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Isolation of Cultivation-Resistant Oomycetes, First Detected as Amplicon Sequences, from Roots of Herbicide-Terminated Winter Rye

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ABSTRACT

The dynamics of microbial communities associated with dying cover crops are of interest because of potential impacts on disease in a subsequent crop, and because of the importance of microbial activity on plant residue to soil organic matter dynamics and nutrient cycling. We used high throughput amplicon sequencing to characterize the composition and structure of oomycete and fungal communities associated with a rye cover crop, and to track their community dynamics in the first several weeks after herbicide was applied to terminate the cover crop. The dominant oomycetes associated with cereal rye roots were *Pythium volutum*, *Pythium* sp. F86 (an unknown species within clade B), and *Lagenidium radicum*. Because *P. volutum* is sensitive to common additives in isolation media, and *L. radicum* is an obligate intracellular parasite, a unique aspect of this work is to reveal the dominance of oomycete taxa that would have been missed entirely under a traditional cultivation-based approach. Based on first detection in an amplicon sequencing survey, we were able to isolate *P. volutum* and *Pythium* sp. F86. We demonstrate that both species are pathogenic on corn, and that corn seedlings grown in the field

following a rye cover crop show elevated rates of infection by *P. volutum*, highlighting a potential disease risk associated with cover cropping. *P. volutum* and *Pythium* sp. F86 exhibited contrasting spatial patterns of abundance, with nearly complete turnover of the dominant species across the field site. In contrast to the strong spatial structuring and low diversity of oomycete communities, fungal communities associated with a cereal rye cover crop were more diverse and dynamic, with some displacement of basidiomycetes by ascomycetes over time. Several plant pathogens, as well as putative beneficial organisms, were detected among fungal communities associated with rye roots. This work sheds light on microbial community dynamics on dying host plants, highlights the power of culture-independent microbial community assessment to yield new insights, and suggests the need for informed management to reduce seedling disease risk in corn following rye cover crops.

Additional keywords: agriculture, ecology, plant pathology, rhizosphere and phyllosphere, soil ecology.

Plants impact the identities and abundances of microbes that are present in the phytobiome and alter the trajectory of microbial

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community development (Bakker et al. 2012). In turn, the microbiota associated with plants perform important services that can impact plant performance (Bakker et al. 2010; Redman et al. 2002). In agricultural systems, plant-driven impacts on soil microbiomes are among the primary motivations for crop rotation. There is a rich body of literature detailing the impacts of living host plants on the composition and structure of microbial communities in the rhizosphere (Peiffer et al. 2013), phyllosphere (Bai et al. 2015), and internal plant tissues (Lundberg et al. 2012). However, there has been less attention paid to the impacts of dying plants and their residues on associated microbiomes.

Cover cropping is an agricultural management practice that expands living plant cover over a larger portion of the year and adds diversity to crop rotations. In one common application of cover cropping, winter cereals are established in late fall, survive the winter, and grow in early spring before active termination with an

herbicide in advance of spring cash crop planting (Kaspar and Singer 2011). This system of winter cover cropping eliminates the traditional winter fallow period and alters the timing of inputs of recently dead plant residue relative to the germination of a subsequent grain crop. Cover cropping has been shown previously to impact soil fungal communities (Nishizawa et al. 2010).

Microbial community dynamics on dead and dying plant tissues, such as those produced through cover cropping, may have functional implications. For instance, microbial activity drives the decomposition of crop residues, with impacts on the formation of soil organic matter and on nutrient availability to subsequent crops (Cotrufo et al. 2013). Furthermore, effects of cover crop residues on the phytobiome may influence rhizosphere development, beneficial microbial interactions, or disease epidemiology of a subsequent crop, because rhizosphere microbiome development is sensitive to characteristics of the initial pool of colonizing microbes (Bakker et al. 2015).

In crop production systems, impacts on the growth, reproduction and fate of plant pathogens are among the most important influences of crop residue. The conditions created by agricultural management with cover crops may provide opportunity for growth and proliferation by some plant pathogens. Bakker et al. (2016) demonstrated that two *Fusarium* species and two *Pythium* species that were pathogenic on corn seedlings were able to increase in density in the roots of herbicide-terminated rye cover crops. Thus, understanding microbial community dynamics on cover crop residues is vital to management decisions aimed at limiting disease risk.

Our primary objectives in this work were as follows. (i) Track the dynamics of oomycete and fungal communities in response to the death of cereal rye plants that had been grown as a winter cover crop in a field setting, and that were actively killed with herbicide. (ii) Relate characteristics of the oomycete and fungal communities associated with cover crops to variables such as time since herbicide application, spatial location across a field, or relevant soil edaphic properties. (iii) Investigate the potential for cover cropping to influence pathogen pressure on a subsequent corn crop using a method that avoids bias associated with ease of cultivation in the laboratory. Based on the community profiles obtained by amplicon sequencing, we isolated cultivation-resistant oomycete taxa associated with rye cover crop roots and assessed their pathogenicity on corn seedlings.

MATERIALS AND METHODS

Site description. A field experiment was conducted on an Iowa State University research farm in Boone County, IA (42°1'15''N, 93°46'26''W). The field used in this experiment was in a corn–soybean rotation system, with both phases of the rotation present each year, and had a history of no-till management since 1995.

The predominant soils series in the field are Clarion loam, Nicollet clay loam, and Webster silty clay loam, all formed from glacial till (Andrews and Diderikson 1981). Controlled traffic had been implemented since establishment; all machinery and foot traffic were restricted to the same interrows each year and row location was maintained from year to year. More detailed descriptions of the field have been reported previously (Parkin and Kaspar 2006). Air and soil temperatures, soil moisture content, and precipitation at the site (Table 1) were calculated using daily values from a weather station located less than 1 km from the field (Iowa Environmental Mesonet 2016).

In the fall of 2013, a cover crop of winter cereal rye (cultivar Elbon) was planted at a rate of 3.125×10^6 seeds ha⁻¹, by broadcast seeding and incorporation with a roller-chopper. The field was divided into five blocks, within which plots (3.8 m × 53 m) were randomly assigned to an early or a late cover crop termination treatment. The following spring (2014), glyphosate (N-(phosphonomethyl) glycine; 0.66 kg of active ingredient ha⁻¹) was applied on 22 April for plots assigned to the early termination treatment, or 5 May for plots assigned to the late termination treatment. The field was managed similarly in the subsequent 2 years.

To investigate relationships between soil properties and oomycete community composition, we collected soil samples from each plot for analysis of soil organic matter (OM) and sand content. These properties are closely related to soil moisture status, which is relevant to oomycete life history. From each plot, two subsamples consisting of three soil cores each (3.2 cm diameter × 5 cm depth) were drawn from the interrow off the center row of the plot. To determine OM, approximately 50 g of oven-dried soil from each subsample was used to determine weight loss on ignition with a programmable muffle furnace (Fisher-Scientific Isotemp 650-126) at 460°C for 16 h. The OM content was considered to be the ash-free change in weight after burning (Kaspar et al. 2006). Sand content was determined on the burned soil samples on a weight basis by dispersing the samples with 250 ml of 5 g liter⁻¹ of sodium hexametaphosphate, shaking for no less than 18 h, and then wet sieving through a 53-μm sieve and weighing the dry sand remaining on the sieve (Cambardella et al. 2001).

Sample collection and processing. Rye roots were sampled from both treatments on each of three different dates: 25 April, 9 May, and 23 May 2014. To collect rye roots, a probe (3.2 cm diameter) was centered over the crown of an individual rye plant and a soil core was collected to a 15 cm depth. At the first two sampling dates, a single core was collected per plot. At the last sampling date, three subsamples were collected per plot. Soil cores were transported to the lab on ice and were stored frozen until processing. Rye roots were collected on a screen while soil was washed away under a high pressure stream of water. Washed roots

TABLE 1
Weather data for the field plot from which roots of cereal rye cover crops were sampled in the spring of 2014

	Mean air temperature (°C)	Mean soil temperature (10 cm depth; °C)	Mean soil water content (30 cm depth; %)	Precipitation (mm)
Month of:				
March	0.4	−0.2	20.8	31.4
April	9.8	9.6	36.6	120.7
May	16.6	23.8	38.7	108.3
Two weeks preceding sample collection on:				
25 April	11.2	11.2	36.8	54.3
9 May	13.1	15.1	38.1	67.4
23 May	14.9	19.8	38.7	79.5

were freeze-dried and pulverized by beating with a tungsten-carbide bead (3 mm diameter; Qiagen) on a MiniBeadbeater (Biospec Products). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). Where multiple samples were collected per plot, DNA extracts were pooled to a single sample per plot.

Amplicon sequencing. For library preparation, DNA extracts were diluted 20× with molecular biology grade water before PCR amplifying the internal transcribed spacer (ITS) region of the rRNA gene for oomycetes, or the 18S rRNA gene for fungi. For the oomycete ITS amplicon, we used the primer pair ITS6 (5'-GAA GGT GAA GTC GTA ACA AGG-3') and ITS7 (5'-AGC GTT CTT CAT CGA TGT GC-3') (Vannini et al. 2013) with appropriate 454 adaptors and a 10 nucleotide barcode in each of the forward and reverse primer constructs. The thermocycler program consisted of 8.5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 59°C, and 1 min at 72°C; and 5 min at 72°C. For the fungal 18S rRNA amplicon, we used the primer pair nuSSU1196 (5'-GGA AAC TCA CCA GGT CCA GA-3') and nuSSU1536 (5'-ATT GCA ATG CYC TAT CCC CA-3') (Borneman and Hartin 2000) with appropriate 454 adaptors and a 10 nucleotide barcode on the forward primer only. The thermocycler program consisted of 8.5 min at 95°C; 35 cycles of 15 s at 95°C, 30 s at 58°C, and 1 min at 72°C; and 5 min at 72°C. The Maxima SYBR Green master mix (ThermoFisher Scientific) was used for all reactions.

Amplified PCR products were purified using the FlashGel double tier electrophoresis system (Lonza Rockland, Inc.) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Six nanograms of DNA from each sample was combined to generate an oomycete and a fungal library for pyrosequencing. Libraries were purified and concentrated using Ampure Beads (Beckman Coulter) and quantified using the Library Quantification Kit (KAPA). Sequencing was performed on the Roche GS Jr. platform (454 Life Sciences). The raw data (.sff files) have been deposited at the NCBI Sequence Read Archive as experiment SRX1518163.

Oomycete data processing. For the oomycete dataset, ITSx version 1.0.11 (Bengtsson-Palme et al. 2013) was used to extract the ITS region from each read. It is important to constrain ITS amplicon sequencing data to just the ITS region (Lindahl et al. 2013), because differential representation of the very highly conserved flanking regions can influence sequence binning or other aspects of data processing. Amplicon sequences flagged by ITSx as likely belonging to taxa other than oomycetes were culled. We used custom scripting to trim quality scores in the corresponding file to include only those base calls that were retained by ITSx.

Additional processing of sequence data was performed with the software Mothur v. 1.38.0 (Schloss et al. 2009). We required a perfect match to the forward barcode sequence, and allowed 1 mismatch to the reverse barcode. Our dual-index strategy required culling any sequences that were not full length, because the 3' barcode was required to assign sequences to their sample of origin. Sequences were trimmed where the average quality score in a sliding window fell below 25, and were culled if they contained ambiguous base calls. Oomycete ITS reads passing these criteria were preclustered, binning together reads that differed in two or fewer positions. Chimeras were identified with UCHIME (Edgar et al. 2011), using both self and the classification database as references, and were culled.

We used distance based greedy clustering (Rognes et al. 2016; Westcott and Schloss 2015) to bin reads into operational taxonomic units (OTUs), at a distance threshold of 0.03. This method requires only pairwise alignments, which alleviates difficulties associated with aligning ITS sequences. Singleton OTUs were excluded from analysis. Classification of sequences used the naïve Bayesian

classifier (Wang et al. 2007) implemented in Mothur, and a custom set of reference sequences with associated taxonomic information. This reference dataset consisted of the NCBI accessions generated in Table S1 of Robideau et al. (2011) and Table 19 of Hyde et al. (2014). Accessions from the two sources were merged and supplemented with an additional accession for *P. volutum* ATCC 58813. The ITS1 region was extracted using ITSx. Initial classifications of the dataset indicated a large number of reads likely belonging to a single taxon within *Pythium* clade B that did not have sufficient homology to any of the reference sequences to be classified. Three random sequences from this group were added to the reference database under the operational taxon name *Pythium* sp. F86, named for the field from which the original samples were drawn.

Fungal data processing. The fungal 18S rRNA amplicon dataset was also processed in Mothur. Raw flow data were trimmed to 450 flows and were culled if there were homopolymeric runs longer than 10 nucleotides. Fungal sequences passing these criteria were denoised using the Mothur implementation of the PyroNoise algorithm (Quince et al. 2011). Denoised data were screened to permit no more than two mismatches to the forward primer, and to require a perfect match to the barcode sequence. It has been demonstrated using mock communities that primer mismatches can result from real biological variation (Gohl et al. 2016).

We used the SILVA small subunit reference NR 99 Release 128 database (Quast et al. 2013), extracting from the complete database only those reads belonging to fungi or to the plant order Poales (to which the host plant in our study belongs). Sequences were culled if they did not start by, or ended before, the position in the alignment by which 95% of sequences started and ended. Sequences that were unclassifiable or that were classified as of plant origin (21.8% of raw reads) were removed. Chimeras were identified using both self and the classification database as references, and were culled. Sequences were binned into OTUs via distance based greedy clustering. The low distance threshold of 0.01 was used, due to the highly conserved nature of the fungal 18S rRNA gene. Singleton OTUs were culled.

A record of commands used to process the raw sequence files into the final datasets is included as Supplementary Material S1.

***P. volutum* infection of corn radicles.** We performed follow up experiments to validate the presence of *P. volutum* at our field site, and to test the potential for *P. volutum* to impact corn seedlings planted following a rye cover crop.

In the spring of 2015, we assessed the density of *P. volutum* in the roots and mesocotyls of corn seedlings grown in the same field from which samples were drawn for amplicon sequencing the prior year. Corn seedlings were collected from plots in which they either followed a rye cover crop or followed a no cover crop control. Corn seedling radicle and mesocotyl tissue were collected by digging up six seedling plants at irregular intervals across each experimental plot ($n = 5$). For each plant, we collected the first 2 cm of radicle and 2 cm of mesocotyl extending away from the seed. Sections of radicle or of mesocotyl from three plants were bulked into a single DNA extraction, leaving two subsamples per plot. Roots and mesocotyls were washed and processed for DNA extraction as described above.

The abundance of *P. volutum* in each tissue sample was measured with quantitative PCR (qPCR) on a CFX96 instrument (Bio-Rad). The PCRs were performed in a 20 µl volume using QuantiTect SYBR Green (Qiagen), forward and reverse primers at 200 nM final concentrations, and 5 µl of template DNA. For our qPCR primers, we used oligos reported by Tambong et al. (2006) to be specific for *P. volutum* (vol201ex: 5'-AAT GTA GTT TAT TCT GTA TGC GCG-3'; vol203rc: 5'-GTC AGC ACT TCT CTC AAA CAC-3').

Both oligos bind within the ITS region and together amplify a fragment of approximately 400 bp. The thermocycler program consisted of 95°C for 15 min; and 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s (plate read); melt curve.

Each qPCR plate included no-template controls and a standard curve of synthesized DNA (sequence matching positions 1875 to 2282 of GenBank accession AY598686.2) spanning 6 orders of magnitude in template copy number. Technical triplicates were run for all samples and standards. Nondetects were assigned a small nonzero value, defined as half of the calculated DNA content at a cycle threshold of 40. Expressing pathogen DNA abundance relative to host DNA abundance minimizes error associated with variation in DNA extraction efficiency. To enable this, we also assessed corn *tua4* gene density in each DNA extract, using the qPCR assay of Mideros et al. (2009) and a standard curve of known copy number derived from a synthesized template (positions 20 to 82 of GenBank accession X73980.1).

***Pythium* isolations and pathogenicity assays.** In the spring of 2016, we isolated *Pythium* strains from cereal rye cover crop roots grown in the same field from which samples were drawn for amplicon sequencing and determination of *P. volutum* load in corn seedling roots over the previous 2 years. Symptomatic sections of live roots of cereal rye, showing discoloration or disease lesions, were washed extensively with running tap water, rinsed briefly in 0.6% NaOCl followed by sterile water, and blotted dry on sterile paper towels. These surface-disinfested root pieces were placed underneath a growth medium to provide opportunity for physical selection against nonfilamentous organisms incapable of growing through the solid medium. Plates were incubated at 14°C. *P. volutum* has been previously reported to be extremely sensitive to several common additives in oomycete-selective growth media (Chamswang and Cook 1985; Kerns and Tredway 2008a). We confirmed this sensitivity with isolate *P. volutum* ATCC 58813 (data not shown). Accordingly, we used a modified isolation media consisting of corn meal agar (17 g liter⁻¹) amended with rifampicin (5 mg liter⁻¹) and nystatin (8 mg liter⁻¹).

Marker gene sequence data were used to identify *Pythium* spp. isolated from rye roots. Marker gene sequences were obtained by PCR amplifying and Sanger sequencing the ITS region of the ribosomal RNA gene, and the cytochrome C oxidase subunit I (COI) gene, using the primers and method of Robideau et al. (2011).

Marker gene sequences of the closest relatives of *P. volutum*, as indicated by the five gene phylogeny of Hyde et al. (2014), were obtained from GenBank. A maximum likelihood phylogeny of our unknown isolates with their closest relatives was generated using the CLC Genomics Workbench (Qiagen). Briefly, sequences were trimmed to the same region and aligned. A phylogram was constructed using the neighbor-joining method and the Jukes-Cantor nucleotide substitution model.

Pathogenicity assays were performed using a known isolate of *P. volutum* (strain ATCC 58813), as well as strains of *P. volutum* and *Pythium* sp. F86 that were isolated in this work. To produce inoculum, equal volumes of sand and vermiculite (1,450 g total) were combined with 100 g of corn meal and 450 ml of tap water in an autoclavable plastic bag with an air exchange patch. The substrate was autoclaved on a 30 min cycle on each of two consecutive days. The sterile substrate was infested with 10 pieces (1 cm²) of colonized agar, heat sealed, and incubated at 15°C for 18 days. After homogenization, this colonized medium was used directly as the potting medium for corn seedling growth. Inoculum strength was estimated by extracting DNA from a 7.5-g subsample of colonized substrate (FastDNA 50-ml SPIN kit, MP Biomedicals) and performing quantitative PCR using the same primers as were used for amplicon sequencing. Pathogen biomass was ranked and the two

isolates having the lowest inoculum strength were dropped from the experiment. Estimated inoculum strength differed by threefold or less among the remaining pathogen isolates (four isolates of *P. volutum*, three isolates of *Pythium* sp. F86), and was not adjusted. The noninoculated control substrate showed no amplification.

Corn seeds of hybrid cultivar PO448 (Dupont Pioneer; no chemical treatment) were surface-disinfested by shaking for 2 min in 70% ethanol, and then 20 min in 0.6% NaOCl plus detergent, and rinsed four times with sterile water. Seeds were pre-imbibed between sheets of moist filter paper for 1 day at room temperature. Two seeds were planted per pot (3.8 cm × 21 cm; Stuewe & Sons, Inc.), with 10 replicate pots per treatment (medium infested with a putative pathogen isolate, or noninfested medium). Pots were grown at a constant 14°C with 12 h days in a controlled environment chamber, in a randomized complete block design. Pots were watered three times a week with 15 ml of deionized water or fertilizer solution (once only). At 21 days after planting, roots were washed and examined for disease symptoms, and radicle lengths were measured. For *P. volutum* ATCC 58813 only, roots were also processed for recovery of the pathogen on selective growth media. Isolates recollected from corn roots were compared morphologically and by marker gene sequence to confirm that the same isolate that was used in inoculation was recovered from the diseased tissue.

A note on sampling. The same field site was sampled at several different points in time across several years. Sampling location within plots was not precisely matched for samples destined for DNA extraction versus for isolations, versus for edaphic characterization.

Statistical analyses. One sample in the oomycete dataset had very low sequence yield, and was excluded from analysis. Sequences were randomly subsampled to the depth of the smallest sample (443 reads for the oomycete dataset, 405 reads for the fungal dataset) prior to estimating diversity and calculating community similarity indices, which formed the basis for both ordinations and statistical tests for differences in community structure. Patterns of similarity in microbial community structure were visualized via principal coordinates analysis (PCoA; function cmdscale in R), using pairwise Bray-Curtis or weighted UniFrac distances (Lozupone et al. 2007) as the input data. The VEGAN package for R (Oksanen et al. 2016) was used to fit soil OM and sand content onto ordinations (envfit function). The significance of differences in community structure among treatments was assessed with analysis of molecular variance (AMOVA), as implemented in Mothur.

To test for differences in the mean proportional abundance of certain oomycete taxa (i.e., *P. volutum* and *Pythium* sp. F86 across experimental blocks, or *L. radicicola* per sampling time point), sequence reads were summed across OTUs assigned to the same taxon. The centered log-ratio transformation (Gloor and Reid 2016) was used to alleviate statistical difficulties associated with compositional data and to improve assumptions of homogeneity of variances. Transformed sequence abundance data, as well as OTU diversity index values, were subjected to analysis of variance (ANOVA) (additive models, incorporating the blocking factor plus the sampling date) with Tukey contrasts. We identified fungal taxa that either increased or decreased over time by ranking OTUs according to the slope of their proportional abundances across the three sampling dates.

In the pathogenicity assays, corn radicle lengths were averaged across the two plants per pot and analyzed by ANOVA (additive model, consisting of blocking factor and *Pythium* isolate) with Tukey contrasts. Measured *P. volutum* densities in corn tissue were expressed as the ratio of *P. volutum* ITS gene copy number to corn *tua4* gene copy number, and then were log₁₀ transformed. Transformed density ratios were analyzed by ANOVA (additive

model, consisting of blocking factor and cover crop treatment) with Tukey contrasts.

RESULTS

Oomycete community membership and dynamics in the roots of a rye cover crop. Our final oomycete amplicon dataset consisted of 32,953 ITS sequence reads, across 230 OTUs. These OTUs could be further binned into a much smaller number of described taxa (Table 2). The vast majority of reads belonged to organisms from *Pythium* clade B: *Pythium volutum* comprised 47.3% of reads, and its close relative, *Pythium* sp. F86, comprised 40.4% of reads. *L. radicola* made up 8.7% of reads, and the remaining 4% of reads were divided among several other *Pythium* and *Globisporangium* species, or could not be confidently assigned an identity (Table 2).

Irrespective of termination timing or sampling date, oomycete communities were strongly structured by spatial position of sample origin within the field (Fig. 1; experimental blocks arranged 1 to 5, west to east). This effect was predominantly the result of a switch in the dominant *Pythium* species from west to east across the field; *P. volutum* dominated the oomycete community in the western half of the field, while *Pythium* sp. F86 dominated the oomycete community in the eastern half of the field (Fig. 2A). Sampling date was also a significant factor for *P. volutum* relative abundance ($P = 0.002$), but was not for *Pythium* sp. F86 ($P = 0.11$). The relative abundance of *P. volutum* increased significantly (ANOVA, $P < 0.05$) between the first sampling date (32.8% of reads) and the later sampling dates (46.2 and 50.1% of reads at the second and third sampling dates, respectively).

Likely explanations for the switching of dominant *Pythium* species across the field site may be associated with soil properties and elevation differences. Soil sand content was significantly related to oomycete community structure (by permutation test, $P = 0.002$ for the fit of sand content onto an ordination based on oomycete community structure; Fig. 1). Organic matter was not significantly related to oomycete community structure ($P = 0.18$). There is an elevation change of approximately 3 m over the field site and a change in slope gradient and direction (Supplementary

Material S2), which is consistent with the change in dominant *Pythium* species. The western portions of the field, where *P. volutum* dominates, have slightly higher elevation, but lower soil sand content and impeded water shedding due to variable direction of slope. The eastern portions of the field, where *Pythium* sp. F86 dominates, have slightly lower elevation, but higher sand content and long slopes of consistent orientation that would facilitate water movement. In sum, the abundances of the two most prevalent oomycete taxa were most strongly related to spatial location in the field.

In contrast, the third most abundant oomycete taxon, *L. radicola*, responded dramatically to host plant death; for both early and late termination treatments, the relative abundance of *L. radicola* dropped quickly with time after herbicide application. In both the early and late termination treatments, *L. radicola* had been significantly reduced in abundance between the first and the last sampling date (Fig. 2B).

Quantitative PCR and isolation of *Pythium* species. In the field, *P. volutum* was not detectable by qPCR in corn seedlings grown following a winter fallow, but was readily detectable in radicles and mesocotyls of corn seedlings grown following a cereal rye cover crop (Fig. 3A). In the fallow control, no corn tissue samples showed amplification with *P. volutum* primers. In contrast, primers selective for *P. volutum* amplified a product in 80% (early termination treatment) to 100% (late termination) of corn radicles, and 60% (late termination) to 80% (early termination) of corn mesocotyls following a rye cover crop. Measured *P. volutum* densities in corn radicles were significantly higher than the control for both termination treatments, and densities in corn mesocotyls were significantly higher than the control for the early cover crop termination treatment (Fig. 3A; ANOVA, $P < 0.05$). Thus, the ability of cereal rye to host *P. volutum* also results in higher loads of this pathogen in corn seedlings following a cereal rye cover crop.

To validate the dominant *Pythium* species as indicated by our amplicon sequencing and qPCR data, we successfully isolated strains of *P. volutum* and of *Pythium* sp. F86 from roots of rye cover crops in the field. Identification of these strains was confirmed by marker gene sequencing. We have deposited COI and ITS sequences for *P. volutum* ATCC 58813, and for isolates of *P. volutum*

TABLE 2
Oomycete species detected in roots of a cereal rye cover crop via amplicon sequencing

Genus	Clade	Species	Mean abundance (% of reads)	Number of OTUs
<i>Lagenia</i>		<i>radicola</i>	8.7	22
<i>Globisporangium</i>	E	<i>acrogynum</i>	<0.1	1
<i>Globisporangium</i>	E	Unclassified	<0.1	1
<i>Globisporangium</i>	F	<i>attrantheridium</i>	0.3	1
<i>Globisporangium</i>	F	<i>recalcitrans</i>	0.3	6
<i>Globisporangium</i>	I	<i>heterothallicum</i>	<0.1	2
<i>Globisporangium</i>	J	<i>perplexum</i>	<0.1	1
<i>Pythium</i>	A	<i>monospermum</i>	<0.1	3
<i>Pythium</i>	B	<i>aristosporum/arrhenomanes</i> ^a	0.2	2
<i>Pythium</i>	B	sp. F86	40.4	71
<i>Pythium</i>	B	<i>volutum</i>	47.3	48
<i>Pythium</i>	B	aff. <i>volutum</i>	0.5	32
<i>Pythium</i>	B	Unclassified	2.1	36
Unclassified <i>Pythiaceae</i>		Unclassified	0.1	2
Unclassified oomycetes		Unclassified	<0.1	2

^a These two described species cannot be distinguished on the basis of internal transcribed spacer (ITS) marker gene sequence.

and *Pythium* sp. F86 collected in this work as GenBank accessions KX266866 to KX266871.

A maximum likelihood phylogeny of ITS gene sequences for *P. volutum* and *Pythium* sp. F86, along with their 11 closest relatives (Fig. 4), yielded a topology consistent with the more extensive five gene phylogeny of Hyde et al. (2014). Our *P. volutum* isolates grouped together with prior collections of this species. However, our isolates of *Pythium* sp. F86 did not cluster with any other species, indicating that this is likely an undescribed species.

The COI gene has been proposed as a useful marker for non-phylogenetic identification of *Pythium* species (Robideau et al. 2011). While this gene does not provide congruent phylogenetic trees compared with several other genes (Hyde et al. 2014), measures of sequence similarity can still be helpful in determining the taxonomic placement of novel isolates. Our isolates of *P. volutum* had a COI sequence that was >99% similar to other *P. volutum* isolates, which supports our identification of this species. In contrast, *Pythium* sp. F86 had a COI sequence that was only 92.4 to 94.0% similar to any of its 12 closest relatives. This further suggests that *Pythium* sp. F86 is likely an undescribed species.

Corn seedlings grown in a medium that had been infested with *P. volutum* or *Pythium* sp. F86 showed reduced rates of emergence, as well as visible symptoms of root disease. In particular, corn radicles uniformly failed to elongate, presumably due to the death of the radicle tip (Fig. 3). Corn seedlings grown in noninfested growth medium under the same conditions showed normal radicle development and elongation. *P. volutum* isolates sharing an identical marker gene sequence to the inoculated strain were recovered from necrotic radicle tips, as well as from asymptomatic seminal roots of

plants grown in an infested medium (data not shown). Corn seedlings grown in infested medium sometimes also failed to properly elongate their first leaves, a symptom which has previously been reported on wheat in response to *P. volutum* infection (Chamswarnng and Cook 1985). *Pythium* sp. F86 appears to be a somewhat less aggressive pathogen than *P. volutum*, allowing some radicle growth before tip necrosis and having less dramatic impacts on emergence (Fig. 3; considering isolates as reps, T-test on corn radicle length for *P. volutum* versus *Pythium* sp. F86; $P = 0.0039$). In sum, these pathogenicity assays indicate that *P. volutum* and *Pythium* sp. F86, the dominant oomycetes in roots of a rye cover crop, are capable root rot pathogens of corn seedlings.

Fungal community membership and dynamics in the roots of a rye cover crop. Our final fungal 18S rRNA gene dataset consisted of 56,783 reads, which were binned into 837 OTUs. The dataset was divided across the Ascomycota (76.3% of sequence reads), Basidiomycota (22.8%), Chytridiomycota (0.7%), Glomeromycota (0.1%), and Mucoromycotina (0.01%), with the remainder of reads not confidently classified. Even after standardizing sampling effort by subsampling to the number of reads in the smallest sample (405 reads), estimated coverage of fungal taxa present in rye roots remained at least 90% for every sample. This indicates that sequencing more deeply would have revealed relatively few additional taxa.

The fungal community in roots of cereal rye grown as a winter cover crop and actively terminated with herbicide went through a successional process over time (Fig. 5A). Significant fungal community change occurred between each sampling date (AMOVA, $P \leq 0.007$). At the coarsest taxonomic resolution, change over time

Fig. 1. A visualization of patterns of similarity in oomycete community structure among samples of cereal rye roots via principal coordinates analysis on Bray-Curtis dissimilarities. Shaded 95% confidence ellipses for the centroid of each group are shown. The axis labels indicate the proportion of the variation explained by each axis. The vector showing correlation of soil sand content was fit onto the ordination via the envfit function in the VEGAN package for R.

was related to the displacement of basidiomycetes by ascomycetes (Fig. 5B). The fungal taxa whose relative abundance increased the most over time were OTU0001 (unclassified within Hypocreales), OTU0013 (*Atractospora*), OTU0007 (unclassified within Dothidiomycetes), OTU0023 (*Bisporella*), and OTU0018 (unclassified with Sordariomycetes). The fungal taxa whose relative abundance decreased the most over time were OTU0003 (*Mrakia*), OTU0004 (unclassified within Agaricomycetes), OTU0008 (unclassified within Tremallales), OTU0006 (unclassified within Pleosporales), and OTU0009 (*Bisporella*). It is notable that distinct OTUs with the same genus (*Bisporella*) responded oppositely over time. This highlights the potential for wide variation in phenotype or ecological role within narrowly defined microbial taxa, which poses a substantial challenge to drawing inferences from community survey data.

The observed number of fungal OTUs ranged from 22 to 71 per sample, after equalizing sampling depth. Phylogenetic diversity ranged from 0.69 to 2.78, Hill's diversity index values ranged from 2.7 to 26.1, and Shannon's evenness index values ranged from 0.32 to 0.77. However, these measures of fungal richness, evenness, and diversity did not differ significantly between sampling time points (ANOVA; $P > 0.05$).

Five fungal OTUs were detected in every plot that was sampled, indicating that these were the most universally present fungi on or in

roots of rye cover crops at our field site. Of these, two could be confidently classified to genus (*Mrakia* spp., and *Tetracladium* spp.). *Mrakia* is a basidiomycetous yeast known for being adapted to growth at cold temperatures (Tsuji et al. 2015), while *Tetracladium* has been characterized as a root endophyte (Sati et al. 2009). The remaining universally present OTUs could not be assigned with confidence to finely resolved taxonomic ranks, but all belonged to Pezizomycotina (Ascomycota).

Sixty-seven of the observed OTUs could be confidently identified to the genus rank (Supplementary Material S3). Several plant pathogens were included among these, including *Aspergillus*, *Cochliobolus*, and *Verticillium*. Other genera of interest, such as *Sarocladium*, have been categorized at times as plant pathogens, and at other times as nonpathogenic, beneficial endophytes (Giraldo et al. 2015). Potential pathogen antagonists were also detected. For instance, *Cryptococcus* has been evaluated for potential as a bio-control agent to protect against plant disease (Schisler et al. 2015).

DISCUSSION

This work improves our understanding of how phytobiomes adjust to host plant death. Because plant death represents a switch in selective pressures acting on the phytobiome, this can be thought of as an instance of the more general process of community change in

A Switching dominant *Pythium* species across the field

B *Lagena radicola* abundance over time

Fig. 2. A, Relative abundance of each of the two most abundant oomycete species by experimental block. The experiment is blocked from west to east across the field. **B**, Relative abundance of *Lagena radicola* within oomycete communities in rye roots, as a function of the interval of time since herbicide application. DPS = days post spraying of herbicide to kill the cereal rye cover crop. In each panel, bars topped by the same letter do not differ significantly (analysis of variance with Tukey contrasts; $P > 0.05$). While raw proportions are presented, statistical analyses were performed on centered log-ratio transformed data.

response to perturbation. There have been several reports of microbial community change over time in plant roots and in the rhizosphere (Cotta et al. 2013; DeAngelis et al. 2009; Sugiyama et al. 2014), but as far as we are aware, this is the first study to report high-resolution microbial community structure over a time series in senescent roots. From an agronomic perspective, this work informs the more practical questions of how plant pathogens respond to plant death and how pathogen communities vary across space.

We hypothesized that oomycete and fungal communities would go through a period of rapid dynamics on their dying host plants as the rye cover crop was actively killed with herbicide. Certain pathogens may be able to grow rapidly on dying host plants (Bakker et al. 2016), due to compromised host defenses and increased resource availability. For instance, tilling in green cover crop shoot material has been shown to elevate *Pythium* population densities in

some cases (Manici et al. 2004). However, pathogens should eventually be displaced from plant litter by more competitive saprotrophs. Yu and colleagues (2012) contrasted fungal communities in roots of *Pisum sativum* at vegetative, flowering, and senescent stages. They observed that mycorrhizal fungi and some putative pathogens declined in relative abundance in senescent roots, while putative saprotrophs increased in abundance. In the present study, *L. radiculicola* served as a model of this process. *L. radiculicola* is believed to be an obligate biotroph (Barr and Desaulniers 1990; Vanterpool and Ledingham 1930), and declined precipitously in abundance within several weeks of herbicide application to the cereal rye cover crop. However, other pathogens, such as *P. volutum*, did not demonstrate the same decline in abundance with host plant death. It is possible that relative abundance among sequence reads does not relate cleanly to the level of

Fig. 3. A, *Pythium volutum* density in corn seedlings grown in the field ($n = 5$). **B**, Corn radicle length at 21 days after planting in a *Pythium*-infested medium or a noninfested control. Four isolates of *P. volutum* and three isolates of *Pythium* sp. F86 were tested. Values above the bars indicate emergence rates ($n = 10$ pots, with two seeds planted to each). **C**, Representative symptoms on corn seedlings after growing in a medium infested with *P. volutum*. Note the stunted radicles with necrotic tips (circled) and the improper leaf elongation. In each panel, bars topped with the same letter do not differ significantly (analysis of variance with Tukey contrasts; $P > 0.05$).

active biomass (Amend et al. 2010; Egge et al. 2013). For instance, much of the *Pythium* DNA present in our extracts may have originated from spores or survival structures. Some pathogens may also be competent saprotrophs, able to survive quite well after host plant death (Bakker et al. 2016).

Fungal biomass, richness and diversity have been previously reported to increase in senescent roots or in the rhizosphere of senescent plants, compared with earlier developmental stages of live plants (Hannula et al. 2012; Lee et al. 2011). While our data did not indicate a significant increase in fungal diversity as plant roots died, this may have been a function of our use of a marker (the 18S ribosomal RNA gene) with fairly limited power to resolve fungal taxa (Schoch et al. 2012). Because multiple described taxa may share an identical 18S rRNA gene sequence, many of the fungal OTUs described here may represent mixtures of distinct species. Thus, our method likely under-reports fungal diversity. There are always tradeoffs involved in choosing a marker gene, as the performance of candidate marker genes for delimiting taxa differs among taxa (e.g., see Table 1 in Hyde et al. 2014). The inability to confidently identify the organism of origin for many sequence reads also limits the ability to resolve fungal community change. Such inability to classify reads may be due to a lack of homologous sequence in the reference database, or to closely matching sequences in the reference database originating from multiple different taxa.

Early termination of the rye cover crop was expected to provide cooler and wetter conditions during the sampling period, while late termination was expected to provide warmer and drier conditions. We anticipated that these differences could impact microbial community dynamics and that testing both conditions might provide an indication of the variability of community dynamics in response to host plant death. The timing of termination also impacted rye growth stage and the total biomass produced, with the late terminated rye beginning to enter reproductive phase and accumulating substantially more biomass (data not shown). We did not include a treatment in which rye plants were sampled over time in the absence of herbicide application. Thus, we cannot state how

stable fungal community structure may have been prior to or in the absence of herbicide application, or how responsive fungal communities may have been to temperature or moisture conditions rather than to host death per se. Any time series in this system (winter cereal rye grown under field conditions and sampled in the spring) would face the issue of confounding time with soil temperature and moisture conditions, and with changes in host plant physiology as the rye enters its reproductive phase. An additional potential source of confounding is the increase in the number of subsamples collected on the third sampling date. While pooling three subsamples per plot on this date likely helped to generate a more accurate plot-level “average” community profile, the decision to increase subsampling may have influenced observed community composition and structure compared with the single subsamples drawn on the first two sampling dates.

One of the striking findings of this work was the indication of the very strong spatial structuring of oomycete communities, while fungal communities showed greater responsiveness to time and to host plant death. *Pythium* species lack the ability for aerial dispersal possessed by many fungi, and this characteristic would contribute to spatial structuring of *Pythium* populations. Our field site also had a long history of management exclusively without tillage. All field operations are carried out in an orientation perpendicular to the spatial gradient in oomycete community structure. That is, oomycete communities change along an east-west gradient, while farm equipment is always operated in a north-south orientation in this field. Thus, the particular history and management of the agricultural field studied here is likely to have encouraged spatial structuring of *Pythium* populations by providing very few opportunities for mixing of populations from west to east. The westernmost end of the field has the highest elevation, less slope gradient, shorter slope length, the highest organic matter content and the lowest sand content. However, there is not a clean gradient of sand or OM content across the field. Historical contingency may have been an important factor, as the westernmost portion of the field (block 1) was under different management from the rest of the field at some time greater than 20 years ago.

Fig. 4. Maximum likelihood phylogeny showing the placement of our *Pythium volutum* and *Pythium* sp. F86 isolates among their closest relatives. The phylogram is based on internal transcribed spacer sequence data, using the Jukes-Cantor nucleotide substitution model and the neighbor-joining construction method. Node labels indicate bootstrap support. GenBank accession numbers are provided in the branch tip labels.

Similar to our findings in rye roots, a previous amplicon sequencing approach to identify oomycetes associated with carrots revealed relatively simple communities dominated by few taxa (Sapkota and Nicolaisen 2015). It was not expected that *P. volutum* would be recovered from our field site, particularly as the dominant member of the oomycete community. None of the several *Pythium* experts and plant disease diagnosticians from around the Upper Midwest that we consulted were familiar with this species. However, the species was originally described as a pathogen of cereals on the Canadian plains (Vanterpool and Truscott 1932) and has also been reported on small grains in the Pacific Northwest (Chamswang and Cook 1985) and in Japan (Ichitani et al. 1986). More recently,

P. volutum has received attention as a pathogen of turf grass (Kerns et al. 2009; Kerns and Tredway 2008b). A key reason for the limited data on *P. volutum* may be that it is extremely sensitive to several chemicals used in the most common semiselective media for oomycetes (data not shown; see also Chamswang and Cook 1985; Kerns and Tredway 2008a). Thus it seems likely that this species has been largely overlooked because of a bias inherent in traditional cultivation approaches for *Pythium* species.

Pythium sp. F86 represents an apparent novel, undescribed taxon closely related to *P. volutum*. Although we do not have the taxonomic expertise necessary for description of novel *Pythium* species, we were able to collect *Pythium* sp. F86 isolates from rye roots.

A Fungal community structure, over time

B Phylum level relative abundances

Fig. 5. A, Principal coordinates analysis plot for the visualization of patterns of similarity in fungal community structure (weighted UniFrac distance) among samples of cereal rye roots. Points are colored by sampling date. Filled symbols represent samples from the early termination treatment, while open symbols represent samples from the late termination treatment. Shaded 95% confidence ellipses for the centroid of each group are shown. The axis labels indicate the proportion of the variation explained by each axis. **B**, The relative abundance of fungal phyla in cereal rye roots, at different intervals since the application of herbicide. DPS = days post spraying of herbicide.

These isolates have ITS and COI marker gene sequences that suggest that they are part of a currently undescribed lineage. Thus, based on first detection in an amplicon sequencing survey, we were able to isolate a probable new species of *Pythium*.

L. radiculicola, another of the most abundant oomycetes in our study, is even less amenable to cultivation, as it is believed to be an obligate intercellular parasite of plant roots (Barr and Desaulniers 1990; Vanterpool and Ledingham 1930). *L. radiculicola* has been reported as very commonly causing stunting of root tips in the small grains, but to date has required microscopic examination of roots for diagnosis (Macfarlane 1970). By adopting an amplicon sequencing approach, our work revealed dominant members of the oomycete community that would likely have been missed entirely by standard cultivation techniques. Notably, a recent study from New Zealand also identified both *P. volutum* and *L. radiculicola* in sequences of amplified oomycete DNA extracted from environmental samples (Dickie et al. 2016), which lends further support to the notion that these species may be of underappreciated importance because of their recalcitrance toward cultivation.

L. radiculicola has been reported to be a pathogen on corn (Vanterpool and Ledingham 1930), and we demonstrate here that *P. volutum* and *Pythium* sp. F86 are able to cause disease on corn. Thus, the influence of cereal rye cover crops on oomycete communities may serve to expand populations capable of causing disease on the subsequent corn crop (Bakker et al. 2016).

In conclusion, a high throughput sequencing survey of fungal and oomycete communities associated with dying cereal rye cover crops revealed differences in the forces structuring oomycete versus fungal communities. The dominant oomycete taxa in this system are not readily cultured. However, guided by the amplicon sequencing survey, we were able to isolate the dominant *Pythium* species associated with cereal rye cover crop, and to demonstrate their pathogenicity on corn. Future work should explore whether these organisms are also abundant in other systems and what their contribution may be to corn seedling disease.

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