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Variation in soybean rhizosphere oomycete communities from Michigan fields with contrasting disease pressures

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ABSTRACT

Although oomycete species can contribute to significant losses in soybean plant density, root mass and yield, they are often underrepresented in high-throughput sequencing studies. In this study, soybean oomycete rhizosphere communities were characterized over two years from locations with and without historical disease pressure. The goals of this research were to examine the effect of location, soybean genotype, and seed treatment on oomycete communities. Soybean oomycete rhizosphere communities were dominated by *Pythium*, but community composition differed depending on the location and year. *Pythium ultimum* var. *ultimum* was the most abundant oomycete OTU accounting on average for > 30% relative abundance in high disease pressure sites. However, sites without historical disease pressure were not devoid of oomycete plant pathogens indicating that historical disease pressure may be due to an imbalance of species, rather than simply the presence or absence of highly pathogenic species. High-disease pressure sites contained more oomycete taxa and were less even. There was no substantial evidence of seed treatment or soybean genotype impacting oomycete community composition or diversity, however, plant density and root biomass increased with the addition of neonicotinoid insecticides. Overall, this study represents an improvement of our understanding of oomycete communities in soybean rhizosphere and the impacts of agronomic factors on oomycete diversity.

1. Introduction

Soybean (*Glycine* max (L.) Merr) is regarded as a critical crop for global food security (Singh, 2007). With a worldwide harvest of 223 million tons, soybean is ranked the fourth most important crop worldwide (Hartman et al., 2011). Successful seed germination and emergence are essential for soybean establishment in fields, but many pathogens can kill soybean plants. Some of the most destructive pathogens are oomycetes, such as the genera *Pythium* and *Phytophthora*, which can infect the host in both the seed (pre-emergence) or seedling (post-emergence) stages. Symptoms of oomycete seeding rot can include dead seeds or seedlings, water-soaked lesions along the hypocotyl and stem, root-mass reduction, seedling stunting, and reduced seedling vigor.

Moreover, even when disease is not severe enough to cause plant death, seedling rot can negatively influence yield (Martin, 2009; Lévesque, 2011). Seedling disease has increased with the move to minimum or no-till production systems and earlier planting dates. Minimum or no-till production systems increases crop residue in fields. However, crop residues also act as a reservoir for pathogen inoculum and slow soil warming. While earlier planting dates increases the growing season and promotes higher yield potential, it also exposes seedlings to cooler soils and unfavorable conditions for growth that can lead to greater seedling disease (Vossenkemper et al., 2016; Pankhurst et al., 1995; Larkin, 2015).

In previous studies, over 80 oomycetes species belonging to genera *Phytophthora*, *Pythium*, *Phytopythium*, and *Aphanomyces* were identified to be associated with soybean seedlings (Rojas et al., 2017a, 2017b; Broders et al., 2009; Zitnick-Anderson and Nelson, 2015). While some oomycetes such as *Phytophthora sojae* or *Pythium ultimum* are well known to be pathogenic, others are weakly pathogenic or may be mycoparasitic or entomopathogenic (Martin and Loper, 1999; Paul, 1999; Su et al., 2001; Scholte et al., 2014; Ribeiro and Butler, 1995) suggesting potential complex and multi-kingdom interactions. Therefore, studying the oomycete community and its association with disease severity and agricultural practices will provide information for improved oomycete disease management.

Traditional culture-based surveys have been used to survey

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oomycete communities and have provided important knowledge of these organisms. However, a significant disadvantage of this methodology is the labor needed for pathogen isolation, characterization, and maintenance (Rojas et al., 2017a; Coffua et al., 2016). Culture-based surveys may also have biases by the isolation protocol or the culture medium used, and some oomycete species are fastidious or hard to culture (Bakker et al., 2017). An alternative methodology is a culture-independent approach using high-throughput amplicon sequencing or metabarcoding, which has been a widely used technique to provide a more comprehensive understanding of microbial community composition. Metabarcoding studies of bacteria or fungi have been applied to understand the association between microbial community and traits of interest, but metabarcoding studies of oomycetes are less common despite their importance in plant disease, ecosystem function, and community assembly (Agler et al., 2016). With a curated oomycete ITS database (Robideau et al., 2011) and improved strategies to preferentially amplify oomycete ITS sequences from environmental samples (Sapkota and Nicolaisen, 2015; Riit et al., 2016; Taheri et al., 2017), there is an increasing interest and ability to characterize oomycete communities using metabarcoding (Rojas et al., 2019; Agler et al., 2016; Coince et al., 2013; Vannini et al., 2013; Sapkota and Nicolaisen, 2015; Singer et al., 2016; Bakker et al., 2017; Riit et al., 2016; Durán et al., 2018; Coffua et al., 2016; Cerri et al., 2017).

It has been recognized that location and edaphic factors are dominant drivers of oomycete community structure (Rojas et al., 2017a, 2017b; Broders et al., 2009; Zitnick-Anderson et al., 2017; Taheri et al., 2017). However other agronomic factors have not been examined in detail. For example, soybean genotypes have been shown to recruit different beneficial bacterial taxa (Mendes et al., 2014). Additionally, there are inter- and intraspecific variation in sensitivity to anti-oomycete chemicals (oomicides) used within soybean seed treatments (Broders et al., 2007; Matthiesen et al., 2016; Noel et al., 2019; Radmer et al., 2016; Weiland et al., 2014) suggesting the possibility of specific oomycete lineages being selected or counter-selected in the soybean rhizosphere in the presence of different oomicide-genotype combinations. Moreover, because soybean seed treatments usually contain oomicides along with many other active ingredients such as fungicides, nematicides, or insecticides, the likelihood of these chemicals to influence seedling rot diseases or shape the structure of oomycete communities is considerable. Soybean seed treatments have been observed to be more effective in field sites in Allegan county of Michigan, where heavy seed and seedling disease has been observed (Rossman et al., 2018). Herein, this study aimed to provide a profile of the oomycete community present in soybean rhizosphere soils and compare the structure of oomycetes communities between high disease pressure fields in Allegan county and low disease pressure field sites in Ingham county of Michigan. To accomplish this we used next generation amplicon sequencing to characterize oomycete communities between these two counties in two years. We investigated the association between oomycetes community and disease severity as well as seed treatments, soybean genotype, and identified the differences in oomycete communities that may link to the difference in disease pressures observed between these sites.

2. Materials and methods

2.1. Experimental design and field setup

Field experiments were set up in two locations, Allegan county (with high disease stress) and Ingham county (with low disease stress) of Michigan, in two years (2015 and 2016). In each location-year combination, a complete randomized factorial design with four soybean genotypes, four treatments, and six replicates was set up in plots (6.10 m by 3.05 m), which resulted in a total of 96 plots in each of four location-year combinations. All Asgrow genotypes used in this study had a Phytophthora root rot tolerance rating of 4 or 5, on a scale of 1–9 where 9 is the best score possible. Additionally, Asgrow varieties were documented to contain the Rps1 gene for resistance to certain *Phytophthora sojae* races. Pioneer varieties had a Phytophthora root rot tolerance of 4 on a scale of 1–9 where 1 is the best score. Pioneer variety P26T76R contained Rps1K gene for resistance to certain *Phytophthora sojae* races. It was unknown if other varieties used in this study contained Rps genes. Full seed treatment formulations and application rates were described in Rossman et al. (2018). In brief, seed treatments used in this study were generalized based on the target pests, herein abbreviated as non-treated control (NTC), fungicides (F), fungicides plus insecticides (FI), and fungicides plus insecticides plus a biological control nematode protectant (FIN) (Supplemental Table 1). The fungicide component contained the oomicides metalaxyl or mefenoxam. Soybean seeds were planted 3.8 cm below ground, in six rows with 38 cm row spacing, and at a seeding rate of 395,000 seeds ha^{-1}. In all locations, the crop planted in the previous growing season was corn. The coordinates, planting dates, plant sampling dates, and precipitation occurring two weeks after planting for each location-year combination along with bulk soil texture and nutrient levels as characterized by the MSU Soil and Plant Nutrient Laboratory were documented (Table 1). Soil classification in Allegan 2015 and 2016 was a Brady Sandy Loam (mesic Aquollic Hapludalfs). In 2015, the soil in Ingham was a Conover Loam (mesic Aquic Hapludalfs), but was a Corunna Sandy Loam (mesic Typic Hapludalfs) in 2016 (Soil Survey Staff, 2010).

2.2. Sample collection

For each location-year combination, three measurements were taken for disease stresses, including plant density, root biomass, and yield. The four middle rows in each six-row plot were harvested for yield quantification at the end of the season, and plant density was measured by counting the number of emerged soybeans in two of the four harvested rows of each plot at the first trifoliate growth stage. Meanwhile, rhizosphere samples were collected from two side rows (non-harvested rows) of ten random emerged plants (excluding plants in 2.74 m from either end of a row) were collected in each non-harvested row. Loosely adhering soil was shaken from the roots, and these twenty plants were pooled to represent a plot and stored together on ice and transported to the lab for processing the following day. Root tissue was determined based on the soil line, and ten random roots were cut, washed with tap water, and oven dried before measuring the root biomass. The remaining ten roots were used for rhizosphere soil collection. Rhizosphere soil was washed from roots by vortexing for 15 s in a 50 ml tube with 35 ml 10 mM NaCl solution (Shakya et al., 2013). Roots were removed from the tube, and the solution was centrifuged for 10 min at 1685 G to pellet rhizosphere soil. The supernatant was decanted, and the rhizosphere pellet was frozen at −20 °C then lyophilized and stored in sterile coin envelopes with a desiccant before DNA extraction.

2.3. Oomycetes ITS1 amplification and sequencing

For rhizosphere soil samples, total DNA was extracted from 0.35 g of lyophilized rhizosphere soils using the Qiagen PowerMag® Soil DNA Isolation Kit (Toronto, ON, Canada) following the manufacturer's recommendations. A DNA extraction negative control and artificial oomycetes community (Rojas et al., 2019) containing the genomic DNA of 15 oomycete species mixed equivalently and adjusted to a final concentration of 0.05 ng μl^{-1} were included in polymerase chain reaction (PCR) amplification for internal transcribed spacer 1 (ITS1) of oomycetes using a three-step PCR program modified based on the protocol from Lundberg et al. (2013) which uses primers with frameshifts to increase nucleotide diversity and avoid a Phix spike-in. In the PCR step one, samples were amplified using primers ITS6 (5′-GAAGGTGAAGTC GTAACAAGG-3′) and ITS7 (5′-AGCGTTCTTCATCGATGT-3′) (Cooke et al., 2000) with an annealing temperature of 59 °C, which

Table 1
Field location, soil properties, and weather description.

Year	Location	Coordinates	Planting date	Date sampled	Soil classification	Soil texture			Soil nutrients			Weather summary two weeks after planting	
						Sand	Silt	Clay	pH	CEC[a]	SOM[b]	Sum precipitation	Minimum average temperature
						%	%	%		meq 100 g^{-1}	%	mm	°C
2015	Allegan	42.70 N, −86.01 W	29-May	16-Jun	Brady sandy loam	56.4	25.7	17.9	6.8	8.7	3.4	46.61	12.13
	Ingham	42.69 N, −84.50 W	23-May	9-Jun	Conover loam	46.3	35.7	18.0	7.1	9.4	3.2	48.8	11.8
2016	Allegan	42.70 N, −86.01 W	19-May	13-Jun	Brady sandy loam	42.4	36.5	21.1	5.5	11.6	3.0	9.05	12.12
	Ingham	43.05 N, −84.40 W	20-May	9-Jun	Corunna sandy loam	66.4	17.4	16.2	5.5	10.6	3.0	9.88	13.33

[a] CEC refers to the cation exchange capacity and is measured in the milliequivalents (meq) per 100 g soil.
[b] SOM refers to the percent soil organic matter.

preferentially amplifies oomycetes ITS1 while minimising fungal ITS amplification (Sapkota and Nicolaisen, 2015). In the PCR step two and step three, ITS1 amplicons were amplified by frameshift primers and then by a 10 bp barcode plus Illumina adapters, respectfully. All PCR steps contained a final concentration of 1× buffer, 0.2 mM dNTP, 0.8 mg ml^{-1} bovine serum albumin (BSA), 0.2 µM primers and 1 U DreamTaq Polymerase (ThermoFisher Scientific, USA) and 2 µl DNA template for the PCR step one and step two. The PCR step three contained 4 µl of aliquots from PCR step two. Thermal cycling conditions for PCR step one were as followed: 95 °C for 5 min followed by 15 cycles of 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s followed by a final extension at 72 °C for 7 min. Thermal cycling conditions for PCR step two were as followed: 95 °C for 5 min followed by 10 cycles of 95 °C for 20 s, 57 °C for 30 s and 72 °C for 35 s followed by a final extension at 72 °C for 7 min. Thermal cycling conditions for PCR step three were as follows: 95 °C for 5 min followed by 10 cycles of 95 °C for 20 s, 63 °C for 50 s and 72 °C for 1 min 20 s followed by a final extension at 72 °C for 7 min. PCR products were normalized using SequalPrep™ Normalization Plate Kit (ThermoFisher Scientific, USA), pooled then concentrated 20:1 with Amicon® Ultra 0.5 mL filters (EMDmillipore, Germany). The amplicon library was purified and size selected with Agencourt AMPure XP magnetic beads at 0.6× sample to bead volume (Beckman Coulter, USA) and subsequently paired-end sequenced (2 × 250 bp) on an Illumina MiSeq using the v2 500 cycles kit (Illumina, USA).

2.4. Data processing

ITS1 paired-end reads were quality evaluated with FastQC and then demultiplexed according to sample barcodes in QIIME 1.9.1 (Caporaso et al., 2010). Primers were removed from reads with Cutadapt 1.8.1 (Martin, 2011), and then quality filtered using USEARCH 9.1.13 (Edgar, 2010) based on read quality and expected error threshold obtained from VSEARCH stats 2.3.2 (Rognes et al., 2016). Qualified reads were then trimmed to equal length and singletons were removed using USEARCH 9.1.13 (Edgar, 2010). De novo OTU clustering was performed based on 97% similarity using the UPARSE algorithm, which includes a chimera detection step (Edgar, 2013). An OTU table was generated using a custom python script and taxonomy was assigned to each OTU using CONSTAX with a confidence threshold of 80% (Gdanetz et al., 2017). This algorithm generates a consensus taxonomy from the Ribosomal Database Project (RDP) naïve Bayesian Classifier (Wang et al., 2007), UTAX (Edgar, 2013), and SINTAX (Edgar, 2016) classifiers. The reference database used for taxonomy assignment included the curated oomycete ITS sequences from Robideau et al. (2011), LéVesque and De Cock (2004), and the UNITE version 7.2 1.12.2017 fungal database (UNITE community, 2017). OTUs that were identified as fungal were removed from further analysis. OTU sequences identified in the phylum Oomycota were BLAST searched against the NCBI nucleotide database (accessed January 2019) to corroborate taxonomy assignments. If CONSTAX assigned an OTU to a species or if the top BLAST matched an OTU sequence to a species with over 90% identity and a bitscore ≥300, the OTU was grouped to oomycete clades according to Robideau et al. (2011) and LéVesque and De Cock (2004). Samples with < 1000 reads were dropped from analysis due to low sequencing coverage.

2.5. Statistical analysis

Data were imported into R 3.2.2 (R core team 2016) and analyzed using the packages 'phyloseq' 1.24.2 (McMurdie and Holmes, 2013) and 'vegan' 2.5.3 (Oksanen et al., 2018). All samples were rarefied to the minimum reads per sample (i.e., 1522 reads) before α-diversity analysis (Supplemental Fig. 1). Alpha-diversity was estimated for each sample, and only OTUs observed more than once were used before estimating α-diversity. OTU richness (S), Shannon diversity (H′), and

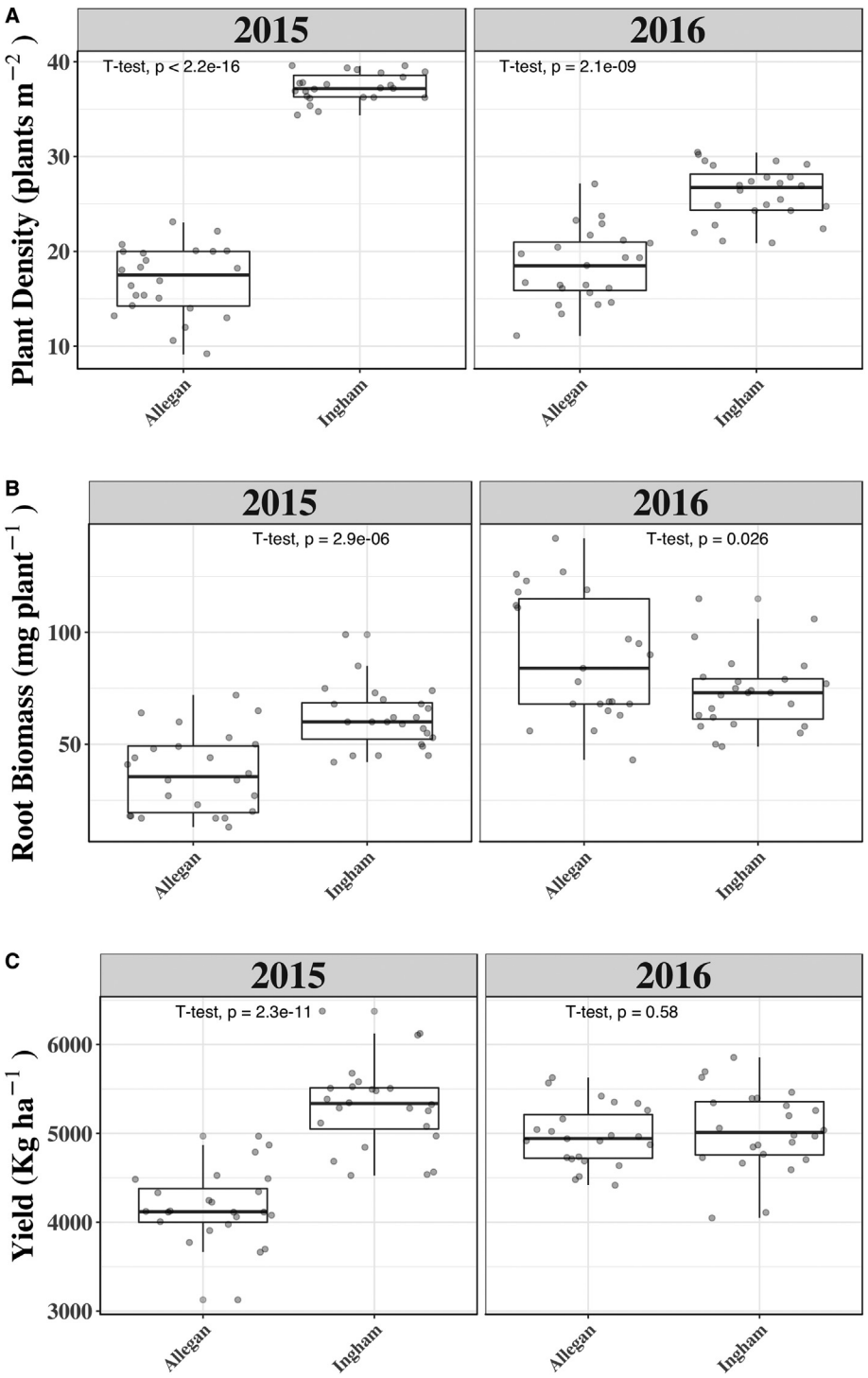

Fig. 1. The effect of location tested within year on plant density (A), root biomass (B) and yield (C) of plants from non-treated seed across all genotypes. *t*-Test *P* value is shown within each figure.

Plieou's evenness (J) were used as α-diversity metrics. Non-metric multidimensional scaling (NMDS) (k = 2) was performed on Bray-Curtis distances to examine differences in beta-diversity (Bray and Curtis, 1957). A permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis distances, was used to test for differences in community centroids due to location, year, seed treatment, soybean genotype, and all interactions using the 'adonis2' function in the package 'vegan'. Differences in community multivariate dispersion were tested with the 'betadisper' function in the R package 'vegan'. Stepwise model building was used to select a constrained model for input into distance-based redundancy analysis (db-RDA) to examine the variation in Bray-Curtis distances due to plant density, root biomass, and yield. A Monte-Carlo permutation test was used to test the significance of constrained factors within db-RDA. Indicator species analyses was performed using the package 'indicspecies' 1.7.6 to identify OTUs significantly associated with covariates (De Caceres and Legendre, 2009).

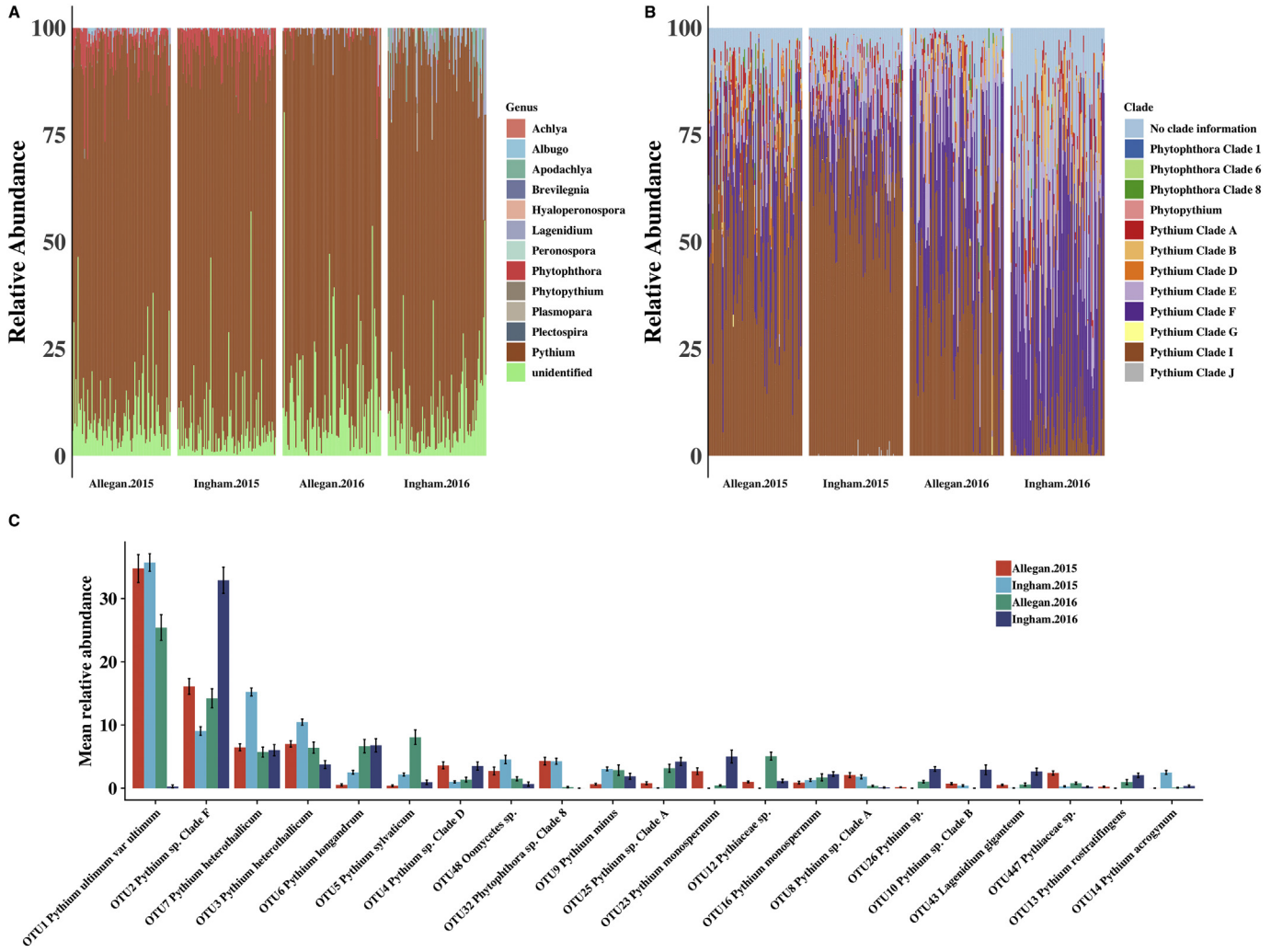

Fig. 2. (A) Genus-level relative abundance of oomycete communities for each year-location combination. (B) Clade-level relative abundance of oomycete OTUs in the genera *Pythium*, *Phytophthora*, and *Phytopythium*. (C) Mean relative abundance of OTUs where the OTU was observed > 2% mean relative abundance at least one site.

2.6. Data availability

OTU table, metadata, and taxonomy files along with code are available on (https://github.com/noelzach/Oomycete-Amplicon-Seq-Soybean-Rhizosphere). Raw sequence data and metadata were deposited in the Harvard Dataverse (https://doi.org/10.7910/DVN/30IEJJ) (Noel, 2019).

3. Results

3.1. Overview of experimental design and factors

A two-year field study in two locations where one with high disease stress (Allegan county of Michigan) and another with low disease stress (Ingham county of Michigan) was established to understand the association among genotypes, seed treatments, and oomycete rhizosphere communities to the severity of soybean root and seed rotting diseases. Among three disease severity measurements, plant density was the most consistent indicator as it was lower in Allegan than Ingham in both years, especially for 2015 where Allegan had on average 17.62 plants m^{-2} compared to Ingham which had on average 31.72 plants m^{-2} (Fig. 1A). Root biomass and yield reflected this tendency, but the reduction of root biomass and yield in Allegan was more evident in 2015 than 2016 (Fig. 1B and C).

In Allegan, plant density and root biomass was significantly higher for the FIN treatment compared to the NTC for all genotypes tested for both years. However, no significant difference in either plant density or root biomass was observed when F was applied in Allegan alone, which indicated the influence of FI or FIN interaction was more important in determining the outcome of plant density and root biomass. There was no significant improvement in plant density, root weight, or yield due to seed treatment in Ingham regardless of the soybean genotype (Supplemental Table 2).

3.2. Oomycete community composition in soybean rhizospheres

In respect to the difference in the disease severity difference between Allegan and Ingham counties (Rossman et al., 2018), an ITS-amplicon sequencing strategy was applied to illuminate the structure and composition of oomycetes communities from 362 rhizosphere samples between these two locations. A total of 2,628,469 quality filtered reads were obtained, and after data processing, reads were clustered into 621 OTUs of which over half (62%) were classified into the Kindom fungi. Among these OTUs, 230 OTUs were assigned to the kingdom Stramenopila, and 219 of the Stramenopila OTUs were classified into phylum Oomycota using CONSTAX. In summary, 219 oomycete OTUs were identified from a total of 361 rhizosphere samples from Allegan and Ingham.

The most abundant genus was *Pythium* at 86.3% across the rhizosphere samples. *Phytophthora* comprised 3.2% and the genera

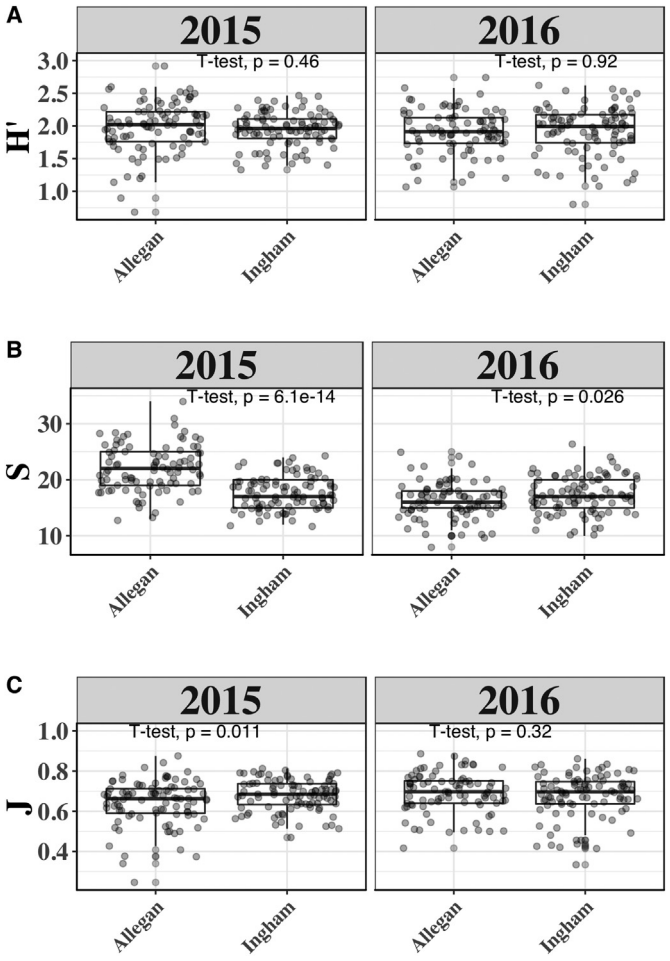

Fig. 3. Influence of location on oomycete alpha diversity within year as estimated by (A) Shannon diversity index (*H'*) and (B) richness (S) and (C) Plieou's evenness (J). *t*-Test *P* value is shown within each figure.

Lagenidium, *Apodachlya*, *Albugo*, *Plasmopara*, *Phytopythium*, *Peronospora*, *Hyaloperonospora*, *Brevilegnia*, *Plectospira*, and *Achlya* together comprised of 1.8% across rhizosphere samples, and 8.7% of the OTUs were not confidently assigned to an oomycete genus (Fig. 2A). *Pythium* clade I (including important pathogenic species like the *Pythium ultimum* species complex, and *Pythium heterothallicum*) was the most abundant clade in Ingham 2015, Allegan 2015, and Allegan 2016 making up 66.3%, 55.0%, and 44.4% across the rhizosphere samples. In Ingham 2016, *Pythium* clade F (including important pathogen species like *Pythium irregulare* and *Pythium sylvaticum*) was most abundant making up 41.1% of the reads (Fig. 2B). The most abundant OTU was identified as *Pythium ultimum* var. *ultimum* (OTU1 in *Pythium* clade I) and was found in Ingham 2015, Allegan 2015, and Allegan 2016 (Fig. 2C), while an unidentified *Pythium* species (OTU2 in *Pythium* clade F) was the most abundant in Ingham 2016 (Fig. 2C). Other frequently observed OTUs were identified as *Pythium heterothallicum* (OTU 3 and 7 in *Pythium* clade I), which was present in all location-year combinations. *Phytophthora sojae* (OTU 102), an important soybean pathogen, was detected in this study but was not highly abundant compared to OTUs within the *Pythium* genus, and was therefore not considered the primary pathogen associated with disease symptoms in this study.

3.3. α-Diversity analysis for Allegan and Ingham

In order to understand the oomycete communities, α-diversity was estimated for each year-location combination. There was no significant difference in Shannon index (H′) between locations (Fig. 3A). However, when the diversity was broken down into Plieou's evenness (J) and richness (S), the richness was significantly higher in Allegan than Ingham in 2015 ($P < 0.0001$) (Fig. 3B) whereas the evenness was significantly lower in Allegan than Ingham in 2015 ($P < 0.01$) (Fig. 3C). Additionally, there were no significant differences in α-diversity metrics due to genotype or seed treatment within any location-year combination, indicating the α-diversity of oomycete communities may be relatively similar and that they are not affected by soybean genotype or seed treatment.

3.4. β-Diversity analysis and identification of unique oomycete communities in Allegan

Rhizosphere communities were highly clustered based on location and year, and the interaction contained significantly different centroids ($P < 0.001$) and multivariate dispersion ($P < 0.001$) (Fig. 4A). Similar to α-diversity, neither soybean genotypes nor seed treatment influenced β-diversity. Most oomycetes OTUs were associated with multiple year-location combinations but, there were 21 OTUs uniquely associated with Allegan 2015 (Fig. 4B; Table 2). These 21 OTUs, unique to Allegan 2015 included OTUs identified as *Pythium ultimum* var. *ultimum*, *Pythium heterothallicum*, *Pythium irregulare*, and *Laganidium giganteum*, and Pythiaceae sp. which added up to 5.61% relative abundance (Table 2).

Focusing on Allegan 2015, a model selection in the distance-based redundancy analysis (db-RDA) pointed out a significant association between oomycetes community and plant density ($P < 0.001$) and root biomass ($P < 0.005$), but not yield based on Monte-Carlo permutation tests. However, only 3.89% of the total variation in oomycete communities could be attributed to plant density and root biomass. Rhizosphere samples from plots with increased plant density and root biomass were associated with positive db-RDA1 scores. Rhizosphere samples from plots with increased root biomass were more associated with positive db-RDA2 scores, whereas samples with increased plant density were more associated with negative db-RDA2 scores (Fig. 5A).

Among plots in Allegan 2015, OTU18 *Pythium* sp. nov (Clade B) was significantly associated with higher plant density and OTU41 *Pythium ultimum var. ultimum* (Clade I) was significantly associated with higher root biomass. On the other hand, OTU135 Saprolegniaceae sp. was significantly associated with lower plant density and OTU71 Oomycete sp. was significantly associated with lower root biomass (Table 3). Among these OTUs identified from indicator species analysis, OTU41 and OTU71 were also found to be unique to Allegan 2015 (Table 2), which indicates their potential importance in the association between oomycetes communities and disease severity at Allegan.

4. Discussion

This study was motivated by the observation of consistent and more severe seedling disease in Allegan field sites compared with Ingham. Therefore, this two-year field study was conducted to profile oomycete communities from over 300 soybean rhizosphere soils, and also to examine the effect of other agronomic factors such as seed treatment and soybean genotype on disease, which have not been examined in detail in previous studies. As observed in Rossman et al. (2018), disease pressure was higher in Allegan than in Ingham, especially in 2015 where plant density and root biomass were significantly reduced compared to Ingham (Fig. 1). Oomycete community profiles were different depending on location and year (Fig. 4A). Disease symptoms most consistent with oomycete disease pressure were most prominent in Allegan 2015 and oomycete communities were associated with variation in plant density and root biomass in Allegan 2015, and unique OTUs associated with high disease pressure were identified.

In all location-year combinations, oomycete communities in soybean rhizosphere samples were dominated by *Pythium*. Notably, this included important pathogenic species like *Pythium ultimum* var.

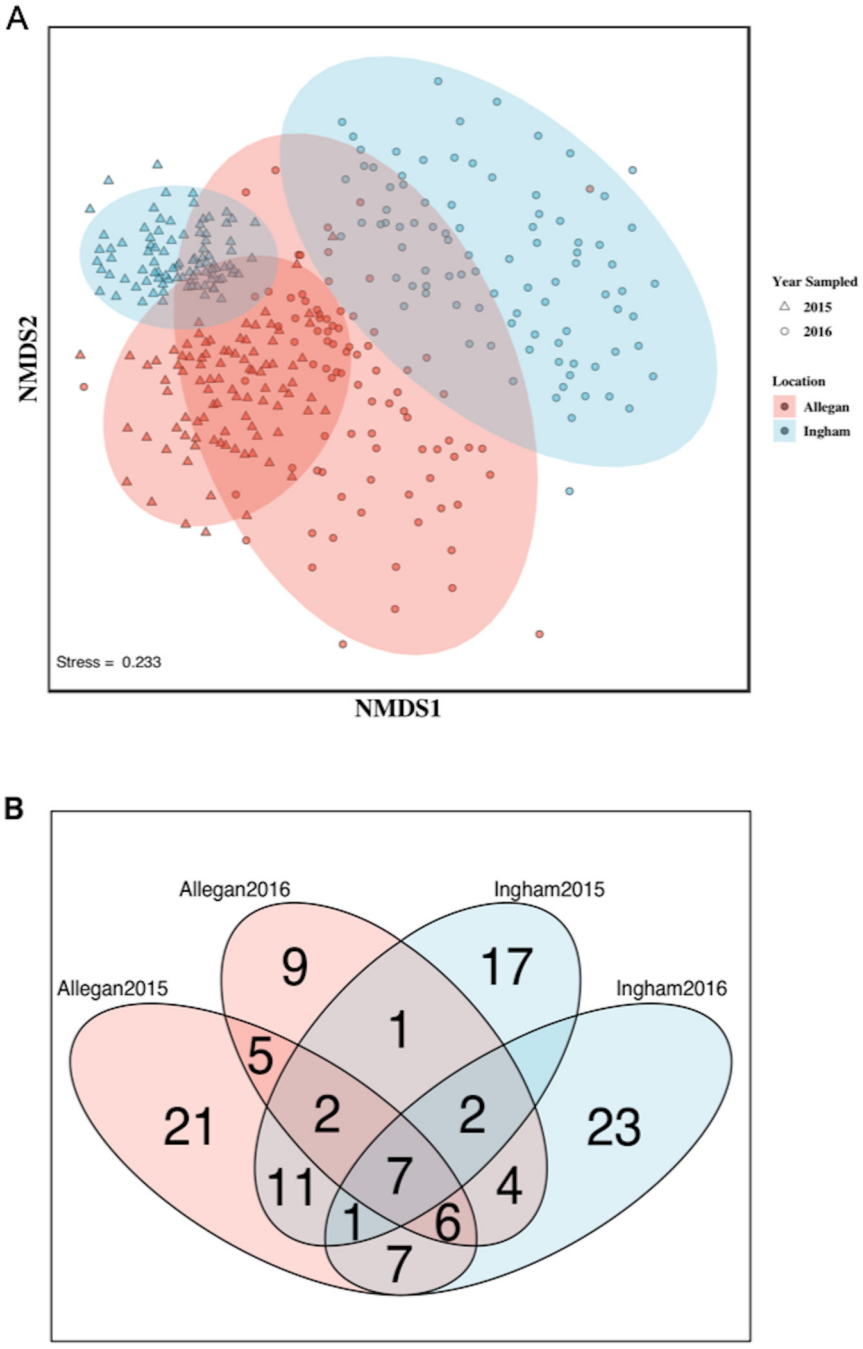

Fig. 4. Between sample diversity of oomycete rhizosphere communities. (A) Non-metric multidimensional scaling ordination of soybean rhizosphere oomycete communities based on log-transformed and Wisconsin double standardized Bray-Curtis distances. Point shapes represent year (2015 or 2016) sampled and color represents location (Ingham or Allegan). Ellipses represent 95% confidence interval of a multivariate normal distribution for each year-location combination. (B) Venn-diagram or the number of OTUs significantly associated with each year-location combination.

ultimum and *Pythium heterothallicum* and putatively beneficial oomycetes. For example, OTU4 was identified as a *Pythium* sp. in clade D with a 100% match to *Pythium oligandrum* and had 3.62 and 3.51% mean relative abundance in Allegan 2015 and Ingham 2016. *Pythium oligandrum*, *Pythium acanthicum*, and *Pythium periplocum* are well-known soil-dwelling antagonists of fungi and oomycetes (Martin and Loper, 1999; Paul, 1999; Ribeiro and Butler, 1995). An OTU identified as *Lagnenidium gigateum*, an entomopathogenic oomycete, was also observed in soybean rhizospheres.

The observation of *Pythium* dominance in the soybean rhizosphere corroborates observations of other culture-based and culture-independent metabarcoding studies where *Pythium* was dominant in agricultural soils (Rojas et al., 2017a, 2017b; Broders et al., 2009; Taheri et al., 2017; Coince et al., 2013; Vannini et al., 2013; Sapkota and Nicolaisen, 2015; Singer et al., 2016; Bakker et al., 2017; Riit et al., 2016; Durán et al., 2018; Coffua et al., 2016; Sapp et al., 2018; Schlatter et al., 2018). Historically, soybean breeding efforts have primarily focused on *Phytophthora sojae* because of its gene-for-gene interaction with soybean R gene products (Dorrance and Grünwald, 2009). Additionally, *Phytophthora sojae* can cause characteristic mid-season stem rot and kill mature soybean plants leading to noticeable losses. There have been few studies focused on *Pythium* resistance breeding (Rosso et al., 2008; Rupe et al., 2011; Kirkpatrick et al., 2006; Lin et al., 2018; Stasko et al., 2016; Ellis et al., 2013) but genetic resistance is not

Table 2
Operational taxonomic units (OTUs) significantly associated uniquely with Allegan 2015.

OTU	Taxonomy	Mean % relative abundance ± SE	Association statistic	*P* value
OTU447	Pythiaceae sp.	2.418 ± 0.327	0.888	0.005
OTU91	*Pythium* sp.	0.168 ± 0.077	0.228	0.005
OTU42	Oomycetes sp.	0.409 ± 0.256	0.616	0.005
OTU81	*Pythium monospermum*	0.350 ± 0.093	0.470	0.005
OTU41	*Pythium ultimum* var. *ultimum*	0.293 ± 0.068	0.492	0.005
OTU34	*Pythium irregulare*	0.311 ± 0.099	0.502	0.005
OTU64	Saprolegniaceae sp.	0.128 ± 0.047	0.340	0.005
OTU90	*Phytopythium litorale*	0.175 ± 0.048	0.409	0.005
OTU111	*Pythium* sp.	0.117 ± 0.039	0.511	0.005
OTU63	*Pythium heterothallicum*	0.277 ± 0.043	0.741	0.005
OTU115	Oomycetes sp.	0.132 ± 0.047	0.351	0.005
OTU71	Oomycetes sp.	0.189 ± 0.058	0.452	0.005
OTU50	Pythiales sp.	0.280 ± 0.189	0.231	0.020
OTU94	*Phytophthora nicotianae*	0.076 ± 0.051	0.233	0.005
OTU130	*Apodachlya brachynema*	0.043 ± 0.016	0.282	0.005
OTU120	*Lagenidium giganteum*	0.028 ± 0.012	0.255	0.005
OTU157	Oomycetes sp.	0.110 ± 0.057	0.390	0.005
OTU124	*Pythium* sp.	0.067 ± 0.047	0.181	0.035
OTU337	*Lagenidium giganteum*	0.004 ± 0.002	0.209	0.015
OTU178	Peronospora sp.	0.029 ± 0.018	0.209	0.010
OTU336	Saprolegniaceae sp.	0.005 ± 0.002	0.233	0.005
Total		5.609		

intentionally applied in the field as it is for *Phytophthora sojae*.

Despite the lack of observed disease pressure in Ingham, it was not due to an absence of pathogenic oomycete species. The most abundant oomycete OTUs in Ingham 2015 was identified as *Pythium ultimum* var. *ultimum*, and *Pythium heterothallicum*, yet little disease was observed. Allegan in 2015 was on average less even than Ingham 2015 indicating that although Allegan 2015 contained more OTUs than Ingham 2015, rhizosphere samples were dominated by fewer taxa. The most abundant OTU in Allegan 2015 was identified as *Pythium ultimum var. ultimum*. This species is a well-known opportunistic plant pathogen notorious for infecting plants at early developmental stages and under stress. According to the PRISM Climate Group database (Prism Climate Group, Oregon State University, 2016), in 2015 Allegan county Michigan experienced 46.61 mm of rain two weeks after planting. Over half of this rain (26.86 mm) occurred two days after planting. A similar amount of rain occurred in Ingham, but it was distributed across two weeks following planting, rather than as a pulse event two days after planting. The weather may have increased favorable conditions for oomycete growth and stressed germinating seeds. The same weather trends did not occur in 2016, when both locations received < 10 mm rain in the two weeks following planting.

There were 21unique oomycete OTUs which were significantly associated with Allegan 2015 based on indicator species analysis. Notably, OTU41, identified as *Pythium ultimum* var. *ultimum* was significantly associated with higher than average plant density in Allegan 2015, perhaps indicating that increased resource availability provided by increased plant density and root mass is attractive to some oomycete taxa. Interestingly, OTU1 was also identified as *Pythium ultimum* var. *ultimum* and was the most abundant in Allegan 2015 but was associated with both high and low plant density. Based on this observation it could be hypothesized that with increased niche space provided by increased plant density allowed for multiple *Pythium ultimum* genotypes to coexist. A pairwise sequence comparison of OTU 1 and OTU 41 indicated that the ITS1 regions sequenced were 9.17% different, indicating considerable genetic differences within the *Pythium ultimum* species complex associated with soybean within the same field location. In Michigan greenhouses 65 multilocus genotypes of *Pythium ultimum* were recovered (Del Castillo Múnera et al., 2019). Future studies could examine this in more detail by with more in-depth population genetics.

The results of the db-RDA indicated that although small, some variation in plant density and root biomass was attributed to oomycete community composition in Allegan 2015. Other edaphic factors such as soil pH and soil temperature could also explain why disease pressure was not observed in Ingham field sites, as these factors can influence pathogenicity (Martin and Loper, 1999; Rojas et al., 2017a). Future studies should include soil chemical properties for each sample to relate specific edaphic factors to oomycete communities. It is possible that disease stress did not merely result from the presence or absence of pathogens; instead, it depends on the evenness of pathogens with the possibility of facilitative interactions between oomycetes and other organisms. An observation to support this statement is plant density, and root biomass was significantly higher in plots with the (FIN) treatment compared to the non-treated control (NTC) but not for the F or FI seed treatments (Supplemental Table 2). On the other hand, there was no significant improvement in plant density, root weight, or yield due to seed treatment in Ingham regardless of the soybean genotype

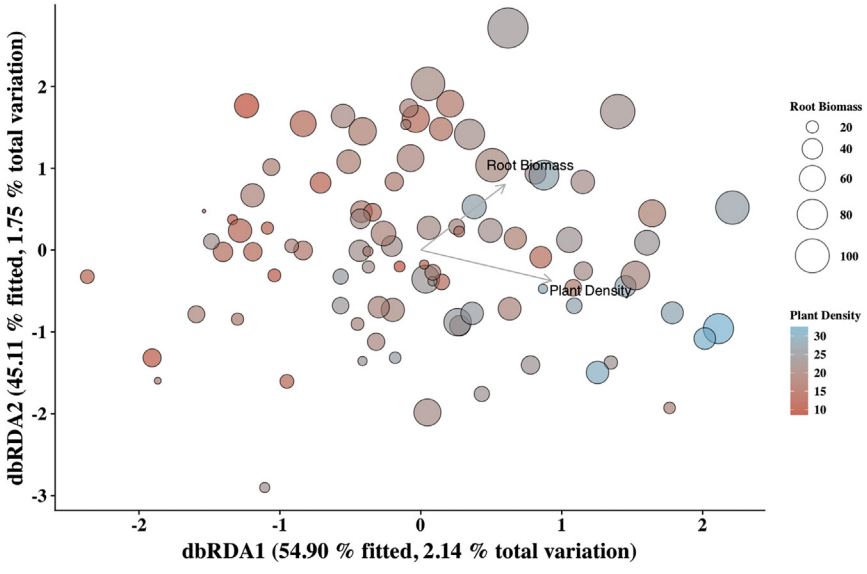

Fig. 5. Association of oomycete communities with plant density and root biomass in Allegan 2015. Distance based redundancy analysis (db-RDA) of rhizosphere oomycete communities based on log-transformed and Wisconsin double standardized Bray-Curtis distances in Allegan 2015. Variation in root biomass and plant density were significantly associated with oomycete community composition based on a Monte Carlo permutation test. Arrows represent direction of increasing root biomass and plant density. Points represent plots sampled are scaled to the mean root biomass per plant and colored by mean plant density.

Table 3
Operational taxonomic units (OTUs) significantly associated with high or lower than average plant density or root biomass in Allegan 2015.

OTU	Taxonomy	Clade	Association	Indicator species		Percent relative abundance		
				Association statistic	*P* value	High	Low	*t*-Test *P* value
OTU18	*Pythium* sp. nov	Clade B	High root biomass	0.593	0.005	1.080 ± 0.352	0.238 ± 0.114	0.026
OTU135	Saprolegniaceae sp.	–	Low root biomass	0.488	0.020	0.006 ± 0.004	0.036 ± 0.011	0.017
OTU41	*Pythium ultimum* var. *ultimum*	Clade I	High plant density	0.604	0.010	0.425 ± 0.113	0.107 ± 0.049	0.012
OTU71	Oomycetes sp.	–	Low plant density	0.529	0.010	0.310 ± 0.028	0.030 ± 0.112	0.030

(Supplemental Table 2). These results indicate that the possibility of soil pests (insects or nematodes) feeding on roots might elevate the risk of oomycete infection and disease stress.

Interestingly, two OTUs identified as *Lagnidium giganteum* were unique to Allegan 2015 (Table 2). Members of the *Lagnidium* genus are known pathogens of animal hosts and the presence of these isolates along with the observation of increased plant stand with insecticides is intriguing. Facilitation of plant death by pathogenic oomycetes may be influenced by the presence of insects or nematode damage allowing more accessible entry into plant tissue (Graham and McNeill, 1972; Willsey et al., 2017). Furthermore, neonicotinoid insecticides can induce systemic acquired resistance (SAR) and prime plant defenses (Ford et al., 2010). Insect larval root feeding injury, presumably from seedcorn maggot (*Delia platura*) has been observed in Allegan field sites, but extensive insect surveys were not conducted since incidence was not above an economic threshold (Rossman et al., 2018). Additional study using metagenome sequencing may reveal other pests or organisms in Allegan, and analyses on multiple organisms together with oomycetes may improve the explanation of variance.

5. Conclusions

Oomycetes are important drivers of community assembly but are often overlooked and an understudied portion of the plant microbiome (Agler et al., 2016). This study represents a 2-year field survey of oomycete communities from a location previously observed to have high disease pressure compared to one that did not. Interestingly and unexpectedly, seed treatments and plant genotype did not have a substantial impact on oomycete community structure, despite their improvement to plant density and root biomass in sites with higher disease pressure. Oomycete communities were different based on location; however, field sites without historical disease pressure were not devoid of pathogenic oomycetes. Therefore, we hypothesize that possible dominance of pathogenic oomycete species, oomycete interactions with edaphic factors, weather conditions at planting, and possible interactions with other soil-dwelling organisms are responsible for the disease pressure observed. In conclusion, this study improves our understanding of oomycete diversity in soybean rhizosphere which will aid in recommendations for plant breeders and oomicide seed treatment recommendations. Future studies are encouraged to integrate oomycetes with fungal, bacteria and soil fauna datasets for understanding disease factors in the plant microbiome.

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Declaration of competing interest

The authors declare no conflicts of interest.

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