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Diversity and Characterization of Oomycetes Associated with Corn Seedlings in Michigan

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ABSTRACT

Corn is a staple feed and biofuel crop with a value close to \$3.7 billion dollars for Michigan's economy. Knowledge about distribution and abundance of seedling pathogens in Michigan corn fields is limited. Here we used a combination of culture-based and next-generation sequencing of soil samples to determine the extent of species associated with diseased corn seedlings and those present in soil. Over 2 years, symptomatic seedlings and associated soil samples were collected from 11 Michigan fields. A total of 170 oomycete cultures were obtained from seedlings using a semiselective medium (CMA-PARPB) and identified using the internal transcribed spacer region. Thirty-three species were isolated, with *Pythium inflatum* (25%; clade B) and *P. sylvaticum* (12%; clade F) being the most abundant species. For the amplicon-based approach, the cytochrome oxidase subunit I marker (COI) mitochondrial region was amplified from soil samples and sequenced using Illumina MiSeq. The dominant *Pythium* clades present in the soil were F, I, D, and B and accounted for at

least 75% of the abundance in all locations. *Pythium* clades F, I, and D were recovered with similar trends with the culture and amplicon approach; however, clade B was highly abundant in plant isolation, but not in soil. The 20 most abundant species were characterized for pathogenicity and fungicide sensitivity. *P. irregulare* and *P. ultimum* var. *ultimum* were the most virulent at both 15 and 20°C. Isolates were tested for their sensitivity to mefenoxam and ethaboxam. Most isolates were sensitive to both chemistries, but *P. rostratifingens* and *P. aff. torulosum* were less sensitive to ethaboxam and *P. ultimum* var. *ultimum* less sensitive to mefenoxam. The survey and isolate characterization provides a better understanding of seedling and root rot disease of corn and opportunities to improve management of this disease complex.

Keywords: amplicon sequencing, corn, ecology, fungicide sensitivity, microbiome, mycology, pathogenicity, root rot, soil ecology

Corn production in Michigan has increased in the last 15 years from around 5 to 8.2 million metric tons (USDA-NASS 2016); however, pre- and postemergence damping off and root rot can result in stunted plants and reduced stand, sometimes resulting in the need for replanting. Weather conditions favorable for disease development such as temperature fluctuations and heavy precipitation events have increased in frequency, exposing

seedlings to conditions that favor infection by soilborne pathogens (Rosenzweig et al. 2001). The shift to early planting dates increases the chance of cold temperature exposure and imbibitional chilling injury (Stolla and Saab 2015). In addition, the move to reduced or no-till and cover crops increases plant residue and the potential for carryover of inoculum and the slowing of soil warming in the spring (Bockus and Shroyer 1998; Licht and Al-Kaisi 2005). These factors favor pathogens commonly associated with seed and seedling root rot.

The cost of corn seed has almost quadrupled in the past 20 years (Bunge and Newman 2018). The production costs are around \$332 dollars per acre, where corn seed represents 30 to 40% of costs. Uniform seed germination and stand establishment are key factors to maximizing yield potential (Nafziger et al. 1991), which emphasizes the importance of management and understanding of seedling diseases. Across the United States from 2012 through 2015, it was estimated that over 152 million metric tons (approximately U.S. \$27 billion) of corn was lost due to disease (Mueller et al. 2016). Of this, almost 10 million metric tons of corn (~US\$1.8 billion) was lost to seedling disease. During seedling

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establishment, seedlings are subject to attack by a number of soilborne pathogens, among these *Pythium* and *Phytophthora* are commonly identified as causal agents (Broders et al. 2007; Rao et al. 1978). Poor plant stands due to disease result in replanting and increased costs to growers. The impact of these soilborne diseases may not only be limited to the beginning of the season, as root infections can occur at later stages, often reducing yield without significant above ground symptoms. Seedling diseases in row crops are usually considered in broad pathogen groups, such as *Pythium*, *Rhizoctonia*, and *Fusarium*; however, knowledge of specific causal agents can aid in the management and control of specific causal agents. For example, the studies conducted by Rojas et al. (2016, 2017) highlighted which species of oomycetes are of primary concern for soybean production and identified regional differences in community composition (Rojas et al. 2016, 2017). This potentially opens the possibility of targeted seed or in-furrow treatments and the screening of germplasm against the most appropriate pathogenic species.

A corn-soybean rotation is common practice, although there are benefits for disease and insect control with this rotation, it has been observed that this scheme has not been effective in reducing soilborne pathogens, as many species are capable of causing disease on both crops (Broders et al. 2007; Radmer et al. 2017; Zhang et al. 1998). Besides the lack of efficacy in reduction of soilborne pathogens with a soybean-corn rotation, there is increased disease incidence related to other cultural practices, such as early planting. These practices and the environment interact and influence the microbial communities present in the soil, causing population shifts in the species present in the soil, which in turn can increase pathogenic species (Arcate et al. 2006; Schlatter et al. 2018).

Statewide and national surveys on the diversity of fungal and oomycete species associated with row crops have provided information on species distribution and potential causal agents (Broders et al. 2009; Paulitz and Adams 2003; Radmer et al. 2017; Rojas et al. 2017; Zitnick-Anderson and Nelson 2014). The combination of surveys with the characterization of isolate aggressiveness and fungicide sensitivity provides information to improve management practices and minimize the impact of these diseases. Classically, surveys based on isolation have been used to characterize the extent of species present. However, soilborne diseases are present in a complex system where soil heterogeneity, geographical location, plant species, root architecture, and a vast number of microbial species interact to suppress or promote disease. From an ecological standpoint, pathogens present in the soil coexist with an extensive number of nonpathogenic fungi, oomycete, and bacterial species. Various disturbances such as crop monocultures and limited rotations can boost the propagation of certain species that can result in the infection and colonization of plant roots, hence causing disease. Thus, next-generation sequencing has been demonstrated as a viable tool to facilitate a deeper understanding of how microbial communities are associated with plants, and can be used to gain a better knowledge of the dynamics of pathogen complexes present in the rhizosphere (Bakker et al. 2017; Gdanetz and Trail 2017; Schlatter et al. 2018). The use of microbial community analysis to study the diversity of oomycetes associated with corn production soils will provide a better understanding of the microorganism complexes that are also present with pathogens.

The knowledge of species causing corn seedling and root rot diseases in Michigan is very limited. Thus, the objectives of this study were to (i) determine the primary oomycete species associated with diseased corn seedlings in Michigan; (ii) identify species abundance in field soils; (iii) characterize the most abundant species for their pathogenicity and virulence; and (iv) determine the fungicide sensitivity of the most abundant species. To achieve these

objectives, we conducted a classic culture-based study and paired it with an amplicon sequencing approach to obtain a greater sampling depth of oomycete species present in the soil environment and determine those affecting corn in Michigan in 2011 and 2012. The use of this combined approach provides a better view of bulk microbial community and the potential pathogens in Michigan fields, and their response to chemistries, which will be important in developing disease management strategies.

MATERIALS AND METHODS

Sample collection, isolation, and identification. In 2011 and 2012, a total of 11 corn fields were surveyed across Michigan (Fig. 1; Supplementary Table S1). Fields were selected based on history of seedling diseases and plant stand issues. A similar sampling protocol as described by Rojas et al. (2016) was conducted, 50 symptomatic corn seedlings were collected from a W-shaped transect across each field. Due to crop rotation practices, diseased corn 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 refrigerated; all plant samples were processed within 24 h post-collection. Seedlings were prepared for isolation by excising the shoots and washing the roots under running tap water for 30 min until all visible soil was removed. Seedling roots were patted dry with sterile paper towel to eliminate excess water and 1-cm root sections of symptomatic tissue were cut-off using a sterile scalpel. Selected root tissue sections from all 50 plants per field were placed onto a semiselective medium, corn meal agar amended with PCNB (50 mg/liter), ampicillin (250 mg/liter), rifampicin (10 mg/liter), pimaricin (5 mg/liter), and benomyl (10 mg/liter) (CMA-PARP) (Jeffers and Martin 1986). Culture plates were incubated for 7 days at room temperature (20°C) and checked daily for hyphal growth and morphology consistent with oomycetes. If oomycete mycelial growth was observed, cultures were transferred to fresh CMA-PARP medium by hyphal tipping. From clean cultures, 5-mm plugs were taken from fresh cultures and transferred to potato carrot agar (PCA) slants and hemp seed vials

Fig. 1. Corn fields sampled in Michigan in 2011 and 2012 and corn planted acres indicated by color intensity at the county level.

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 incubated for 7 to 10 days at room temperature without agitation. Mycelia were harvested from broth cultures, lyophilized overnight, and ground for DNA extraction. For DNA extraction, 100 mg of ground mycelia were resuspended in 800 μ l of cetyltrimethylammonium bromide (CTAB) lysis buffer (AutoGen AG00121, AutoGen Inc.) and incubated for 1 h at 65°C. A phenol-chloroform automated DNA extraction was performed using the AutoGen 850 system (AutoGen Inc., Holliston, MA). DNA was resuspended in 200 μ l of TE buffer, incubated on an orbital shaker for 1 h, and then transferred to 1.5 ml tubes, and stored at –20°C.

Isolates were identified using the internal transcribed spacer (ITS) 1 and 2 of rDNA by amplification with primers ITS6 and ITS4

(Cooke et al. 2000). The PCR amplification reactions consisted of a final concentration of 1 $\times$ DreamTaq buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 μ M ITS6 and 0.2 μ M ITS4, bovine serum albumin (BSA) at 4 μ g/ml, 1U DreamTaq polymerase (Thermo Scientific, Waltham, MA), and 1 μ l 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 μ l of a mixture of 3 U of exonuclease I and 0.5 U of FastAP thermosensitive alkaline phosphatase (Thermo Scientific, Waltham, MA). 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 (accession numbers MK326392 to MK326561).

Soil DNA extraction and amplicon library. Bulk soil samples were collected from all the fields in which the corn seedlings were collected, except location MICO_1 for which no soil was collected. A standard sampling procedure was followed by collecting 15 to 20 soil cores to a depth of 15 cm in a W-shaped transect across each field. Soil cores were mixed together to form a composite soil sample and a 50-ml soil subsample was taken and stored at –80°C until processed for DNA extraction.

For soil DNA extractions, three replicates of 500 mg of soil were taken from each sample and extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, U.S.A.) with a modified protocol (Bilodeau and Robideau 2014). Briefly, 200 μ l of a solution of 100 mM aluminum ammonium sulfate dissolved in 0.1 M sodium phosphate buffer, pH 8.0, was added to Lysing Matrix E tube containing the soil, mixed and then 778 μ l of sodium phosphate buffer from the original kit and 122 μ l of the original MT buffer were added and the protocol was followed as recommended by the manufacturer. DNA was stored at –20°C until used for amplification.

The *coxI* gene of the mtDNA (COI) was amplified using a two-step PCR method. A first PCR used COI primers amplify the target region and the second PCR used the Fluidigm CS adapters linked to the Illumina adapters and barcodes allowing the multiplexing of samples (Supplementary Table S2). Prior to the first amplification, DNA was diluted 10-fold with molecular grade water. Amplification mix consisted of 1 $\times$ Q5 reaction buffer, 0.2 mM dNTPs, 0.5 μ M of each primer, 1 U of Q5 hot-start high fidelity polymerase (NEB, Ipswich, MA), and 1 μ l of DNA in a total volume of 25 μ l. The thermal cycling program consisted of 94°C for 30 s, 25 to 30 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 40 s, and final extension at 72°C for 10 min. All the primers were modified by adding a 2 bp pad-link, followed by the Fluidigm CS adapters at the 5' end. Amplicons were purified by adding 5 μ l of a mixture of 3 U of exonuclease I and 0.5 U of FastAP thermosensitive alkaline phosphatase (Thermo Scientific, Waltham, MA), followed by 45 min at 37°C, and enzymes were inactivated by incubation at 85°C for 10 min. A non-template control and mock community containing DNA from 15 oomycete species were included in the analysis (*Phytophthora cactorum*, *Phytophthora citricola*, *Phytophthora nicotiana*, *Phytophthora sansomeana*, *Phytophthora sojae*, *Phytophthora litorale*, *Pythium aff. dissotocum*, *Pythium aff. torulosum*, *Pythium attrantheridium*, *Pythium heterothallicum*, *Pythium irregulare*, *Pythium lutarium*, *Pythium oopapillum*, *Pythium ultimum* var. *sporangiiferum*, and *Pythium ultimum* var. *ultimum*). All species were mixed together at final concentration of 0.5 ng/ μ l, and the mock community was diluted 1:10 before PCR.

Fig. 2. Number of isolates recovered from symptomatic corn seedlings collected in Michigan in 2011 and 2012 using semiselective medium.

PCR products were submitted to the Research Technology Support Facility Genomics Core at Michigan State University to carry out a second PCR using the dual index paired-end approach for the Illumina MiSeq as described by Kozich et al. (2013). The Research Technology Support Facility Genomics Core performed secondary amplification using dual barcoded primers. After secondary PCR, the products were normalized using Invitrogen SequelPrep DNA Normalization plates and normalized DNA products were pooled into a single library. After QC and quantitation of the pooled DNA, they were loaded on an Illumina MiSeq v3 flow cell and sequenced in a PE300 format using a v3 600 cycle reagent kit. Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ with Illumina Bcl2fastq v1.8.4. The raw sequences were deposited in NCBI SRA database (Bioproject PRJNA540309).

Amplicon analysis. FastQ files were trimmed based on quality using trimmomatic (Bolger et al. 2014), eliminating sequences with a length less than 150 bp, and removing Illumina adapters if present. After quality processing, reads were assembled using pandaseq (Masella et al. 2012), utilizing a simple Bayesian algorithm and setting parameters to remove primers before assembling and a threshold quality of at least 0.8 for alignment of the overlapping region. After assembling the oomycete *coxI* region of the mtDNA, the assembled sequences were processed with Qiime 1.9.0 (Caporaso et al. 2010) using USEARCH 6.1 (Edgar 2010). The molecular operational taxonomic units (OTUs) were selected employing a de novo approach with clustering at 97%, filtering low abundance clusters (less than 4 members) and removing chimeras with a reference database. Taxonomy was assigned using the *assign_taxonomy.py* from Qiime applying blast with default parameters, and reference sequences obtained from the BOLD systems (Ratnasingham and Hebert 2007), taxonomy reference files were constructed manually (taxonomy and reference sequence files available at https://github.com/alejorjas2/Corn_Michigan_data). An OTU table was constructed adding metadata collected from all field locations to create a biom file for downstream analyses.

An OTU table as a biom file was imported into R (R Core Team 2018, Vienna, Austria) using phyloseq (McMurdie and Holmes 2013). Estimates for within-group or field diversity (alpha diversity) were calculated using the “vegan” (Oksanen et al. 2007) package in

R. These included sample size, richness, Shannon-Wiener index, Simpson index, and evenness (Shannon index divided by natural logarithm of total species per sample) and the data were summarized by field. In order to evaluate the community structure, OTU tables were constructed and normalized as relative abundance to determine among-group diversity (beta diversity) using Bray-Curtis distances to compare communities pairwise. Data from culture analysis was integrated with amplicon data for comparison of the recovery of the different oomycete species. All analyses for diversity and other parameters are available online (https://github.com/alejorjas2/Corn_Michigan_data).

Pathogenicity. Pathogen inoculum was prepared by growing the respective oomycete isolates on millet in spawn bags. Two plates of 4- to 7-day-old cultures were cut into 10 mm size plugs for each isolate and transferred into the spawn bags containing sterile millet, and incubated at room temperature (21 to 23°C) for 14 days. The millet inoculum was mixed regularly to assure full colonization and to loosen and separate grains. Seedling assays were performed in 355 ml capacity waxed paper cups (IC12-J7534, Solo cup, Lake Forest, IL) with three 0.5-cm-drainage holes in the bottom. Cups were layered from bottom to top with 200 ml of medium vermiculite, 7 g of colonized millet, 70 ml of medium vermiculite, six corn seeds of a commercial DeKalb corn hybrid 52-61, and 70 ml of medium vermiculite. The vermiculite substrate was initially moistened to water-holding capacity, and thereafter, plants were watered every other day with half-strength Hoagland solution. Cups were maintained in a growth chamber (BioChambers, Manitoba, Canada) with a light regime of 14 h light ($375 \mu\text{E m}^{-2} \cdot \text{s}^{-1}$) and 10 h dark, at 98% humidity, and 15 or 20°C for 14 days. Isolates were grouped by species and assigned into two sets that were used as a block. Every isolate had five cup replicates per experiment, and each experiment was conducted twice for every set. Two controls were included within every experiment, a control with noninoculated autoclaved millet and a non-millet control to account for any effects of the millet on the seedlings. At the completion of the experiment, plant height was measured, and plant roots were washed with tap water to remove debris for evaluation. The plant dry weight was determined by drying roots in an oven at 50°C for 48 to 72 h. Dry weight of nongerminated seed were removed from the samples to account for only plant mass after germination.

TABLE 1
Alpha (α) diversity and evenness measures for soil oomycete communities from corn fields in Michigan during 2011 and 2012^a

Sample ID	County	Year	N ^b	Observed operational taxonomic units	Shannon	Simpson	Evenness ^c
MICO_2	Allegan	2011	3	290.33 ± 106.27 ^d	4.07 ± 0.19	0.91 ± 0.01	0.73 ± 0.02
MICO_3	Allegan	2011	3	385.00 ± 44.51	4.25 ± 0.21	0.93 ± 0.01	0.72 ± 0.02
MICO_4	Clinton	2011	3	217.00 ± 53.84	4.00 ± 0.42	0.93 ± 0.03	0.74 ± 0.05
MICO_5	St. Joseph	2011	3	403.67 ± 145.03	4.39 ± 0.15	0.96 ± 0.01	0.74 ± 0.05
MICO_6	St. Joseph	2011	3	232.67 ± 13.32	4.48 ± 0.05	0.98 ± 0.00	0.82 ± 0.01
MICO2_1	Livingston	2012	3	480.00 ± 36.17	4.65 ± 0.05	0.96 ± 0.01	0.75 ± 0.01
MICO2_2	St. Joseph	2012	3	284.00 ± 103.97	4.31 ± 0.05	0.95 ± 0.02	0.77 ± 0.04
MICO2_3	Clinton	2012	3	315.00 ± 52.72	4.90 ± 0.17	0.98 ± 0.01	0.85 ± 0.01
MICO2_4	Allegan	2012	3	413.00 ± 56.05	4.73 ± 0.18	0.97 ± 0.02	0.79 ± 0.04
MICO2_5	Ionia	2012	3	615.00 ± 32.19	4.97 ± 0.08	0.96 ± 0.01	0.78 ± 0.01

^a Observed Shannon and Simpson indices are reported with standard deviation.

^b N = number of samples analyzed per location.

^c Pielou's evenness: Shannon-Wiener diversity index divided by the natural logarithm of total species in a sample.

^d Mean ± standard deviation for each location sampled.

For statistical analyses, a cup was a replication unit and dry weight and plant height was estimated as the average of plants in the cup. Each variable plant trait measured (plant height and dry weight) were analyzed independently at each temperature tested. For each case the same model was used, where species was treated as a fixed effect while set was treated as a random effect. Dunnett's test was used to determine those species significantly different from the non-millet control. Data were analyzed employing R version 3.4.4 (R Core Team 2018, Vienna, Austria) with packages 'nlme' and 'lsmeans', and graphs were generated with the package 'ggplot2'.

Fungicide sensitivity. Fungicide sensitivity was conducted on 61 isolates representing 20 species using the method of Noel et al. (2019). Isolates were grown for 2 to 5 days on quarter-strength PCA (25 ml of potato carrot concentrate, 975 ml of distilled water, and 5 g of agar). Five 16.5-mm plugs from isolates growing on PCA were homogenized by passing them through a 20G needle into a 15-ml tube. A 96-well plate containing 200 μ l of half-strength V8 broth (V8B) per well was inoculated with 50 μ l of homogenized inoculum. Plates containing half-strength V8B plus amendment were prepared for each the concentrations tested for mefenoxam and ethaboxam (0, 0.01, 0.1, 0.5, 1, 10, and 100 μ g/ml). Each 96 well plate contained a maximum of 30 isolates replicated three times, three wells containing half-strength V8B plus fungicide and three wells only with V8B as background noise control. The 96 well plates were placed in a sealed plastic bin and incubated at 24°C for 24 to 48 h.

Optical density was measured at 600 nm after 10 s of orbital shaking with a Tecan plate reader (Tecan Group Ltd., Männedorf,

Switzerland). Percent relative growth, for each isolate, was calculated by dividing the mean optical density of each fungicide concentration by the mean optical density of nonamended growth and multiplied by 100. Each isolate was repeated at least once. Effective concentration at 50% relative growth (EC₅₀) values were estimated by solving for the point, on a four-parameter log-logistic curve, where 50% relative growth occurred by regressing the percent relative growth against the log fungicide concentrations and specifying type = "absolute" in the "ED" function of the R package "drc" (Ritz and Streibig 2005). This was done to eliminate inaccurate EC₅₀ estimation when the lower parameter of the curve was not below 50% relative growth (Noel et al. 2017).

RESULTS

Oomycete isolates recovered from diseased corn seedlings. A total of 170 oomycete cultures were obtained from the corn seedlings over the 2 years of the survey (Fig. 2). From these isolates, 33 oomycete species were identified which were composed primarily of *Pythium* spp. ($n = 29$), but also included three *Phytophthora* spp. (*Phytophthora sansomeana*, *Phytophthora inundata*, and *Phytophthora drechsleri*) and one *Phytopyrium* sp. (*Phytopyrium litorale*). In terms of abundance, *P. inflatum* (25%), *P. sylvaticum* (12.8%), *P. tardicrescens* (6.1%), *P. irregulare* (5.4%), *P. aff. torulosum* (5.4%), and *P. lutarium* (4.27%) were the most common species over 2011 and 2012. These species represent *Pythium* clades B, F, and I. Variation in isolation frequency was

Fig. 3. Relative abundance of taxonomic groups associated with oomycete operational taxonomic units from soil samples collected in Michigan corn fields during 2011 and 2012. Amplicon results are based on cytochrome oxidase subunit I marker. Clades or genera are shaded according to the legend from top to bottom.

detected between years, for example *P. inflatum* (23.17%) was the most abundant species isolated in 2011, decreasing to 1.83% in 2012. Whereas *P. irregulare* (4.27%) was the most abundant species in 2012 (Fig. 2). The two most abundant species, *P. inflatum* and *P. sylvaticum* were isolated from 36 and 73%, respectively, of the 11 fields sampled. Whereas, *P. tardicrescens* distribution was more limited, with isolates cultured from only two fields. The number of isolates per field varied from 3 to 37, where 70% of the cultures obtained were isolated from samples collected in 2011, in contrast isolate recovery was much lower in 2012 due to dry environmental conditions (Supplementary Table S3).

Soil community analysis based on COI locus. A greater diversity of oomycete taxa was identified using the amplicon sequencing approach, most likely due to the sampling depth that amplicon sequencing affords, but also possibly due to the removal of isolation bias and detecting the microbial soil bank before the host filter. A total of 1.7 million paired-end reads, an average of $31,659 \pm 7,675$ reads in 2011 and $27,253 \pm 2,891$ in 2012 samples (Supplementary Fig. S1). After quality trimming, an average of $13,260 \pm 4,247$ reads and $10,712 \pm 1,083$ reads were obtained in 2011 and 2012, respectively. From the filtered and trimmed reads, 40% were assigned to known oomycete reference sequence. Overall, the soil samples had similar levels of alpha diversity within each year. Observed OTUs ranged from 217 to 403 in 2011, and 284 to 615 in 2012 (Table 1). The evenness of the different soil samples ranged among similar magnitudes, but in 2012 were slightly higher. Shannon and Simpson indices for soil samples collected in 2012 displayed a similar trend, being slightly higher than 2012 (Table 1, Supplementary Fig. S2). Analysis of variance of observed OTUs and Shannon index by year and sample indicated significant differences between years ($P = 0.0145$). Differences across years in community structure ($P = 0.029$, $R = 0.1128$) were detected with permutation analysis (anosim). Similarly, the ADONIS test using year as factor resulted in significant differences ($P = 0.001$); however, only 7% of the variation is explained by this factor. Most of the variability comes from the fields, nesting the fields within years explaining 63% of the variation ($P = 0.001$). The community composition shows similar levels of clade abundance (Fig. 3), but samples in 2011 were dominated by OTUs in *Pythium* clade F. Only two samples in 2011 were not dominated by clade F.

Taxa identified in the amplicon sequencing not found in the isolations included the genera *Aphanomyces*, *Achlya*, and *Saprolegnia* (Fig. 3). Other clades/genera represented in the amplicon approach were *Phytophthora* and *Phytophthora* clade 8, which have been reported as root rot pathogens. *Pythium* clades F, I, D, and B were the most abundant in the soil samples. The abundance of oomycete clades identified from the corn seedling isolations and amplicon sequencing of soil samples was for the most part concordant (Fig. 4). *Pythium* clade B was the most abundant in the oomycete isolates recovered, but less abundant in the soil sequences. The top 20 OTUs recovered from soil in corn fields (Fig. 5), showed that OTUs assigned to *P. ultimum* and *P. heterothallicum* both in clade I, followed by *P. attrantheridium* and *P. sylvaticum* from clade F are abundant in most fields. The taxonomy assignment of OTUs corresponded in some cases with the species isolated from corn seedlings (Fig. 5), supporting the species-level assignment of certain OTUs in certain cases.

Pathogenicity–virulence of oomycete isolates. To examine the pathogenicity and virulence of the oomycetes recovered in the survey, representative isolates of 20 oomycete species were screened in a cup assay at two temperatures. Three isolates per species or any isolates available, if less than three isolates, were collected during the survey. At 15°C, *P. irregulare* and *P. ultimum* var. *ultimum* caused significant height reduction compared with the noninoculated

control. At 20°C an additional 11 species caused significant plant height reduction (Fig. 6A). In terms of weight per plant at 15°C, *P. irregulare*, *P. ultimum* var. *ultimum*, *P. orthogonon*, *P. tardicrescens*, and *P. heterothallicum* reduced plant weight. Interestingly at 15°C *P. lutarium* increased plant weight compared with the control, but it was not significant ($P = 0.0539$). At 20°C, an additional 11 species significantly reduced plant weight compared with the control (Fig. 6B). All the species that reduced plant weight at 15°C, also reduced plant weight significantly at 20°C. Germination and emergence were affected by the same species; in general, emergence ranged from 35 to 96% at 15°C and 75 to 98% at 20°C, in both cases the lowest values were associated with *P. irregulare* and *P. ultimum* sensu lato (Supplementary Table S4).

Fungicide sensitivity. Most oomycete species tested were very sensitive to mefenoxam with EC_{50} of less than 1 $\mu\text{g/ml}$. However, isolates of *P. lutarium* and *P. ultimum* var. *ultimum* were less sensitive to mefenoxam with average EC_{50} of 2.4 $\mu\text{g/ml}$ and greater than 100 $\mu\text{g/ml}$, respectively (Table 2). However, the *P. ultimum* var. *ultimum* isolates tested varied from 0.074 to >100 $\mu\text{g/ml}$ (Table 2). A different profile of fungicide sensitivity was observed for ethaboxam, with most species with calculated EC_{50} values <2 $\mu\text{g/ml}$. *Phytophthora litorale* had an average EC_{50} of 3 $\mu\text{g/ml}$ and *P. rostratifingens* and *P. aff. torulosum* with an EC_{50} > 100 $\mu\text{g/ml}$. These species belong to clades known to be less sensitive to ethaboxam (Noel et al. 2019).

DISCUSSION

Significantly this is the first survey of oomycetes associated with diseased corn seedlings conducted in Michigan. A total of 33

Fig. 4. Relative abundance of taxonomic groups recovered by isolation and amplicon sequencing from Michigan corn fields in 2011 and 2012. Amplicon results are based on cytochrome oxidase subunit I marker (COI).

oomycete species were identified from a collection of 170 cultures recovered from corn seedlings; however, only a portion, 16 out of the 20 species were determined to be pathogenic in the growth chamber assay, based on being significantly different from the controls. Interestingly, there are concordant results in a survey of oomycete species associated with corn in Ohio, where Broders et al. (2007) reported *P. dissotocum* as the most frequent, followed by *P. sylvaticum* as the second most abundant. In this study, *P. dissotocum* was far less abundant, while *P. inflatum* and *P. sylvaticum* were the most abundant. Differences may be due to regional variation in oomycete communities as highlighted previously (Rojas et al. 2016, 2017). In addition, *P. sylvaticum* has been found to be highly prevalent in the Midwest in both soybean root, rhizosphere and soil samples (Jiang et al. 2012; Marchand et al. 2014; Rojas et al. 2017).

The subset of oomycete isolates used to represent different *Pythium* and *Phytophthora* species showed varied responses for the different plant phenotypic traits measured. *P. irregulare* and *P. ultimum* var. *ultimum* were the only two species with consistent reduction of germination, emergence, plant height and weight, being pathogenic at both temperature conditions tested. Broders et al. (2007) also reported *P. ultimum* sensu lato as pathogenic on corn seed, while the current study focused on seedling disease, the result from both seed and seedling were found to be consistent. *P. irregulare* was also pathogenic and it was the most virulent species in the seedling assay used, however Broders showed that *P. irregulare* was less virulent on seed in comparison with *P. ultimum*. Radmer et al. (2017) also reported *P. irregulare* as a virulent species causing reduction of root length, and corroborated the aggressiveness of *P. ultimum* var. *ultimum* observed in this study.

Fig. 5. Top 20 operational taxonomic units (OTUs) from soil samples collected in corn fields in Michigan analyzed from amplicon sequencing data. Taxa are sorted based on average abundance across samples, and *Pythium* or *Phytophthora* clade and species are displayed. Numbers on cells and color range are based on percent read abundance per sample. The color scale is based on $\log_{10}$ values of percent read abundance. OTU taxonomy descriptions in bold were also found by isolation with semiselective media.

Other species that were consistent with the Radmer et al. (2017) study, was *P. sylvaticum*, which expressed less virulence behavior at lower temperatures. Most of the *Pythium* species in the present study have been identified as pathogenic on other field crops, such as soybean and dry beans (Rojas et al. 2017; Rossman et al. 2017). In fact, the species *P. irregulare*, *P. ultimum* sensu lato, and *P. sylvaticum* were also reported as pathogens on representative cultivars of Andean and Middle American gene pools of dry beans. Among these, *P. sylvaticum* caused only root rot, while the other two species, *P. irregulare* and *P. ultimum*, were seed and seedling pathogens, and similar effects were observed in other hosts (Rojas et al. 2017; Rossman et al. 2017). In contrast, other species

identified in the current study, *P. attrantheridium*, *P. inflatum*, and *P. aff. torulosum* demonstrated a limited reduction of biomass on corn plants, but no significant reduction was observed on dry beans (Rossman et al. 2017). Host species are expected to have variable responses to different oomycete species; however, *P. irregulare* and *P. ultimum* var. *ultimum* consistently caused disease across host species (soybean, corn, and dry bean) in this and other studies and under the different conditions tested (Rojas et al. 2017; Rossman et al. 2017).

Temperature plays an important role on the life cycle of *Pythium* species, having been shown to modify the organism's response to fungicides and their ability to cause disease (Matthiesen et al. 2015).

Fig. 6. Corn plant **A**, mean height and **B**, mean dry weight after challenged with 20 *Pythium* species and *Phytophthora sansomeana* at 15 and 20°C. Solid circles denote a statistical difference relative to the noninoculated control as determined by Dunnett's test ($P < 0.05$).

Temperature can affect growth rate of oomycetes, also seed germination and seedling development. For instance, soybean seed under cold stress (4 to 10°C) alters their development causing delayed germination and root exudation (Serrano and Robertson 2018). Also, the pathogen growth rates are also affected by low temperatures, which can influence and ultimately change the outcome of pathogenicity screening. In contrast, warmer temperatures (18 to 20°C or higher) decrease root exudates leaked, but provide adequate conditions to activate spores and enhance growth, which in turn can increase virulence of certain *Pythium* spp. (Serrano and Robertson 2018; Vančura 1967). In the current study, the temperatures used in the pathogenicity test were five degrees apart, but not cold enough to cause plant stress. As a result, there were a number of species that shifted their virulence or became pathogenic as a response to the temperature under the conditions tested. When measuring dry weights, it was necessary to remove the attached seed because the weight of a nongerminated (rotted) seed was often greater than or equal to germinated seed on a healthy seedling, thus masking the true effect of seed rot pathogens. A healthy seedling utilizes the seed-derived nutrient resources that are essential for plant establishment and growth, thus causing a reduction in seed weight. This should be taken into consideration for other studies, or acknowledge how weight data were collected, since the rotted seed weight could hinder the identification of pathogenic species.

To our knowledge, this is the first report of *P. tardicrescens* as a pathogen on corn, in the present study this species reduced plant weight at 15 and 20°C, but only reduced plant height at 20°C. The

screen resulted in a 9% reduction in seed germination compared with the control. The biggest impact was 18% emergence reduction when compared with the control, the germinated seed exhibited minimal and stubby root development and rotting symptoms on the roots. The host range of *P. tardicrescens* as a pathogen includes *Glycine max*, turfgrass, *Pinus*, and *Eucalyptus* (Abad et al. 1994; Linde et al. 1994; Rojas et al. 2017). The distribution of *P. tardicrescens* in the Corn Belt of the United States is unknown, and association with soybean has been only reported in Kansas and Michigan (Rojas et al. 2017).

Amplicon sequencing data of the bulk soil and oomycete isolates cultured from corn plants revealed a similar pattern of abundance by clade. However, *Pythium* clade B was highly represented in the isolations from corn plants as opposed to the soil, most likely indicating host filtering of the soil community. *Pythium* species belonging to clade B include *P. inflatum*, *P. tardicrescens*, and *P. aff. torulosum*, which were among the top five most abundant species isolated from corn roots and were all pathogenic and with varying levels of virulence. Examining diversity by field using the amplicon data, *P. ultimum* and *P. heterothallicum* from clade I and *P. sylvaticum* and *P. attrantheridium* from clade F were highly prevalent across most fields. In terms of diversity and community composition, the greatest variability was observed between fields. These differences were most likely driven by the environment differences between sampling years, 2012 was characterized by reduced precipitation and warmer temperatures overall. However, fields sampled in 2012 had slightly higher diversity and were different in composition from fields in 2011. In 2011, three fields

TABLE 2
Relative effective control to 50% growth inhibition (EC₅₀) in poison plate assays of most abundant *Pythium* species isolated in 2011 and 2012 from Michigan corn fields challenged with mefenoxam and ethaboxam

Species	N ^a	Ethaboxam µg/ml		Mefenoxam µg/ml	
		Mean	Range	Mean	Range
<i>Pythium acanthicum</i>	1	0.59	–	0.074	–
<i>Pythium</i> aff. <i>dissotocum</i>	3	0.54	0.10–0.87	0.256	0.06–0.58
<i>Pythium</i> aff. <i>torulosum</i>	4	>100	12.73–>100	0.095	0.07–0.12
<i>Pythium amasculinum</i>	2	1.08	0.84–1.32	0.087	0.06–0.11
<i>Pythium arrhenomanes</i>	1	0.02	–	0.010	–
<i>Pythium attrantheridium</i>	1	0.36	–	0.082	–
<i>Pythium heterothallicum</i>	3	0.97	0.84–1.11	0.028	0.02–0.04
<i>Pythium inflatum</i>	4	0.25	0.19–0.33	0.638	0.03–1.30
<i>Pythium irregulare</i>	4	0.68	0.13–2.04	0.453	0.03–1.66
<i>Phytophythium litorale</i>	1	3.39	–	0.091	–
<i>Pythium lutarium</i>	4	1.81	0.05–5.44	2.360	0.23–7.74
<i>Pythium oopapillum</i>	1	0.72	–	0.119	–
<i>Pythium orthogonon</i>	3	0.21	0.08–0.46	0.240	0.12–0.47
<i>Pythium perillium</i>	4	0.36	0.20–0.58	0.094	0.04–0.14
<i>Pythium perplexum</i>	3	0.44	0.08–1.11	0.702	0.46–0.94
<i>Phytophthora sansomeana</i>	3	0.19	0.12–0.22	0.079	0.04–0.11
<i>Pythium pleroticum</i>	2	0.48	0.28–0.68	0.051	0.02–0.09
<i>Pythium rostratifingens</i>	3	>100	14.37–>100	0.084	0.01–0.22
<i>Pythium sylvaticum</i>	3	0.70	0.09–1.87	0.404	0.04–0.99
<i>Pythium tardicrescens</i>	4	0.25	0.20–0.27	0.169	0.09–0.38
<i>Pythium ultimum</i>	2	0.12	0.10–0.14	0.302	0.13–0.47
<i>Pythium ultimum</i> var. <i>ultimum</i>	5	0.66	0.21–1.43	>100	0.07–>100

^a Number of isolates tested.

out of the five sampled showed a high abundance of *Pythium* species belonging to clade F, which is known to primarily contain plant pathogenic species (Lévesque and de Cock 2004). The OTUs identified in this clade, include species, *P. attrantheridium*, *P. sylvaticum*, *P. irregulare*, *P. sp. balticum*, and *P. intermedium*. Most of the species isolated are also well represented in the amplicon sequencing approach, emphasizing the value of this approach to characterize communities associated with specific cropping systems. In the present study, soil samples were used to reveal the microbial bank that could potentially infect corn plants. Such information can and has been used for targeted isolation methods to address the presence of specific species and their role in disease (Bakker et al. 2017).

The resolution of the amplicon markers may be limited due to the size of the amplicon and the locus used, however, the results of these prevalent species are supported by the isolation data (Figs. 4 and 5). Species-level designation of OTUs in fungi and oomycetes either using ITS or COI is not ideal, since it has been shown the barcoding gap depends on the group studied (Robideau et al. 2011). Nonetheless, we found concordance between species isolated from plant tissue and species-level taxonomy assignment of the OTUs from soil samples. The species-level identification for OTUs should be approached carefully, especially for those found in low abundance. The amplicon sequencing approach enabled us to estimate the relative abundance of the oomycete species present in the soil at a much greater sampling depth than traditional culture-based isolation. Amplicon sequencing significantly reduces the labor and cost associated with culturing and use of Sanger sequencing for identification. However, the community view that amplicon sequencing provides can also be complemented by limited or focused culturing. Cultures are often essential for phenotyping variables such as, pathogenicity, virulence, and fungicide sensitivity, among other traits. Isolate collections and their phenotyping will remain an invaluable resource for the interpretation of amplicon and metagenomic data for a more thorough understanding of oomycete ecology, community structure, and disease epidemiology.

A study by Deep and Lipps (1996), showed that *P. arrhenomanes* was associated with reduced yield and present in all corn fields sampled that had a history of continuous cropping with no tillage. Among the top 20 OTUs detected by the amplicon approach, an OTU was classified as *P. arrhenomanes* and was present in all the fields sampled, but from the culture data, it was only isolated from two fields in 2011. The isolates of *P. arrhenomanes* isolates were not pathogenic under conditions tested, but the prevalence in the community analysis indicates the association of this species with corn. This might also be the case for other oomycete species, such as *Phytophthora sansomeana* which was reported in corn (Zelaya-Molina et al. 2010), causing stunting and has also been reported on soybean. *Phytophthora sansomeana* may be an example of an emergent, but not widely recognized species that causes root damage (Chang et al. 2017; McCoy et al. 2018). The high prevalence of *P. sylvaticum* in the Midwest, could be a result of management practices or the lack of effective rotation systems. Stunting is a common symptom in the field and often overlooked, this symptom is often associated with infection by a soilborne pathogen, such as oomycetes. However, there is limited information on the impact of soilborne pathogens during the season on plant health and yield.

The two fungicides evaluated had the capacity to inhibit the growth of the *Pythium*, *Phytophthora*, and *Phytophythium* species in this study. With respect to mefenoxam, EC₅₀ values were below 3 µg/ml for 21 of the 22 species tested; however, isolates of *P. ultimum* var. *ultimum* exhibited different responses, with three out of the five isolates evaluated showing a response higher than 50 µg/ml, suggesting resistance against mefenoxam. In contrast,

Broders et al. (2007) reported sensitivity of *P. ultimum* based on two concentrations of mefenoxam (5 and 100 µg/ml), with a very restricted growth at both concentrations. Their study did, however, find that *P. dissotocum* was able to grow on 100 µg/ml, whereas *P. aff. dissotocum* showed sensitivity in our study. In addition, Broders et al. (2007) identified two *Pythium*, which were designated at that time as Group 3 and Group 6, which could be reclassified as *Phytophythium*. In our study, we recovered and evaluated one isolate of *Phytophythium litorale*, which was less sensitive to mefenoxam, with an EC₅₀ of 3.39. Similar findings were reported by Radmer et al. (2017), showing slight growth at 100 µg/ml. The EC₅₀ values to ethaboxam were mostly below 4 µg/ml. Only *P. aff. torulosum* and *P. rostratifingens* had an EC₅₀ values out of the range of concentrations tested (>100 µg/ml). These *Pythium* are classified in clade B1 (*P. aff. torulosum*) and clade E (*P. rostratifingens*), which have been suggested are clades that contain species inherently insensitive to ethaboxam (Noel et al. 2019).

By determining which specific pathogens are causing seedling and root rot disease of corn we can examine the efficacy of management practices, such as fungicide seed treatments, crop rotation, and variety resistance. Many of the oomycete species that were abundant and pathogenic on corn are also pathogens of soybean, hence a corn-soybean rotation may not be of great value in reducing disease pressure. For example, it has been demonstrated that a simple soybean-corn rotation does not reduce soybean sudden death syndrome. However, with the use of additional crops to create an extended and diverse rotation, disease pressure was reduced (Leandro et al. 2018). Extended rotations to other crops may not be economically viable due to market prices or lack of available land. The use of cover crops may play a role in diversifying the microbiome and managing disease. However, studies are still needed to determine the impact of cover crops on pathogenic and nonpathogenic members of the microbiome.

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