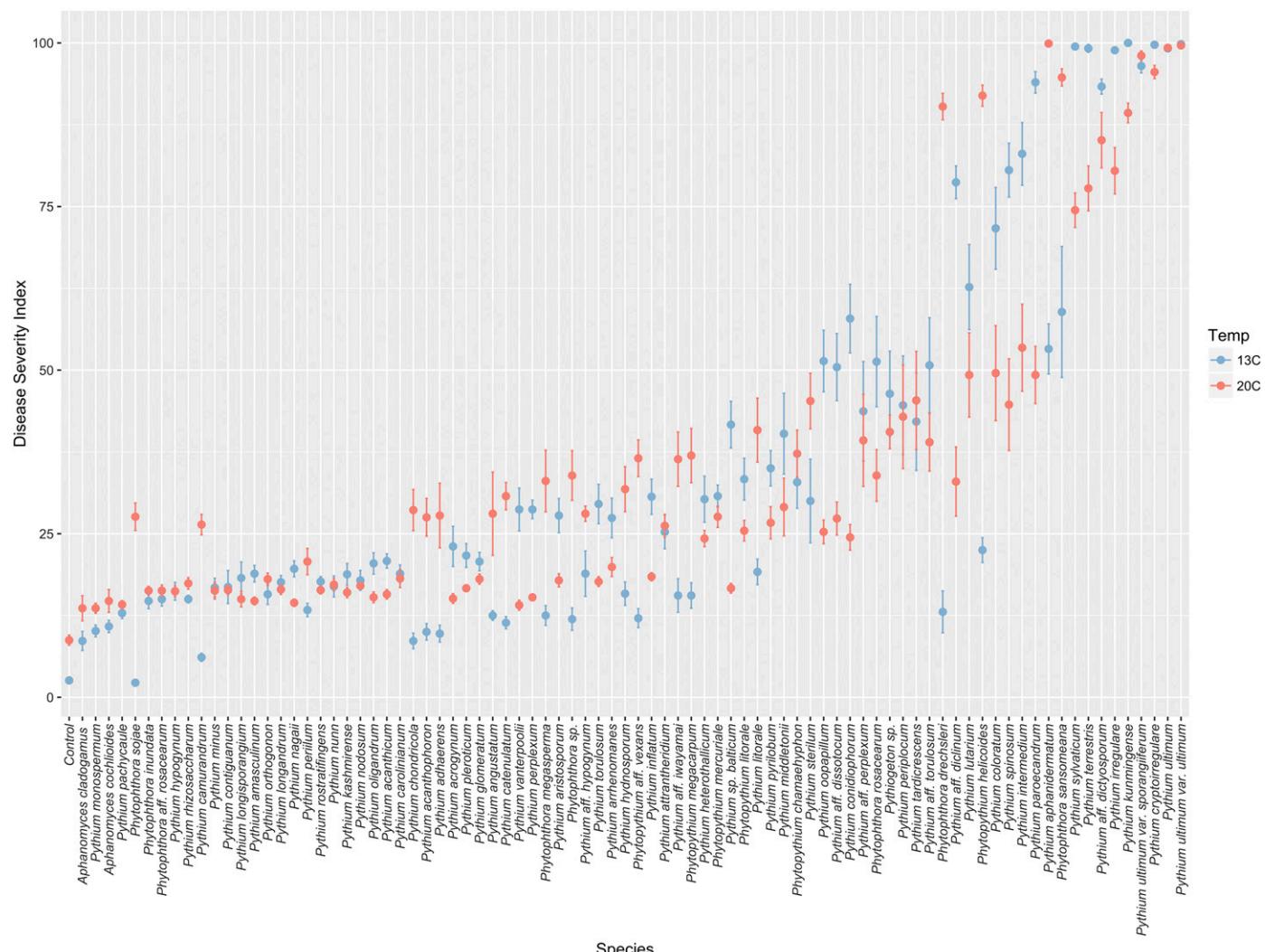


Fig. 4. Mean disease severity index (DSI) of 84 oomycete species screened in a seed-rot assay at 13 and 20°C. Bars represent standard error and DSI values from 0 = nonpathogenic to 100 = highly virulent.

Of the 43 species that were significantly different from the non-rice control in the MANOVA analysis, there were 22 species that were significant in only two or one of the univariate analyses, including members of the *Pythium* clades B, F, I and E as well as different species of genera *Phytophytum* and *Aphanomyces* (Table 2).

The remaining 41 oomycete species did not have a significant effect on seedlings, based on root area, root length, and dry weight per root (Supplementary Table S3). Interestingly, *Pythium* Clade B, which contains most of the pathogenic species, also included nonpathogenic species (*P. inflatum*, *P. catenulatum*, *P. angustum*). In addition to clade B, other clades were also represented, including *Pythium* clade E (*P. acrogynum*, *P. middletonii*, *P. pleroticum*), clade J (*P. perplexum*, *P. nodosum*, *P. orthogonon*), and clade D

(*P. amasculinum*, *P. oligandrum*, *P. acanthicum*) (Fig. 5). Among the less-frequent nonpathogenic species, *Pythium* clade F and A were represented with three species each and clade I and G with one species each. Apart from *Pythium* spp., three species from *Phytophthora* clade 6 were nonpathogenic, followed by two *Phytophytum* spp. and one species of the genus *Pythiogeton*.

Koch's postulates were completed by reisolation from inoculated seedlings and identification of isolates via SSCP. All of the species designated as pathogenic on the seedlings were isolated and confirmed by SSCP, having two or more successful isolation events. Of the nonpathogenic species, most of the species used in the seedling cup assay were recovered at least once, except *Phytophthora megasperma*, *P. adhaerens*, and *P. chondricola*, which we failed to reisolate.

TABLE 2. Forty-three oomycete species highly virulent on soybean cv. Sloan in the seedling root-rot assay measured as root area, root length and dry weight per root^a

Species	No. ^b	MANOVA P value ^c	Root area (cm ²) ^d		Root length (cm) ^d		Dry weight per root (mg) ^e	
			Mean ± SE ^f	P value ^g	Mean ± SE	P value	Mean ± SE	P value
<i>Aphanomyces cladogamus</i>	1	<0.001	2.33 ± 0.50	<0.001	31.65 ± 7.18	0.002	28.67 ± 4.85	0.044
<i>Aphanomyces coelchioides</i>	1	0.019	4.38 ± 0.61	0.019	53.98 ± 9.33	1.000	38.00 ± 6.00	1.000
<i>Phytophthora drechsleri</i>	1	<0.001	2.26 ± 0.58	<0.001	21.70 ± 5.61	<0.001	16.44 ± 2.86	<0.001
<i>Phytophthora rosacearum</i>	3	<0.001	4.97 ± 0.63	<0.001	58.23 ± 8.62	0.031	42.00 ± 2.38	1.000
<i>Phytophthora sansomeana</i>	2	<0.001	0.26 ± 0.06	<0.001	2.25 ± 0.56	<0.001	2.33 ± 0.52	<0.001
<i>Phytophthora sojae</i>	3	<0.001	2.15 ± 0.39	<0.001	28.18 ± 5.32	<0.001	20.93 ± 3.65	<0.001
<i>Phytophytum</i> aff. <i>vexans</i>	2	0.001	4.81 ± 0.44	0.001	54.68 ± 4.39	0.388	38.33 ± 1.96	0.587
<i>Phytophytum</i> <i>chamaephphon</i>	3	<0.001	4.64 ± 0.35	<0.001	54.82 ± 3.55	0.163	42.07 ± 2.07	1.000
<i>Phytophytum</i> <i>helicoides</i>	2	<0.001	3.79 ± 0.32	<0.001	41.13 ± 2.73	0.016	36.11 ± 1.70	0.251
<i>Phytophytum</i> <i>litorale</i>	3	0.002	5.08 ± 0.35	0.002	63.17 ± 4.45	0.677	53.48 ± 1.73	1.000
<i>Phytophytum</i> <i>mercuriale</i>	3	0.005	5.29 ± 0.36	0.005	65.89 ± 4.23	1.000	54.82 ± 1.76	1.000
<i>Pythium</i> aff. <i>diclinum</i>	3	<0.001	1.77 ± 0.43	<0.001	18.60 ± 4.56	<0.001	28.74 ± 4.58	<0.001
<i>P. aff. dictyosporum</i>	2	<0.001	2.19 ± 0.50	<0.001	22.22 ± 5.38	<0.001	18.78 ± 4.11	<0.001
<i>P. aff. dissotocum</i>	3	<0.001	2.72 ± 0.30	<0.001	38.13 ± 4.28	<0.001	27.93 ± 2.55	<0.001
<i>P. aff. torulosum</i>	3	<0.001	2.89 ± 0.35	<0.001	38.98 ± 5.00	<0.001	28.22 ± 2.54	<0.001
<i>P. aphanidermatum</i>	3	<0.001	4.07 ± 0.35	<0.001	51.26 ± 4.60	0.016	56.74 ± 2.91	1.000
<i>P. attrantheridium</i>	3	<0.001	2.05 ± 0.25	<0.001	27.77 ± 3.46	<0.001	23.26 ± 2.37	<0.001
<i>P. coloratum</i>	3	<0.001	4.32 ± 0.52	<0.001	51.89 ± 6.57	0.003	56.59 ± 3.76	1.000
<i>P. conidiophorum</i>	3	0.002	5.52 ± 0.62	0.002	64.87 ± 6.97	0.343	59.48 ± 3.86	1.000
<i>P. contiguum</i>	3	0.019	5.44 ± 0.47	0.019	66.42 ± 4.92	1.000	62.07 ± 2.86	1.000
<i>P. cryptoirregularis</i>	1	<0.001	1.74 ± 0.22	<0.001	17.28 ± 2.34	<0.001	19.56 ± 2.38	<0.001
<i>P. heterothallicum</i>	3	<0.001	2.43 ± 0.25	<0.001	34.06 ± 3.96	<0.001	27.56 ± 2.35	<0.001
<i>P. hypogynum</i>	3	0.002	5.16 ± 0.38	0.002	62.81 ± 4.85	0.339	53.19 ± 2.23	1.000
<i>P. intermedium</i>	3	<0.001	3.41 ± 0.63	<0.001	42.13 ± 7.78	<0.001	41.85 ± 5.69	<0.001
<i>P. irregularare</i>	3	<0.001	1.62 ± 0.29	<0.001	19.66 ± 3.98	<0.001	20.96 ± 2.33	<0.001
<i>P. kunningense</i>	2	<0.001	1.30 ± 0.11	<0.001	10.64 ± 1.17	<0.001	13.67 ± 1.51	<0.001
<i>P. litorale</i>	1	0.023	4.60 ± 0.62	0.023	53.38 ± 6.66	1.000	38.00 ± 3.99	1.000
<i>P. longandrum</i>	3	0.049	5.58 ± 0.40	0.049	67.10 ± 4.51	1.000	59.26 ± 2.70	1.000
<i>P. longisporangium</i>	3	0.004	5.84 ± 0.58	0.004	61.18 ± 5.41	0.139	44.67 ± 3.26	0.894
<i>P. lutarium</i>	3	<0.001	2.54 ± 0.33	<0.001	33.43 ± 4.88	<0.001	26.15 ± 2.54	<0.001
<i>P. minus</i>	3	0.001	5.08 ± 0.37	0.001	58.63 ± 3.78	0.451	48.44 ± 2.73	1.000
<i>P. nagaii</i>	3	0.026	5.60 ± 0.32	0.026	71.57 ± 3.60	1.000	52.89 ± 2.11	1.000
<i>P. nunnii</i>	3	0.039	5.76 ± 0.41	0.039	69.77 ± 4.60	1.000	61.56 ± 2.41	1.000
<i>P. oopapillum</i>	3	<0.001	2.67 ± 0.37	<0.001	36.52 ± 4.98	<0.001	27.93 ± 2.97	<0.001
<i>P. periilium</i>	3	0.002	5.54 ± 0.56	0.002	59.44 ± 5.38	0.186	49.56 ± 4.09	1.000
<i>P. periplocum</i>	3	<0.001	5.00 ± 0.72	<0.001	52.74 ± 7.38	<0.001	38.49 ± 4.39	0.003
<i>P. sylvaticum</i>	3	<0.001	2.00 ± 0.24	<0.001	26.12 ± 3.42	<0.001	28.67 ± 3.03	<0.001
<i>P. tardicrescens</i>	3	<0.001	4.75 ± 0.63	<0.001	57.75 ± 7.89	0.001	43.70 ± 4.01	0.104
<i>P. terrestris</i>	1	<0.001	2.38 ± 0.37	<0.001	23.48 ± 4.14	<0.001	36.22 ± 4.58	1.000
<i>P. ultimum</i>	3	<0.001	0.18 ± 0.04	<0.001	1.54 ± 0.43	<0.001	5.11 ± 1.40	<0.001
<i>P. ultimum</i> var. <i>sporangiferum</i>	3	<0.001	1.34 ± 0.24	<0.001	15.20 ± 3.17	<0.001	19.20 ± 2.50	<0.001
<i>P. ultimum</i> var. <i>ultimum</i>	3	<0.001	0.48 ± 0.07	<0.001	4.23 ± 0.74	<0.001	8.26 ± 1.25	<0.001
<i>P. vanterpoolii</i>	3	0.022	6.02 ± 0.57	0.022	70.14 ± 7.03	1.000	47.70 ± 2.31	1.000
Control		0.339	7.50 ± 0.28	0.339	92.33 ± 3.15	1.000	59.14 ± 1.71	1.000
Non-rice control		NA ^g	10.09 ± 0.34	NA	111.72 ± 3.12	NA	68.32 ± 2.13	NA

^a Only species with significant differences from the non-rice control are represented. Data were analyzed by multivariate analysis of variance (MANOVA) combining all the parameters: root area, root length and weight per root; and followed by univariate analysis for each of the measured parameters. Plants were grown at 20°C in a growth chamber for 2 weeks.

^b Number of isolates tested per species.

^c P value based on MANOVA, significantly different from the non-rice control ($\alpha = 0.05$).

^d Root area and length were determined by using ASSESS 2.0 (American Phytopathological Society).

^e Dry weight per root was established after drying plants at 50°C for 48–72 h.

^f SE = standard error; NA = not applicable.

^g P value for univariate analysis based on Dunnett's test, significantly different from the non-rice control ($\alpha = 0.05$).

Comparison of seed and seedling root-rot assays. Among the species evaluated, *P. paroecandrum* and *P. spinosum* were the only species that caused seed rot and did not cause significant damage to plants in the seedling assay. The remaining species determined to be pathogenic with the seed-rot assay were also

identified as pathogenic with the seedling assay (Fig. 5). Among the species pathogenic on seed and seedling, *P. terrestris*, *P. aphanidermatum*, and *Phytophytium helicoides* were the only species that were not significant for root weight univariate analysis, the rest of species were significant for all analyses. Taking

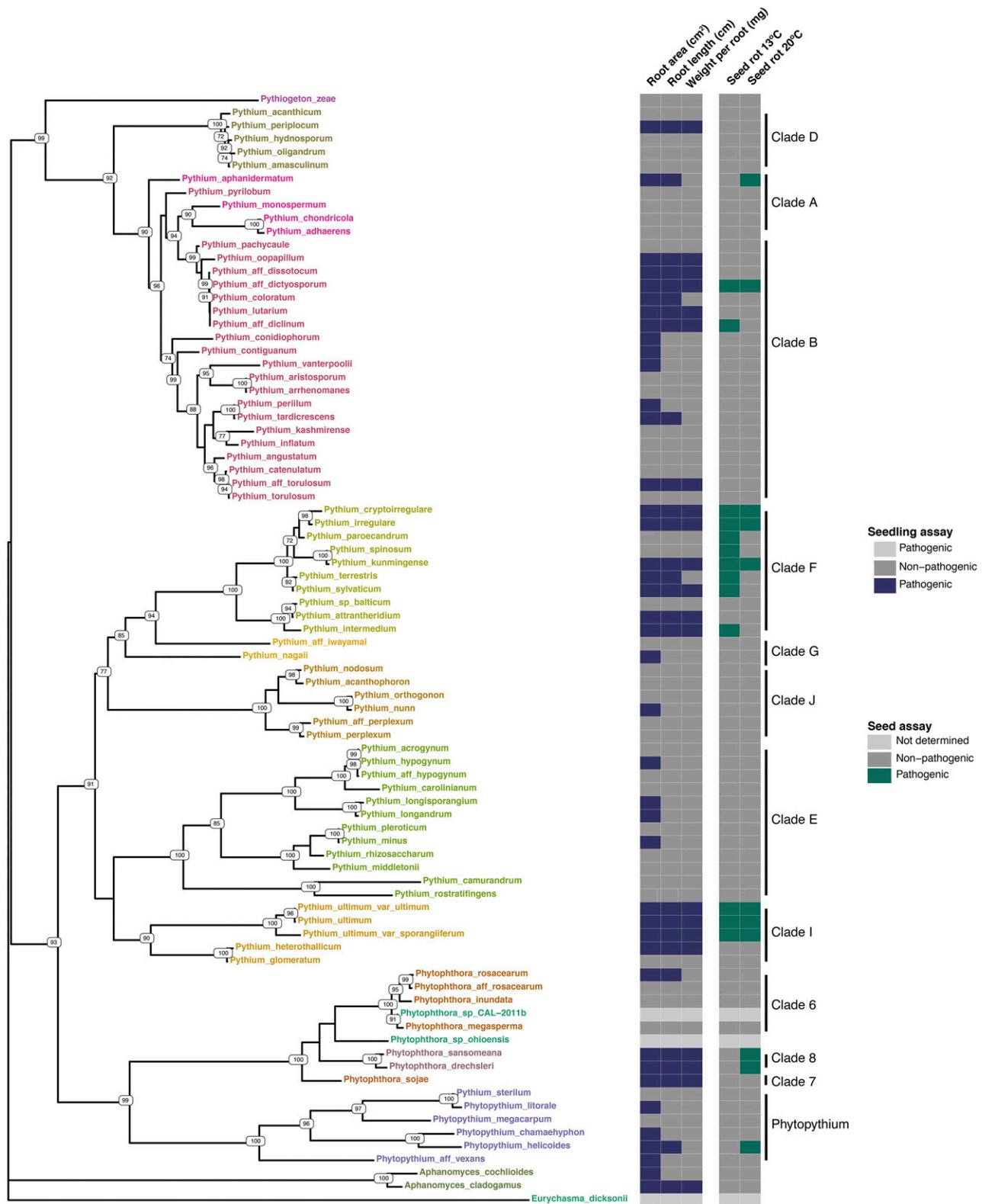


Fig. 5. Maximum likelihood phylogeny of the internal transcribed spacer sequences of the rDNA for oomycete species found during the survey. Numbers on the branches indicated bootstrap values for 1,000 replicates (>70). Phylogenetic distribution of pathogenicity traits mapped to taxa represented in the tree. Tip shades indicate members of different clades. Parameters in light gray represent taxa not isolated in the study.

pathogenicity into account, the prevalence of these species was evaluated by state, using the data from the survey (Fig. 6). The species *P. sylvaticum*, *P. heterothallicum*, *P. ultimum* sensu lato, *P. oopapillum*, and *P. aff. dissotocum* were pathogenic and also prevalent across most states sampled during the survey (Fig. 6). Among the nonpathogenic species under the conditions of this study, *P. perplexum*, *P. rostratifringens*, and *P. inflatum* were the most prevalent across the sampled states.

DISCUSSION

The current study was undertaken to acquire a greater understanding of the oomycete communities associated with and potentially involved in soybean seedling diseases across the major United States soybean-producing states and Ontario, Canada. A total of 84 oomycete species were identified out of 3,416 isolates collected primarily from diseased soybean seedlings over the years 2011 and 2012. The 84 species belonged to the genera *Pythium* (94.85%), *Phytophthora* (4.15%), *Phytophytium* (0.91%), *Aphanomyces* (0.06%), and *Pythiogeton* (0.03%). Of the 84 species, 43 were determined to be pathogenic to seeds or seedlings, with the majority of isolates being pathogenic to both seeds and seedlings. The majority of the isolates recovered were pathogenic on soybean belonging to *Pythium* clades F, B, and I, which are known to contain the majority of pathogenic *Pythium* species (Lévesque and De Cock 2004).

Between years 2011 and 2012, the number of isolates changed considerably, despite using the same sampling approach. In 2011, a

total of 2,380 isolates were collected, whereas in 2012 only 1,038 were collected. The difference in recovery of oomycetes could be due to the drought and temperature differences between years. For instance, in 2011, in the midwestern United States, from April to June, 5% of the region was identified as experiencing moderate drought conditions, with an additional 3.5% ranked as abnormally dry. However, from April to June in 2012, by comparison, 18% of the region experienced moderate drought and 47% was classified as abnormally dry (<http://droughtmonitor.unl.edu/>). The average environmental temperature in this same region for the period from April to June in 2011 was 16°C and in 2012 was 18°C (<http://www.ncdc.noaa.gov/temp-and-precip/climatological-rankings>). These dry conditions could have impacted the recovery of species due to reduced infection, since soil water serves as a carrier for chemical root stimulants and provides conditions for oospore germination, sporangia formation, and zoospore locomotion (Martin and Loper 1999).

One of the goals of the second year was to increase the number of *Phytophthora* spp. recovered; therefore, a second medium was included to improve recovery. The medium was amended with hymexazol to inhibit *Pythium* spp.; however, it is known that it can also affect some *Phytophthora* spp. (Jeffers 1986). In general, the V8-RPBH medium reduced the recovery of *Pythium* spp., but a small percentage of isolates were still recovered from most *Pythium* clades (Supplementary Fig. S6). Aiming to increase the recovery of *Phytophthora* spp., samples were plated into two different medium that could also affect the number of oomycetes recovered, in addition to the other environmental factors mentioned above. The

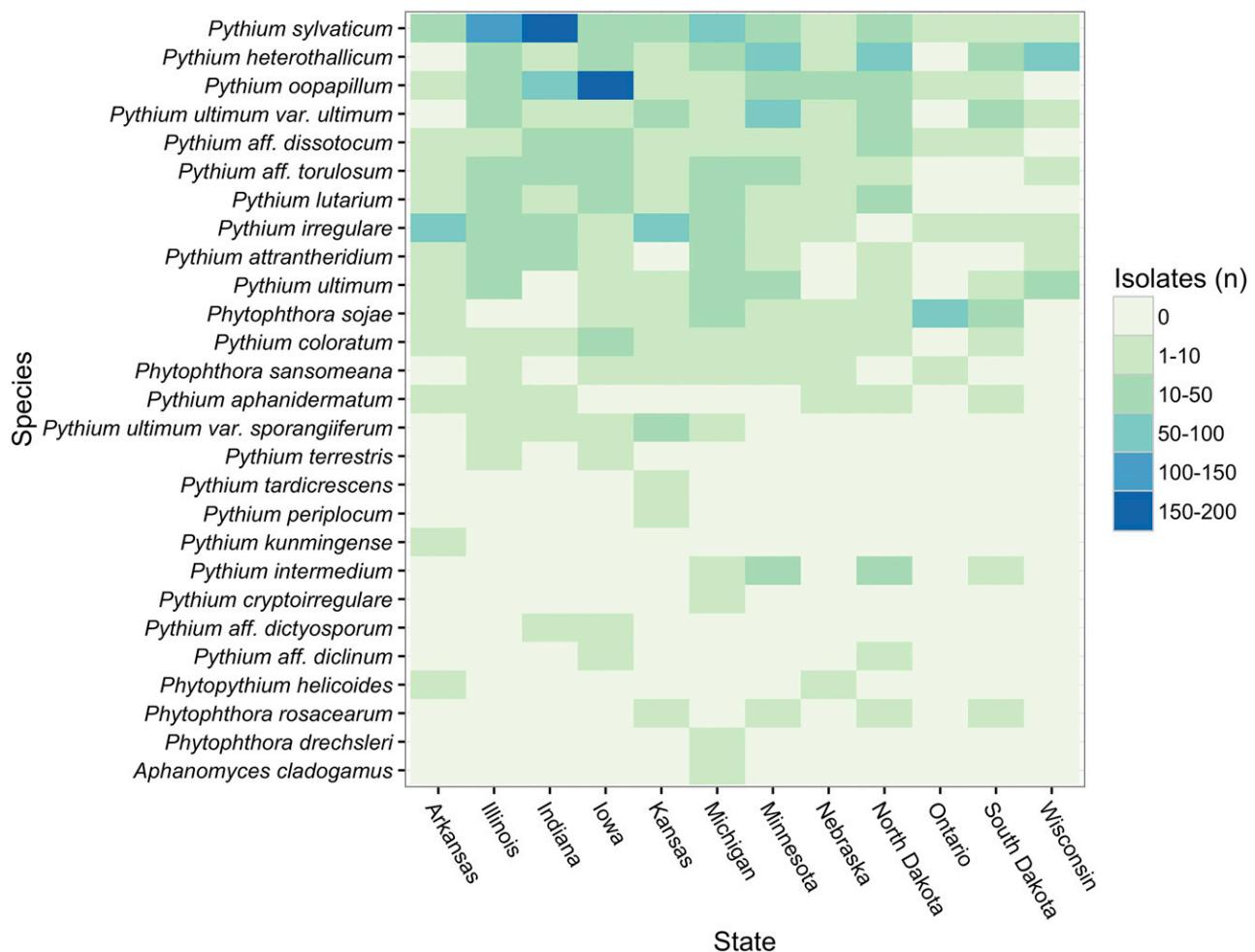


Fig. 6. Prevalence of pathogenic oomycete species, designated based on seedling assay data, across the states sampled during the current study. Color gradient indicates number of isolates per species collected per state during 2011 and 2012.

medium did increase the recovery of *Phytophthora* spp., but the numbers in comparison with those of *Pythium* spp. were still low. The frequency of isolation of *Phytophthora* spp. is affected by fast-growing species, such as members of genera *Pythium* and *Mortierella*, which can still be recovered in the presence of hymexazol (Tsao and Guy 1977). In *Phytophthora* clade 7, which contains *Phytophthora sojae*, recovery increased 20% on the V8-RPBH medium in comparison with the CMA-PARPB medium. Interestingly, this medium also increased the recovery of other genera such as *Phytophytium* and *Pythiogeton*. Ontario showed a biased recovery of *Phytophthora sojae* due to modification in the isolation protocol, which utilized baiting and modified conditions such as soil moisture saturation, to increase the recovery of this pathogen.

Previous surveys examining the diversity of *Pythium* spp. associated with symptomatic soybeans characterized a range of 11 to 27 different species present in individual states (Broders et al. 2007, 2009; Zitnick-Anderson and Nelson 2015). Most species found in this multistate survey were in agreement with other studies that focused on soybean root rot, including common species such as *P. ultimum* sensu lato, *P. sylvaticum*, and *P. irregularare*. However, other species such as *P. echinulatum* and *P. graminicola* were not isolated during our survey but have been reported from soybean fields in Ohio (Broders et al. 2007). In North Dakota, an extensive survey reported similar species to the ones found in our study, especially the most abundant species like *P. ultimum*, *P. heterothallicum*, and *P. sylvaticum*. However, there were differences in the least abundant species, as indicated by *P. debaryanum* and *P. violae*, which were not recovered in our study (Zitnick-Anderson and Nelson 2015). Other studies in which the soil and the rhizosphere of soybean fields were sampled in order to characterize *Pythium* spp. recovered species similar to the ones in our study (Jiang et al. 2012; Marchand et al. 2014).

Previous studies have demonstrated the potential for multiple oomycete species to be present within a single root system of soybean plants (Broders et al. 2007; Zitnick-Anderson and Nelson 2015). It is important to distinguish which of the multiple oomycete species may have a detrimental effect on the root system. Seed rot and seedling root-rot assays have been used in several studies to characterize the pathogenicity of oomycete species. Broders et al. (2007) used two different assays to evaluate the pathogenicity of several *Pythium* spp. to corn and soybean. These assays have been used in various studies to evaluate the pathogenicity and virulence of oomycete species (Matthiesen et al. 2016; Zitnick-Anderson and Nelson 2015) in which both seed rot and seedling assays were scored using a qualitative visual assessment. In this study, we used both assays. However, for the seedling assay, quantitative data were collected using dry weights and software image analysis to quantify root area and root length, in order to measure the effect of the potential pathogenic species on soybean seedlings. Based on our results, root area was the most informative parameter to identify a greater number of pathogenic species. Similar approaches, including the determination of root area and dry weight of roots and shoots, have been used to characterize root-rot pathogens on cucurbits and other plants (Biernacki and Bruton 2001; Bock et al. 2010; Higginbotham et al. 2004).

The 84 oomycete species identified in this study were characterized for pathogenicity and virulence using a subset of up to three isolates per species for pathogenicity on seed and seedlings. Overall, the variability of virulence per species was low, but further characterization of more isolates, particularly with species such as *P. luteum* and *P. aff. torulosum*, is needed. This variability is expected in some species, due to the degree of genetic diversity and potential species complexes, as was reported for *P. ultimum* (Higginbotham et al. 2004). The seedling assay based on quantitative measurements captured a broad range of effects of the different species in a susceptible cultivar of soybean, identifying 43 species as pathogenic with different levels of virulence. A total of 21 species had

a detrimental effect in reducing all parameters measured, and the remaining 22 species reduced either one or two of the parameters, as compared with the non-rice control (Fig. 5). Most of the species identified as seedling pathogens were also characterized as seed pathogens. Only *P. paroecandrum* and *P. spinosum* caused seed rot at 13°C but did not cause root rot on seedlings. The remaining 41 of the 84 oomycete species did not significantly increase root rot compared with the control and were designated as nonpathogenic.

Environmental conditions often influence the outcome of the interaction of different *Pythium* spp. with soybean seedlings, since it has been observed that different species have temperature-mediated virulence (Matthiesen et al. 2016). In our study, this was observed in the seed-rot assay, where multiple species had a virulence shift based on temperature, being more virulent at either low or high temperatures. Similar behavior was reported for *P. torulosum* on seeds and seedlings, being nonpathogenic or having reduced virulence at temperatures of 18 and 23°C but increased virulence at 13°C (Matthiesen et al. 2016). The pH can also impact virulence; for instance, *P. debaryanum* is more virulent below pH 6.6, and some species increase their saprophytic activity around pH 7 (Martin and Loper 1999).

It has been suggested that plants infected with *Pythium* spp. have reduced vigor (Gilbert 2002; Paulitz et al. 2002; Pieczarka and Abawi 1978). The reduced vigor is often observed as stunted plants, necrotic root lesions, and leaf yellowing (Kirkpatrick et al. 2006). Therefore, measuring various root parameters is an approach to characterize and parse the effects of different species on the root system of soybean plants. The use of two controls, one with noninoculated rice and one of a non-rice control, were intended to rule out any negative effects of rice by itself on seedling and root development. However, we did not see statistical differences between the two controls in any of the tests conducted. Some of the species designated as nonpathogenic produced lesions in the seedlings but, based on the statistical analysis, their effect was negligible when compared against a non-rice control. Although several of the species were nonpathogenic in the assays used, it is possible that they may not have fully expressed their virulence due to the lack of certain conditions, such as temperature, pH, or interaction with other organisms (Becker et al. 2012; Littrell and McCarter 1970; Mondal and Hyakumachi 2000). On the other hand, the designation of nonpathogenic species based on our analysis also overlapped with the previous reports in which species like *P. nunn*, *P. orthogonon*, and *P. torulosum*, among others, did not cause significant symptoms on soybean plants (Zitnick-Anderson and Nelson 2015). Some of these species have been reported as mycoparasites or competitors. This is the case with *P. nunn*, which has niche overlap with *P. ultimum*, being a colonizer of organic matter without causing plant disease and parasitizing hyphae (Martin and Loper 1999). Therefore, the isolation of these species could be the result of niche overlap or these species could be parasitizing certain pathogenic *Pythium* spp. In addition, some of the species in our study resulted in observable (not significant) positive effects on the root parameters, resulting in values higher than the control. It has been observed that *P. oligandrum* and other *Pythium* spp. produced auxin-like products that could increase root formation or cause irregular root development (Le Floch et al. 2003).

Several of the species reported as pathogens in the USDA-ARS fungal-host database and reported here were prevalent in most of the states surveyed, including *P. sylvaticum*, *P. heterothallicum*, and *P. oopapillum* (Fig. 6). The species were present in most states, but their abundance varied across the different fields. Other pathogenic species were less prevalent but still present in low numbers in more than four states; such is the case with *P. ultimum*, *P. aff. dissotocum*, and *P. aff. torulosum*. In regards to *Phytophthora* spp., both *Phytophthora sojae* and *P. sansemeana* were recovered in low numbers in most states; however, this could be an artifact of sampling method and timing.

Due to the large number of isolates recovered in the study, we utilized ITS sequencing and BLASTn searches against a curated set of sequences recently compiled by Robideau et al. (2011) and sequences deposited in the Consortium for the Barcode of Life database (www.barcoding.si.edu/). Previous studies have utilized SSCP or a combination of morphological and sequence data for species identification. However, the resolution at species level of SSCP is limited, since it may not always capture the diversity, as the region utilized is not informative for all species. Although, conducting SSCP is cost effective, it does require the use of isolate standards or the additional sequencing or morphological identification of those isolates resulting in unique SSCP patterns. Sequence data provides an easily searchable and archive-ready data format. However, caution should be exercised when searching against the GenBank DNA sequence database, as the sequences are not highly curated and there is a high error rate in species labels (Kang et al. 2010). Zitnick-Anderson and Nelson (2015) used sequencing of the rDNA aided by morphological characterization of *Pythium* spp., which helped correct some of the molecular misidentifications based on poor sequence data in GenBank. It has been discussed previously that one gene might not reflect the species boundaries and caution should be used when setting a blast threshold (Kang et al. 2010). However, some precautionary measures can be used to reduce error, such as the length of the alignment and the database used. The ITS of rDNA and cytochrome c oxidase subunit I have been designated as barcodes for the oomycetes and, in some cases, either barcode gene is not enough to resolve some species, but these regions do have the most complete set of curated sequences and, if possible, sequencing both barcodes typically increases the confidence of the species designation (Kang et al. 2010; Robideau et al. 2011).

Previous to this study, a total of 24 oomycete species had been reported as root pathogens of soybean, 16 of which were also isolated in our study. In the present study, we report 13 oomycete species that are pathogens of soybean causing a detrimental effect on seedling roots that have not previously been associated with this crop. These included *P. drechsleri*, *P. cryptotrichum*, *P. kunmingense*, *P. periplocum*, *P. conidiophorum*, *P. longisporangium*, *P. contiguum*, *P. vanterpoolii*, *P. nagaii*, *P. longandrum*, *Phytophytium* aff. *vexans*, *Phytophytium* *litorale*, and *Aphanomyces* spp. However, the number of reported pathogenic species could be higher, but we are assuming that the affinity species were overlooked due to the lack of sequence resources to clearly identify this species and have previously been reported as the actual species. These include *P. aff. diclinum*, *P. aff. dictyosporum*, *P. aff. dissotocum*, and *P. aff. torulosum*, which were collected and characterized as pathogenic/virulent under this study's conditions. The current study provides an overview of characteristics and prevalence of the different oomycete species associated with seedling diseases in the major soybean-producing states. The diversity of species identified and characterized provides a valuable resource for the testing of different management strategies, evaluating fungicide resistance, and in selecting a pool of candidate pathogens to aid breeding programs focused on screening for resistance to oomycete pathogens.

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